Androgen inactivation and steroid-converting enzyme expression in abdominal adipose tissue in men

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Abstract

We examined 5α-dihydrotestosterone (5α-DHT) inactivation and the expression of several steroid-converting enzymes with a focus on aldoketoreductases 1C (AKR1C), especially AKR1C2, in abdominal adipose tissue in men. AKR1C2 is mainly involved in the conversion of the potent androgen 5α-DHT to its inactive forms 5α-androstane-3α/β,17β-diol (3α/β-diol). Subcutaneous (s.c.) and omental (Om) adipose tissue biopsies were obtained from 21 morbidly obese men undergoing biliopancreatic derivation surgery and 11 lean to obese men undergoing general abdominal surgery. AKR1C2 mRNA and 5α-DHT inactivation were detected in both s.c. and Om adipose tissue. After incubation of preadipocytes with 5α-DHT, both 3α-diol and 3β-diol were produced through 3α/β-ketosteroid reductase (3α/β-HSD) activity. In preadipocyte cultures, 3α-reductase activity was significantly predominant over 3β-reductase activity in cells from both the s.c. and Om compartments. Expression levels of AKR1C1, AKR1C3 and of the androgen receptor were significantly higher in s.c. versus Om adipose tissue while mRNA levels of 17β-HSD-2 (hydroxysteroid dehydrogenase type 2) and 3(α→β)-hydroxysteroid epimerase were significantly higher in Om fat. 3α/β-HSD activity was mainly detected in the cytosolic fraction, suggesting that AKR1C may be responsible for this reaction. Experiments with isoform-specific AKR1C inhibitors in preadipocytes showed that AKR1C2 inhibition significantly decreased 3α-HSD and 3β-HSD activities (3α-HSD: 30±24% of control for s.c. and 32±9% of control for Om, 3β-HSD: 44±12% of control for s.c.). When cells were incubated with both AKR1C2 and AKR1C3 inhibitors, no significant additional inhibition was observed. 5α-DHT inactivation was significantly higher in mature adipocytes compared with preadipocyte cultures in s.c. adipose tissue, as expressed per microgram total protein (755±830 versus 245±151 fmol 3α/β-diol per μg protein over 24 h, $P<0{\cdot}05$ $n=10$ cultures). 5α-DHT inactivation measured in tissue homogenates was significantly higher in the s.c. depot compared with Om fat (117±39 versus 79±38 fmol 3α/β-diol per μg prot over 24 h, $P<0{\cdot}0001$). On the other hand, Om 3α/β-HSD activity was significantly higher in obese men (body mass index (BMI) ≥30 kg/m^2) compared with lean and overweight men (84±37 versus 52±30 fmol 3α/β-diol per μg protein over 24 h, $P<0{\cdot}03$). No difference was found in s.c. 3α/β-HSD activity between these groups. Positive correlations were found between s.c. 5α-DHT inactivation rate and circulating levels of the androgen metabolites androsterone-glucuronide ($r=0{\cdot}41$, $P<0{\cdot}02$) and 3α-diol-glucuronide ($r=0{\cdot}38$, $P<0{\cdot}03$) and with the adrenal precursor androstenedione ($r=0{\cdot}42$, $P<0{\cdot}02$). In conclusion, androgen inactivation was detected in abdominal adipose tissue in men, with higher 3α/β-HSD activity in the s.c. versus Om depot. Higher Om 5α-DHT inactivation rates were found in obese compared with lean men. Further studies are required to elucidate whether local androgen inactivation in abdominal adipose tissue is involved in the modulation of adipocyte metabolism and regional fat distribution in men.

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Introduction

The physiological importance of excess adipose tissue accumulation in the etiology of type 2 diabetes and cardiovascular disease is of growing interest in the context of the obesity epidemic prevailing in affluent societies (Must *et al.* 1999, Lewis *et al.* 2002, Ravussin & Smith 2002). A predominantly abdominal fat distribution with increased fat accumulation within the abdominal cavity (visceral obesity) has been identified as a critical correlate of obesity-related metabolic alterations leading to adverse health outcomes including insulin resistance, hyperinsulinemia, a dyslipidemic state and proinflammatory/prothrombotic alterations (Després *et al.* 1990, Lemieux *et al.* 2001, Juhan-Vague *et al.* 2002).

Epidemiological evidence indicated that androgens may be involved in the regulation of obesity and body fat distribution

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patterns (Khaw & Barrett-Connor 1992, Tchernof *et al.* 1995, Gapstur *et al.* 2002). Testosterone administration to female-to-male transsexuals leading to supraphysiological plasma hormone concentration induced a significant increase in visceral adipose tissue area assessed by magnetic resonance imaging (Elbers *et al.* 1999, 2003). However, endogenous plasma testosterone levels are negatively associated with visceral fat accumulation in healthy men (Khaw & Barrett-Connor 1992, Tchernof *et al.* 1995), and treatment with physiological androgen doses improves the metabolic profile possibly through reductions in visceral fat accumulation (Mårin *et al.* 1992, Boyanov *et al.* 2003). In addition, plasma 5α-androstan-3α,17β-diol-glucuronide (3α-diol-glucuronide), a marker of androgen metabolism (inactivation) in peripheral tissues (Labrie *et al.* 1997), was positively correlated with total adiposity and visceral adipose tissue accumulation in men, suggesting that local androgen conversion may be related to adipose tissue metabolism (Tchernof *et al.* 1997).

The notion that adipose tissue is a complex and metabolically active organ which possesses endocrine, paracrine, and autocrine hormonal properties is increasingly recognized (Mohamed-Ali *et al.* 1998, Kershaw & Flier 2004). In this regard, several steroid-converting enzymes involved in local sex steroid metabolism were detected in adipose tissue, including aromatase, 3β-hydroxysteroid dehydrogenase (3β-HSD), type 1 11β-HSD, type 2 and type 3 17β-HSD, 7α-hydroxylase, 17α-hydroxylase, 5α-reductase, and uridine diphosphate (UDP)-glucuronosyltransferase 2B15 (reviewed by Bélanger *et al.* 2002). Our group also described the presence of three members of the aldoketoreductase 1C (AKR1C) family, namely AKR1C1 (20α-HSD), AKR1C2 (type 3 3α-HSD), and AKR1C3 (type 5 17β-HSD) in whole adipose tissue samples and preadipocytes obtained from women (Blouin *et al.* 2003, Blanchette *et al.* 2005).

AKR1C1, AKR1C2 and AKR1C3 display ketosteroid reductase activities (3α-HSD, 20α-HSD, and 17β-HSD), but in different proportions. Experiments in intact cells have shown that AKR1C2 is mainly involved in the inactivation of the most potent androgen 5α-androstane-17β-ol-3-one (5α-dihydrotestosterone, 5α-DHT) into the weak androgen 5α-androstan-3α,17β-diol (3α-diol) through its 3α-HSD activity. This enzyme also possesses a low 17β-HSD activity (~2–3% of its 3α-HSD activity) and a moderate 20α-HSD activity (~10% of its 3α-HSD activity) (Zhang *et al.* 2000, Dufort *et al.* 2001). AKR1C1 mainly inactivates progesterone into 20α-hydroxyprogesterone through its 20α-HSD activity, but also forms testosterone from 4-androsten-3,17-dione (D4-dione) (~3–4% of its 20α-HSD activity) and inactivates 5α-DHT into 3α-diol (~8% of its 20α-HSD activity). AKR1C3 also mainly inactivates progesterone into 20α-hydroxyprogesterone, but also shows significant 17β-HSD activity leading to testosterone formation from D4-dione (~35% of its 20α-HSD activity), and 3α-HSD activity (~7–8% of its 20α-HSD activity; Dufort *et al.* 1999, Zhang *et al.* 2000). Purified enzyme assays have shown that AKR1C1, AKR1C2 and AKR1C3 also possess 3β-HSD activity leading to the formation of 5α-androstan-3β,17β-diol (3β-diol) from 5α-DHT (Steckelbroeck *et al.* 2004).

We previously observed positive correlations between visceral adipose tissue area assessed by computed tomography and 3α/β-HSD and 20α-HSD activities as well as AKR1C mRNA levels measured in Om adipose tissue homogenates in women (Blouin *et al.* 2003, 2005, Blanchette *et al.* 2005). The aim of the present study was to examine 3α/β-HSD activity (5α-DHT inactivation) and the expression of several enzymes involved in androgen metabolism in s.c. and omental (Om) adipose tissue obtained from lean to morbidly obese men, and to further investigate the relationship between 3α/β-HSD activity and obesity. We tested the hypothesis that AKR1C enzymes would be detected in abdominal adipose tissue depots and 5α-DHT inactivation would be related to obesity in men.

Materials and Methods

Subjects

Men 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 12 men aged 38·6–57·1 years (body mass index (BMI) 30·9±5·0 kg/m^2, range 24·6–39·1 kg/m^2) undergoing general abdominal surgery. Reasons for surgeries were: umbilical hernia (n=7), endocholecystectomia (n=2), giant parastomal hernia (n=1), and sigmoid restriction (n=2). In the analysis, 22 men aged 22·6–61·2 years (BMI 51·8±9·2 kg/m^2, 40·6–79·1 kg/m^2) undergoing biliopancreatic derivation surgery for morbid obesity were also included. None of the subjects were taking hormonal treatments except for thyroid hormones (n=2). Excluding these two subjects from the analyses did not alter the present results. One subject was treated with domperidone and one subject was taking an anti-obesity drug. Some subjects also received medication for diabetes (n=10), hypertension (n=16) and dyslipidemia (n=6). BMI and waist circumference were measured according to standardized procedures (Lohman *et al.* 1988). Approbations by the medical ethics committees of Laval University, Laval Hospital and Laval University Medical Center were obtained. All subjects provided written informed consent before their inclusion in the study.

Sex steroid hormones, C_{19} steroid precursors and androgen metabolite measurements in plasma and culture media

Concentrations of D4-dione, testosterone, 5α-DHT, 5α-androstan-3α-ol-17-one (androsterone), 3β-diol, estrone, and 17β-estradiol were measured using high performance gas chromatography and negative chemical ionization mass spectrometry (GC–MS). The intra- and inter-assay coefficients of variation did not exceed 5·9% for these

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measurements. Androsterone-glucuronide and 3α-diol-glucuronide levels were determined using liquid chromatography and mass spectrometry (LC–MS) using a PE Sciex API 300 tandem mass spectrometer (Perkin-Elmer, Foster City, CA, USA) equipped with a Turbo ionspray source. The intra- and inter-assay coefficients of variation did not exceed 6·4% for these measurements. HPLC was used for the identification and the relative quantification of ^{14}C-5α-DHT metabolites after 24-h incubations with Om preadipocytes. Briefly, ^{14}C-labeled steroids were analysed using a Zorbax cyano normal-phase HPLC column (4·6×250 mm, 5 μm). The mobile phase was hexane/tetrahydrofuran (96/4 v/v), with a flow rate of 1·5 ml/min. Radioactivity was monitored in the eluent using a Beckman 171 HPLC Radioactivity Monitoring System. ^{14}C-steroids (5α-DHT, 3α-diol, 3β-diol, 5α-androstan-3,17-dione (A-dione)), used as standards, were HPLC-purified in the laboratory in the same conditions.

Adipose tissue sampling

Paired Om and s.c. adipose tissue samples were collected during the surgical procedure and immediately carried to the laboratory in 0·9% saline preheated at 37 °C. A portion of the biopsy was used for adipocyte and preadipocyte isolation and the remaining tissue was immediately frozen at −80 °C for subsequent analyses.

Separation of cytosolic and membrane fractions

Separation of whole adipose tissue cytosolic and membrane fractions was performed according to a previously published method with some modifications (Cormont *et al.* 1993). Briefly, frozen adipose tissue samples were homogenized 10 times in 10 mM Tris, 1 mM EDTA, 250 mM sucrose, and 1 mM phenylmethylsulphonyl fluoride, using a Potter device. Homogenates were centrifuged at 288 ***g*** to remove lipids. Following a 75 min ultracentrifugation at 210 000 ***g***, cytosol (supernatant) was harvested. The pellet was homogenized again using the same procedure and ultracentrifuged for 75 min at 210 000 ***g***. The pellet (total membrane fraction) was resuspended in buffer. Isolated cytosolic and membrane fractions were used for 3α/β-HSD activity determination.

Preadipocyte isolation and primary cultures

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 (1964) method. Adipocyte suspensions were filtered through nylon mesh and washed thrice with KRH buffer. Preadipocytes were isolated using a modification of the method previously described by Hauner (Hauner 1990, Hauner *et al.* 2001). Briefly, the residual KRH buffer of the adipocyte isolation was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal bovine serum, 0·25 μg/ml amphotericin B, and 50 μg/ml gentamicin. The 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. The medium was changed every 2–3 days.

Cell size measurements

Mature adipocyte suspensions were visualized using a contrast microscope attached to a camera and computer interface. Pictures were taken and the Scion Image software (Scion Corporation, Frederick, MA, USA) was used to measure the size of 250 adipocytes.

Lipolysis and lipoprotein lipase (LPL) activity

Basal lipolysis experiments were performed by incubating isolated cell suspensions for 2 h at 37 °C. Glycerol release in the medium was quantified by bioluminescence using the nicotinamide adenine dinucleotide hydroxide-linked bacterial luciferase assay (Kather *et al.* 1982), a Berthold Microlumat plus bioluminometer (LB 96 V) and the WinGlow software (EG&G, Bad Wildbad, Germany). The average coefficient of variation for duplicate glycerol release measurements was 11·5%. Lipid weight of the cell suspension was measured by performing Dole's extraction, and lipolysis results were expressed as a function of adipocyte surface area (nanomoles glycerol/2 h×10^8 μm^2).

Heparin-releasable (HR)-LPL activity activity was determined in 30–50-mg frozen adipose tissue samples by the method of Taskinen *et al.* (1980). Tissue eluates were obtained by incubating the sample in Krebs–Ringer phosphate buffer and heparin at 28 °C for 90 min. The eluates were then incubated with excess concentrations of unlabeled and ^{14}C-labeled triolein in a Tris–albumin buffer 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 was used as a source of Apo-CII to stimulate LPL activity, and unpasteurized cow's milk was used as an internal LPL activity standard for inter-assay variations. The activity results were expressed in nanomoles oleate/10^6 cells/h.

Real-time RT-PCR

Total RNA was isolated using Rneasy kit (Qiagen). First strand cDNA synthesis was accomplished using 2 μg isolated RNA in a reaction containing 200 units of Superscript II Rnase H-reverse transcriptase (Invitrogen), 300 ng oligo dT18, 500 μM dNTP, 10 mM dithiothreitol, and 34 units porcine RNase inhibitor (Amersham Pharmacia) in a final volume of 50 μl incubated at 42 °C for 2 h. The resulting products were then treated with 1 μg Rnase A for 30 min at 37 °C and purified thereafter with Qiaquick PCR purification kits (Qiagen). A Light-Cycler PCR (Roche

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Diagnostics) was used to measure quantitative expression using sets of primers shown in Table 1. The FastStart DNA Master SYBR green kit (Roche Diagnostics) was used in a final reaction volume of 20 µl containing 4 mM $MgCl_2$, 20 ng of each primer and 20 ng of the cDNA template. The PCR was carried out according to the following conditions: 95 °C/10 min, 40 cycles (95 °C/10 s, 62 °C/5 s, 72 °C/11 s, 81 °C/3 s), and temperature transition was 3 °C/s for all reactions. PCR results were normalized according to subunit O of ATP synthase expression levels. A universal standard curve was generated with ATPase 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 (Cp) to calculate the amount of copies in initial cDNA specimens were determined with the double-derivative method (Luu-The *et al.* 2005). For each sample, the Cp value of the gene quantified 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 the samples examined in the present experiment (Luu-The *et al.* 2005). PCR data are expressed as normalized numbers of copies per microgram total RNA.

Enzymatic activities

3α/β-HSD activity was measured in preadipocyte primary cultures, in mature adipocytes, in whole tissue homogenates, and in isolated cytosolic and membrane fractions. Preadipocyte cultures were grown in 12-well culture plates. Culture medium was changed for fresh medium containing 87 nM (or 100 nM, depending on lot specific activity) 5α-DHT (Perkin-Elmer Life Sciences Inc.) as substrate for 3α/β-HSD activity and cells were incubated for 3, 6, 12, and 24 h. For experiments using AKR1C-specific inhibitors, cultures were preincubated 2 h with inhibitor(s) prior to the addition of radioactive 5α-DHT. AKR1C2 was inhibited using 100 µM 5β-cholanic acid-3α,7α-diol (5β-chol) and AKR1C3 with 20 µM indomethacin (Indo). For measures in adipose tissue homogenates, tissue samples were homogenized with a Polytron in 50 mM sodium phosphate buffer (pH 7·4), 20% glycerol, 1 mM EDTA, and 1 mM NADPH. For measures in adipose tissue homogenates as well as in cytosolic and membrane fractions, ^{14}C-labeled 5α-DHT was added and reactions were performed at 37 °C in a final volume of 1 ml for 24 h. For mature adipocytes, incubation with radiolabeled 5α-DHT was performed in BSA-free KRH buffer at 37 °C in a final volume of 1 ml for 24 h. Steroids from culture media and tissue homogenates were extracted twice with one volume ether as described previously (Dufort *et al.* 2001). For mature adipocytes, the steroid extraction with ether was preceded by two extractions with three volumes ethanol:acetone (1:1) to remove lipids. The organic phases were pooled and evaporated to dryness. The steroids were solubilized in 50 µl dichloromethane (reference standards were diluted in ethanol) and applied to Silica Gel 60 thin layer chromatography (TLC) plates (Merck) using 10 µl calibrated micropipets. The separation was done either by migration in toluene-acetone (4:1, does not allow for the separation of 3α-diol and 3β-diol) or in ether:ethyl acetate (1:1) to separate 3α-diol and 3β-diol. The radioactivity was detected using a Storm 860

Table 1 Oligonucleotides used in real-time RT-PCR quantification

Gene	Oligonucleotide sequence
AKR1C1	5′-CCTATAGTGCTCTGGGATCCCAC-3′
	5′-AGGACCACAACCCCACGCTGT-3′
AKR1C2	5′-CCGTCAAATTGGCAATAGAAGCC-3′
	5′-CAACTCTGGTCGATGGGAATTGCT-3′
AKR1C3	5′-CAACCAGGTAGAATGTCATCCGTAT-3′
	5′-ACCCATCGTTTGTCTCGTTGA-3′
3β-HSD-1	5′-CTTCAACCGCCACATAGTCACATT-3′
	5′-AGGAGGGTGGAGCTTGATGACAT-3′
17β-HSD-2	5′-GCGCCTCTCGGTGCTCCAAATG-3′
	5′-CGGCCATGCATTGTTTGTAGTCAGTCA-3′
17β-HSD-3	5′-GCGATGGAATTGGGAAAGCGTACT-3′
	5′-CTCCCTGTAGTCCGCTCGATCTCTG-3′
3(α→β)-Hydroxysteroid epimerase	5′-TTCGTGGGCCTGTACTACCTTCTGC-3′
	5′-CAGGTTCCCAAAGCCCGAGTCACA-3′
11-*cis*-Retinol dehydrogenase (RDH5)	5′-CTGATCTGTGACCCGGACCTAA-3′
	5′-GGGGCAGAAATAAATCAAAGTCCTT-3′
5α-Reductase-1	5′-TGGCGATTATGTTCTGTACCTGTA-3′
	5′-AACCACAAGCCAAAACCTATTAGA-3′
P450 aromatase	5′-CGACAGGCTGGTACCGCATGCTC-3′
	5′-AAGAGGCAATAATAAAGGAAATCCAGAC-3′
Androgen receptor	5′-AGCCATTGAGCCAGGTGTAGTGT-3′
	5′-CATCCTGGAGTTGACATTGGTGAA-3′
ATP synthase O subunit	5′-ATTGAAGGTCGCTATGCCACAG-3′
	5′-AACGACTCCTTGGGTATTGCTTAA-3′

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PhosphorImager (Amersham Pharmacia Biotech Inc.) and quantification was done using the ImageQuant software version 5.1 (Amersham Pharmacia Biotech Inc). Proteins were quantified by the BCA method for the comparison between preadipocytes and mature adipocytes or by the method of Lowry for other experiments. Total proteins were used in the calculation of activity values.

Statistical analyses

A paired *t*-test procedure was used to compare enzyme activitiy or expression in s.c. versus Om adipose tissue. 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 compare the means between the groups. When normality could not be reached, *a posteriori* mean contrasts were used for comparison. The Bonferroni correction was used to adjust for multiple comparisons. The nonparametric Wilcoxon rank-sum test was used to compare means between lean and obese subjects. Cut-off for obesity (Fig. 9) was defined as a BMI ≥ 30 kg/m^2. Correlation analyses were performed in the entire sample and also excluding men with class III obesity (BMI ≥ 40 kg/m^2; WHO guidelines). Spearman rank correlation coefficients were computed to quantify associations. The analyses were performed using the JMP statistical software (SAS Institute, Cary, NC, USA).

Results

Steroid-converting enzyme mRNA expression in adipose tissue

Table 2 shows the real-time RT-PCR quantification of several enzymes involved in androgen metabolism, including members of the aldoketoreductase 1C family, and the androgen receptor. The expression of AKR1C1, AKR1C3, and androgen receptor was significantly higher in s.c. versus Om adipose tissue ($P<0{\cdot}05$). The expression of 17β-HSD-2 and 3(α→β)-hydroxysteroid epimerase was higher in Om versus s.c. adipose tissue ($P<0{\cdot}05$). AKR1C enzymes were strongly expressed in both adipose tissue depots when compared with other mRNAs measured.

Androgen metabolites in preadipocyte primary cultures

Figure 1 shows a representative thin layer chromatogram of steroid products obtained when incubating s.c. or Om preadipocyte primary cultures with radiolabeled 5α-DHT for 24 h. When incubating cells with 5α-DHT, 3α/β-diol and A-dione were detected, with 3α-diol and 3β-diol being the major reaction products (Fig. 1, lanes 1–4). Separation of 3α-diol and 3β-diol was not possible using this chromatography method (Fig. 1, lanes 6–7). HPLC separation in Fig. 2 shows that, in addition to A-dione, both 3α-diol and 3β-diol were produced when Om preadipocytes were incubated with 5α-DHT for 24 h. Figure 3 shows the GC/LC–MS quantification of steroid metabolites produced after incubation of preadipocytes with D4-dione (Fig. 3A) or 5α-DHT (Fig. 3B). When cells were incubated with the adrenal precursor D4-dione, testosterone, 5α-DHT and androsterone were produced (Fig. 3A). When cells were incubated with 5α-DHT, high levels of 3β-diol were produced (Fig. 3B) which represented approximately 25% of total steroids. Quantification of 3α-diol was not possible using this method. Figure 4 shows the quantification of 3α-diol and 3β-diol in s.c. and Om preadipocytes. Average 3α-reductase activity was significantly higher when compared with 3β-reductase activity in both depots. 3β-reductase activity was not significantly different between s.c. and Om preadipocytes. However, there was a trend for higher 3α-reductase activity in

Table 2 Expression levels of steroid-converting enzymes and nuclear receptor involved in androgen metabolism and action in subcutaneous and omental adipose tissue in 13 men. Data are means ± S.D

	mRNA levels (10^3 copies/μg total RNA)		
	s.c.	Om	***P* value**
Aldoketoreductases			
AKR1C1	1029·0 ± 553·5	485·1 ± 149·1	<0·05
AKR1C2	802·5 ± 714·6	430·9 ± 179·4	<0·09
AKR1C3	488·2 ± 334·1	253·4 ± 96·4	<0·05
Short-chain dehydrogenases			
3β-HSD-1	0·2 ± 0·2	0·2 ± 0·4	NS
17β-HSD-1	0·01 ± 0·04	0·3 ± 0·4	<0·05
17β-HSD-3	1·2 ± 2·4	2·4 ± 4·2	NS
3(α→β)-Hydroxysteroid epimerase	0·2 ± 0·2	13·4 ± 12·9	<0·05
11-*cis*-Retinol dehydrogenase (RDH5)	152·3 ± 108·8	130·2 ± 105·2	NS
Others			
5α-Reductase-1	1·2 ± 1·5	1·0 ± 0·8	NS
P450 aromatase	0·6 ± 1·3	0·1 ± 0·2	NS
Androgen receptor	42·6 ± 29·5	20·8 ± 12·6	<0·05

NS, not significant.

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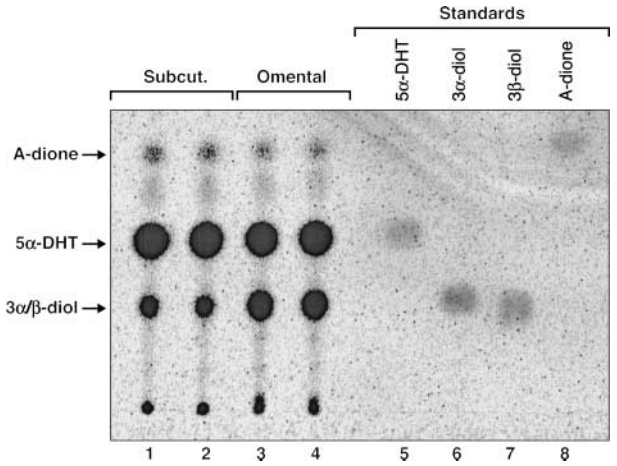

Figure 1 Thin layer chromatogram showing steroid products obtained when incubating s.c. or Om preadipocyte primary cultures with radiolabeled 5α-DHT. Results are representative of experiments performed in at least seven cultures from each fat depot, in duplicate.

s.c. preadipocytes. In order to evaluate whether the 3α/β-HSD activity originated from AKR1C enzymes, which are cytosolic, assays were performed in isolated cytosolic and membrane fractions. Figure 5 shows that 3α/β-HSD activity

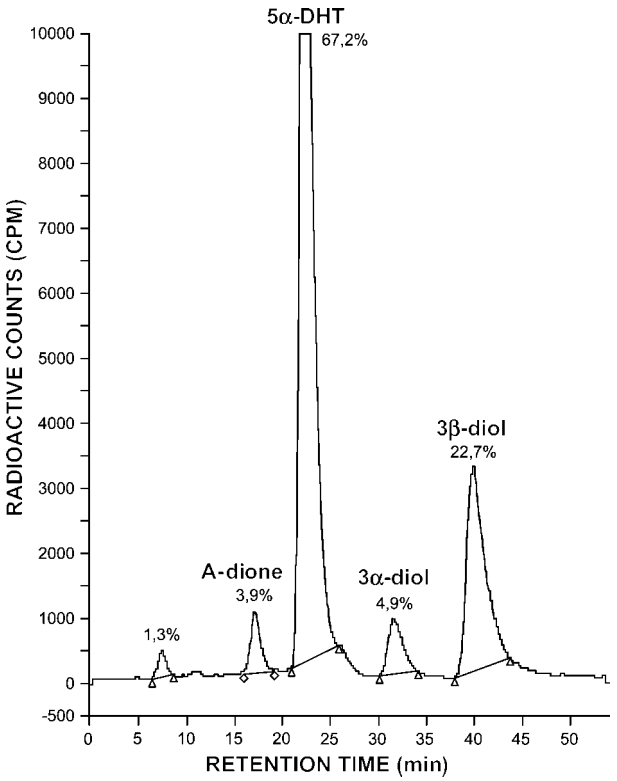

Figure 2 Identification and relative quantification by HPLC of 5α-DHT metabolites, namely 3α-diol, 3β-diol and A-dione. ^{14}C-5α-DHT was incubated for 24 h in the presence of Om preadipocytes, in duplicate. Percentage of steroid products are shown above each peak.

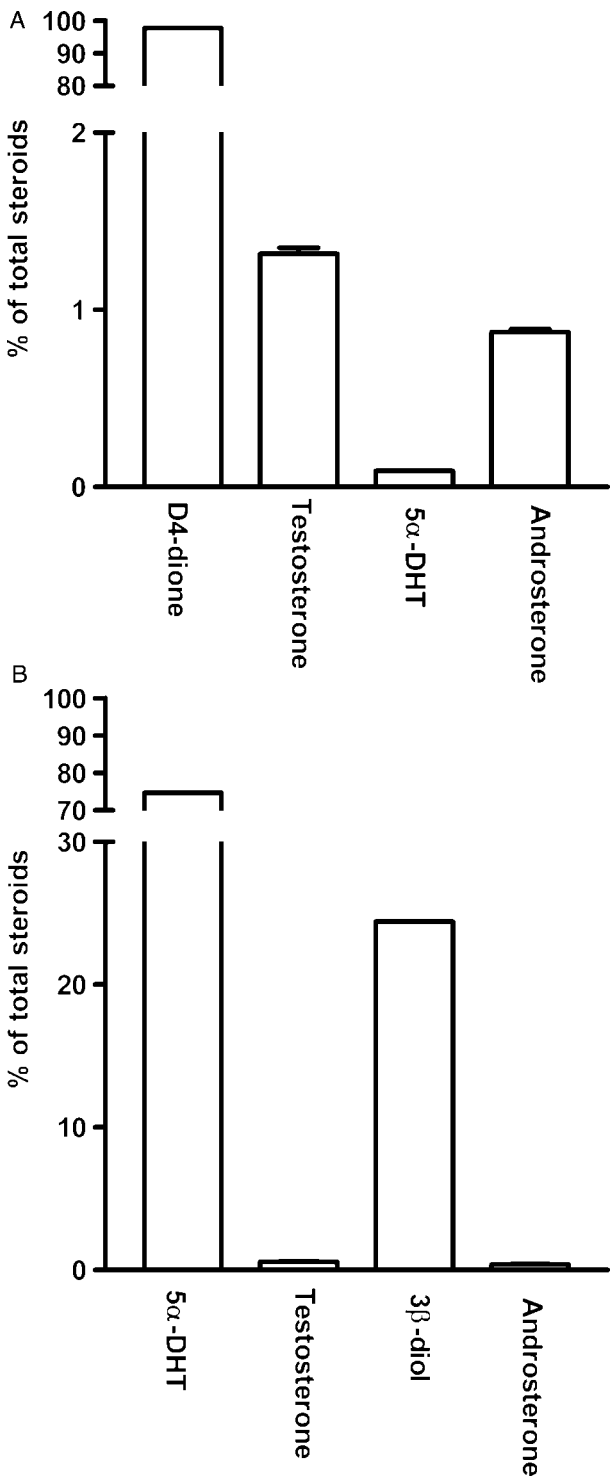

Figure 3 Quantification of steroid products by GC/LC–MS after incubation of Om preadipocytes with (A) 0·35 μM D4-dione or (B) 5α-DHT. Experiments were performed in duplicate. Means ± S.E.M. are shown.

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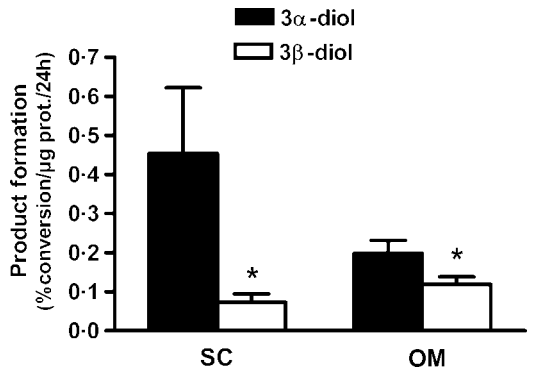

Figure 4 Measurement of 3α-reductase and 3β-reductase activities in s.c. and Om peadipocyte cultures using TLC in ether:ethyl acetate (1:1). The results were obtained in s.c. cultures from four subjects and in Om cultures from five subjects, in duplicate. Means±S.E.M. are shown. *$P<0.05$, 3α-diol versus 3β-diol formation.

was mostly present in the cytosol (approximately fourfold). Figure 6 shows the effects of specific AKR1C inhibitors on the formation of 3α-diol and 3β-diol. The strongest inhibition of 3α-diol and 3β-diol production was observed when cells were incubated with 100 µM 5β-cholanic acid-3α,7α-diol (3α-HSD: 30±24% of control for s.c. and 32±9% of control for Om, 3β-HSD: 44±12% of control for Om). When cells were incubated with both 5β-cholanic acid-3α,7α-diol, and indomethacin, no significant additional inhibition was observed. Figure 7 shows the results of time-course and dose–response experiments in preadipocyte cultures. Maximal stimulation of 3α/β-HSD activity was not reached at 1 µM substrate (Fig. 7A). Time course showed a linear 3α/β-diol formation over 24 h incubations (Fig. 7B). Om and s.c. preadipocyte cultures were not different.

3α/β-HSD activity in preadipocytes and mature adipocytes

Figure 8 shows that 5α-DHT inactivation through 3α/β-diol formation in the s.c. depot was significantly higher in mature adipocytes compared with preadipocyte cultures, as expressed

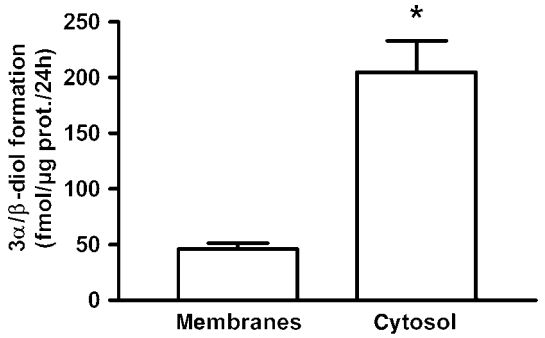

Figure 5 Quantification of 3α/β-HSD activity in adipose tissue cytosolic and membrane fractions. Data were obtained from six s.c. and five Om fat samples. *$P<0·0001$ membranes versus cytosol. Means±S.E.M. are shown.

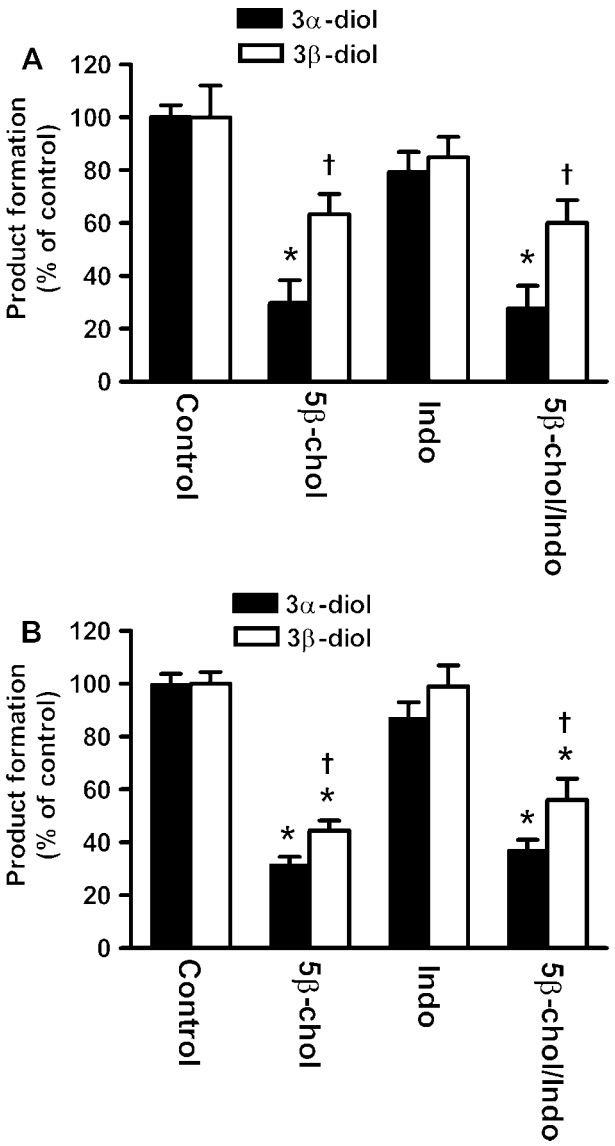

Figure 6 The effects of the specific AKR1C inhibitors 5β-cholanic acid-3α,7α-diol (5β-chol; inhibits AKR1C2) and indomethacin (Indo; inhibits AKR1C3) on the conversion of 5α-DHT into 3α-diol and 3β-diol after a 2-h preincubation period with the inhibitors in s.c. (A) and Om (B) preadipocyte cultures. Results are expressed as percentage of control. Data were obtained in s.c. cultures from four subjects and in Om cultures from five subjects, in duplicate. *Different from control ($P<0·05$) by *a posteriori* mean contrasts and Bonferroni correction. †$P<0·05$ 3α-diol versus 3β-diol formation by paired *t*-test. Means±S.E.M. are shown.

in femtomoles of 3α/β-diol formed per microgram total protein over 24 h (755±830 versus 245±151 fmol 3α/β-diol per µg protein over 24 h, $P<0·05$ $n=10$ cultures). No significant difference was observed between preadipocytes and mature adipocytes in Om adipose tissue ($n=11$ mature adipocyte cultures and $n=6$ preadipocyte cultures) and between the s.c. and Om depots for each given cell type.

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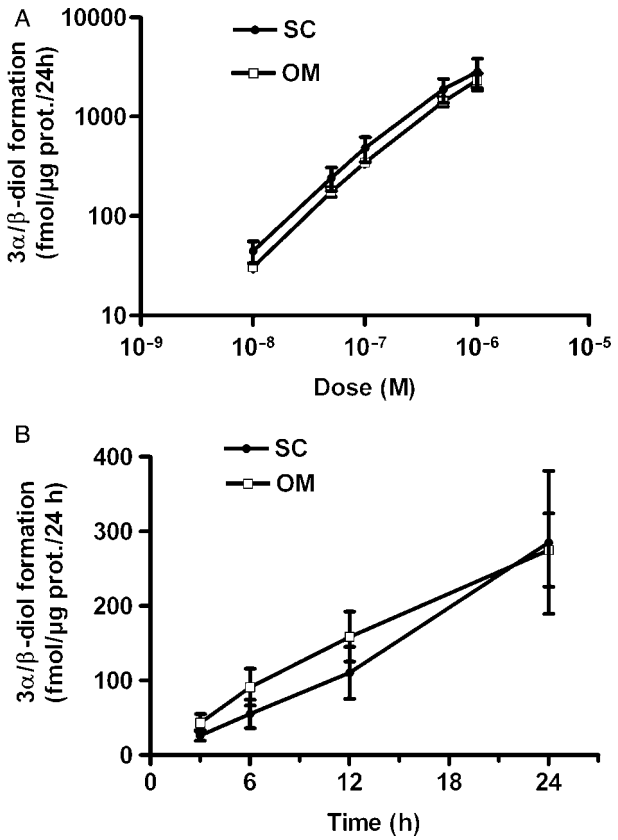

Figure 7 (A) Dose–response for 3α/β-HSD activity in preadipocyte primary cultures. (B) Time-course for 3α/β-HSD enzymatic activity in preadipocyte primary cultures. Experiments were performed in three cultures from each depot, in duplicate. Means ± S.E.M. are shown.

3α/β-HSD activity in adipose tissue homogenates

Table 3 shows characteristics of the sample of men in which adipose tissue homogenate 3α/β-HSD activity measures were performed. Subjects covered a wide range of BMI values (24·6–79·1 kg/m^2). Om HR-LPL activity was significantly higher than s.c. HR-LPL activity ($P<0·05$). Basal lipolysis as well as adipocyte diameter were not significantly different between the two adipose tissue compartments.

Table 3 Physical characteristics of the sample of 34 men

	Mean ± S.D.	Range
Characteristic		
Age (years)	43·8 ± 10·0	22·6–61·2
Weight (kg)	138·7 ± 46·9	70·4–265·0
BMI (kg/m^2)	44·4 ± 12·9	24·6–79·1
Waist circumference (cm)	136·1 ± 27·4	91·5–190·0
Subcutaneous		
HR-LPL activity	41·9 ± 19·7	14·5–110·8
Basal lipolysis	79·9 ± 58·0	10·0–301·3
Adipocyte diameter (µm)	109·3 ± 11·2	85·8–131·3
Omental		
HR-LPL activity	52·3 ± 24·0	22·4–102·2
Basal lipolysis	85·2 ± 104·3	21·2–568·1
Adipocyte diameter (µm)	110·0 ± 9·6	94·2–129·1

HR-LPL activity expressed as nmol FFA/h × 10^6 cells. Lipolysis expressed as nmol glycerol/2 h × 10^8 µm^2; $n=33$ for waist circumference, $n=30$ for HR-LPL activity, $n=24$ for s.c. lipolysis, $n=26$ for Om lipolysis.

3α/β-HSD activity measured in s.c. adipose tissue homogenates was significantly higher than that of the Om depot (117 ± 39 versus 79 ± 38 fmol 3αβ-diol/µg protein/24 h, $P<0·0001$) in the entire group. Figure 9 shows that 3α/β-HSD activity measured in Om adipose tissue was significantly higher in obese compared with lean or overweight men (84 ± 37 versus 52 ± 30 fmol 3α-diol/µg protein/24 h, $P<0·05$). 5α-DHT inactivation in s.c. adipose tissue was not different between lean/overweight and obese men.

No significant correlation was observed between 3α/β-HSD activity in Om adipose tissue and Om adipocyte size as well as LPL activity when all the subjects were considered. When extremely obese subjects were excluded (class III obesity, 40 kg/m^2), a significant positive association was observed between Om 3α/β-HSD activity and adipocyte

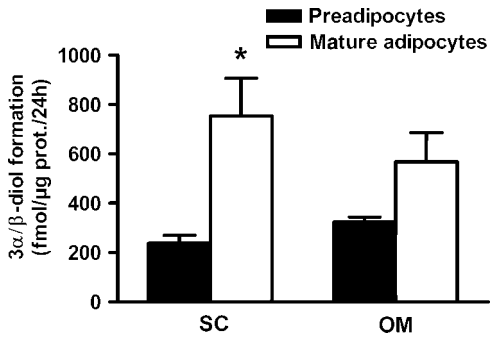

Figure 8 Comparison of 3α/β-HSD activity measured in s.c. and Om mature adipocytes and preadipocyte cultures. Data obtained from 10 s.c. and 11 Om samples for mature adipocyte cultures and from 10 s.c. and 6 Om samples for preadipocyte cultures. Measures were performed in triplicate for mature adipocytes and in duplicate for preadipocytes. *$P<0·05$ preadipocytes versus mature adipocytes by Welch ANOVA. Means ± S.E.M. are shown.

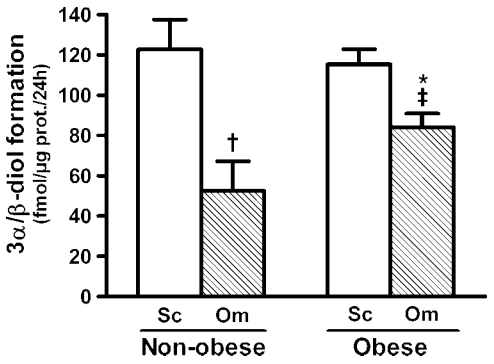

Figure 9 Comparison of 3α/β-HSD activity measured in s.c. and Om adipose tissue homogenates in non-obese (BMI < 30 kg/m^2, $n=6$) or obese (BMI ≥ 30 kg/m^2, $n=28$) men. *$P<0·05$ non-obese versus obese by Wilcoxon rank-sum test; †$P<0·05$, ‡$P<0·0005$ s.c. versus Om by paired *t*-test. Means ± S.E.M. are shown.

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diameter ($r=0{\cdot}69$, $P<0{\cdot}02$). No significant association was observed between s.c. 3α/β-HSD activity and s.c. adipocyte diameter.

We examined associations between plasma steroid hormones and 3α/β-HSD activity values in the two adipose tissue depots. Plasma 5α-DHT and testosterone were not related to 3α/β-HSD activity measured in any fat compartment. Figure 10 shows significant positive correlations of s.c. 3α/β-HSD activity with androsterone-glucuronide ($r=0{\cdot}41$, $P<0{\cdot}02$, $n=34$), 3α-diol-glucuronide ($r=0{\cdot}38$, $P<0{\cdot}03$, $n=34$), and D4-dione ($r=0{\cdot}42$, $P<0{\cdot}02$, $n=34$) levels. No association was observed between circulating steroid levels and 3α/β-HSD activity measured in Om adipose tissue.

Discussion

The aim of the present study was to examine 5α-DHT inactivation as well as the expression of enzymes involved in androgen metabolism in s.c. and Om adipose tissue obtained from normal weight to morbidly obese men, and to investigate the relationship between 5α-DHT inactivation and obesity. We tested the hypothesis that AKR1C enzymes would be detected in abdominal adipose tissue compartments and 5α-DHT inactivation would be related to obesity in men. AKR1C2 mRNA and 5α-DHT inactivation were, indeed, detected in both s.c. and Om adipose tissues, with whole s.c. adipose tissue having higher values of 5α-DHT inactivation and a trend for higher AKR1C2 mRNA levels. Both 3α-diol and 3β-diol were detected following incubation of preadipocytes with 5α-DHT. The androgen receptor and several enzymes involved in androgen metabolism were also expressed in adipose tissue. Consistent with our hypothesis, Om 3α/β-HSD activity in tissue homogenates was significantly higher in obese men compared with men with BMI <30 kg/m^2. 3α/β-HSD activity was detected mostly in the cytosolic fraction of whole adipose tissue. 5α-DHT inactivation was significantly higher in mature adipocytes compared with preadipocytes in the s.c. depot. This is the first study to report on the presence of AKR1C and 5α-DHT inactivation in abdominal adipose tissue obtained in lean and obese men. Future studies are needed to clarify the potential physiological importance of local androgen inactivation in the modulation of regional adipose tissue distribution.

This study in men was prompted by the recent results obtained in women. We reported higher 5α-DHT inactivation in female s.c. adipose tissue compared with Om adipose tissue (Blouin *et al.* 2003, 2005). We also found that women with high visceral adipose tissue area (assessed by computed tomography) had higher AKR1C1 and AKR1C2 mRNA levels as well as higher 5α-DHT inactivation rates in Om adipose tissue homogenates compared with women with low visceral adipose tissue area (Blouin *et al.* 2003, 2005). Significant positive correlations were also observed between 5α-DHT inactivation (and mRNA levels of AKR1C1 and AKR1C2) and visceral adipose tissue accumulation or Om adipocyte diameter (Blouin *et al.* 2003, 2005). Together, our results show that 3α/β-reduction of 5α-DHT is detected in abdominal fat compartments of both men and women, and that Om reaction rates are positively associated with adiposity

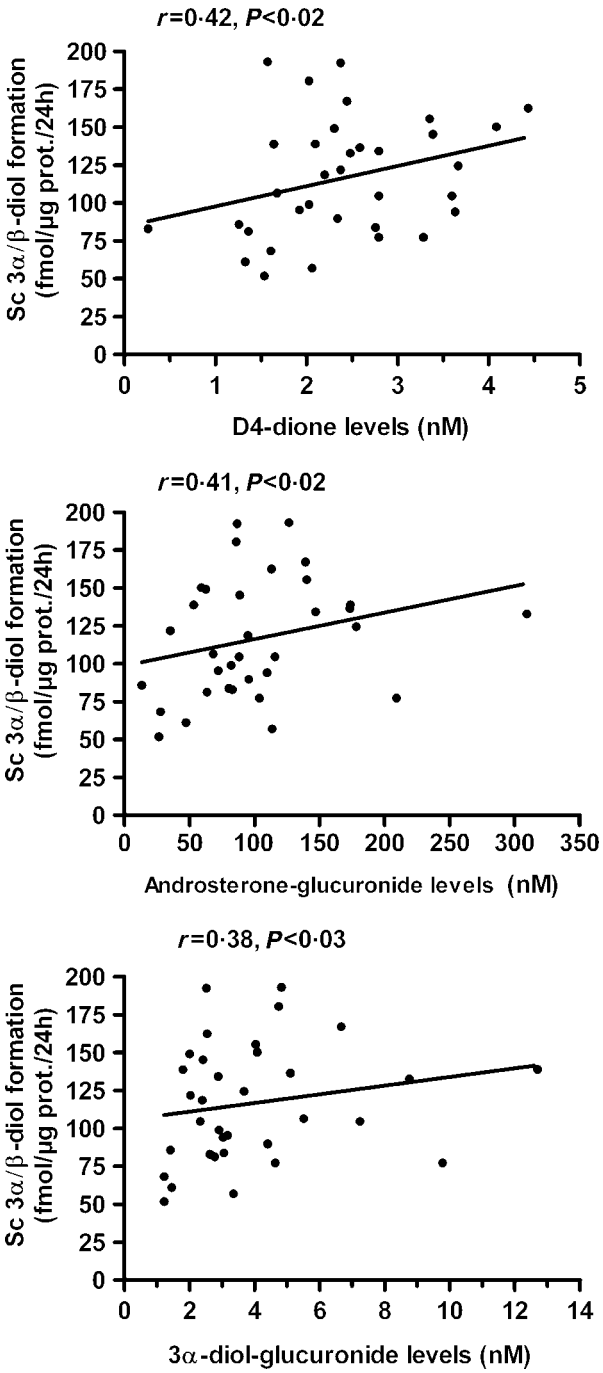

Figure 10 Correlations between 3α/β-HSD activities measured in s.c. adipose tissue homogenates and plasma measurements of D4-dione, androsterone-glucuronide and 3α-diol-glucuronide levels. Analysis performed on $\log_{10}$-transformed values for 3α/β-HSD activity.

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measures in both sexes, at least in the lean to moderately obese range. Mechanisms common to men and women may be involved in the regulation of androgen processing and inactivation in abdominal adipose tissue.

The study of 5α-DHT inactivation in adipose tissue is relevant only in the context of the global pathway of androgen metabolism and action in this tissue. Obviously, androgens and androgen precursors must be present in adipose tissue, which was shown by several groups (Feher & Bodrogi 1982, Deslypere *et al.* 1985, Szymczak *et al.* 1998) including ours (Bélanger *et al.* 2006). In the present study, the expression of several enzymes involved in androgen metabolism was detected in fat samples, including 5α-reductase-1, which is necessary for 5α-DHT formation. Incubation of preadipocytes with the adrenal precursor D4-dione led to the formation of testosterone, 5α-DHT, and androsterone (or epiandrosterone) as assessed by GC/LC–MS. The expression of the androgen receptor was also detected and mRNA levels were higher in s.c. versus Om adipose tissue. Together, these data support the relevance to study 5α-DHT inactivation in adipose tissue. Interestingly, the expression of P450 aromatase was relatively low and neither 17β-estradiol nor estrone was detected using GC–MS when D4-dione was incubated with Om preadipocytes for 24 h (not shown). Accordingly, a previous study has shown that real-time RT-PCR-measured mRNA levels of P450 aromatase in mature adipocytes were very low, and sometimes undetectable (Dieudonné *et al.* 2006). We measured P450 aromatase mRNA levels in whole adipose tissue samples of obese men, which were most likely enriched in mature adipocytes. Thus, our finding of only moderate aromatase expression in such tissue samples is not surprising. Killinger *et al.* (1990) also demonstrated that percent conversion of androstenedione to estrone ranged between 0·01 and 0·8% in abdominal s.c. adipose stromal cells and near 0·01% for Om preadipocytes. In the conditions of the present study, the most sensitive method used (GC–MS) would have allowed for the detection of approximately 0·25% estrone formation. The low rates of estrone formation in these cells (Killinger *et al.* 1990) could not have been detected using our method. Altogether, our results do not seem to be in disagreement with other previous studies regarding aromatization in adipose tissue.

The expression of the 3 AKR1C enzymes was particularly high compared with other enzymes measured in the present study, with marked depot differences. We also report that 3α/β-HSD activity originated mainly from the cytosolic fraction. Since AKR1C enzymes are known to be cytosolic enzymes, and AKR1C are highly expressed in fat samples, we can suggest that AKR1C, and especially AKR1C2, are actually involved in androgen inactivation in adipose tissue in men. To assess this issue, experiments were performed with specific AKR1C inhibitors in preadipocyte cultures. Incubation with a specific AKR1C2 inhibitor showed that this enzyme seems to be responsible for the generation of approximately 70% of the 3α-diol and 40–60% of the 3β-diol. When the cells were incubated with both AKR1C2 and AKR1C3 inhibitors, no significant additional inhibition was observed indicating that AKR1C1 could be responsible for most of the remaining 3α-HSD and 3β-HSD activities.

From the physiological standpoint, there is a growing interest for the study of local androgen synthesis or inactivation in adipose tissue. For example, Corbould *et al.* (2002) found that BMI and waist circumference were positively associated with the ratio of type 3 17β-HSD to aromatase mRNA measured in intra-abdominal adipose tissue, suggesting that androgen formation may be higher than its inactivation through aromatization in intra-abdominal adipose tissue of abdominally obese women (Corbould *et al.* 2002). More recently, Quinkler *et al.* (2004) studied several steroid-converting enzymes involved in adipose tissue local androgen metabolism during the differentiation of preadipocyte primary cultures. We hereby demonstrate that 5α-DHT inactivation is an important reaction that could also contribute to limited exposure of adipose cells to 5α-DHT in men through a pre-receptor regulatory mechanism. The finding that 5α-DHT is produced locally at relatively low rates, but is strongly and rapidly converted into other steroids supports this hypothesis.

Cross-sectional analyses cannot help in establishing the cause and effect relationships. However, a few possibilities can be raised to explain the higher Om 3α/β-HSD activity in obesity. Androgen effects on adipose tissue are postulated to be at least partly mediated through the androgen receptor, as male knock-out mice for this receptor develop late onset obesity (Sato *et al.* 2003). It is possible that increased androgen inactivation in the omentum of obese men through AKR1C enzymes represents a pathological condition underlying visceral obesity. According to this hypothesis, increased androgen inactivation in visceral adipose tissue could have led to decreased exposition to active androgens, which have been shown to inhibit adipose tissue LPL activity and to accelerate lipid turnover in men (Mårin *et al.* 1995). Androgens could also exert their effects directly through the control of adipogenesis. Accordingly, androgens were found to inhibit adipogenesis in C3H 10T1/2 mouse pluripotent cells as well as in the mouse preadipocyte cell line 3T3-L1 (Singh *et al.* 2003, 2006). This hypothesis is also consistent with higher AKR1C enzyme expression and 3α/β-HSD activity in s.c. adipose tissue, since this depot is generally larger than the Om/visceral fat depot. Consistent with this hypothesis and the higher s.c. versus Om androgen inactivation rates, we also found that adipose tissue 5α-DHT concentrations are higher in the Om than in the s.c. depot (Bélanger *et al.* 2006).

On the other hand, the regional depot difference in androgen inactivation rates and androgen inactivating enzymes could be a consequence of obesity, or other characteristics of the adipose tissue compartments examined. We found significantly higher 5α-DHT inactivation rates in mature adipocytes compared with preadipocytes in s.c. adipose tissue. Thus, depot or obesity-related differences in stromal cell, preadipocyte, or mature adipocyte content could

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explain the regional difference observed in androgen inactivation. However, the question of preadipocyte number in various fat depots and in obesity is not completely resolved, a possible reason being that the assessment of preadipocyte number is considered labor intensive and error prone (Bakker *et al.* 2004). No significant depot difference was found in one study, whereas an examination of breast adipose tissue indicated that BMI was not correlated to the ratio of stromal cells/mature adipocytes (van Harmelen *et al.* 2003), suggesting that the degree of obesity does not influence cell population proportions at least in that depot. Further studies are required to establish whether 3α/β-HSD activity and androgen inactivation in adipose tissue is a consequence of obesity-related characteristics of adipose tissue such as differences in cell populations or is causally related to abdominal obesity.

We have shown that appreciable amounts of both 3α-diol and 3β-diol were produced when preadipocytes were incubated with 5α-DHT. This finding could be of physiological importance because 3β-diol is known to stimulate the estrogen receptor β-1 (ERβ-1) (Pak *et al.* 2005). Pedersen *et al.* have found previously that ERβ-1 mRNA and protein were expressed in abdominal s.c. and intra-abdominal adipose tissue in men and women with significantly higher levels found in s.c. adipose tissue in both sexes (Pedersen *et al.* 2001). Interestingly, we found that the expression of 3(α→β)-hydroxysteroid epimerase was low, but was dramatically different between s.c. and Om adipose tissue. Further investigations are needed to evaluate the relative importance of active androgens and their metabolites, including 3β-diol on adipose cell metabolism and differentiation.

The higher 5α-DHT inactivation found in mature adipocytes also suggests that 3α/β-HSD activity and local 5α-DHT concentration could be involved in the modulation of preadipocyte differentiation, an increasing 5α-DHT inactivation during differentiation preventing exposure to 5α-DHT in mature adiocytes. This hypothesis is supported by the observed *in vitro* inhibition of mouse pluripotent cell and 3T3-L1 adipogenesis by 5α-DHT (Singh *et al.* 2003, 2006). However, caution is needed when interpreting our comparison of preadipocytes and mature adipocytes because it is based on a protein normalization. DNA normalization may have been preferable because it may be more representative of the number of cells examined. Moreover, differences in the lipid content between cell types and the added lipid extraction step in mature adipocytes may have interfered with the determination of 3α/β-HSD activity and/or total protein content. These results need further validation in differentiating adipocytes.

We observed no association between plasma steroid levels and 3α/β-HSD activity measurements with the exception of the 3α-reduced androgen metabolites androsterone-glucuronide and 3α-diol-glucuronide, and with D4-dione, which were positively associated with s.c. 3α/β-HSD activity. Om 3α/β-HSD activity was not related to circulating androgen metabolite levels. These data suggest that steroidogenesis taking place in s.c. adipose tissue may contribute to circulating steroid concentrations due to a mass action effect, whereas Om local steroid conversion may have little or no impact on circulating hormone levels given the relatively small size of this compartment. Quinkler *et al.* (2004) also suggested that s.c. adipose tissue may contribute significantly to systemic androgen production based on their observation that s.c. adipose tissue predominantly activated androgens, whereas Om adipose tissue predominantly inactivated androgens. However, other studies have shown that local steroid metabolism in adipose tissue may not necessarily reflect circulating steroid levels due to the fact that other tissues, including the liver, also express steroidogenic and/or steroid-inactivating enzymes (Dufort *et al.* 2001, Rask *et al.* 2002, Blouin *et al.* 2005).

In conclusion, 5α-DHT inactivation was detected in abdominal adipose tissue in men, and higher Om adipose tissue 3α/β-HSD activity was found in obese men. The expression of several enzymes involved in local androgen metabolism was also detected, with AKR1C1, AKR1C2, and AKR1C3 having especially high expression levels and large depot-differences compared with other enzymes measured. Higher 5α-DHT inactivation was found in mature adipocytes compared with preadipocytes, and 5α-DHT inactivation rates were higher in Om fat from obese men. Further studies are required to elucidate whether increased local androgen inactivation in Om adipose tissue is directly involved in the modulation of adipocyte metabolism and regional fat distribution in men.

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