

Androgen generation in adipose tissue in women with simple obesity – a site-specific role for 17 β -hydroxysteroid dehydrogenase type 5

Marcus Quinkler, Binayak Sinha, Jeremy W Tomlinson, Iwona J Bujalska, Paul M Stewart and Wiebke Arlt

Division of Medical Sciences, University of Birmingham, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom
(Requests for offprints should be addressed to P M Stewart; Email: p.m.stewart@bham.ac.uk)

Abstract

Women with polycystic ovary syndrome (PCOS) have high circulating androgens, thought to originate from ovaries and adrenals, and frequently suffer from the metabolic syndrome including obesity. However, serum androgens are positively associated with body mass index (BMI) not only in PCOS, but also in simple obesity, suggesting androgen synthesis within adipose tissue. Thus we investigated androgen generation in human adipose tissue, including expression of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) isozymes, important regulators of sex steroid metabolism. Paired omental and subcutaneous fat biopsies were obtained from 27 healthy women undergoing elective abdominal surgery (age range 30–50 years; BMI 19.7–39.2 kg/m²). Enzymatic activity assays in preadipocyte proliferation cultures revealed efficient conversion of androstenedione to testosterone in both subcutaneous and omental fat. RT-PCR of whole fat and preadipocytes of subcutaneous and omental origin showed expression of 17 β -HSD types 4 and 5, but no relevant expression of 17 β -HSD types 1, 2, or 3. Microarray analysis confirmed this expression pattern (17 β -HSD5>17 β -HSD4) and suggested a higher expression of 17 β -HSD5 in subcutaneous fat. Accordingly,

quantitative real-time RT-PCR showed significantly higher expression of 17 β -HSD5 in subcutaneous compared with omental fat ($P<0.05$). 17 β -HSD5 expression in subcutaneous, but not omental, whole fat correlated significantly with BMI ($r=0.51$, $P<0.05$). In keeping with these findings, 17 β -HSD5 expression in subcutaneous fat biopsies from six women taking part in a weight loss study decreased significantly with weight loss ($P<0.05$). A role for 17 β -HSD5 in adipocyte differentiation was further supported by the observed increase in 17 β -HSD5 expression upon differentiation of stromal preadipocytes to mature adipocytes ($n=5$; $P<0.005$), which again was higher in cells of subcutaneous origin. Functional activity of 17 β -HSD5 also significantly increased with differentiation, revealing a net gain in androgen activation (androstenedione to testosterone) in subcutaneous cultures, contrasting with a net gain in androgen inactivation (testosterone to androstenedione) in omental cultures. Thus, human adipose tissue is capable of active androgen synthesis catalysed by 17 β -HSD5, and increased expression in obesity may contribute to circulating androgen excess.

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Introduction

Hyperandrogenaemia is the principal biochemical finding in the polycystic ovary syndrome (PCOS) (Dunaif & Thomas 2001). In addition, women with PCOS frequently present with obesity and have an increased risk of developing the metabolic syndrome including type 2 diabetes mellitus and hypertension (Dunaif & Thomas 2001, Elting *et al.* 2001). High circulating androgens in PCOS have been thought to originate from increased androgen generation in ovaries and adrenals (Franks 1995). However, there is evidence for a significant contribution of peripheral steroidogenesis to androgen synthesis

and activation in PCOS (Stewart *et al.* 1990, Fassnacht *et al.* 2003). Interestingly, serum androgens are positively associated with body mass index (BMI) not only in PCOS, but also in women with simple obesity (Taponen *et al.* 2003). This, together with a fall in luteinising hormone levels with increasing BMI (Holte *et al.* 1994), suggests that androgen synthesis may take place not only in adrenals and ovaries but also in adipose tissue.

Recent research focused on sex steroid conversion in human adipose tissue, specifically investigating the expression and activity of 17 β -hydroxysteroid dehydrogenases (17 β -HSDs), which represent a major switch regulating sex steroid activation and inactivation at the pre-receptor

level (Adamski & Jakob 2001, Luu-The 2001). By employing RNA protection assays and Southern blot, Corbould *et al.* (1998) detected low mRNA expression levels of 17 β -HSD isozymes 2 and 3, but not of 17 β -HSD1, in human adipose tissue. In another study, Corbould *et al.* (2002) found that preadipocytes readily converted androstenedione to testosterone and that the ratio of 17 β -HSD3 to aromatase expression in omental adipose tissue was positively correlated with BMI. However, in recent years 17 β -HSD5 (AKR1C3) has been described as a major player in androgen interconversion (Dufort *et al.* 1999, Penning *et al.* 2000, Luu-The *et al.* 2001). Recently, Blouin *et al.* (2003) described mRNA expression of 17 β -HSD5 (AKR1C3) and 3 α -HSD type 3 (AKR1C2) in human female adipose tissue. They reported an increased inactivation of 5 α -dihydrotestosterone by AKR1C2 in omental fat tissue from women with visceral obesity and suggested that local androgen inactivation is a predominant reaction in female abdominal tissue.

Here, we studied androgen generation in human female adipose tissue, particularly focusing on the site-specific expression of 17 β -HSD5 (AKR1C3) in omental and subcutaneous fat, and investigated the impact of changes in BMI. In addition, we explored 17 β -HSD5 expression and androgen conversion across adipocyte differentiation.

Subjects and Methods

Subjects

Paired subcutaneous and omental biopsies Paired subcutaneous (sc) and omental (om) adipose tissue biopsies were obtained from 27 female patients undergoing elective abdominal hysterectomy for myoma or fibroma (median age 43 years, range 30–50 years; median BMI 27.8 kg/m², range 19.7–39.2 kg/m²). All were premenopausal, non-diabetic, and none of them had been treated with glucocorticoids within the preceding twelve months or had been on any hormonal treatment, e.g. contraceptives. In 24 of the 27 subjects sufficient material for both activity and expression studies was obtained, while biopsy material in the remaining three subjects was only sufficient for expression analysis.

Subcutaneous biopsies (weight loss study) Six healthy women (median age 45 years, range 23–57 years; median BMI 35.2 kg/m², range 30.4–38.3 kg/m²) with no significant past medical history were recruited for a weight loss study as previously reported (Tomlinson *et al.* 2004). A sc buttock biopsy was performed under local anaesthetic to obtain 1–2 g adipose tissue. After a median duration of 10 weeks of a weight loss program with a very low calorie diet (425 kcal/day) and a weight loss of more than 10% of the initial body weight, the patients returned to a normal

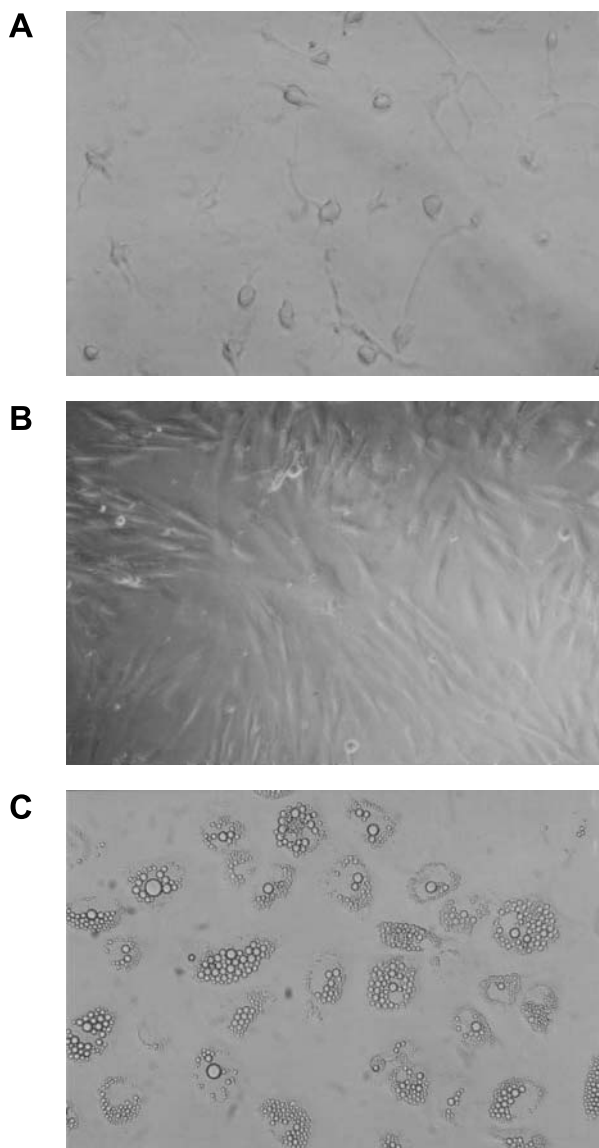


Figure 1 Human primary preadipocyte cell cultures. (A) Stromal preadipocytes on day 1, (B) proliferation cultures with confluent preadipocytes on day 7, (C) differentiation cultures with preadipocytes fully differentiated into mature adipocytes (day 14). Magnification $\times 200$.

diet for at least 1 week before they received a second sc buttock biopsy (Tomlinson *et al.* 2004).

All studies had the approval of the local research ethics committee and written informed consent was obtained in every case.

Tissue and primary cell culture

Samples of whole sc and om fat tissue were used for RNA extraction and for the isolation of preadipocytes as previously reported (Bujalska *et al.* 1997). Briefly, adipose

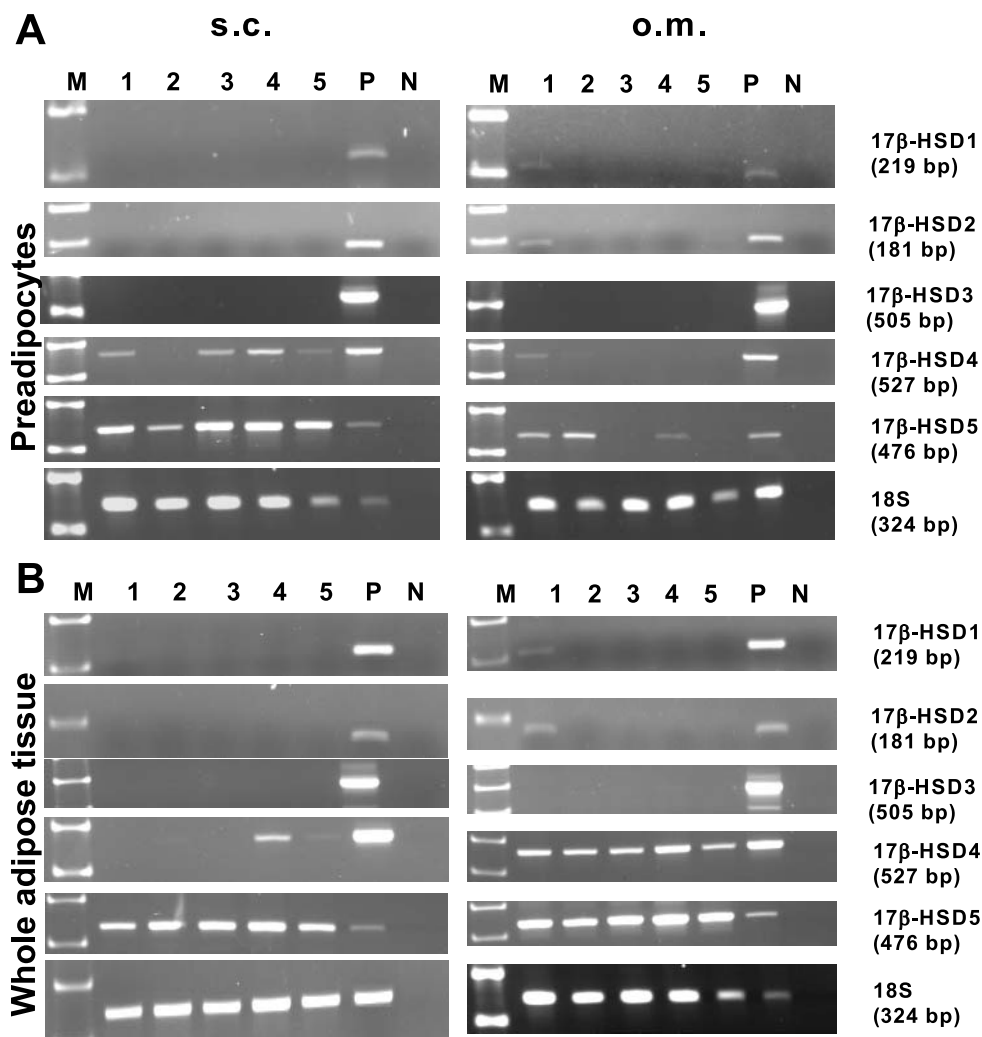


Figure 2 PCR analysis of 17 β -HSD isozyme expression in subcutaneous (sc) and omental (om) preadipocytes (A) and whole fat tissue (B) RNA extracted from five women undergoing paired sc and om biopsies (1–5). M, ladder; P, positive control (human testis for 17 β -HSD isoforms 1, 3 and 4; human liver for 17 β -HSDs 2 and 5); N, negative control; 18S, 18S ribosomal RNA as internal loading control.

tissue biopsies were washed in PBS, then chopped and digested with collagenase class 1 (2 mg/ml; Worthington Biochemical Corp., Reading, Berks, UK) in 1 \times Hank's balanced salt solution (Life Technologies Inc., Paisley, Strathclyde, UK) for 60 min at 37 $^{\circ}$ C in a shaking water bath. Samples were centrifuged at 90 \times g for 1 min, the top adipocyte layer was removed and then centrifuged again at 90 \times g for 5 min. The pellet containing preadipocytes was removed, and cells were washed with DMEM/Nutrient Mixture F-12 (Life Technologies Inc.) containing 15% fetal calf serum (Life Technologies Inc.), penicillin (50 000 U/l) and streptomycin (50 000 μ g/l), and seeded in 24-well plates. Cells were left overnight and washed the following day with 1 \times Hank's balanced salt solution (Fig. 1A). Subsequently, preadipocyte

cultures were either cultured towards confluence to study proliferated preadipocytes or subjected to differentiation stimuli to study the effects of maturation of stromal preadipocytes to adipocytes.

For preadipocyte proliferation cultures, human preadipocytes were maintained in DMEM/F-12 medium containing 15% fetal calf serum until confluence, which was achieved after 5 and 7 days of cell culture (Fig. 1B). Preadipocytes were then used for enzyme assays and RNA extraction.

For preadipocyte differentiation assays, human subcutaneous and omental preadipocytes were cultured for 14 days in media known to promote adipocyte differentiation (Hauner *et al.* 1987) following an established protocol (Bujalska *et al.* 1999) (Fig. 1C). On days 1 and 14,

Table 1 Expression of 17 β -HSD isozymes according to cDNA arrays with RNA extracted from preadipocytes and whole fat tissue of both subcutaneous (sc) and omental (om) origin. For each cell compartment the corresponding specific RNAs of 5 women were pooled

Enzyme (Gene)	Subcutaneous		Omental		Fold change sc vs om
	Detection	Signal	Detection	Signal	
Preadipocytes					
17 β -HSD1 (HSD17B1)	A	—	A	—	—
17 β -HSD2 (HSD17B2)	A	—	A	—	—
17 β -HSD3 (HSD17B3)	A	—	A	—	—
17 β -HSD4 (HSD17B4)	P	263	P	222	—
17 β -HSD5 (AKR1C3)	P	511	P	73	6.5
Whole fat tissue					
17 β -HSD1 (HSD17B1)	A	—	A	—	—
17 β -HSD2 (HSD17B2)	A	—	A	—	—
17 β -HSD3 (HSD17B3)	A	—	A	—	—
17 β -HSD4 (HSD17B4)	P	330	P	303	—
17 β -HSD5 (AKR1C3)	P	1525	P	428	3.4

Detection: qualitative measure indicating if the transcript is reliably detected (present, P), not detected (absent, A). Signal: quantitative measure of the relative abundance of a transcript. Fold change: change in expression level for a transcript between sc and om.

total RNA was extracted and differentiation of adipose stromal cells to adipocytes was confirmed by analysing lipoprotein lipase and glycerol-3-phosphate dehydrogenase expression, as described previously (Bujalska *et al.* 2002b). In addition, sc and om preadipocyte cultures were used for enzymatic activity assays on day 1 and day 14 of differentiation.

Enzyme assays

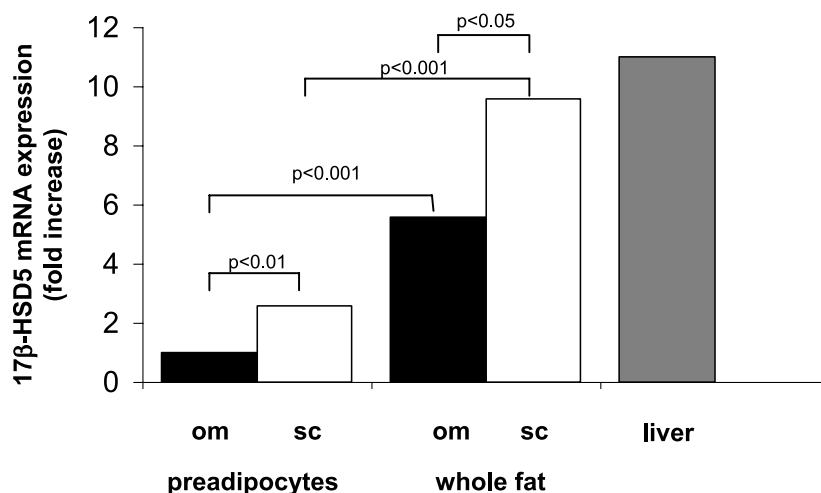
Paired sc and om preadipocyte proliferation cultures from 19 women were incubated in serum-free DMEM without phenol for 20 h with 40 nM androstenedione containing 100 000 c.p.m. [1,2,6,7-³H] androstenedione (specific activity 25 Ci/mmol; Perkin-Elmer, Cambridge, Cams, UK).

Paired sc and om preadipocyte differentiation cultures from 5 women were incubated on day 1 and day 14 in serum-free DMEM without phenol for 20 h with 40 nM androstenedione containing 100 000 c.p.m. [1,2,6,7-³H] androstenedione or with 40 nM testosterone containing 100 000 c.p.m. [1,2,6,7-³H] testosterone (specific activity 78.5 Ci/mmol; Perkin-Elmer).

Steroids were extracted in 5 vols dichloromethane, concentrated by evaporation, and separated by thin layer chromatography (TLC) on silica gel/aluminium foil-coated TLC plates (Fluka Chemika, Buchs, Switzerland) with chloroform/ethylacetate (80:20) as the solvent system. Fractional conversion of steroids was quantified using a Bioscan 2000 Imager (LabLogic, Sheffield, UK). Steroids were identified by referring to co-migration of unlabelled steroid standards (Sigma, UK) visualised by Liebermann-Burchard reagent as previously described (Quinkler *et al.* 1999). Cell protein content was determined with a modified Bradford method (Bio-Rad Laboratories, Munich, Germany), and enzyme activities were expressed as pmol or fmol product formed per mg protein per h. All incubations were carried out at least in triplicate.

RNA extraction and RT

Total RNA was extracted from whole fat tissue and preadipocyte cultures using a single step extraction method (Tri reagent, Sigma, UK (preadipocytes), or Genelute total mammalian RNA extraction kit, Sigma, UK (whole adipose tissue)). RNA integrity was assessed by gel



Sample Type	Mean dCT	± SD
om preadipocytes	13.95	1.09
sc preadipocytes	12.59	1.31
om whole fat	11.47	1.02
sc whole fat	10.69	1.40
liver	10.46	0.20

Figure 3 Quantitative mRNA expression of 17 β -HSD5 according to real-time PCR in omental (om) and subcutaneous (sc) preadipocytes and whole fat of 19 women, and in liver (3 women). Expression in om preadipocytes was set at 1 and other expressions were compared with it as fold increase. Mean Δ CT (dCT) is shown beneath the graphs.

electrophoresis and concentrations were measured by spectrophotometry at A_{260} and checked for purity using the ratio $A_{260/280}$. Reverse transcription of RNA was performed as previously described (Bujalska *et al.* 1999) employing reagents from Promega (Southampton, UK).

PCR for 17 β -HSD isozymes

Qualitative PCR analysis for expression of 17 β -HSD isozymes was carried out using cDNA generated from paired sc and om fat samples of 5 women, employing the following primers: 17 β -HSD1 (HSD17B1), forward: 5'-TTTCAATGACGTTTATTGCGCCAGCA-3', reverse: 5'-TTGCTGTGGGCGAGGTATTGGTAGAA-3'; 17 β -HSD2 (HSD17B2), forward: 5'-CTGGTGACAGGTGGTGA TTG-3', reverse: 5'-TTATCTGCACTGGCTTCGTG-3'; 17 β -HSD3 (HSD17B3), forward: 5'-TCCTGAACGCA CCGGATGAAAT-3', reverse: 5'-TACCTGACCTTG GTGTTGAGCTTCAG-3'; 17 β -HSD4 (HSD17B4), forward: 5'-AGTTCTCTCTCTTCTTGTGGCTC TGGA-3', reverse: 5'-GCGTCCTATTTCTCAA TACAAAGGTACTCT-3'; 17 β -HSD5 (AKR1C3), forward: 5'-ACTTCATGCCTGTATTGGGATTTG-3', reverse: 5'-CTGCCTGCGGTTGAAGTTTGACA-3'. 17 β -HSD5 primers differ in two bases (forward primer) and three bases (reverse primer) respectively, from the AKR1C2 sequence (see underlined bases above) and are specific for 17 β -HSD5 (AKR1C3) as previously shown by Bumke-Vogt *et al.* (2002). In addition to the primer sequences for 17 β -HSD isozymes 1, 2, and 3 given above,

we also performed specific PCR reactions with the 17 β -HSD primers used by Corbould *et al.* (1998). Specific PCR reactions (20 μ l) were carried out in 1 \times PCR buffer (Promega) containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100, 2 mM MgCl₂, 0.2 μ M of each dNTP, 0.6 μ M of forward and reverse primer and 1 U Taq DNA polymerase (Promega). Samples were amplified using initial denaturation at 94 $^{\circ}$ C (5 min) followed by 34 cycles at 94 $^{\circ}$ C (30 s), 60 $^{\circ}$ C (30 s), and 72 $^{\circ}$ C (30 s) with final elongation at 72 $^{\circ}$ C (7 min). After gel electrophoresis, PCR products of reactions with 17 β -HSD5 primers were excised under UV light, extracted with QIAquick Gel Extraction Kit (Qiagen) and subjected to direct sequencing.

Microarray

Total RNA was extracted from paired sc and om whole fat tissue as well as from preadipocyte proliferation cultures from five women. Biotin-labelled cRNA was reverse transcribed from 10 μ g total RNA (pool of 2 μ g total RNA from each cell preparation) according to the Affymetrix technical protocol (GeneChip Expression Analysis, Technical Manual, <http://www.affymetrix.com/support/index.affx>). Integrity of the samples was tested on Affymetrix Test3 array and samples were accepted if their 3'/5' ratio of human housekeeping genes was lower than 3, referring to the house-keeping genes beta-actin (ACTB, Affymetrix probe HSAC07/X00351) and glyceraldehyde-3-phosphate dehydrogenase (GAPD,

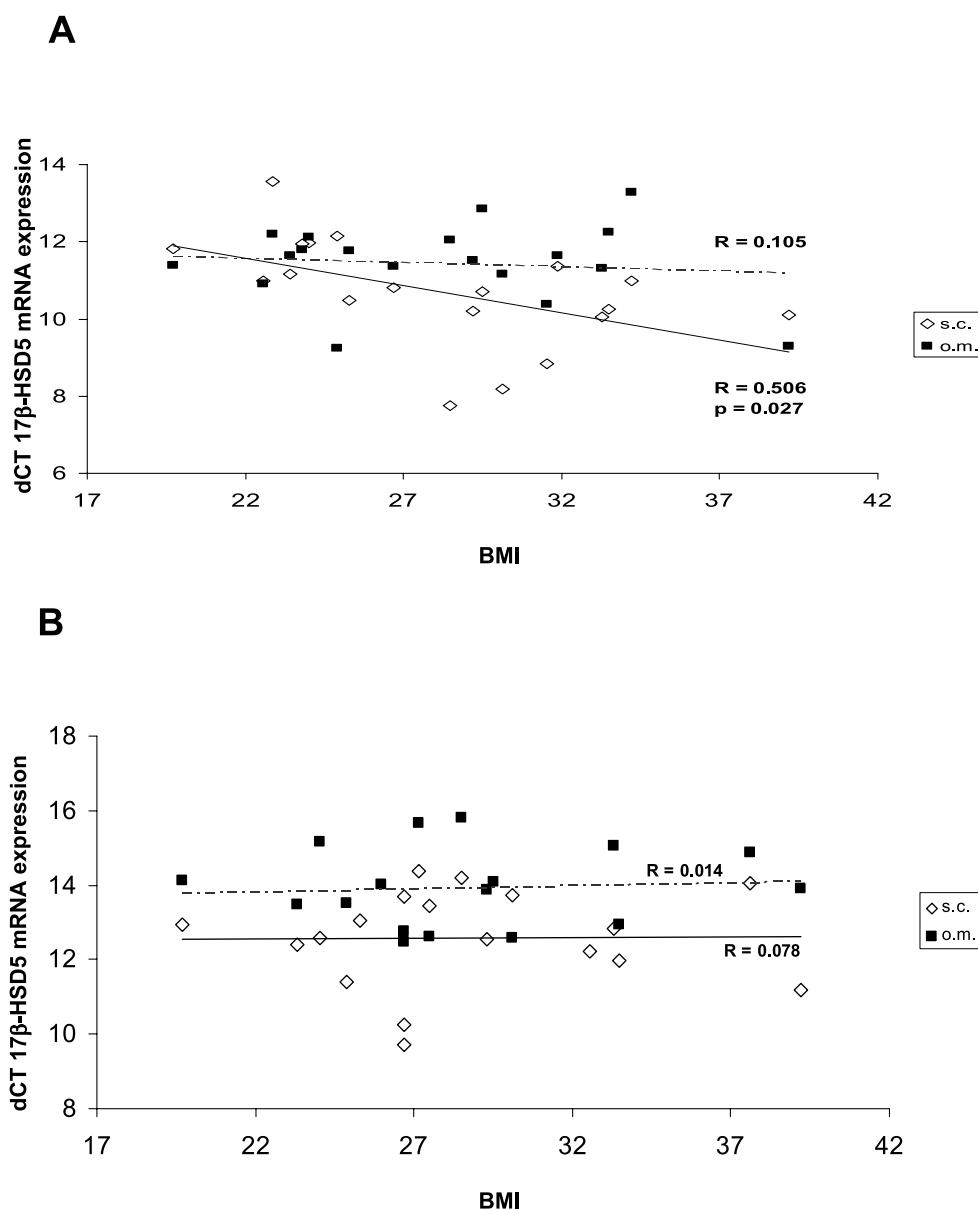


Figure 4 Quantitative mRNA expression of 17β-HSD5 according to real-time PCR reported as Δ CT (dCT) values (high values represent low values of expression) in correlation to body mass index (BMI) in 19 women. (A) Expression in omental (om, ■) and subcutaneous (sc, ◇) whole fat. (B) Expression in om (■) and sc (◇) preadipocytes.

Affymetrix probe HUMGAPDH/M33197) were accepted. Fragmented cRNA (15 µg) was hybridised to each of the array of the Human Genome U133 set (Affymetrix, High Wycombe, UK). Affymetrix software (Data Mining Tool, Version 3.0) and Microarray Data Suite 5.0 (Affymetrix) were used for analysing microarray data. Accordingly, results are presented as qualitative (=detection) and quantitative (=signal) measures of expression level, which represent arbitrary data. The inter-assay variability be-

tween microarrays was below 5%. The probe set for 17β-HSD5 (209160_at) on the HG U133A chip includes 11 probes, all matching the published sequence. Due to the sequence difference between 17β-HSD5 and other 17β-HSD enzymes, none of the probes coding for 17β-HSD5 match the probe sets of 17β-HSD isozymes 1, 2, 3 or 4. The enzymes AKR1C1 (probe set 204151_x_at) and AKR1C2 (probe set 209699_x_at) have a high homology to 17β-HSD5 (AKR1C3); however only 2 and 3 probes,

respectively, out of 11 probes matched the 17 β -HSD5 sequence.

Quantitative PCR

17 β -HSD5 mRNA expression was analysed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK) that employs TaqMan chemistry for highly accurate mRNA quantification as previously described (Bujalska *et al.* 2002a). Reactions were performed in 25- μ l vol on 96-well plates, in buffer containing TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 25 ng cDNA template. All reactions were multiplexed with the housekeeping gene 18S (Perkin-Elmer). Reactions were as follows: 50 °C for 2 min, 95 °C for 10 min, and then 44 cycles at 95 °C for 15 s and 60 °C for 1 min. Oligonucleotide primers and a Taqman probe for 17 β -HSD5 were as follows: forward, GGGATCTCAACGAGACAAACG; reverse, AAAGGACTGGGTCCTCCAAGA; probe, TGG ACCCGAACTCCCCGGTG. Data were expressed as CT values (=cycle number at which logarithmic PCR plots cross a calculated threshold line) and were used to determine Δ CT values (=CT of the target gene minus CT of the housekeeping gene; high Δ CT values represent low levels of expression). Fold changes were calculated using transformation [fold increase = $2^{-\text{difference in } \Delta\text{CT}}$].

Statistical analysis

Data are expressed as means \pm S.D or S.E.M., as specified. Statistical analysis on real-time PCR data was performed on mean Δ CT values to exclude potential bias owing to averaging data that had been transformed through the equation $2^{-\Delta\text{CT}}$. Statistical analysis of comparisons between groups was undertaken using paired and unpaired *t*-tests where appropriate, otherwise the Mann-Whitney Rank Sum Test was used. Linear regression analysis was performed using Pearson's correlation coefficient.

Results

PCR for 17 β -HSD isozymes

The conversion of androstenedione to testosterone and vice versa requires the presence of reductive and oxidative 17 β -HSD activity. Thus, to identify the enzymes responsible for testosterone synthesis and inactivation in adipose tissue, we performed RT-PCR analysis employing specific primers for the amplification of human 17 β -HSD isozymes. When analysing preadipocyte and whole adipose mRNA obtained from sc and om depots of five individual patients, abundant expression of 17 β -HSD4 and 17 β -HSD5 mRNA was detected (Fig. 2). The data suggest that 17 β -HSD4 expression is higher in om than

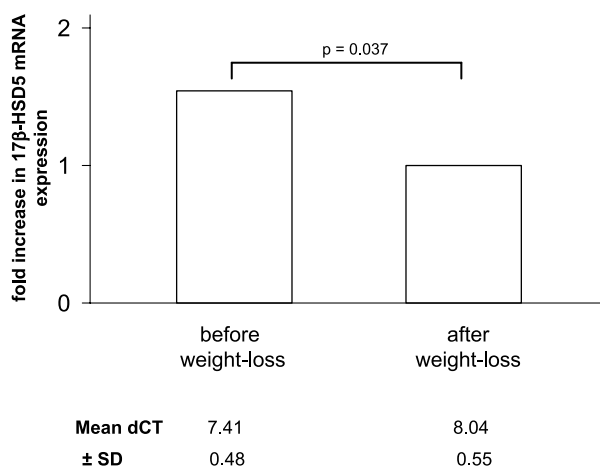


Figure 5 Quantitative mRNA expression of 17 β -HSD5 according to real-time PCR in subcutaneous whole fat buttock biopsies from six women before and after weight loss, who underwent a weight loss study (>10% of initial body weight). Mean Δ CT (dCT) is shown beneath the graphs.

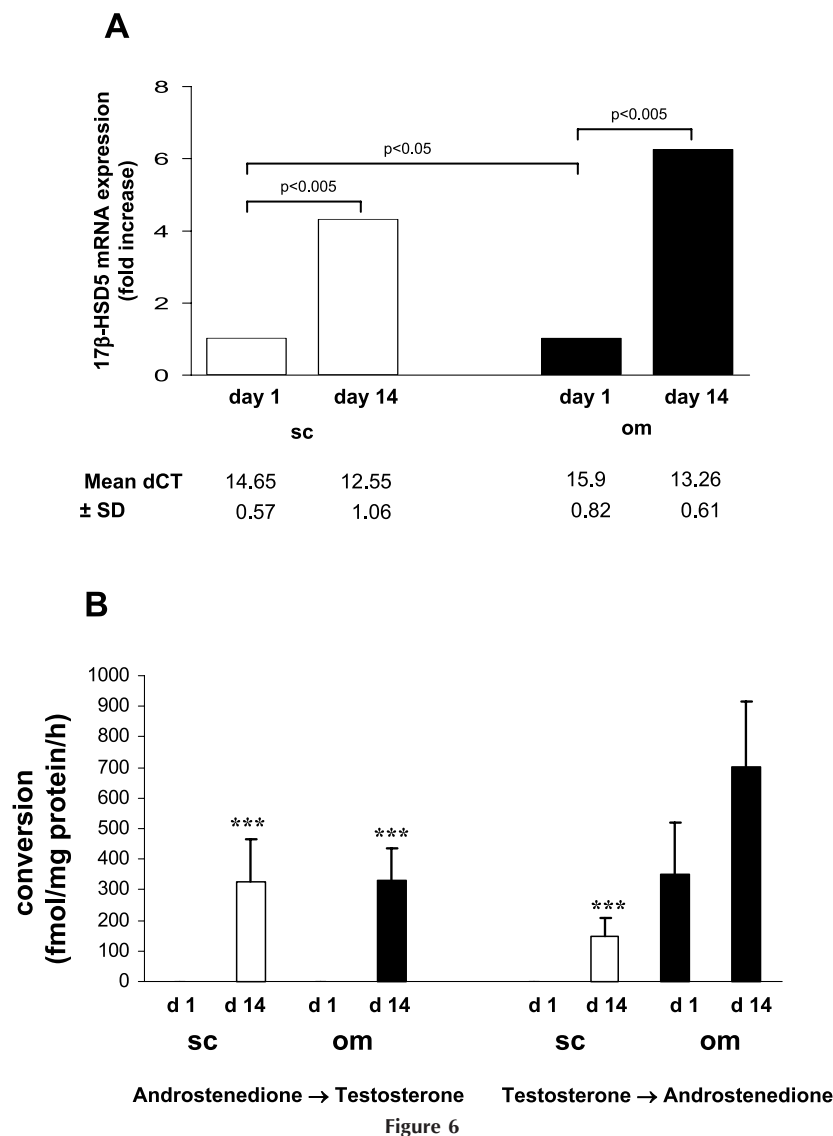
sc whole adipose tissue. 17 β -HSD5 expression appears to be weakest in om preadipocytes. In whole fat and preadipocytes of both sc and om origin no relevant expression of 17 β -HSD1, 2 or 3 was detected, except for a weak band for 17 β -HSD1 and 2 in omental tissue of one subject (Fig. 2). Using the primers and methods reported by Corbould *et al.* (1998) we were not able to detect mRNA expression of 17 β -HSD isozymes 1, 2 or 3, either in whole fat or in preadipocytes, despite a strong band in the positive control lane (data not shown).

Microarray

Microarray analysis confirmed the absence of 17 β -HSD1, 2 and 3 and the presence of 17 β -HSD4 and 5 in preadipocytes and whole fat tissue of sc and om origin (Table 1). 17 β -HSD5 microarray expression signals were higher in sc than in om fat (511 vs 73 in preadipocytes, $P < 0.001$; 1525 vs 428 in whole fat, $P < 0.001$), with an apparently higher abundance in whole fat than in preadipocytes of sc origin. By contrast, 17 β -HSD4 expression signals did not differ significantly between sc and om fat, either in preadipocytes (263 vs 222) or in whole fat tissue (330 vs 303). The expression levels of AKR1C1 and AKR1C2 were very high (2800 and 2200 respectively) and exceeded those of 17 β -HSD5 (AKR1C3) in whole fat tissue with no significant difference between sc or om tissue.

Enzyme assays in preadipocyte proliferation cultures

Enzyme activity studies in primary preadipocyte proliferation cultures from 19 patients revealed significant androgen generation, as assessed by the conversion of androstenedione to testosterone, with no apparent



difference between sc and om preadipocyte cultures (0.83 ± 0.24 vs 0.86 ± 0.23 pmol/mg/h).

Quantitative PCR for site-specific expression of 17 β -HSD5

For validation of the results on 17 β -HSD5 expression in fat achieved by microarray analysis, we performed quantitative real-time PCR on cDNA generated from paired sc and om fat biopsies of 19 women. In keeping with the microarray data, 17 β -HSD5 mRNA expression levels were significantly higher in sc than in om fat, both in preadipocytes ($P < 0.01$) and in whole fat ($P = 0.05$) (Fig. 3). However, in both sc and om depots, 17 β -HSD5 mRNA expression was significantly higher in whole fat tissue than in preadipocytes ($P < 0.001$) (Fig. 3). This

suggests a much higher abundance of 17 β -HSD5 expression in mature adipocytes than in preadipocytes.

A potential site-specific role for 17 β -HSD5, in the light of higher abundance of expression in sc than in om fat, was also supported by correlation of real-time 17 β -HSD5 mRNA expression with BMI in the 19 patients studied (Fig. 4A and B). 17 β -HSD5 mRNA expression in whole adipose tissue was significantly correlated with BMI in the sc compartment ($r = 0.51$, $P = 0.027$) but not in the om depot ($r = 0.11$) (Fig. 4A). By contrast, expression of 17 β -HSD5 in preadipocytes, which was considerably lower than in whole fat tissue, did not correlate with BMI, either in cells of om ($r = 0.078$) or sc ($r = 0.014$) origin (Fig 4B). There was no significant correlation between 17 β -HSD5 mRNA expression and age in whole fat or preadipocytes.

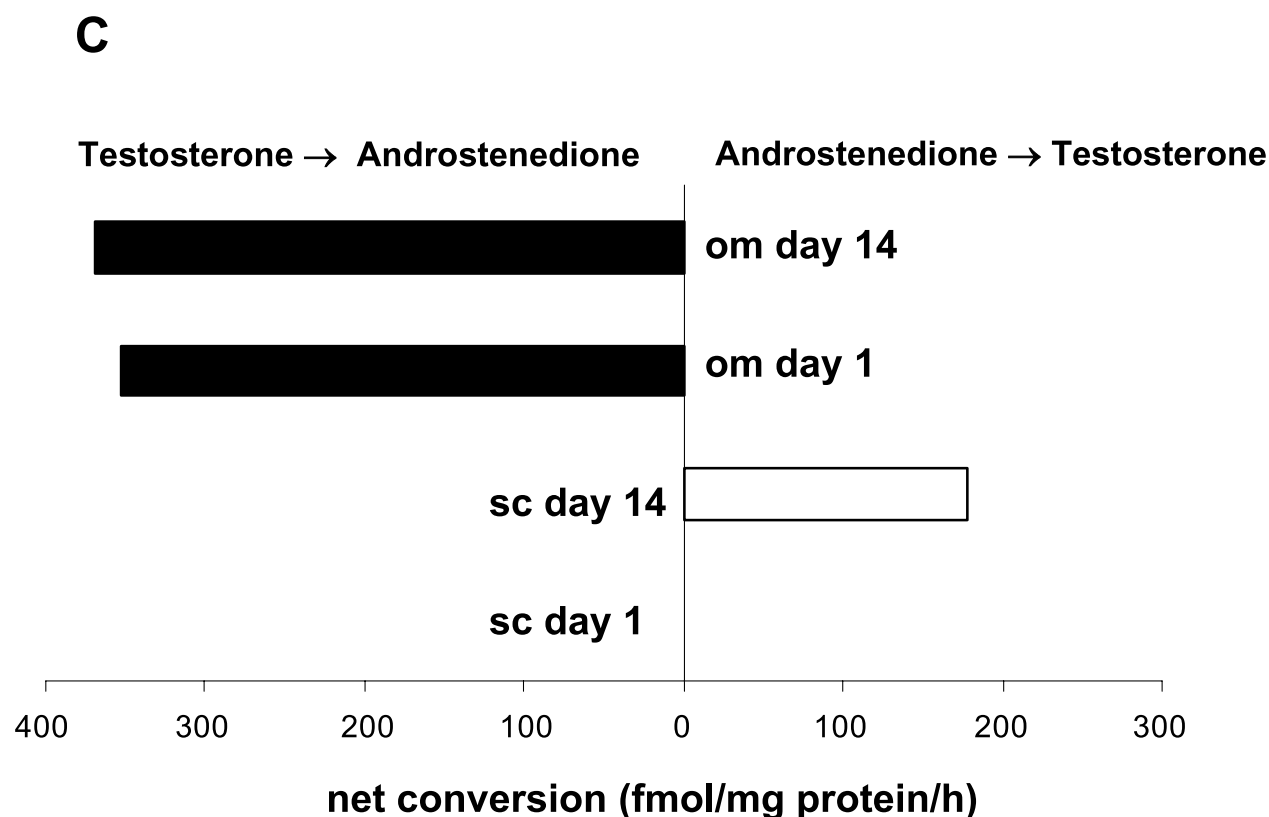


Figure 6 Continued.

Figure 6 Androgen conversion and quantitative mRNA expression of 17 β -HSD5 across the differentiation of preadipocytes. (A) Quantitative mRNA expression of 17 β -HSD5 according to real-time PCR in omental (om) and subcutaneous (sc) preadipocytes before (day 1) and after (day 14) differentiation to adipocytes, reported as Δ CT (dCT) values (high values represent low values of expression) ($n=8$). (B) Conversion (fmol/mg protein/h) of androstenedione to testosterone and testosterone to androstenedione in om and sc preadipocytes before (day 1, d1) and after (day 14, d14) differentiation to adipocytes ($n=5$). Results are expressed as means \pm S.E.M. *** $P<0.001$ comparing day 14 to day 1. (C) Net conversion (fmol/mg protein/h) of testosterone to androstenedione and vice versa in om and sc preadipocytes before (day 1) and after (day 14) differentiation to adipocytes.

Quantitative PCR for 17 β -HSD5 expression in weight loss study

We performed quantitative real-time PCR on cDNA generated from subcutaneous buttock biopsies of six women who underwent a weight loss study. The mRNA expression of 17 β -HSD5 was significantly reduced ($P<0.05$) in the biopsies taken after the weight loss compared with those taken before the weight loss (Fig. 5).

Quantitative PCR for 17 β -HSD5 expression across differentiation

To further explore the expression of 17 β -HSD5 across differentiation, primary preadipocyte cultures from sc and om fat of eight individuals were differentiated to mature adipocytes in adipogenic medium. 17 β -HSD5 mRNA levels were assessed by real-time RT-PCR and again were found to be significantly higher ($P<0.05$) in sc than in om

preadipocytes on day 1. Upon differentiation to adipocytes (day 1 compared with day 14), 17 β -HSD5 mRNA levels in both om and sc cultures increased significantly ($P<0.005$) (Fig. 6A).

Enzyme assays in preadipocyte differentiation cultures

We studied the conversion of androstenedione to testosterone and vice versa across differentiation using primary preadipocyte differentiation cultures from sc and om fat of five women. On day 1 we could not detect any conversion of androstenedione to testosterone in sc and om preadipocytes (Fig. 6B). Omental, but not subcutaneous, preadipocytes readily converted testosterone to androstenedione on day 1. After differentiation to mature adipocytes on day 14, om and sc cultures exhibited similar conversion rates of androstenedione to testosterone, whereas the reverse conversion of testosterone to androstenedione was much

stronger in the om than in cultures of sc origin (Fig 6B). When calculating the net conversion in preadipocyte differentiation cultures, om preadipocytes predominantly inactivated testosterone to androstenedione both before (day 1) and after 14 days of differentiation (Fig. 6C). By contrast, in differentiated cells (day 14) of sc origin net conversion revealed a significant gain in androgen activation, i.e. conversion of androstenedione to testosterone (Fig. 6C).

Discussion

Conversion of androstenedione to testosterone in human adipose tissue may represent an intracrine source of androgen synthesis, possibly also contributing to the previously described positive correlation between circulating androgens and BMI (Taponen *et al.* 2003). It is important to recognize that this may follow a sex-specific pattern as this correlation has been found in women only (Taponen *et al.* 2003). Epidemiological studies have found that circulating levels of dehydroepiandrosterone (DHEA) sulphate, the sulphate ester of the crucial androgen precursor DHEA, are positively correlated with truncal fat in women (Williams *et al.* 1993) whilst there is an inverse correlation in men (Haffner *et al.* 1993). Thus androgen metabolism within male adipose tissue may differ from the pattern we report here in women.

Previous studies by Corbould *et al.* (1998, 2002) suggested that 17 β -HSD3, which primarily shows gonadal expression, is also expressed in female adipose tissue. They concluded that 17 β -HSD3 may be responsible for the conversion of androstenedione to testosterone, because no functional 17 β -HSD1 enzyme was found in adipose tissue (Corbould *et al.* 1998). However, we were not able to detect any 17 β -HSD3 mRNA expression in our fat tissue samples, even using the primers described by Corbould *et al.* and highly sensitive microarray techniques. There are some differences between the study presented by Corbould *et al.* (1998) and our study, in particular with regard to tissue handling and preadipocyte cultures. We have grown the preadipocytes in proliferation culture until confluence, i.e. for 5–7 days, before isolating RNA, whereas Corbould *et al.* used preadipocytes immediately after isolation and without further culturing. However, Corbould *et al.* did not investigate 17 β -HSD5 expression in their fat samples, which is a likely candidate enzyme for the catalysis of the conversion of androstenedione to testosterone (Dufort *et al.* 1999, El-Alfy *et al.* 1999, Qiu *et al.* 2004). Very recently, 17 β -HSD5 has been shown to be expressed in human adipose tissue (Blouin *et al.* 2003). We did not observe expression of 17 β -HSD3, but did define abundant expression of 17 β -HSD5. Thus we consider it highly likely that 17 β -HSD5 represents the main reductive 17 β -HSD isozyme responsible for conversion

of androstenedione to testosterone in human fat. The only other 17 β -HSD enzyme we found to be expressed in adipose tissue was 17 β -HSD4, which only catalyses the oxidative reaction and therefore cannot convert androstenedione to testosterone (Adamski & Jakob 2001, Luu-The 2001, Mindnich *et al.* 2004).

According to our microarray data and quantitative real-time PCR results comparing whole fat with preadipocytes, we found 17 β -HSD5 to be predominantly expressed in mature adipocytes, suggesting a role for this enzyme in adipocyte differentiation (Singh *et al.* 2003). This is further supported by the outcome of our preadipocyte differentiation assays, which showed a significant increase in 17 β -HSD5 expression upon differentiation from stromal preadipocytes to mature adipocytes. However, it is not clear if the increased expression of 17 β -HSD5 represents cause or consequence of ongoing differentiation. The increase in intracrine generation of androgens via the conversion of androstenedione to testosterone may influence differentiation (Singh *et al.* 2003). 17 β -HSD5 also catalyses the conversion of DHEA to androstenediol, which has been shown to occur in female adipose tissue following incubation with radio-labelled DHEA (Schindler & Aymar 1975). Androstenediol has very similar structural characteristics to 5 α -androstenediol, which recently has been shown to exhibit differentiating effects in the prostate via selective activation of the oestrogen receptor β (ER β) (Weihua *et al.* 2002).

The results of the enzymatic activity assay in our preadipocyte differentiation cultures revealed predominant androgen inactivation, i.e. conversion of testosterone to androstenedione, which is likely to be catalysed by 17 β -HSD4, as we did not detect expression of the other oxidative 17 β -HSD isozyme, 17 β -HSD2. Blouin *et al.* (2003) previously suggested that omental adipose tissue represents an androgen inactivating rather than an androgen activating tissue. This is further supported by their finding of increased inactivation of 5 α -dihydrotestosterone by 3 α -HSD activity, possibly catalysed by AKR1C2 (Blouin *et al.* 2003).

In contrast to the predominant androgen inactivation in om cultures, calculation of net conversion in our preadipocyte differentiation cultures of sc origin revealed significant androgen activation, i.e. conversion of androstenedione to testosterone, suggesting that sc fat depots participate in systemic androgen production.

Our findings strongly suggest a site-specific role for 17 β -HSD5. First, 17 β -HSD5 expression was significantly higher in sc than in om adipose tissue. Secondly, we found a significant positive correlation of BMI with 17 β -HSD5 expression in sc, but not in om fat in our female cohort. Conversely, we could show that 10% weight loss resulted in a significant decrease in 17 β -HSD5 expression in sc fat. This lends some support to the view that 17 β -HSD5 expression plays a site-specific role in female obesity.

However, the weight loss data should be interpreted with some caution; in this study we have used gluteal sc adipose tissue, which may differ from abdominal sc adipose tissue with respect to both enzymatic expression patterns and differentiation behaviour.

Interestingly, our findings with regard to 17 β -HSD5 expression exactly mirror previous observations on the expression of the androgen receptor (AR) in human adipose tissue, describing an increased expression in om compared with sc fat and a decrease in AR expression with differentiation of preadipocytes to mature adipocytes (Dieudonne *et al.* 1998). Higher conversion to more active androgen in subcutaneous adipose tissue may reflect a compensatory mechanism to counteract the lower AR expression. Our findings therefore highlight the importance of understanding site-specific regulation of adipogenesis by sex steroids.

In conclusion, we have shown that human female preadipocytes are capable of active androgen generation with the conversion of androstenedione to testosterone most likely catalysed by 17 β -HSD5. We observed a site- and cell type-specific expression pattern for 17 β -HSD5 with predominant expression in sc mature adipocytes, therefore suggesting a possible role for this enzyme in the regulation of adipocyte differentiation. We have shown that 17 β -HSD5 expression increases across differentiation from stromal preadipocytes to mature adipocytes and that the interconversion of androstenedione and testosterone is shifted towards testosterone generation with ongoing differentiation of sc preadipocytes. In addition, we observed a positive correlation between BMI and sc 17 β -HSD5 expression and a decrease in 17 β -HSD5 expression with weight loss. This supports the hypothesis that human adipose tissue may represent an intracrine source of androgen synthesis, possibly also contributing to the previously described positive correlation between circulating androgens and BMI. Further studies are warranted to explore adipose tissue-specific metabolism of sex steroids in both males and females and to elucidate the clinical significance of these findings, bearing in mind the importance of regional fat distribution for cardiovascular complications of obesity.

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