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SERUM 5-ANDROSTENE-3β,17β-DIOL SULPHATE AND 5α-ANDROSTANE-3α,17β-DIOL SULPHATE IN HIRSUTE FEMALES WITH POLYCYSTIC OVARIAN DISEASE

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Summary—Serum sulphates of 5-androstene-3β,17β-diol (5-ADIOL-S), 5α-androstane-3α,17β-diol (3α-DIOL-S) and dehydroepiandrosterone (DHEA-S), unconjugated androstenedione (AD) and testosterone (T), sex hormone binding globulin (SHBG), free androgen index (FAI), 17α-hydroxyprogesterone (17OHP), luteinising hormone (LH) and follicle stimulating hormone (FSH) were measured by specific radioimmunoassay in 28 hirsute women with polycystic ovarian disease (PCO) and in normal women ($n = 73$). Mean levels of steroids measured were significantly elevated, and SHBG significantly depressed, in the women with PCO with values (mean ± SE) for 5-ADIOL-S (516 ± 51 vs 267 ± 10 nmol/l), 3α-DIOL-S (130 ± 9 vs 52 ± 2 nmol/l), DHEA-S (7.3 ± 0.5 vs 4.4 ± 0.2 μmol/l), AD (11.3 ± 1.1 vs 3.4 ± 0.2 nmol/l), T (3.3 ± 0.2 vs 1.5 ± 0.1 nmol/l) and 17OHP (5.1 ± 0.8 vs 2.8 ± 0.2 nmol/l). SHBG levels were 31 ± 2.9 vs 65 ± 2.5 nmol/l, and the free androgen index [100 × T (nmol/l) ÷ (SHBG nmol/l)] was 12.5 ± 1.4 vs 2.4 ± 0.1. The mean LH to FSH ratio was also elevated at 2.8 ± 0.3. These studies suggest that the measurement of 5-ADIOL-S and DHEA-S may indicate adrenal gland involvement in PCO while 3α-DIOL-S appears to be a reflection of peripheral androgen metabolism. A comprehensive biochemical profile of PCO should thus include the analysis of these sulphoconjugates as well as unconjugated steroids.

INTRODUCTION

The polycystic ovary syndrome (PCO) may be best defined as functional gonadotropin-dependent ovarian hyperandrogenism and results from a primary increase in the ratio of the serum concentrations of luteinizing hormone (LH) to follicle stimulating hormone (FSH) or the ratio of the intra-ovarian concentrations of androgen to oestrogen, or from follicular atresia [1]. Females with PCO in its full blown state may present with oligo/amenorrhoea, infertility, hirsutism and obesity, [1–3], but not all patients have the typical syndrome.

About 75% of adolescents and adults with PCO have abnormal serum gonadotrophin levels reflecting an elevated LH to FSH ratio [4–7]. Studies from several laboratories have indicated a significant increase in androgen levels in PCO [8–20], as well as increased 17α-hydroxyprogesterone [14, 15, 17, 19, 20] and suppressed sex hormone binding globulin levels [20, 21]. In addition, Lobo has measured blood 5α-androstane-3α,17β-diol glucuronide as a marker of peripheral androgen overproduction in hirsute females with PCO [22].

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The role of the adrenal gland in the aetiology of PCO remains unclear; the fact that DHEA-S, a 17-ketosteroid of almost exclusive adrenal origin, is elevated in about 50% of patients with PCO [19, 20, 23, 24] suggests that excess adrenal androgen production occurs in addition to the increased ovarian secretion of testosterone and androstenedione. Other studies [17, 25, 26] have also shown that the increased production of adrenal androgens is not due to a classical enzyme deficiency.

The aims of the present study were to determine, by specific RIA, levels of the 19-carbon sulphoconjugated steroids 5-ADIOL-S and 3α-DIOL-S in patients with PCO, in addition to the steroids which are normally measured in PCO. A second, subsidiary aim was to establish a comprehensive biochemical profile in blood to aid in the diagnosis of PCO, and a better appreciation of androgen abnormalities in this condition.

EXPERIMENTAL

Patients

Twenty-eight patients with excessive facial and/or body hair and PCO, confirmed by ultrasound or laparoscopy to have enlarged ovaries and/or multicystis ovaries, age range 13–34 years, were studied. A

group of normal women, ($n = 73$) age range 21–40 years, without menstrual dysfunction, hirsutism or alopecia and not receiving any hormonal medication acted as controls. Blood from both patients and controls was drawn between 0900 hours and 1600 hours. Obesity was noted in 50% of the patients with PCO, while menstrual dysfunction occurred as irregular periods in 40%, oligomenorrhoea in 35% and amenorrhoea in 23%.

Materials

All organic solvents were analytical reagent grade, purchased from British Drug Houses (Port Fairy, Victoria, Australia). Unlabelled steroids were purchased from Research Plus Inc., Bayonne, N.J.) or from Sigma Chemical Company (St Louis, Mo.).

Antisera. The 5-ADIOL antiserum was purchased from Bio-Mega Diagnostic Inc. (Montreal, Canada), and the 3α-DIOL antiserum from Serono (Coinsins, Switzerland). Antisera to both AD and 17OHP was purchased from Miles Scientific (Mulgrave, Victoria, Australia); the testosterone (T) antiserum prepared against T-3-carbodiimomide-BSA was kindly provided by the Chemical Pathology Department, Prince Henry's Hospital, (Melbourne, Victoria, Australia). Normal rabbit serum was from the Animal House, Royal Children's Hospital, and used at a dilution of 1:150. Sheep anti-rabbit antiserum was purchased from Silenus ICI (Melbourne, Victoria, Australia) and used at a dilution of 1:15.

Labelled ligands. [1,2,^{3}H(*N*)]androstene-3β,17β-diol, 40–60 Ci/mmol, and [7-^{3}H]DHEA-sulphate ammonium salt, 40–60 Ci/mmol, were both purchased from Du Pont NEN Research Products (Sydney, N.S.W., Australia); 5α-[1α,2α(n)-^{3}H]androstane-3α, 17β-diol, 40–60 Ci/mmol, [1,2,6,7-^{3}H]testosterone, 80–110 Ci/mmol and [1,2,6,7-^{3}H]androst-4-ene-3,17-dione, 80–110 Ci/mmol, were purchased from Amersham Australia, (North Ryde, N.S.W.). These tritiated labelled steroids were purified over Sephadex LH 20 before use to ensure maximum purity, and then checked monthly. The testosterone tracer [^{125}I]testosterone) was prepared as T-3-iodo [^{125}I]histamine and was kindly provided by the Department of Chemical Pathology, Prince Henry's Hospital. The [^{125}I]17OHP tracer was purchased from Baxter, E.I.R. (Wurenlingen, Switzerland).

Buffers and solutions. Phosphate-buffered saline 0.01 M, pH 7.4 (PBS) was prepared by dissolving 0.45 g of $NaH_2PO_4 \cdot 2H_2O$, 1.3 g $Na_2HPO_4 \cdot 2H_2O$ 9.0 g NaCl, 0.1 g sodium azide (NaN_3) in 1 l of distilled water, with the pH adjusted to 7.4. PBS with 0.2% bovine serum albumin (PBS–BSA) was prepared by addition of 2.0 g/l of BSA, Conn fraction V, purchased from Miles Scientific. Other buffer reagents were purchased from Ajax Chemicals (Sydney, Australia). Polyethylene glycol solution (PEG) was prepared by mixing 60 g of PEG (BDH, Kilsyth, Victoria, Australia) with 9 g NaCl and 0.1 g NaN_3. Sephadex LH 20 gel and dextran T70 were purchased from Pharmacia, Uppsala, Sweden, Norit A charcoal from Fisher Scientific Company (N.J.) and PCS scintillation fluid from Amersham Australia (Sydney, Australia). Sodium acetate buffer 0.2 M, pH 4.6 was prepared by mixing glacial acetic acid (12 ml to 1 l with distilled water) and sodium acetate trihydrate (27 g/l) in proportion of 3:2 (acid:acetate). Dextran-coated charcoal (DCC) was prepared by mixing 0.5% charcoal and 0.05% dextran T70 in PBS.

Radioimmunoassays

DHEA-S. DHEA-S was measured in duplicate with kits purchased from Radioassay Systems Laboratories Inc. (Carson City, Calif.) which allows direct assay of diluted serum. Values so obtained correlate very well with the tritiated assay of Buster and Abraham[27], and this correlation was confirmed by preliminary validation studies in our laboratory. Blank values determined by assay of water and sera from prepubertal (1–5 yr) children showed levels below the lowest standard (and detection limit) of 0.25 μmol/l. In our hands, the inter- and intra-assay variation was 5.9% ($n = 30$) and 4.8% ($n = 17$) at a level of 3.5 μmol/l, and 6.2% ($n = 30$) and 3.3% ($n = 10$) at a level of 8.3 μmol/l. Cross-reactivity with the antiserum was: DHEA 58%, androstenedione 39%, androsterone 30%, testosterone 0.6% and 5α-dihydrotestosterone <0.01%. Since the level of DHEA-S in blood is about 3 orders of magnitude higher than those of these other unconjugated steroids, their interference in the DHEA-S assay is negligible.

SHBG. SHBG was measured in duplicate with kits purchased from Farmos Diagnostica (Oulunsalo, Finland), allowing an immunoradiometric assay directly on diluted serum. Buffer (blank) values were immeasurably low, and in our hands the inter- and intra-batch variation was 8.8% ($n = 13$) and 2.5% ($n = 10$) at a level of 19 nmol/l, and 3.9% ($n = 12$) and 2.8% ($n = 10$) at 93 nmol/l. No other human serum proteins are known to cross-react with the combination of antibodies employed in this assay.

Androstenedione (AD). In this procedure, 300 μl of serum was extracted with 3 ml of iso-octane and the organic phase evaporated to dryness and then taken up in 300 μl of PBS. The RIA was performed in duplicate with 100 μl of tritiated AD (diluted in PBS to given approximately 15,000 cpm/100 μl), 100 μl of AD antiserum (diluted according to manufacturer's instructions) and 300 μl of PBS. Samples were incubated overnight at room temperature, and bound and free fractions separated by incubation with 500 μl of cold DCC for 5 min, followed by cold centrifugation at 3000 rpm for 10 min. The bound fraction (supernatant) was counted in a Packard beta counter for 1 min. The efficiency of extraction was monitored by spiking pooled sera with tracer, with recoveries of 70 ± 3% (mean ± SD; $n = 85$); patient values were corrected accordingly. This extraction procedure utilising a non-polar solvent such as

iso-octane significantly reduces the extraction efficiency of more polar steroids which may have some cross-reactivity with the antiserum, thus obviating the need for prior chromatographic purification of the samples. In preliminary studies, chromatography of samples on Sephadex LH20 confirmed the specificity method. Female samples, with values of 4.5, 4.0, 3.6, 0.8, 5.8 and 3.9 nmol/l after iso-octane extraction, gave values of 4.4, 3.4, 3.9, 1.1, 5.4 and 2.8 respectively after extraction and chromatography on LH20 with benzene:methanol (95:5) as the eluting solvent ($r = 0.945$). Accuracy was determined by spiking female plasma to levels of 0.35, 0.70, 1.75, 3.5, 7.0, 10.5 and 17.5 nmol/l of pure AD standard, equilibrating for a minimum of 30 minutes and then taking the samples through the entire procedure. Recoveries of 83–103% were obtained ($r = 0.998$). Buffer blanks were negligible, and inter- and intra-assay variation was 9.5% ($n = 23$) and 7.5% ($n = 10$) at a level of 3.8 nmol/l, and 12% ($n = 23$) and 6.8% ($n = 10$) at 7.7 nmol/l. The limit of detection was 15 pg/tube. Cross-reactivities were 5α-androstanedione 37%, testosterone 1%, and DHEA 2%.

Testosterone (T). In this procedure, 150 μl of serum was extracted with 1.5 ml of diethyl ether, the organic phase evaporated to dryness and then taken up in 300 μl of PBS. The RIA was performed in duplicate with 100 μl of extract, 100 μl of antibody at a 1:2000 dilution, 100 μl of [^{125}I]T (20,000 cpm), 100 μl of sheep antirabbit serum and 100 μl of normal rabbit serum. Samples were incubated overnight at room temperature, and bound and free fractions separated by the addition of 2.0 ml of cold 6% PEG and cold centrifugation for 15 min at 3000 rpm. The supernatant was discarded and the radioactivity in the pellet determined in an LKB Wallac multigamma II gamma counter for 1 min. The extraction efficiency was tested by addition of tritiated T to pooled sera. Recovery values of >90% were consistently obtained. The inter- and intra-assay variation was 11.7% ($n = 12$) and 5.9% ($n = 10$) at 0.57 nmol/l, and 10.0% ($n = 18$) and 5.6% ($n = 10$) at 3.0 nmol/l. The limit of detection was 5 pg/tube, and buffer blanks were <5 pg/tube. Cross-reactivities for this antiserum were 5α-DHT 22%, and DHEA, androsterone, androstenedione and 17OHP all <1.0%. In preliminary studies, as with the AD assay, chromatographic purification on LH 20 confirmed the specificity of the antiserum, thus obviating the need for routine chromatography. Female serum samples with values of 2.3, 1.0, 2.1, 2.9, 0.7, 0.4, 1.7, 2.6, 1.3, 0.4, 1.9, 2.1 and 2.1 nmol/l of testosterone on solvent extraction only gave T values of 2.1, 0.9, 1.9, 2.5, 0.5, 0.3, 1.5, 2.2, 1.4, 0.3, 2.0, 2.3 and 2.1 respectively on LH20 chromatography ($r = 0.973$). Accuracy was determined by spiking female plasma with levels of 0.42, 0.83, 1.25, 2.0, 4.2 and 8.3 nmol/l of pure T and taking the spiked samples through the entire procedure. Recoveries of 85–115% were obtained ($r = 0.998$).

17α-Hydroxyprogesterone (17OHP). 17OHP was determined by extraction of 50 μl of serum with 1.5 ml of petroleum ether plus 2% absolute ethanol. The organic phase was evaporated to dryness and then taken up in 500 μl of PBS. The RIA was performed in duplicate with 100 μl of extract, 100 μl of [^{125}I]17OHP (12,000 cpm), 100 μl of antibody (diluted according to manufacturer's instructions), 100 μl of rabbit serum and 100 μl of sheep anti-rabbit antiserum. Samples were incubated overnight at room temperature, bound and free fractions separated by the addition of 2.0 ml of cold PEG and centrifugation at 3000 rpm, and the radioactivity in the pellet determined. The extraction efficiency was 85 ± 3.0% ($n = 80$) and the patient values were corrected accordingly. The inter- and intra-assay variation was 8.1% ($n = 17$) and 6.5% ($n = 10$) at 3.0 nmol/l and 5.2% ($n = 12$) and 3.4% ($n = 10$) at 19.4 nmol/l. The limit of detectability was 5 pg/tube, and buffer blanks were routinely less than 5 pg/tube. The cross-reactivities were progesterone 1.5%, 5α-pregnane-3,20-dione 0.3%, 20α-dehydroprogesterone <0.1% and cortisol <0.1%.

C-19 Diol Sulphates. 5-ADIOL-S and 3α-DIOL-S were measured by specific RIA following acid solvolysis and Sephadex LH20 chromatography according to the procedure described by Habrioux *et al.*[28] with some modifications. In this procedure, 100 μl of serum was first extracted with diethyl ether to remove the unconjugated steroids; a trace amount of tritiated 5-ADIOL (approximately 2000 dpm) was added and left to equilibrate for 30 min, to monitor the procedural losses. Acetate buffer (100 μl; pH 4.4, 0.2 M) and 0.15 g of sodium chloride were added followed by 200 μl of 1 M sulphuric acid. Ethyl acetate (4.0 ml) was then added and the mixture extracted for 5 min on a multivortex shaker. The extracts were then transferred to fresh glass tubes and incubated at 65°C for 2.5 h to allow solvolysis of the steroid sulphates. After cooling, the extracts were washed once with 1 ml of sodium bicarbonate (68 g/l), twice with distilled water and then evaporated to dryness. Chromatographic purification was then performed on Sephadex LH20 in benzene:methanol (95:5) on glass columns (120 × 12 mm) fitted with fritted glass discs (porosity 1); 3 g of gel was used, giving a column height of 34 mm. The dried extracts were dissolved in 2 × 300 μl of solvent mixture and applied to the column. The first 6 ml of eluate containing the less polar steroids such as AD, androsterone, DHEA, T and DHT was discarded and the following 16 ml containing the two diols was collected and evaporated to dryness. PBS–BSA buffer (2.0 ml) was added and 0.5 ml was counted to determine the recovery thoughout the procedure. Recoveries 65 ± 5% ($n = 20$), and the patient values corrected accordingly.

3α-DIOL-S. 3α-DIOL-S was determined with 100 μl of buffer extract, 100 μl of antiserum (diluted

according to manufacturer's instructions), 100 μl of tritiated 3α-DIOL (12,000 cpm) and 200 μl of PBS-BSA. The mixture was incubated at 4°C overnight; free and bound steroid were separated with 500 μl of cold DCC and the bound fraction counted. The detection limit was 20 pg/tube. The inter- and intra-assay variation were 14.9% ($n = 12$) and 8.3% ($n = 8$) at a level of 40 nmol/l. Accuracy was determined by spiking female plasma with levels of 70, 140 and 280 nmol/l of pure 3α-DIOL and then taking these through the entire procedure. The recoveries at the three levels were 106, 93 and 94% respectively ($r = 0.999$). The cross-reactivities of other steroids were androstenedione 3.5%, 5β-androstane-3α,17β-diol 2.2%, DHT 0.1%, 5α-androstane-3,17-dione <0.1%, epiandrosterone <0.1%, 5-androstene-3β,17β-diol <0.1%, and 5α-androstane-3β,17β-diol <0.1%.

5-ADIOL-S. 5-ADIOL-S was determined in duplicate on 20 μl of buffer extract, 100 μl of antiserum (diluted according to manufacturer's instructions), 100 μl of tritiated 5-ADIOL (12,000 cpm) and 280 μl of PBS-BSA. The mixture was incubated at 4°C overnight, with separation of free from bound steroid by cold DCC. The limit of sensitivity was 20 pg/tube and buffer blanks were routinely <20 pg. The inter- and intra-assay variation was 17.5% ($n = 14$) and 8.6% ($n = 10$) at 300 nmol/l and 14.3% ($n = 10$) and 6.8% ($n = 10$) at a level of 1100 nmol/l. Accuracy was determined by spiking female plasma with levels of 70, 140 and 280 nmol/l of pure 5-ADIOL and taking these spiked samples through the entire procedure. Recoveries at these levels were 101, 112 and 83% respectively ($r = 0.970$). The cross-reactivities of other steroids were 5α-androstane-3β,17β-diol 2.5%, testosterone 2%, 5α-DHT 1%, 5α-androstane-3α,17β-diol <0.1%, pregnenolone 1%, 17OH pregnenolone 0.4%, DHEA <0.1% and androstenedione <0.1%.

The efficiency of solvolysis was determined routinely with [7-^{3}H]DHEA-S ammonium salt which following solvolysis and extraction gave 85% recovery. The solvolysis efficiency of diolic sulphates was determined with [7-^{3}H]5-androstene-3β,17β-diol sulphate which was synthesized from [7-^{3}H]DHEA-S according to the procedure of Payne and Mason[29] as follows: 300×10^6 dpm of [7^3-H]DHEA-S was dissolved in 500 μl of pure methanol and cooled in ice; 2 mg of sodium borohydride was added and left to stand overnight at room temperature. Acetone (80 μl) was added to decompose the excess borohydride and after the addition of 25 μl of water, the mixture was placed in the freezer (−20°C). The residue was recrystallized from methanol:ether (1:1). The reduced sulphate was then purified on Sephadex LH20 according to the method of Huhtaniemi *et al.*[30], to separate mono- and disulphated steroids in the solvent system chloroform:methanol (1:1) with 0.01 M sodium chloride. The peak elution volume, determined by collection of 2 ml fractions, was found to be 4–8 ml, and this fraction was collected and subjected to solvolysis. The solvolytic efficiency was 90%. Following solvolysis, the free steroid was subjected to LH20 chromatography in the same system as described above for the measurement of 5-ADIOL and 3α-DIOL to verify the identity of synthesized tritiated 5-ADIOL-S. Again 2 ml fractions were eluted and counted; the results showed that at least 90% of the radioactive material eluted in the same polar zone (6–22 ml) as authentic [1,2,^{3}H(n)] androstene-3β,17β-diol, therefore confirming its identity. Excellent efficiency of cleavage of diol sulphates is thus obtained by this method of solvolysis.

Serum LH and FSH were determined with kit reagents purchased from Amersham Australia (North Ryde, N.S.W.). In our hands the inter- and intra-assay variations ($n = 10$) was 9.8 and 4.6% for LH at a level of 22 μ/l respectively, and 5.0 and 3.5% for FSH at a level of 16.8 μl/ respectively. Cross-reactivities for the LH assay were: FSH 0.8%, TSH 3.8% and HCG 22.9% and for the FSH assay: LH < 0.1%, TSH < 0.1% and HCG < 0.1%.

Statistical analysis

All results are presented as mean ± SEM; in addition, the 95% confidence limits are given for the control population. The Mann–Whitney Test (unpaired) for non-parametric distribution of data was used to determine statistical significance when data were compared. All statistical analysis was performed with a Minitab programme on an IBM personal computer.

RESULTS

The mean serum levels of DHEA-S, 5-ADIOL-S, 3α-DIOL-S, AD, T, SHBG, FAI, 17OHP, LH, FSH and LH to FSH ratio are shown in Table 1.

Table 1. Serum levels of steroids, SHBG, FAI, LH and FSH in PCO patients and controls (mean ± SE). Values in parentheses are 95% confidence limits for the control subjects

Hormone	Controls	PCO
DHEA-S (μmol/l)	4.4 ± 0.2, $n = 73$ (1.2–7.6)	7.3 ± 0.5* $n = 28$
5-ADIOL-S (nmol/l)	267 ± 10, $n = 50$ (122–412)	516 ± 51* $n = 28$
3α-DIOL-S (nmol/l)	52 ± 2, $n = 57$ (26–78)	130 ± 8.5* $n = 28$
AD (nmol/l)	3.4 ± 0.2, $n = 50$ (1.2–5.8)	11.3 ± 1.1* $n = 26$
T (nmol/l)	1.5 ± 0.1, $n = 59$ (0.7–2.3)	3.3 ± 0.2* $n = 28$
SHBG (nmol/l)	65 ± 2.5, $n = 51$ (30–100)	31 ± 2.9* $n = 28$
FAI	2.4 ± 0.1, $n = 51$ (0.5–4.3)	12.5 ± 1.4* $n = 28$
17OHP (nmol/l)	2.8 ± 0.2, $n = 36$ (<5.8)	5.1 ± 0.8** $n = 22$
LH (U/l)	3–18 (Ref. range)	17.5 ± 2.7 $n = 24$
FSH (U/l)	2–10 (Ref. range)	5.4 ± 0.5 $n = 24$
LH:FSH ratio	1.0–1.6 (Ref. range)	2.8 ± 0.3 $n = 24$

*$P < 0.001$; **$P < 0.01$.

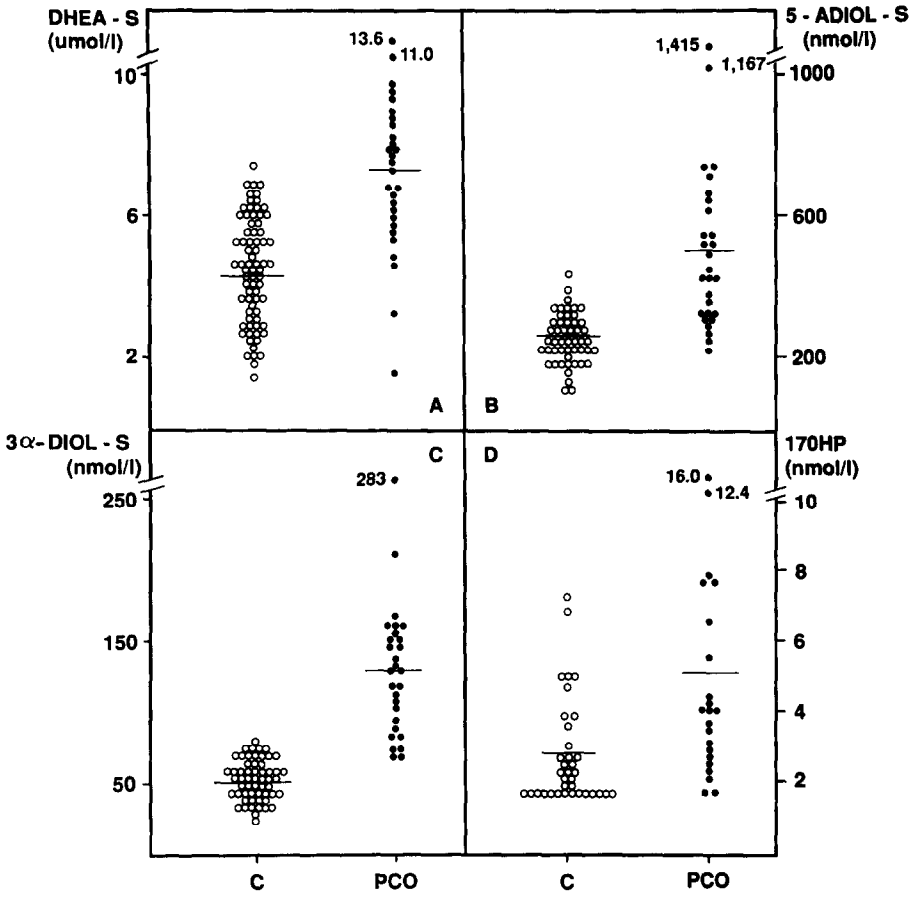

Fig. 1. Concentrations of serum DHEA-S, 5-ADIOL-S, 3α-DIOL-S and 17OHP in normal women (open circles) and women with PCO (closed circles). The horizontal lines indicate the mean values.

Individual values for all steroids measured and SHBG are shown in Figs 1 and 2.

The mean levels of the sulphoconjugated steroids DHEA-S, 5-ADIOL-S and 3α-DIOL-S were all significantly elevated in PCO patients compared with control, as were the mean levels of the unconjugated steroids AD, T and 17OHP. Of the 28 patients with PCO, DHEA-S was elevated (>2 SD) in 14, while 5-ADIOL-S was elevated in 16, with considerable overlap of these two steroids with the normal population, as was also the case for 17OHP. The mean SHBG level was significantly depressed, and the mean FAI significantly raised, in PCO patients.

Levels of LH and FSH were within the reference range for normal women during the follicular or luteal phase of the menstrual cycle. In contrast, however, the mean ratio of LH to FSH in patients with PCO was twice that of normal women [31].

DISCUSSION

Most *in vitro* studies have shown that in PCO the ovaries, follicular fluid, and isolated theca and stroma contain or produce increased amounts of testosterone, androstenedione and dehydroepiandrosterone, so that PCO is classically characterised by elevated blood androgen levels [1]. The elevated mean levels of AD and T in our group of patients with hirsutism and PCO is in agreement with these studies. What remains unclear in PCO is the extent of involvement of the adrenal gland, and the pathogenic mechanisms involved in the hirsutes. Lobo *et al.*[32] have shown that while hyperandrogenaemia is a hallmark of PCO it need not necessarily be accompanied by hirsutism. The ovary and/or adrenal clearly provides substrates for androgen action, and the manifestation of hirsutism depends upon peripheral utilisation of these androgen substrates in target tissues such as skin, under the action of 5α-reductase, as reflected by elevation of 5α-androstane-3α,17β-diol glucuronide [22, 33]. Kutten *et al.*[34] have also suggested that the clinical expression of hirsutism depends mainly on the capacity of the skin to utilise circulating androgens, whatever their levels.

To examine androgen production by the peripheral compartment, we have studied the level of 3α-DIOL-S as a possible marker of peripheral androgen metabolism in target tissues such as the skin. Habrioux *et al.*[28] in a study of 9 hirsute women measured both free and conjugated 3α-DIOL and 5α-androstane-3β,17β-diol, and concluded that

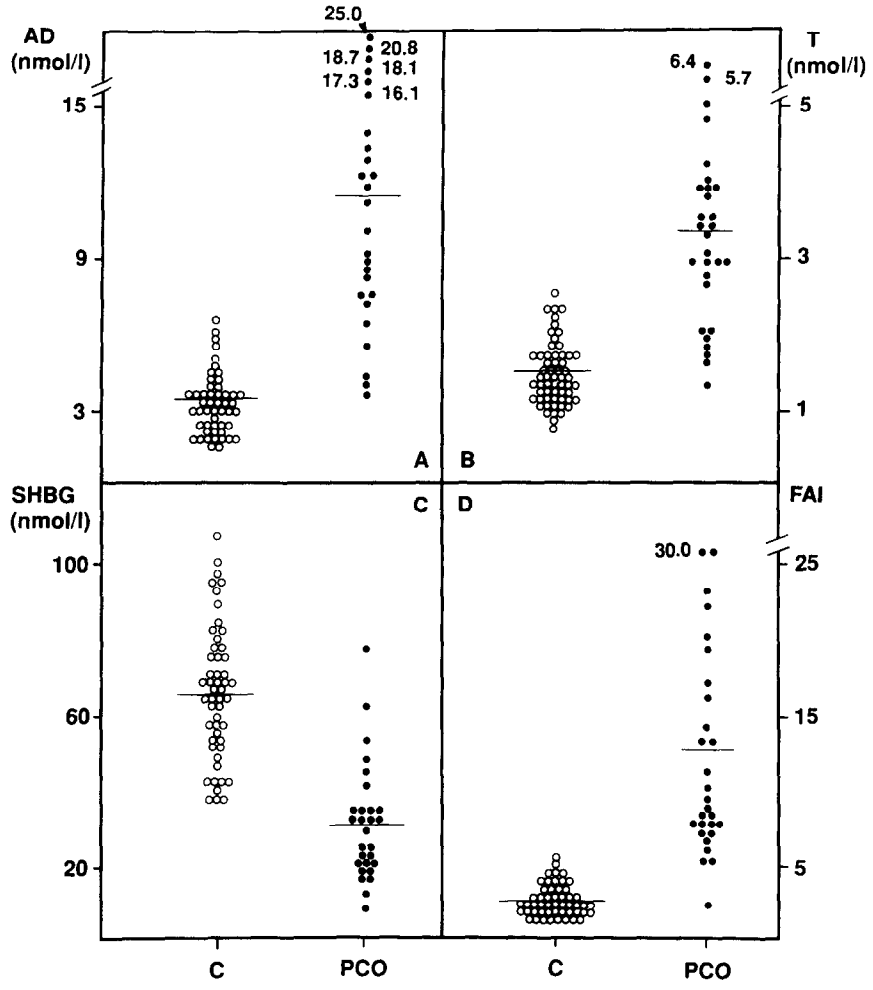

Fig. 2. Concentrations of serum AD, T, SHBG and FAI in normal women (open circles) and women with PCO (closed circles). The horizontal lines indicate the mean values.

3α-DIOL-S gave more information than the unconjugated form in pathological cases. Matteri *et al.*[35] measured 3α-DIOL-S in normal women and men, and found similar female levels (60 ± 13, M ± SD) as we did (52 ± 13); they also found that 3α-DIOL-S and C-19 sulphates are directly formed in skin. In our group of PCO patients, 24 of the 28 showed an elevated level of 3α-DIOL-S (> M + 2 SD), while the remaining four had 3α-DIOL-S levels at the upper limit of normal. 3α-DIOL-S is formed from 5α-DHT under the action of 5α-reductase, and 3α-DIOL may then be sulphated by tissue steroid sulphohydrolase to form the sulphoconjugate. As for 3α-DIOL-glucuronide, 3α-DIOL-S may therefore also be an important marker of peripheral androgen metabolism in PCO patients with hirsutism. Whether 3α-DIOL-S may play a role in the aetiology of the hirsutism is yet unclear; in this context, Wright *et al.*[36] found that in the subcellular microsomal fraction of pubic skin of women, there was some back conversion of DHT to 3α-DIOL, and that 3α-DIOL may be an active androgen in itself rather than merely an end metabolite of DHT. In this regard, it is of interest to speculate that 3α-DIOL-S, in addition to its synthesis in peripheral tissues, may also undergo solvolysis by steroid sulphatase releasing free 3α-DIOL to tissues.

The mean levels of both DHEA-S and 5-ADIOL-S were significantly elevated in PCO patients compared with control. There was considerable overlap of both DHEA-S and 5-ADIOL-S with normal, but serum DHEA-S was clearly above normal limits in 14 of the 28 patients, while 5-ADIOL-S was raised in 16 of 28, and both were raised in 11 of 28 PCO patients. These findings indicate a close association of the two sulphated steroids, lending support to the studies of Baulieu *et al.*[37] who reported a direct oxidation *in vivo* of 5-ADIOL-S to DHEA-S without splitting the ester link. It is generally accepted that DHEA-S is almost exclusively of adrenal origin and has been utilised as a marker of adrenal androgen synthesis. Our results indicate that 5-ADIOL-S may also be of adrenal origin and as such may be a new marker of adrenal androgen production, so that the measurement of 5-ADIOL-S and DHEA-S may provide a more reliable assessment of adrenal involvement in

patients with PCO. Goldzieher *et al.*[38] suggested that the androstenedione derivative, 11β-hydroxyandrostenedione (11OHAD) might serve as a unique marker of adrenal androgen production. Studies by Polson *et al.*[39], however, found normal levels of 11OHAD in PCO patients, while Putz *et al.*[40] found somewhat higher levels than normal in their PCO patients; neither study reported DHEA-S levels as a comparison. As suggested by Horrocks *et al.*[18], it seems possible that the adrenal abnormality leading to elevated levels of DHEA-S in PCO may be a secondary phenomenon, reflecting a relative deficiency of 3β-hydroxysteroid dehydrogenase due to elevated oestrogen concentrations. If such is the case, an elevation of neither AD nor 11OHAD would be expected in PCO. Alternatively, if the adrenal abnormality is primarily one of trophic factors, such as cortical androgen stimulating hormone, then an elevation of 11OHAD might be expected. The usefulness of 11OHAD as a marker of adrenal androgen production may then lie in its ability to point to specific mechanisms of overstimulation, which to date have been not completely resolved.

The presence and measurement of 5-ADIOL-S in blood was first reported by Vihko[41] using gas chromatography and mass spectrometry. Kalliala *et al.*[42] measured 5-ADIOL-S in human ovarian vein blood and concluded that normal ovaries neither secrete nor trap steroid sulphates such as 5-ADIOL-S and DHEA-S. Sirinathsinghji and Mills[43] found no change in 5-ADIOL-S production on LH stimulation of women with PCO, and suggested that it was therefore unlikely that the ovaries are a source of production of 5-ADIOL-S; Laatikainen and Vihko[44] studied 5-ADIOL-S in normal women during the luteal and follicular phases of the cycle and found no significant difference between them, again suggesting that normal ovaries have little effect on this steroid sulphate. Wallace and Silberman[45], however, have reported that unconjugated 5-ADIOL could be converted to its sulphate, but only in trace amounts, by homogenates of normal human ovaries. The sulphated steroids DHEA-S and 5-ADIOL-S may also play a role in the aetiology of hirsutism in PCO. Kaufman *et al.*[46] have demonstrated sulphatase activity in genital skin of adult men and women, and concluded that despite a low conversion ratio of DHEA-S to DHT, it may become significant with pathological elevations of DHEA-S.

In summary, the majority of patients with PCO showed elevated levels of AD, T, FAI, and 3α-DIOL-S, plus depressed SHBG, which reflect biochemical abnormalities in the ovarian and peripheral compartments; approximately half of our patients with PCO demonstrated elevated levels of DHEA-S and 5-ADIOL-S, reflecting adrenal hyperandrogenism. Hence, a comprehensive steroid profile, enabling the identification of androgen abnormality in the ovarian, adrenal and peripheral compartments should include the evaluation of each of these hormones, as well as 3α-androstanediol glucuronide, as previously suggested [22].

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REFERENCES

1. Barnes R. and Rosenfield R. L.: The polycystic ovary syndrome: pathogenesis and treatment. *Ann. Intern. Med.* **110** (1989) 386–399.
2. Yen S. S.: The polycystic ovary syndrome. *Clin. Endocr.* **12** (1980) 177–208.
3. Goldzieher M. W. and Green J. A.: The polycystic ovary. Its clinical and histological features. *J. clin. Endocr. Metab.* **22** (1962) 235–238.
4. Moll G. W. Jr and Rosenfield R. L.: Plasma free testosterone in the diagnosis of adolescent polycystic ovary syndrome. *J. Pediat.* **102** (1983) 461–464.
5. Rebar R., Judd H. L., Yen S. S. C., Rakoff J., Vandenberg G. and Naftolin F.: Characterization of the inappropriate gonadotropin secretion in polycystic ovary syndrome. *J. Clin. Invest.* **57** (1976) 1320–1329.
6. Givens J. R., Anderson R. N., Umstot E. S. and Wiser W. L.: Clinical findings and hormonal responses in patients with polycystic ovarian disease with normal versus elevated LH levels. *Obstet. Gynec.* **47** (1976) 388–394.
7. Berger M. J., Taymor M. L. and Patton W. C.: Gonadotrophin levels and secretory patterns in patients with typical and atypical polycystic ovarian disease. *Fert. Steril.* **26** (1975) 619–626.
8. Bardin C. W., Hembree W. D. and Lipsett M. D.: Suppression of testosterone and androstenedione production rates with dexamethasone in women with idiopathic hirsutism and polycystic ovaries. *J. Clin. Endocr. Metab.* **28** (1968) 1300–1306.
9. Rosenfield R. L., Ehrlich E. N. and Gleary R. F.: Adrenal and ovarian contributors to the elevated free plasma androgen levels in hirsute women. *J. Clin. Endocr. Metab.* **34** (1972) 92–98.
10. Abraham G. E. and Chakamakjian Z. H.: Plasma steroids in hirsutism. *Obstet. Gynec.* **44** (1974) 171–175.
11. Tulchinsky D. and Chopra I. J.: Estrogen-androgen imbalance in patients with hirsutism and amenorrhea. *J. Clin. Endocr. Metab.* **39** (1974) 164–169.
12. De Vane G. W., Czekala W. M., Judd H. L. and Yen S. S. C.: Circulatory gonadotrophins, estrogens and androgens in polycystic ovarian disease. *Clin. Obstet. Gynec.* **121** (1975) 496–500.
13. Kirschner A. and Jacobs J.: Combined ovarian and adrenal catheterization to determine the site(s) of androgen overproduction in hirsute women. *J. Clin. Endocr. Metab.* **33** (1971) 199–209.
14. Givens J. R., Anderson R. N., Ragland J. B., Wiser W. L. and Umstot E. S.: Adrenal function in hirsutism. Diurnal changes and response of plasma androstenedione, testosterone, 17-hydroxyprogesterone, cortisol, LH and FSH to dexamethasone and ACTH. *J. Clin. Endocr. Metab.* **40** (1975a) 988–1000.
15. Judd H. L., McPherson R. A., Rakoff J. S. and Yen S. S. C.: Correlation of the effects of dexamethasone administration on urinary 17-ketosteroid and serum androgen levels in patients with hirsutism. *Am. J. Obstet. Gynec.* **128** (1977) 408–417.
16. Horton R. and Neisler J.: Plasma androgens in patients with polycystic ovary syndrome. *J. Clin. Endocr. Metab.* **28** (1968) 479–484.

17. Lachelin G. C. L., Barnett M., Hopper B. R., Brink G. and Yen S. S. C.: Adrenal function in normal women and women with polycystic ovary syndrome. *J. Clin. Endocr. Metab.* **49** (1979) 892–898.
18. Horrocks P. M., Kandeel F. R., London D. R., Butt W. R., Lynch S. S., Holder G. and Logan-Edwards R.: ACTH function in women with the polycystic ovarian syndrome. *Clin. Endocr.* **19** (1983) 143–150.
19. Carmina E., Rosato F. and Janni A.: Increased DHEA-S levels in PCO syndrome. Evidence for the existence of two subgroups of patients. *J. Endocr. Invest.* **9** (1986) 5–9.
20. Loughlin T., Cunningham S., Moore A., Culliton M., Smyth P. P. A. and McKenna T. J.: Adrenal abnormalities in polycystic ovary syndrome. *J. Clin. Endocr. Metab.* **62** (1986) 142–147.
21. Moll C. W. Jr., Rosenfield R. L. and Helke J. H.: Estradiol-testosterone binding interactions and free plasma estradiol under physiological conditions. *J. Clin. Endocr. Metab.* **52** (1981) 868–874.
22. Lobo R. A.: Polycystic ovary syndrome and hirsutism. In *Progress in Endocrinology* (Excerpt. Med. Congr. Series 799) (Edited by H. Imura, K. Shizume and S. Yoshida). Elsevier, Amsterdam (1988).
23. Hoffman D. I., Klove K. and Lobo R. A.: The prevalence and significance of elevated dehydroepiandrosterone sulfate levels in anovulatory women. *Fert. Steril.* **42** (1984) 76–81.
24. Lobo R. A.: The role of the adrenal in polycystic ovary syndrome. *Semin. Reprod. Endocr.* **2** (1984) 251–262.
25. Givens J. R., Anderson R. N., Ragland J. B., Wiser W. L. and Umstot E. S.: Adrenal function in hirsutism. Diurnal change and response of plasma androstenedione, testosterone, 17α-hydroxyprogesterone, cortisol, LH and FSH to dexamethasone and 0.5 unit of ACTH. *J. Clin. Endocr. Metab.* **40** (1975) 988–1000.
26. Child R. F., Bu'lock D. E. and Anderson D. C.: Adrenal steroidogenesis in hirsute women. *Clin. Endocr.* **12** (1980) 595–601.
27. Buster J. E. and Abraham G. E.: Radioimmunoassay of plasma dehydroepiandrosterone sulphate. *Analyt. Lett.* **5** (1972) 543–551.
28. Habrioux G., Desfosses B., Condom R., Faure B. and Jayle M.: Simultaneous radioimmunoassay of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol unconjugated and conjugated in human serum. *Steroids* **32** (1978) 61–71.
29. Payne A. H. and Mason M.: Conversion of dehyroepiandrosterone sulphate to androst-5-endiol-3-sulphate by soluble extracts of rat testis. *Steroids* **6** (1965) 323–337.
30. Huhtaniemi I., Luukkainen T. and Vihko R.: Identification and determination of neutral steroid sulphates in human foetal adrenal and liver tissue. *Acta Endocr.* **64** (1970) 273–286.
31. Aono T., Miyake A., Shioji T., Kinugasa T., Onishi T. and Kurachi K.: Impaired LH release following endogenous oestrogen administration in patients with amenorrhea-galactorrhea syndrome. *J. Clin. Endocr. Metab.* **42** (1976) 696–702.
32. Lobo R. A., Goebelsmann J. and Horton R.: Evidence for the importance of peripheral tissue events in the development of hirsutism in polycystic ovary syndrome. *J. Clin. Endocr. Metab.* **57** (1983) 393–397.
33. Serafini P., Ablan F. and Lobo R. A.: 5α-reductase activity in the genital skin of hirsute women. *J. Clin. Endocr. Metab.* **60** (1985) 349–355.
34. Kutten F., Couillin P., Girard F., Billoud L., Vincens M., Boucekkine C., Thalabard J. C., Maudelonde T., Spritzer P., Mowszowicz I., Boue A. and Mauvais-Jarvis P.: Late-onset adrenal hyperplasia in hirsutism. *New Engl. J. Med.* **313** (1985) 224–231.
35. Matteri R. K., Stanczyk F. Z., Kaufman F. K., Delgado C., Gentzchein E. and Lobo R. A.: *Program of the 70th Annual Meeting of the Endocrine Society*, New Orleans, Louisiana (1988) p. 207 (Abstr.).
36. Wright F. and Giacomini M.: Reduction of dihydrotestosterone to androstanediols by human female skin *in vitro*. *J. Steroid. Biochem.* **13** (1980) 639–643.
37. Baulieu E. E., Corpechot C. and Emiliozzi R.: H^3-androstene-3β,17β-diol-S^{35}-3-sulfate to H^3-dehydroisoandrosterone-S^{35}-sulfate *in vivo*: an example of "direct metabolism of a steroid conjugate". *Steroids* **4** (1963) 429–451.
38. Goldzieher J. W., de la Pena A. and Aivaliotis M. M.: RIA of androstenedione, testosterone and 11β-hydroxy-androstenedione after chromatography on Lipidex 5000. *J. Steroid Biochem.* **9** (1978) 169–173.
39. Polson D. W., Reed M. J., Franks S. S., Scanlon M. J. and James V. H. T.: Serum 11β-hydroxyandrostenedione as an indicator of the source of excess androgen production in women with polycystic ovaries. *J. Clin. Endocr. Metab.* **66** (1988) 946–950.
40. Putz Z., Hampel R., Veleminsky J., Kreze A., Sulcova J. and Starka L.: Radioimmunoassay of 11β-hydroxy-androstenedione in laboratory diagnosis of selected endocrine disorders. *J. Clin. Chem. Clin. Biochem.* **25** (1987) 723–727.
41. Vihko R.: Gas Chromatographic-mass spectrometric studies on solvolyzable steroids in human peripheral plasma. *Acta Endocr.* **109** (Suppl.) (1966) 1–67.
42. Kalliala K., Laatikainen T., Luukkainen T. and Vihko R.: Neutral steroid sulphates in human ovarin vein blood. *J. Clin. Endocr.* **30** (1970) 533–535.
43. Sirinathsinghji D. J. S. and Mills I. H.: Effect of human pituitary luteinizing hormone administration on plasma levels of dehydroepiandrosterone, androstenediol and their sulphates and testosterone in women with secondary amenorrhea. *J. Endocr.* **98** (1983) 201–210.
44. Laatikainen T. and Vihko R.: Plasma neutral steroid sulphates and the menstrual cycle. *J. Steroid Biochem.* **2** (1971) 173–177.
45. Wallace E. and Silberman N.: Biosynthesis of steroid sulphates by human ovarian tissue. *J. Biol. Chem.* **239** (1964) 2809–2812.
46. Kaufman F. R., Matteri R. K., Stanczyk F. Z., Gentzchein E., Delgado C. and Lobo R. A.: *Program of the American Pediatric Society and Pediatric Research Society*, Washington, D.C. (1988) p. 279A (Abstr.).