Tissue-specific dysregulation of 11 β -hydroxysteroid dehydrogenase type 1 in overweight/obese women with polycystic ovary syndrome compared with weight-matched controls

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Abstract

Context: Abnormal cortisol metabolism in polycystic ovary syndrome (PCOS) has been invoked as a cause of secondary activation of the hypothalamic–pituitary–adrenal axis and hence androgen excess. However, this is based on urinary excretion of cortisol metabolites, which cannot detect tissue-specific changes in metabolism and may be confounded by obesity.

Objective: To assess cortisol clearance and whole-body and tissue-specific activities of 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1 (HSD11B1)) in PCOS.

Design: Case–control study.

Setting: Medical center.

Patients: A total of 20 overweight–obese unmedicated Caucasian women with PCOS, aged 18–45 years, and 20 Caucasian controls matched for age, BMI, body fat distribution, and *HSD11B1* genotypes (rs846910 and rs12086634).

Main outcome measures: Cortisol metabolites were measured in 24 h urine. During steady-state 9,11,12,12-²H₄-cortisol infusion, cortisol clearance was calculated and whole-body HSD11B1 activity was assessed as the rate of appearance of 9,12,12-²H₃-cortisol (d3-cortisol). Hepatic HSD11B1 activity was quantified as the generation of plasma cortisol following an oral dose of cortisone. Subcutaneous adipose HSD11B1 activity and *HSD11B1* mRNA were measured, *ex vivo*, in biopsies.

Results: Urinary cortisol metabolite excretion, deuterated cortisol clearance, and the rate of appearance of d3-cortisol did not differ between patients with PCOS and controls. However, hepatic HSD11B1 conversion of oral cortisone to cortisol was impaired ($P < 0.05$), whereas subcutaneous abdominal adipose tissue *HSD11B1* mRNA levels and activity were increased ($P < 0.05$) in women with PCOS when compared with controls.

Conclusions: Tissue-specific dysregulation of HSD11B1 is a feature of PCOS, over and above obesity, whereas increased clearance of cortisol may result from obesity rather than PCOS.

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Introduction

An important level of control of glucocorticoid action in humans is exerted by peripheral metabolism of cortisol by tissue-specific enzymes, in particular A-ring reductases (5 α and 5 β -reductase) in the liver and 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2 (HSD11B2)) in the kidney (1). The metabolic clearance rate of cortisol is also influenced by the extent of regeneration of cortisol from inactive cortisone by 11 β -HSD type 1 (11 β -HSD1 (HSD11B1)) in liver and adipose tissue (1, 2).

Adipose tissue, as well as liver, is a key target tissue in which glucocorticoid action influences susceptibility to obesity and metabolic complications. Transgenic mice over-expressing HSD11B1 selectively in adipose tissue develop obesity, particularly in visceral depots, insulin resistance, and all the features of the metabolic syndrome (3). A similar level of overexpression of *Hsd11b1* selectively in liver does not induce obesity but impairs insulin sensitivity, promotes dyslipidemia, and increases blood pressure (4). Conversely, *Hsd11b1* knockout mice fed on a high-fat diet are protected from obesity and its metabolic complications (5).

In human obesity, the metabolic clearance rate of cortisol is increased in the liver, secondary to enhanced cortisol inactivation by A-ring reductases (6) and impaired regeneration of cortisol from cortisone by HSD11B1 (7). However, intra-adipose cortisol generation by HSD11B1 is increased (7). This probably balances the down-regulation of HSD11B1 in the liver (7), as whole-body HSD11B1 activity, measured *in vivo* by the rate of appearance of trideuterated cortisol (9,12,12-²H₃-cortisol, d3-cortisol) during tetradeuterated cortisol infusion (9,11,12,12-[²H]₄-cortisol, d4-cortisol) (8), is not altered in obesity (9). However, the selective increase in HSD11B1 activity in the adipose tissue seems to contribute to the association between obesity and metabolic complications. Cortisol is pivotal in adipogenesis by promoting the differentiation of stromal cells (preadipocytes to adipocytes) and plays a crucial role in modulating glucose homeostasis, insulin sensitivity, and energy balance (10, 11).

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder, characterized by androgen excess, ovulatory dysfunction, and polycystic ovaries. Obesity and insulin resistance are also frequently present and contribute to the pathogenesis of PCOS (12). In addition, women with PCOS frequently develop metabolic dysregulations at early ages, particularly type 2 diabetes (13). One hypothesis to explain the association of obesity with PCOS is that altered cortisol metabolism contributes to the activation of the hypothalamic–pituitary–adrenal axis and induces

ACTH-dependent androgen excess. This has been supported by evidence that women with PCOS excrete a higher proportion of cortisol metabolites in urine as 5 α - and 5 β -reduced steroids (14, 15, 16, 17), suggesting that liver A-ring reductase activity is enhanced, although the ratio of cortisol:cortisone metabolites is not altered, from which it has been inferred that HSD11B1 is not dysregulated in PCOS. However, some of these studies (14, 15) have not distinguished the effects of PCOS from potentially confounding effects of coexistent obesity. Moreover, all these studies have relied on ratios of cortisol metabolites in 24-h urine with inconsistent results (14, 15, 16, 17, 18, 19, 20), perhaps explained by complex changes in the balance between many enzymes that influence urinary steroid excretion and by the insensitivity of urinary steroid measurements to detect tissue-specific changes in cortisol metabolism. To assess HSD11B1 activity directly *in vivo*, a tracer for cortisol regeneration must be used (8).

The purpose of this study was to investigate cortisol metabolism, including whole-body and tissue-specific activities of HSD11B1, in PCOS using the ‘gold standard’ methods. To do this, cortisol clearance was measured during d4-cortisol infusion; whole-body HSD11B1 activity was measured by the rate of appearance of d3-cortisol during d4-cortisol infusion; hepatic HSD11B1 activity was assessed by measuring conversion of an oral dose of cortisone into cortisol on first-pass metabolism in the liver; and subcutaneous adipose HSD11B1 was assessed, *ex vivo*, by measuring enzyme activity and mRNA in subcutaneous abdominal fat biopsies. To eliminate the confounding effect of obesity, we matched patients having PCOS with controls for BMI and body fat distribution. In addition, we have recently demonstrated that *HSD11B1* genotypes influence enzyme activity *in vivo* (20). In particular, we found that the combination of SNPs rs846910 A and rs12086634 T confers increased *HSD11B1* transcript levels in adipose tissue and increased whole-body regeneration of cortisol from cortisone. Therefore, to eliminate the potential confounding effect of *HSD11B1* genotypes, we also matched patients having PCOS with controls for the combination of the two SNPs rs846910 and rs12086634.

Subjects and methods

Study design

We investigated 20 overweight–obese unmedicated Caucasian Italian women with PCOS, aged 18–45 years, and 20 Caucasian controls matched for age, BMI, body fat

distribution, estimated by waist circumference and by a computerized tomography (CT) scan at the level of L4–L5 (21), and for *HSD11B1* genotypes (rs846910 and rs12086634). Women with PCOS had at least two of the following symptoms: i) chronic oligoovulation–anovulation (luteal serum progesterone below 2 ng/ml (22)); ii) hirsutism (modified Ferriman–Gallwey score ≥ 8 (23)) or elevated serum total testosterone levels (24); and iii) polycystic ovarian morphology at ultrasound, according to the Rotterdam consensus conference criteria (25). Hyperprolactinemia, Cushing's syndrome, congenital adrenal hyperplasia, and androgen-secreting tumors were excluded by laboratory analysis (26). Controls had no signs of hyperandrogenism and regular ovulatory menstrual cycles (progesterone levels ≥ 8 ng/ml during the luteal phase (22)). None of the participants had thyroid dysfunction, cardiovascular, renal, or liver diseases on clinical examination and routine laboratory tests.

These women attended on three further study days. On the first day, they completed a 24-h urine collection and attended at 0800–0830 h after overnight fast. Basal blood samples for hormonal (luteinizing hormone (LH), androstenedione, DHEA-S, SHBG, cortisol, adiponectin) and metabolic (total cholesterol, HDL cholesterol, triglycerides, ALT, AST, γ GT) measurements were collected before a 75 g oral glucose tolerance test (OGTT) with blood samples taken after 30, 60, 90, 120, and 180 min for glucose and insulin determinations. On the same day, fat-free mass and fat mass were estimated with a single-frequency 50 kHz bioelectrical impedance analyzer (BIA 101 RJL, Akern Bioresearch, Florence, Italy), according to the standard tetrapolar technique, with the subject in supine position and the electrodes placed on the dorsal surface of the right foot and ankle, and the right wrist and hand, and using the software provided by the manufacturer (27, 28). On the second day at 0800 h, after overnight fast, a ~ 300 mg s.c. needle aspiration biopsy of subcutaneous adipose tissue from the anterior abdominal wall was obtained and stored at -80°C as described previously (29). After the biopsy procedure, d4-cortisol (Cambridge Isotopes, Andover, MA, USA) was infused at 20 molar percent excess of cortisol (Flebocortid Richer, Sanofi-Aventis S.p.A., Milano, Italy) at 1.74 mg/h after a priming bolus dose of 3.5 mg, and blood sampled at intervals for 4 h (8). The evening before the third assessment day, subjects took 1 mg oral dexamethasone (Decadron, Visufarma S.p.A., Roma, Italy) at 2300 h, to suppress endogenous cortisol production, and fasted overnight. At 0800 h, an oral dose of 25 mg cortisone acetate (Cortone Acetato, Giuseppe Rende S.r.L., Roma, Italy)

was administered and blood samples obtained at intervals for 4 h to measure plasma cortisol.

Samples were immediately chilled on ice and centrifuged; 24-h urine and serum were stored at -20°C and plasma at -80°C . Studies were performed between days 5 and 10 of the menstrual cycle, or during amenorrhea, after excluding pregnancy by appropriate testing. The study protocol was approved by the ethics committee (Ref. 154/2005/U/Sper) and informed consent was obtained from the patients.

Laboratory assays

Hormone and biochemical assays ► The assays for hormonal and biochemical measurements have been reported elsewhere (26). Serum adiponectin was measured by the RIA kit (Linco Research, Inc., St Charles, MO, USA) using ^{125}I -labeled adiponectin and polyclonal antiserum. All samples were measured in a single assay. The intra-assay coefficient of variation values at low (12.2 ng/ml) and high (60.4 ng/ml) levels were 9.7 and 8.5% respectively. LDL cholesterol was calculated by the Friedewald calculation (30), and the free androgen index (FAI) was calculated as the ratio between total testosterone and SHBG (31). Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) (32) and the insulin sensitivity index during the OGTT (ISI) (33).

Analysis of d4-cortisol and its metabolites ► Plasma d4-cortisol, d3-cortisone, and d3-cortisol were measured by liquid chromatography/tandem mass spectrometry as described previously (34) and tracer kinetics was calculated using the mean of five measurements in steady state between 180 and 240 min of d4-cortisol infusion. The rate of appearance of d3-cortisol was calculated as (d4-cortisol infusion rate)/(d4-cortisol:d3-cortisol ratio). The rate of appearance of cortisol was calculated as ((d4-cortisol infusion rate)/(d4-cortisol:cortisol ratio)) – (cortisol infusion rate). Clearance of d4-cortisol was calculated as (d4-cortisol infusion rate)/(d4-cortisol).

Analysis of urinary steroids ► Cortisol and its metabolites (5 β -tetrahydrocortisol (5 β -THF), 5 α -THF, 5 β -tetrahydrocortisone (5 β -THE), cortols, cortolones, and cortisone) were measured in urine by electron impact gas chromatography–mass spectrometry (GC–MS) as described previously (20). Total cortisol excretion was calculated from the sum of 5 β -THF, 5 α -THF, 5 β -THE, cortols, and cortolones. The balance of HSD11B1 and HSD11B2 activities in all tissues was assessed as the ratio of

(5 α -THF+5 β -THF)/5 β -THE, and renal HSD11B2 activity was assessed as urinary cortisol:cortisone ratio. Relative 5 α - and 5 β -reduction of cortisol was assessed by Ulick's A-ring reduction quotients, 5 α -THF/cortisol and 5 β -THF/cortisol (35). The balance of 5 β - and 5 α -reductases was also assessed by the ratio 5 β -THF:5 α -THF.

Analysis of adipose tissue *HSD11B1* mRNA and activity ► Approximately 500 mg of fat were homogenized in 1 ml TRIzol (Invitrogen), RNA was quantified by spectrophotometry, and RNA integrity was checked by agarose gel electrophoresis. RNA of 1 μ g was reverse transcribed using the Invitrogen Reverse Transcription System and transcript levels of *HSD11B1* were quantified by real-time PCR with primer–probes sets from PE Applied Biosystems: 5'-GGAATATTCAGTGTCCAGGGTCAA-3' (forward), 5'-TGATCTCCAGGGCACATTCCT-3' (reverse), and 5'-6-FAM-CTTGGCCTCATAGACACAGAAACAGCCATAMRA-3' (probe). Human cyclophilin A (PE Applied Biosystems) was used to normalize the *HSD11B1* transcript levels. A standard curve was generated in triplicate by serial dilution of cDNA pooled from several subjects. Each sample was run in duplicate, and the mean of the duplicates was expressed as a fold difference in RNA level vs an internal control sample. RT negative controls and intron spanning primers were used to demonstrate the lack of genomic DNA contamination and further prevent its amplification respectively.

Adipose tissue HSD11B1 activity was measured in the dehydrogenase direction, which is the more stable *in vitro* and under conditions of prolonged incubation to obtain measurable conversion with the low available protein concentrations without exhausting substrate supply, as described previously (36). Briefly, 250 mg of tissue were homogenized in KREBS buffer. Total protein of 400 μ g/ml was incubated with 2 mM NADP, 0.2% glucose, and 100 nM cortisol (of which 10 nM is 1,2,6,7-[³H]₄-cortisol) at 37 °C for 30 h. Aliquots were withdrawn at intervals (6, 24, and 30 h) and conversion to 1,2,6,7-[³H]₄-cortisone was measured by HPLC with online scintillation detection. Results are expressed as picomole of product generated per microgram of homogenized tissue.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Normal distribution of variables was tested by means of the Kolmogorov–Smirnov test. General linear models were applied in order to analyze data. In particular, multivariate Hotelling's test was applied to groups of homogeneous variables, one-way

repeated measures ANOVA was used to compare patients with PCOS and controls, while two-way repeated measures ANOVA was applied to variables evaluated at different time points (i.e. liver and adipose HSD11B1 activities). One-way repeated measures ANCOVA was applied in order to relate subcutaneous adipose tissue *HSD11B1* mRNA levels and subcutaneous and liver HSD11B1 activities, expressed as AUC values, with biochemical parameters. No *post-hoc* analysis was performed. Statistical analyses were performed using the IBM SPSS Statistics (Version 21 for Windows, IBM Co., Armonk, NY, USA) software package. Two-tailed $P < 0.05$ were considered statistically significant.

Results

Women with PCOS and controls were of similar age, BMI, and body fat distribution and had similar plasma transaminases, lipids, and LH and cortisol levels (Table 1). However, as expected, PCOS cases had higher levels of androgens (total testosterone, FAI, androstenedione, and DHEA-S), higher insulin response to the OGTT, and lower levels of adiponectin in plasma when compared with controls (Table 1). In addition, women with PCOS had a higher HOMA-IR and a lower ISI, reflecting lower insulin sensitivity when compared with controls (Table 1).

Cortisol metabolites were measured in urine to assess HSD11B1 activity *in vivo* (Table 2). Women with PCOS did not differ from controls in terms of (5 α -THF+5 β -THF)/5 β -THE ratio, suggesting a similar process of whole-body conversion of cortisone to cortisol. The A-ring reduction quotients reflecting 5 α - (5 α -THF/cortisol) and 5 β -reductase activity (5 β -THF/cortisol) did not differ between patients with PCOS and controls. Similarly, the 5 β -THF/5 α -THF ratio, that reflects the balance of 5 β - and 5 α -reductases, was similar between PCOS cases and controls.

During d4-cortisol infusion, steady state was achieved between 180 and 240 min. Mean plasma measurements at steady state are given in Table 3. Cortisol, d4-cortisol and d3-cortisone concentrations, and d4-cortisol clearance rate were not different between PCOS cases and controls. Women with PCOS and controls had similar whole-body rates of appearance of cortisol, reflecting the combination of adrenal cortisol secretion and net regeneration of cortisol by HSD11B1, and of d3-cortisol, reflecting exclusively the whole-body contribution of HSD11B1.

Tissue-specific dysregulation of HSD11B1 in PCOS was found. Liver HSD11B1 activity, measured as appearance of cortisol on first-pass conversion after an oral dose of cortisone, was significantly lower in PCOS cases (Fig. 1).

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Table 1 Clinical and biochemical characteristics of PCOS cases and controls. Data are expressed as means $\pm$ S.E.M.

Variables	PCOS	Controls	P values
Matching design variables			0.98 ^a
Age (years)	28.4 $\pm$ 1.6	28.7 $\pm$ 1.4	0.74
BMI (kg/m ²)	35.0 $\pm$ 1.2	34.7 $\pm$ 0.9	0.86
Waist circumference (cm)	100.9 $\pm$ 2.7	101.3 $\pm$ 2.2	0.92
Body fat (%)	42.0 $\pm$ 1.6	40.7 $\pm$ 1.9	0.46
CT scan			0.61 ^b
Total adipose tissue (cm ²)	584 $\pm$ 45	546 $\pm$ 49	0.81
Subcutaneous adipose tissue (cm ²)	483 $\pm$ 37	464 $\pm$ 44	0.97
Visceral adipose tissue (cm ²)	101 $\pm$ 11	82 $\pm$ 10	0.70
Visceral:subcutaneous ratio	0.21 $\pm$ 0.02	0.18 $\pm$ 0.02	0.71
Fasting 0800–0830 h plasma			<0.05 ^a
ALT (U/l)	22.2 $\pm$ 2.7	15.9 $\pm$ 1.0	0.06
AST (U/l)	30.5 $\pm$ 5.9	19.6 $\pm$ 2.1	0.13
γ GT (U/l)	22.7 $\pm$ 3.3	16.9 $\pm$ 2.6	0.20
HDL cholesterol (mmol/l)	1.35 $\pm$ 0.07	1.53 $\pm$ 0.10	0.09
Triglycerides (mmol/l)	1.29 $\pm$ 0.13	1.08 $\pm$ 0.12	0.32
LDL cholesterol (mmol/l)	2.53 $\pm$ 0.19	2.85 $\pm$ 0.20	0.32
Total testosterone (nmol/l)	2.27 $\pm$ 0.31	1.04 $\pm$ 0.13	<0.01
Free androgen index	3.50 $\pm$ 0.41	1.34 $\pm$ 0.22	<0.001
Androstenedione (nmol/l)	13.2 $\pm$ 1.19	6.25 $\pm$ 0.61	<0.001
DHEA-S (μ mol/l)	6.51 $\pm$ 0.65	3.41 $\pm$ 0.61	<0.01
LH (mU/ml)	7.50 $\pm$ 1.06	8.68 $\pm$ 1.59	0.81
SHBG (nmol/l)	20.8 $\pm$ 2.4	28.5 $\pm$ 3.3	0.08
Cortisol (nmol/l)	376 $\pm$ 26	343 $\pm$ 29	0.64
Adiponectin (μ g/ml)	9.84 $\pm$ 0.89	12.03 $\pm$ 1.43	<0.05
Oral glucose tolerance test			<0.05 ^a
Fasting glucose (mmol/l)	4.84 $\pm$ 0.32	4.11 $\pm$ 0.09	<0.05
Fasting insulin (μ U/ml)	21.6 $\pm$ 4.1	13.9 $\pm$ 1.8	0.33
Glucose _{AUC} (mmol/l per min)	1188 $\pm$ 92	1083 $\pm$ 41	0.17
Insulin _{AUC} (μ U/ml per min)	24 296 $\pm$ 5163	12 795 $\pm$ 1788	<0.05
Insulin resistance indices			0.23 ^a
HOMA-IR	4.98 $\pm$ 1.09	2.58 $\pm$ 0.36	<0.05
ISI	3.30 $\pm$ 0.62	4.79 $\pm$ 0.65	<0.05
Adipose <i>HSD11B1</i> mRNA ^c	−0.129 $\pm$ 0.072	−0.401 $\pm$ 0.056	<0.05

^aHotelling's multivariate tests of significance (all parameters).

^bHotelling's multivariate tests of significance (total adipose tissue and visceral:subcutaneous ratio were excluded because these variables are linearly dependent on subcutaneous and visceral adipose tissues).

^cExpressed as a difference to cyclophilin internal control.

By contrast, in subcutaneous abdominal adipose tissue, both *HSD11B1* mRNA levels (Table 1 and Fig. 2A) and 11 β -HSD1 activity, measured *ex vivo* by the conversion of cortisone from cortisol (Fig. 2B), were significantly higher in PCOS cases when compared with controls.

The AUC of liver HSD11B1 activity was 60 338 $\pm$ 6409 nmol/l per min for PCOS cases and 86 935 $\pm$ 10 211 nmol/l per min for controls, whereas the AUC of *ex vivo* 11 β -HSD1 activity in subcutaneous fat was 271 $\pm$ 41 pmol/ μ g.h for PCOS cases and 152 $\pm$ 30 pmol/ μ g.h for controls. These were used to perform the one-way ANCOVA of liver and adipose HSD11B1 activities and adipose *HSD11B1* mRNA adjusted for biochemical parameters was significantly different between PCOS cases and controls as covariates and the regression analysis (Table 4). In the one-way ANCOVA, liver HSD11B1 activity

persisted lower in PCOS cases when compared with controls only after the adjustment for total testosterone and androstenedione, and adipose HSD11B1 activity persisted higher in PCOS cases when compared with controls only after the adjustment for FAI and androstenedione. *HSD11B1* mRNA persisted higher in PCOS cases when compared with controls only after the adjustment for androstenedione. In regression analysis, liver HSD11B1 activity and subcutaneous adipose tissue HSD11B1 expression and activity did not correlate significantly with any parameter investigated within controls (Table 4). However, within PCOS, liver HSD11B1 activity correlated negatively with total testosterone, subcutaneous adipose tissue *HSD11B1* mRNA levels correlated positively with total testosterone and HOMA-IR, and *ex vivo* HSD11B1 activity in subcutaneous

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Table 2 24-h urine cortisol metabolites of PCOS cases and controls (Hotelling's multivariate tests of significance, $P < 0.05^a$). Data are expressed as means $\pm$ s.e.m.

Variables	PCOS	Controls	P values
Cortisol (μ g/day)	127 $\pm$ 12	142 $\pm$ 13	0.48
Cortisone (μ g/day)	188 $\pm$ 20	264 $\pm$ 27	<0.05
5 β -Tetrahydrocortisol (5 β -THF, μ g/day)	2394 $\pm$ 304	2200 $\pm$ 199	0.66
5 α -Tetrahydrocortisol (5 α -THF, μ g/day)	2170 $\pm$ 351	2716 $\pm$ 365	0.37
5 β -Tetrahydrocortisone (5 β -THE, μ g/day)	4572 $\pm$ 606	3657 $\pm$ 377	0.29
Total cortisol metabolites (μ g/day) ^b	13 811 $\pm$ 1489	13 938 $\pm$ 1259	0.96
Cortisol/cortisone	0.774 $\pm$ 0.085	0.595 $\pm$ 0.051	0.06
(5 α -THF + 5 β -THF)/5 β -THE	1.10 $\pm$ 0.10	1.70 $\pm$ 0.46	0.21
5 α -THF/cortisol	17.6 $\pm$ 2.7	19.6 $\pm$ 2.1	0.61
5 β -THF/cortisol	19.8 $\pm$ 2.1	16.8 $\pm$ 1.6	0.29
5 β -THF/5 α -THF	1.80 $\pm$ 0.45	0.99 $\pm$ 0.11	0.08

^aTotal cortisol metabolites, cortisol/cortisone, (5 α -THF + 5 β -THF)/5 β -THE, 5 α -THF/cortisol, 5 β -THF/cortisol, and 5 β -THF/5 α -THF were excluded because these variables are linearly dependent on the previous ones.

^bTotal cortisol metabolites = 5 β -THF + 5 α -THF + 5 β -THE + cortols + cortolones.

fat correlated positively with insulin response to the OGTT and with androstenedione. No other significant correlations were found with subcutaneous adipose tissue *HSD11B1* mRNA levels or *ex vivo* HSD11B1 activity (Table 4).

Discussion

This is the first study to undertake comprehensive functional evaluation of HSD11B1 activity in overweight-obese women with PCOS and weight-matched controls. The novelty of this study is the method used to analyze the *in vivo* overall activity of HSD11B1 and the measurement of tissue-specific activities of HSD11B1 in PCOS. We found that the overall activity of HSD11B1 in PCOS is similar to that of controls, thus confirming previous results (14, 15, 20). However, to measure the whole-body activity of 11 β -HSD1, we used not only ratios of cortisol metabolites in 24-h urine, a method used in all the studies performed so far, but also d4-cortisol infusion test, that is the 'gold standard' method, because it is able to discriminate the activity of 11 β -HSD1 from the activities of 11 β -HSD type 2 and A-ring reductases. In addition, we confirmed in this study what was previously demonstrated by others (37), in particular that transcript levels of *HSD11B1* in subcutaneous adipose tissue are increased in PCOS cases when compared with controls. However, we added for the first time information about subcutaneous HSD11B1 adipose tissue activity, which was increased in PCOS cases, and about hepatic HSD11B1 activity, which was decreased in PCOS cases when compared with controls. The availability of a control group matched for body fat makes it possible to conclude that these

differences cannot be attributed to obesity. Therefore, the data reveal that alterations in cortisol metabolism in PCOS do exist and include increased HSD11B1 activity in adipose tissue and decreased HSD11B1 activity in liver, albeit that these alterations appear to be balanced such that whole-body HSD11B1 activity is not measurably altered.

By contrast, in this group of women with PCOS, there was no evidence of the previously reported increase in activities of the A-ring reductases (14, 15, 17, 18), 5 β - and

Table 3 Steady-state concentrations and ratios during d4-cortisol infusion in PCOS cases and controls (Hotellings multivariate tests of significance, $P = 0.39^a$). Data are expressed as means $\pm$ s.e.m. The mean data for each participant from samples obtained between 180 and 240 min during the d4-cortisol infusion was used to calculate steady-state concentrations.

Variables	PCOS	Controls	P values
Cortisol (nmol/l)	228.2 $\pm$ 23.6	259.9 $\pm$ 42.4	0.52
D4-cortisol (nmol/l)	38.8 $\pm$ 4.9	42.1 $\pm$ 7.0	0.71
D4-cortisol clearance (l/min)	0.575 $\pm$ 0.114	0.502 $\pm$ 0.054	0.56
D4-cortisol:cortisol ratio	0.177 $\pm$ 0.012	0.176 $\pm$ 0.013	0.94
D4-cortisol:d3-cortisol ratio	1.31 $\pm$ 0.08	1.32 $\pm$ 0.07	0.95
D3-cortisone (nmol/l)	6.70 $\pm$ 0.78	5.42 $\pm$ 0.59	0.18
Rate of appearance of cortisol (nmol/min)	31.4 $\pm$ 9.3	32.7 $\pm$ 9.1	0.93
Rate of appearance of d3-cortisol (nmol/min)	12.1 $\pm$ 0.7	12.1 $\pm$ 0.8	0.99

^aD4-cortisol:cortisol ratio and d4-cortisol:d3-cortisol ratio were excluded because these variables are linearly dependent on the previous ones.

Figure 1

Liver HSD11B1 activity in PCOS cases and controls. Data are expressed as mean $\pm$ S.E.M. Patients with PCOS (closed square). Controls (open square). By repeated measures ANOVA, PCOS had an overall lower liver HSD11B1 activity ($P < 0.05$). * $P < 0.05$ between PCOS and controls at each time point.

5 α -reductase, and no change in cortisol clearance. Confounding effects of obesity and/or reliance on urinary steroid ratios, which may be unreliable because they are influenced by the activity of other enzymes, could explain inconsistencies in previous studies (14, 15, 16, 17, 18, 19, 20).

Whole-body regeneration of cortisol by HSD11B1 can be measured specifically using the d4-cortisol stable isotope tracer (8, 34). Consistent with the lack of difference in urinary cortisol:cortisone metabolite ratios, the rate of appearance of d3-cortisol was similar in our population of women with PCOS when compared with controls, thus demonstrating that whole-body HSD11B1 activity is not altered in overweight–obese women with PCOS. However, in obesity, a similar lack of change in whole-body HSD11B1 activity (9) is accompanied by substantial tissue-specific changes in cortisol regeneration by HSD11B1 (1, 36). The major source of extra-adrenal cortisol regeneration is the liver (2, 38), but recent data have confirmed that there is a contribution from adipose tissue (34). In this study, we quantified liver HSD11B1 activity by administering oral cortisone and measuring the initial rate of appearance of cortisol in peripheral plasma after first-pass hepatic metabolism (38). This test has potential limitations, because levels of cortisol in plasma reflect not only the amount generated from cortisone by HSD11B1 activity but also the extent of inactivation and clearance of cortisol by 5 α - and 5 β -reductases in the liver. However, measurement of the rate of appearance of cortisol in plasma during the early time points (up to 30 min) after cortisone ingestion has been validated by

measurements of tracer dilution in the hepatic vein, at least in healthy subjects (38). Although similar validation has not been undertaken in patients with PCOS, our data suggest that overweight–obese women with PCOS are characterized by a decrease in HSD11B1 activity in the liver when compared with weight-matched controls. In subcutaneous adipose tissue, the same women were characterized by higher *HSD11B1* transcript levels and a higher enzyme activity, measured *ex vivo*. Similar results for adipose tissue transcript levels of *HSD11B1* were previously observed in omental adipose tissue obtained during bariatric surgery of obese women with and without PCOS (39) and in subcutaneous adipose tissue obtained from lean patients with PCOS compared with

Figure 2

Adipose HSD11B1 in PCOS cases and controls. Data are expressed as mean $\pm$ S.E.M. (A) *HSD11B1* mRNA levels in subcutaneous adipose tissue, expressed as a ratio to cyclophilin internal control. By ANOVA, PCOS had increased transcript levels of *HSD11B1* ($P < 0.05$). (B) *Ex vivo* HSD11B1 activity in subcutaneous adipose tissue, expressed as conversion of cortisol to cortisone. By repeated measures ANOVA, PCOS had a higher HSD11B1 activity ($P < 0.05$). * $P < 0.05$ between PCOS and controls at each time point.

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Table 4 Results of one-way ANCOVA of liver and adipose HSD11B1 activities and adipose *HSD11B1* mRNA adjusted for different biochemical parameters as covariates. The *P* values of the various effects taken into account are shown in the table. The positive or negative relationships are also indicated within parentheses.

Covariate	PCOS vs controls adjusted for regression	Regression within PCOS	Regression within controls	Interaction: regression within PCOS vs regression within controls
Liver HSD11B1 activity				
Total testosterone	<0.05	<0.05 (–)	0.43 (–)	0.18
Free androgen index	0.09	0.70 (–)	0.13 (–)	0.17
Androstenedione	<0.05	0.07 (–)	0.30 (–)	0.13
DHEA-S	0.30	0.10 (–)	0.77 (–)	0.18
Adiponectin	0.97	0.38 (–)	0.69 (–)	0.35
Fasting glucose	0.99	0.12 (–)	0.75 (–)	0.89
Insulin _{AUC}	0.37	0.83 (–)	0.64 (–)	0.74
HOMA-IR	0.64	0.73 (+)	0.44 (+)	0.49
ISI	0.20	0.64 (+)	0.71 (–)	0.57
Adipose HSD11B1 activity ^a				
Total testosterone	0.13	0.44 (+)	0.74 (+)	0.56
Free androgen index	<0.05	0.29 (+)	0.16 (+)	0.16
Androstenedione	<0.01	<0.05 (+)	0.29 (+)	0.05
DHEA-S	0.06	0.10 (–)	0.35 (–)	0.46
Adiponectin	0.19	0.84 (+)	0.72 (+)	0.57
Fasting glucose	0.68	0.25 (+)	0.33 (+)	0.81
Insulin _{AUC}	0.60	<0.05 (+)	0.54 (+)	0.88
HOMA-IR	0.74	0.39 (+)	0.53 (–)	0.39
ISI	0.38	0.40 (–)	0.67 (–)	0.67
Adipose <i>HSD11B1</i> mRNA ^b				
Total testosterone	0.57	<0.05 (+)	0.59 (+)	0.06
Free androgen index	0.33	0.57 (+)	0.99 (+)	0.81
Androstenedione	<0.05	0.15 (+)	0.16 (+)	0.09
DHEA-S	0.69	0.35 (–)	0.89 (–)	0.07
Adiponectin	0.65	0.32 (–)	0.13 (–)	0.99
Fasting glucose	0.39	0.77 (+)	0.44 (+)	0.49
Insulin _{AUC}	0.11	0.96 (+)	0.17 (+)	0.26
HOMA-IR	0.26	<0.05 (+)	0.40 (+)	0.61
ISI	0.44	0.22 (–)	0.35 (–)	0.63

^aEx-vivo HSD11B1 activity in subcutaneous adipose tissue, expressed as conversion of cortisol to cortisone.

^b*HSD11B1* mRNA levels in subcutaneous adipose tissue, expressed as a ratio to cyclophilin internal control.

lean controls (37). Overall, these data show that the tissue-specific dysregulation of HSD11B1, well established in obesity in rodents and humans (1, 36), is also observed in women with PCOS compared with weight-matched controls.

Previous studies have described increased excretion of 5 α - or 5 β -reduced cortisol metabolites in urine and inferred that increased cortisol clearance might impair negative feedback suppression of the hypothalamic–pituitary–adrenal axis, thereby contributing to ACTH-dependent adrenal androgen excess (16, 17). However, our data suggest that there is no added effect of PCOS on cortisol clearance or on urinary cortisol metabolite excretion over and above the presence of obesity. Increased cortisol clearance is a consistent finding with obesity (9, 40, 41), but our data suggest that it is not a

feature of PCOS *per se*. However, the results relying on cortisol metabolites in the urine have to be considered with caution because they are influenced by the activity of many enzymes.

The cross-sectional design of the study does not allow us to evaluate what it is about PCOS that causes changes of HSD11B1 over and above obesity. However, we can speculate that two factors might be involved: insulin and androgens. In humans, insulin infusion acutely increases intra-adipose HSD11B1 activity, probably through a post-transcriptional mechanism (42, 43). A stimulatory effect of insulin on adipocyte HSD11B1 that involves activation of the p38 MAPK signaling pathway has been demonstrated (44). In addition, insulin decreases HSD11B1 in hepatocytes *in vitro* (45) while, *in vivo*, a reduction of fasting insulin concentration by a low-carbohydrate diet is

associated with an increased hepatic HSD11B1 activity (29). Therefore, chronic hyperinsulinemia, as so frequently observed in PCOS, particularly in the overweight-obese phenotype (12), might be responsible for both increased adipose 11 β -HSD1 and decreased hepatic HSD11B1 activities. Accordingly, we found a positive correlation between subcutaneous *HSD11B1* mRNA expression and HOMA-IR and between subcutaneous HSD11B1 activity and insulin responsiveness to the glucose load. These findings, in particular that subcutaneous HSD11B1 expression and activity in humans is negatively correlated with insulin sensitivity, are consistent with previous observations in the general population (46) and in PCOS cases (37). Increased activity of adipose HSD11B1 in PCOS could also reflect a regulatory effect of androgen excess; it has been demonstrated that testosterone induces the expression and activity of intra-adipose HSD11B1 in humans, in omental more than subcutaneous depots (47). Indeed, we found a positive correlation between androstenedione and subcutaneous HSD11B1 activity in PCOS. In addition, we found a negative correlation between testosterone and liver HSD11B1 activity, consistent with an inhibitory effect of androgens on hepatic HSD11B1.

It is impossible from this observational study to establish whether these alterations in cortisol metabolism are primary or secondary to the development of PCOS. On one hand, increased intra-adipose cortisol production due to primary HSD11B1 overactivity could induce the differentiation of adipose stromal cells to adipocytes, particularly in visceral depots, with a consequent increase in visceral adiposity, and the development of insulin resistance and hyperinsulinemia. On the other hand, testosterone-induced up-regulation of HSD11B1 could increase intra-adipose cortisol regeneration, with a consequent increased preadipocyte differentiation and amplification of the negative effect of excess body fat on the PCOS phenotype.

In conclusion, PCOS-specific overactivity of HSD11B1 within adipose tissue could influence fat distribution, contribute to metabolic consequences, and perpetuate the syndrome. These findings reinforce the importance of HSD11B1 as a pathophysiological mediator in PCOS and suggest that approaches aimed at reducing glucocorticoid action in adipose tissue in this condition may be valuable.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A Gambineri contributed to data acquisition, revision, and interpretation, drafted and critically revised the article, and wrote and approved the final version of the manuscript. F Fanelli, F Tomassoni, A Munarini, and R Andrew performed laboratory analysis, critically revised the article for intellectual content, and approved the final version of the manuscript. B R Walker, and R Pasquali contributed to data revision and interpretation, drafted and critically revised the article, and wrote and approved the final version of the manuscript.

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