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URINARY 5-ENE-STEROID EXCRETION IN NON-CLASSICAL CONGENITAL ADRENAL HYPERPLASIA DUE TO 3β-HYDROXYSTEROID DEHYDROGENASE DEFICIENCY (NC-3BHSD)

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Summary—The diagnosis of non-classical 3β-hydroxysteroid dehydrogenase deficiency (NC-3BHSD) is made either on the basis of significantly elevated serum levels of basal and post-ACTH 5-ene-steroids or by the presence of elevated urinary 5-ene-steroid metabolites. There has been only one report to date describing a single patient where the diagnosis was based on both serum and urinary 5-ene-steroid levels. We, therefore, measured both serum 5-ene-steroid responses to ACTH 1–24 (by RIA) and urinary 5-ene-steroid metabolites (GC–MS) in 42 hirsute premenopausal women. While the serum 5-ene-steroid profile was consistent with NC-3BHSD in 5 women, only 2 of them had increased excretion of 5-ene-steroid metabolites. Elevated 5-ene-steroid excretion was also observed in several patients with normal serum 5-ene-steroids. Detection of NC-3BHSD by either elevated serum 5-ene-steroids or increased urinary excretion of their metabolites in isolation may not therefore be reliable.

INTRODUCTION

Classical Congenital Adrenal Hyperplasia (CAH) due to 3BHSD deficiency may result in the development of ambiguous genitalia, severe salt-wasting and is a potentially life threatening adrenal disorder [1, 2]. The diagnosis of this rare specific enzyme deficiency was originally made on the basis of markedly elevated urinary excretion of 5-ene-steroids [1]. However, Shackleton *et al.*[3] have also measured significant excretion of saturated steroid metabolites, such as pregnanetriol, in patients with this defect thereby indicating intact peripheral 3BHSD activity [4, 5].

More recently, a milder defect of adrenal 3BHSD activity has been described in women with peri-pubertal onset of hirsutism, acne and oligomenorrhoea [2, 6, 7] and it has been suggested that, like non-classical 21-hydroxylase deficiency (NC-21OH), mild 3BHSD deficiency is an allelic variant of the classical defect [8]. However, while the diagnosis of NC-21OH by both serum (pre- and post-ACTH 1–24 stimulation) and urinary steroid analysis is well established [9] and may be confirmed by HLA typing and restriction fragment length polymorphisms of genomic DNA using cDNA probes [10], the diagnosis of NC-3BHSD remains relatively poorly defined. Consequently, there is marked variability in the prevalence estimates for this defect [6, 8, 11, 12].

While the diagnosis of several enzyme defects including NC-3BHSD, solely on the basis of urinary steroid excretion profiles in a group of hyperandrogenic women has recently been described [13], there has been only one report of a combined serum and urinary 5-ene-steroid profile of this defect in a single 17-year-old girl [14].

We therefore decided to adopt the post-ACTH 1–24 stimulated serum steroid criteria recently described by Pang *et al.*[8], from a comprehensive investigation of a large group of hyperandrogenic women, to detect mild adrenal 3BHSD deficiency in hirsute pre-menopausal women presenting at several clinics in Australia. The aim of the study was to compare the level of urinary 5-ene-steroid excretion in these women with the serum levels of 5-ene-steroids.

EXPERIMENTAL

Subjects

A Synacthen (Ciba) stimulation test (blood drawn before and 60 min following 250 μg bolus i.v. ACTH

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Abbreviations: DHEA, dehydroepiandrosterone = 3β-hydroxyandrost-5-en-17-one. 16-OHDHEA, 16-hydroxy-dehydroepiandrosterone = 3β,16(α & β)-hydroxyandrost-5-en-17-one. Adiol, androstenediol = androst-5-ene-3β,17β-diol. PT16, pregnene-16α-triol = pregn-5-ene-3β,16α,20α-triol. PT17, pregnene-17α-triol = pregn-5-ene-3β,17α,20α-triol. Pregnanetriol = 3α,17α,20α-trihydroxy-5β-pregnan-11-one. 5β,17-hydroxypregnanolone = 3α,17α-dihydroxy-5β-pregnan-20-one.

1–24) was performed on 42 hirsute (Ferriman–Gallwey score >10) premenopausal women (aged 16–35 years) of varying ethnic origin and ideal body weight (body mass index <25) who presented at several clinics throughout Australia over a period of 6 months. A 24 h urine sample was obtained from each patient prior to the test. These women were selected on the basis of no evidence of either polycystic ovarian syndrome (PCOS, by pelvic sonography and serum LH levels) or NC-21OH. One-third of the women had menstrual irregularity and one in four presented with marked cystic acne and/or female pattern androgenic alopecia. 24 h urine samples were also collected from 18 normal premenopausal women (aged 22–31 years) of similar body mass index. The normal range of serum steroid responses to ACTH stimulation were similarly determined by a standard Synacthen test following urine collection.

To compare the urinary 5-ene-steroid profile in non-classical 3BHSD patients with that of the classical variants a 24 h urine sample was also collected from a 22-year-old male patient with classical CAH due to 3BHSD deficiency before and following a chronic ACTH stimulation test (i.v. infusion of 4 mg Synacthen).

Methods

Serum steroid measurements. Serum concentrations of 17-hydroxyprogesterone (17OHP) and progesterone (P) were measured using RIA kits (Mallinkrodt Ltd, Sydney, Australia). 17-hydroxypregnenolone (17Pe), dehydroepiandrosterone (DHEA), DHEA-sulphate (DHEAS) and cortisol (F) were also measured by RIA kit methods (Radioassay Systems Laboratories Inc., Calif.). Intra- and inter-assay variation for serum 17OHP, P, DHEA, DHEAS and F were <5 and <7% respectively. The radioimmunoassay of androstenedione (adione) and testosterone (T) has recently been described in detail by Montalto *et al.*[15]. Intra-assay variation for both steroids was <7%, while the level of inter-assay variation was <9%. The accurate determination of 17Pe concentrations necessitated the use of Lipidex 5000 (Packard, Zurich, Switzerland) column chromatography in order to isolate this steroid from 17OHP which cross reacts significantly (32.4%) with the 17Pe antibody. The method is described elsewhere [16] and yields quantitative recovery of both 17Pe and 17OHP. Intra- and inter-assay variation for serum 17Pe was 4.7% ($n = 18$) and 8.1% ($n = 8$).

Urinary steroid analysis by gas chromatography. Steroid metabolites were extracted from 10 ml aliquots of urine using Sep-pak C18 columns (Waters Associates, Milford, Mass) as previously described [17]. 5-ene-steroids are predominantly excreted as sulphate conjugates and it has recently been shown that solvolysis of urinary steroids is a necessary pre-requisite for the detection of 3BHSD deficiency [18]. All urine samples therefore underwent acid solvolysis using 1 M HCL by an improved method recently reported by Khan and Taylor[19]. Internal standards were added to each sample and methoxamine and trimethylsilylimidazole (TMSI) derivitization was performed overnight. The derivitized products were purified using lipidex 5000 [20] and the methanol eluates evaporated under nitrogen and reconstituted in pyridine:hexamethyldisilizane:cyclohexane (1:1:100). Steroid separation and quantitation was carried out using a Varian 3400 gas chromatograph (GC). The samples (500 μl) were injected by an autosampling device into a 25 M × 0.22 mm (i.d.) bonded phase fused silica BP-1 column (Waters Associates) with a temperature gradient set from 180 to 310°C at 3°C/min. The split ratio of the injection system was 40:1, inlet pressure was 30 KPa, carrier gas (helium) flow at 24 ml/min and make up gas (nitrogen) at 35 ml/min. A flame ionization detector was used and the data analysed by a Varian Chromatography Data System 402, which identified all steroids by retention time and quantitated their concentrations by peak area comparison with the internal standards. The appropriate response factors were calculated from the standard samples comprising known concentrations of 16-hydroxy-DHEA (16-OHDHEA), pregn-5-ene-3β,16α,20α-triol (PT16), pregn-5-ene-3β,17α,20α-triol (PT17), androstenediol (Adiol) and DHEA purchased from Sigma Chemical Company (St Louis, Mo.). Confirmation of steroid identity and further analysis was by GC–mass spectrometry (GC–MS) using a Finnigan-Matt 212 mass spectrometer and a GC separation similar to that described.

Statistics

The data were normally distributed (according to the Wilks–Shapiro test) and analysed by Student's t-test.

RESULTS

The diagnosis of NC-3BHSD was based on criteria derived from a recent description of NC-3BHSD in a large group of hyperandrogenic women [8]. These criteria were 60 minute post-ACTH serum levels of 17Pe (>50 nmol/l), DHEA (>60 nmol/l), 17Pe/17OHP (>5.5) and DHEA/Adione (>13.0). 5 of the 42 hirsute women (Ferriman–Gallwey score >10) fulfilled all these criteria, with the exception of patient 4, whose adione response to ACTH was such that the DHEA-adione ratio fell just below the "cut off point", as shown in Table 1. While post-ACTH 1–24 (Synacthen) serum levels of the 5-ene-steroids and 5-ene/4-ene steroid ratios for those hirsute women who were not diagnosed with NC-3BHSD (on the basis that they failed to meet any one of these criteria) fell within the ranges of the normal group of women, also shown in Table 1, the levels for our 5 patients were significantly higher than normal.

Table 1. Post-synacthen serum levels of 5-ene-steroids and 5-ene/4-ene-steroid ratios in the NC-3BHSD patients and normal premenapausal women

Patient	17Pe	DHEA	DHEAS	DHEA/ADIONE	17Pe/17OHP
1	70.6	62.5	7.7	13.0	5.1
2	60.8	67.3	14.1	15.8	5.9
3	53.4	77.3	2.7	21.5	5.4
4	56.4	68.6	5.6	10.1	5.8
5	63.6	67.7	12.1	13.5	7.5
Normal women	30.8 ± 9.1	26.9 ± 5.3	4.5 ± 1.6	5.5 ± 3.7	3.6 ± 1.1

Mean $\pm$ SD. 17Pe and DHEA (nmol/l), DHEAS (μmol/l).

Table 2. Urinary 5-ene-steroid excretion (μmol/24 h) profiles for the NC-3BHSD patients and normal premenopausal women

Patient	DHEA	ADIOL	16-OHDHEA	PT17	PT16
1	0.50	0.30	<0.10	0.79	<0.10
2	4.51	2.94	1.10	0.70	<0.10
3	0.30	0.14	0.45	0.57	<0.10
4	0.62	0.16	0.63	0.49	<0.10
5	5.68	2.87	0.85	1.44	<0.10
Normal women	1.12 ± 0.55	0.46 ± 0.14	1.95 ± 1.59	0.41 ± 0.25	<0.10

Mean $\pm$ SD.

Table 3. Baseline and post chronic ACTH stimulated urinary excretion of 5-ene-steroid metabolites (μmol/24 h) for an adult male with classical adrenal 3BHSD deficiency

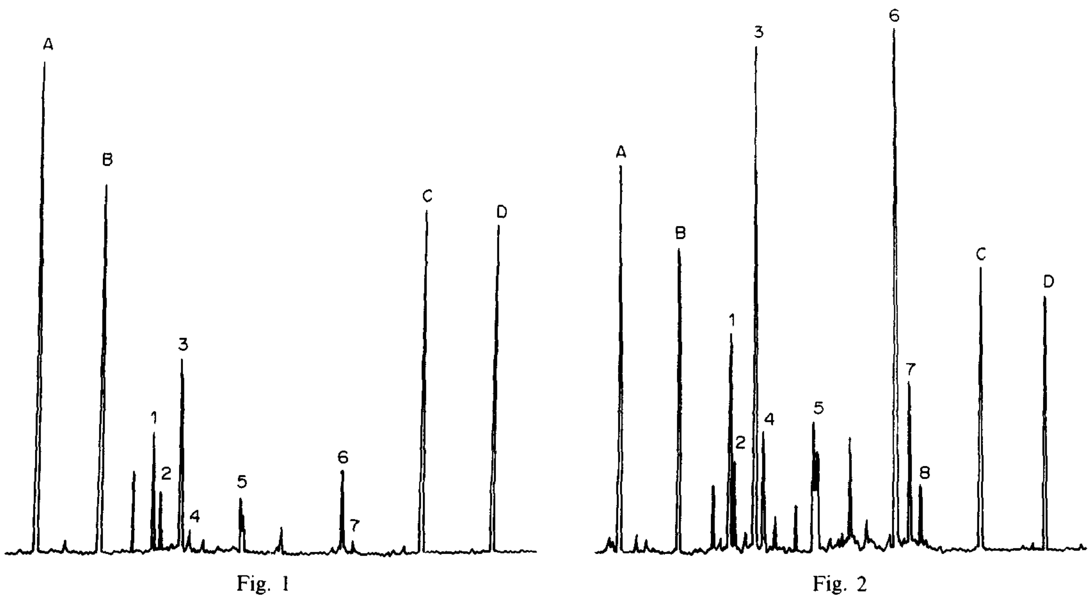
	DHEA	ADIOL	16-OHDHEA	PT17	PT16
Pre-ACTH	23.0	3.3	2.7	4.0	<0.1
Post-ACTH	54.0	6.0	6.9	97.2	12.6

The urinary excretion of DHEA, 16-OHDHEA (doublet peak on GC profile), Adiol, PT17 and PT16 for the normal women (mean $\pm$ SD) together with the individual 5-ene-steroid profiles of the 5 NC-3BHSD patients is shown in Table 2. The urinary 5-ene-steroid profiles for patients 1, 3 and 4 were normal. However, while levels of PT17 for patient 1 were just below the upper limit of the normal range (95% confidence limits), only patients 2 and 5 exhibited significantly elevated excretion of two or more urinary 5-ene-steroids, i.e. DHEA and Adiol. All 5 patients, however, had normal excretion of the 16-hydroxy metabolites, 16-OHDHEA and PT16. Moreover, we also noted increased urinary 5-ene-steroid levels in a number of hirsute women for whom serum 5-ene-steroid concentrations before and following an ACTH stimulation test were well within the normal range (data not shown). These observations contrast markedly with the baseline 24 h urinary steroid profile of an adult male patient diagnosed by our laboratory as suffering from classical CAH due to 3BHSD deficiency and illustrated in Fig. 1. Baseline 5-ene-steroid excretion (Table 3), with the exception of PT16 was significantly higher than normal in this patient. The marked 5-ene-steroid metabolite responses to a chronic ACTH infusion test, also described in Table 3 and illustrated by Fig. 2, unequivocally defines the site of this enzyme defect.

DISCUSSION

The measurement of urinary steroid excretion by either RIA or by means of gas liquid chromatography is a well established method for the diagnosis of a number of steroid hormone disorders [21–24], including the enzyme deficiencies underlying the considerable clinical heterogeneity of CAH [3]. The diagnosis of classical CAH due to 3BHSD deficiency can be made on the basis of significantly elevated levels of 5-ene-steroids in 24 h urine samples [1]. However, while the detection of NC-21OH by basal and post-synacthen serum concentrations of 17OHP and adione may be confirmed by measuring urinary excretion of pregnanetriol, pregnanetriolone and 5β,17-hydroxypregnanolone, the diagnostic criteria for the milder non-classical variant of adrenal 3BHSD deficiency are not so well defined. The diagnosis of NC-3BHSD has been predominantly based on varying criteria for both baseline and post ACTH stimulation levels of serum 5-ene-steroids [5–9, 11, 12]. This inconsistency may be responsible for the wide range of prevalence estimates for this disorder amongst the hyperandrogenic female population (1.8–16%). None of these reports, however, described the urinary 5-ene-steroid profile in women diagnosed with NC-3BHSD on the basis of these serum criteria.

Redmond *et al.* [13] however, have defined the diagnosis of NC-3BHSD solely in terms of the presence of elevated urinary 5-ene-steroid excretion [13]. The prevalence of this disorder amongst the hyperandrogenic female population was found to be approximately 9% but the authors made no mention of which specific urinary 5-ene-steroid levels were increased in these women. There has been only one report to date describing the diagnosis of



Figs 1 and 2. Urinary steroid gas chromatograph profile for a 22-year-old male patient with classical CAH due to 3BHSD deficiency (not receiving therapy) before (Fig. 1) and following (Fig. 2) chronic ACTH stimulation test. The urinary steroids underwent acid solvolysis as described previously [19] and were subsequently chromatographed as methoxamine-TMSI derivatives on a 25 M × 0.22 mm (i.d.) bonded phase fused silica BP-1 capillary column. Internal standards: A = 5α-androstane-3α,17β-diol, B = n-tetracosane, C = n-diacontane, D = cholesterol butyrate. Steroids: 1 = androsterone, 2 = etiocholanolone, 3 = dehydroepiandrosterone (DHEA), 4 = androstenediol, 5 = 16-hydroxyDHEA (doublet peak), 6 = 5-ene-pregnene-3β,17α,20α-triol, 7 = 5-ene-pregnene-3β,16α,20α-triol, 8 = tetrahydrocortisol.

NC-3BHSD (in a 17-year-old girl) by combined analysis of serum and urinary 5-ene-steroid concentrations [14].

The results from this study clearly show that the abnormal 5-ene-steroid responses to a short ACTH test, defined by Pang *et al.*[8] as indicative of NC-3BHSD, may not be associated with elevated urinary excretion of 5-ene-steroid metabolites. This may be a consequence of the mildness of the adrenal enzyme defect compared with that for NC-21OH. However, since peripheral conversion of 5-ene to 4-ene-steroids is a necessary prerequisite for the formation of active androgen at target sites and it has been shown to be intact in patients with classical 3BHSD deficiency [1, 4, 5, 14], it is likely that such peripheral enzyme activity is also intact in NC-3BHSD thereby resulting in relatively normal excretion of 5-ene-steroid metabolites despite mild adrenal hypersecretion of DHEA and 17Pe. The increased excretion of DHEA and Adiol in 2 of our patients may reflect a similar heterogeneity of this enzyme deficiency to that of NC-21OH. However, the presence of elevated 5-ene-steroid metabolites in 24 h urine samples from hyperandrogenic women exhibiting normal serum steroid responses to an ACTH test (data not shown), suggests that there may be an uncoupling between circulating levels of 5-ene-steroids and their respective metabolism to sulpho-conjugates prior to urinary excretion.

It is well established that significantly elevated urinary 5-ene-steroid excretion is associated with the diagnosis of adrenal carcinoma [25, 26]. However, the markedly increased levels of urinary 5-ene-steroids following an ACTH infusion test, shown by our patient with classical adrenal 3BHSD deficiency, leaves no doubt as to the site of this defect. A similar test may therefore be required to confirm the post-ACTH serum 5-ene-steroid diagnosis of NC-3BHSD.

In summary, we suggest that while GC and GC–MS analysis of urinary steroids may accurately diagnose several specific enzyme abnormalities, the detection of NC-3BHSD by the measurement of either 5-ene-steroids in serum or their metabolites in a basal urine sample may not be reliable in isolation.

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