

Variations of Steroid Hormone Metabolites in Serum and Urine in Polycystic Ovary Syndrome after Nafarelin Stimulation: Evidence for an Altered Corticoid Excretion*

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ABSTRACT

To evaluate the clinical relevance of testing pituitary-ovarian responses in patients suffering from polycystic ovary syndrome (PCOS) with the GnRH agonist nafarelin, a 1.2-mg dose of nafarelin was given intranasally to 19 women with PCOS and 15 healthy premenopausal women. The subsequent analysis of steroids in both serum and urine during the test was carried out at several time points for up to 24 h.

Serum levels of 17α -hydroxyprogesterone were elevated at all time points of the test in PCOS patients vs. controls [at baseline, 3.5 ± 0.2 vs. 1.8 ± 0.1 nmol/L ($P < 0.001$); at 24 h, 9.9 ± 0.9 vs. 4.9 ± 0.3 nmol/L ($P < 0.001$)]. Basal levels of androstenedione were higher in the patient group, but there was no significant change during the test in either group. Serum testosterone levels were also found to differ in PCOS patients compared with the control values at baseline (2.2 ± 0.2 vs. 1.5 ± 0.1 nmol/L; $P < 0.05$) and after nafarelin treatment (at 24 h, 3.2 ± 0.4 vs. 1.8 ± 0.2 nmol/L; $P < 0.05$). Serum estradiol levels rose significantly in both groups during the test; the posttest levels were significantly higher in PCOS than in controls.

The PCOS patients displayed a significant increase in androgen

and gestagen metabolites as well as in glucocorticoid metabolites excreted in the urine during the 24 h. In the control subjects, except for 17α -hydroxypregnanolone, which rose significantly, none of the urinary steroids investigated showed relevant changes during the nafarelin test. The posttest excretion of allo-tetrahydrocortisol (1.4 ± 0.2 vs. 0.3 ± 0.1 μ mol/g creatinine; $P < 0.001$) and the increase in 17α -hydroxypregnanolone excretion (1.4 ± 0.2 vs. 0.3 ± 0.1 μ mol/g creatinine; $P < 0.001$) were distinctly higher in PCOS patients than in the controls; the diagnostic sensitivity of the combination of both parameters was 89% at a 93% specificity. Thus, measurements of 17α -hydroxyprogesterone levels in serum and of urinary allo-tetrahydrocortisol and 17α -hydroxypregnanolone after nafarelin treatment make this stimulation test a valuable diagnostic tool for identifying PCOS patients. The significant changes in the excretion of urinary androgen and gestagen metabolites, unmasked by GnRH agonist stimulation, suggest a functional alteration of the pituitary-ovarian axis. The reason for the increased excretion of glucocorticoid metabolites after nafarelin stimulation remains to be clarified. (*J Clin Endocrinol Metab* 80: 280–288, 1995)

POLYCYSTIC ovary syndrome (PCOS) is an endocrinopathy commonly associated with menstrual irregularities; anovulatory infertility; skin manifestations such as

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A: androstenedione (androst-4-ene-3,17-dione); AD: androstenediol ($3\alpha,17\beta$ -dihydroxy-5 α -androstane); ADG: androstane-diol glucuronide ($3\alpha,17\beta$ -dihydroxy-5 α -androstane 3-glucuronide); ADT: androsterone (3α -hydroxy-5 α -androstane-17-one); ADTG: androsterone glucuronide (3α -hydroxy-5 α -androstane-17-one 3-glucuronide); CORT: cortolone ($3\alpha,17\alpha,20\alpha,21$ -tetrahydroxy-5 β -pregnan-11-one); β CORT: β -cortolone ($3\alpha,17\alpha,20\alpha,21$ -tetrahydroxy-5 β -pregnan-11-one); DHEA: dehydroepiandrosterone (3β -hydroxyandrost-5-en-17-one); DHEAS: dehydroepiandrosterone-sulfate (3β -hydroxyandrost-5-en-17-one 3-sulfate); E: cortisone ($17\alpha,21$ -dihydroxypregn-4-ene-3, 11, 20-trione); E2: 17β -estradiol ($3, 17\beta$ -dihydroxyestra-1, 3, 5(10)-triene); ET: 3α -etiocolanolone (3α -hydroxy-5 β -androstane-17-one); F: cortisol ($11\beta, 17\alpha, 21$ trihydroxypregn-4-ene-3, 20-dione); FAI: free androgen index; IRMA: immunora-

acne, alopecia, or hirsutism; hyperinsulinemia; and obesity (1–5). The syndrome is clinically heterogeneous, exhibiting a broad spectrum of phenotypes (4, 5). The diagnosis of PCOS has been made by histological identification of multiple follicular cysts and thecal cell hyperplasia (2), usually in enlarged ovaries (4), although this is not a requirement for the clinical diagnosis. Polycystic ovaries are easily recognized by ultrasound, but as they are found in other disorders as well as in normal women, the finding of polycystic ovaries alone is not sufficient to make the diagnosis of PCOS.

diometric assay; PD: pregnenediol (5 β -pregnane-3 $\alpha,20\alpha$ -diol); PROG: progesterone (pregn-4-ene-3,20-dione); 17HPROG: (17α -hydroxyprogesterone (17 α -hydroxypregn-4-ene-3,20-dione); 17HPREG: 17α -hydroxy-pregnanolone ($3\alpha,17\alpha$, dihydroxy-5 β -pregnan-20-one); PT: ($3\alpha, 17\alpha, 20\alpha$ -trihydroxy-5 β -pregnane); RIA: radioimmunoassay; SHBG: sex hormone-binding globulin; T: testosterone (17β -hydroxyandrost-4-en-3-one); THE: tetrahydrocortisone ($3\alpha,17\alpha,21$ -trihydroxy-5 β -pregnane-11,20-dione); THF: tetrahydrocortisol ($3\alpha,11\beta,17\alpha,21$ -tetrahydroxy-5 β -pregnan-20-one); aTHF: allo-tetrahydrocortisol ($3\alpha,11\beta,17\alpha,21$ -tetrahydroxy-5 α -pregnan-20-one); THS: tetrahydro-11-deoxyxortisol ($3\alpha,17\alpha, 21$ -trihydroxy-5 β -pregnan-20-one).

The etiology of the disorder is still a subject of controversy. There are lines of evidence suggesting that PCOS is a form of functional ovarian hyperandrogenism, triggered by gonadotropin-dependent mechanisms (6, 7). One explanation of this phenomenon is the estrone hypothesis (8, 9), according to which permanent hyperestronemia is thought to sensitize the pituitary (9, 10) and cause an increase in LH secretion. As a result, thecal-interstitial cells are stimulated to hyperplasia, leading to an excess secretion of androstenedione (A) and other androgens. An elevation in unbound or free estradiol (E₂) has also been suggested to be a contributory factor (11). These and other theories are not mutually exclusive. More recently, defects in different enzyme systems regulating the steroid pathway have been thought to initialize the pathophysiological conditions for PCOS. One of the underlying defects is thought to be an increase in the activity of skin and hepatic 5 α -reductase (12, 13), resulting in an augmented conversion of testosterone (T) to dihydrotestosterone (14). In gas chromatography steroid profiles, this results in higher ratios of 5 α -tetrahydrocortisol (aTHF)/5 β -tetrahydrocortisol (THF) and 5 α -androsterone (5 α ADT)/5 β -etio-cholanolone (5 β ET) in urine (14). Rodin *et al.* (15) found an increased adrenal secretion of cortisol due to an augmented adrenocortical activity in women with PCOS caused by increased activity of 11 β -hydroxysteroid dehydrogenase (11 β HSDH), entailing an altered interconversion of cortisol (F) to cortisone (E). Thus, the enhanced cortisol clearance rate brings about an increase in the synthesis of adrenal androgens. Another explanation for the functional ovarian hyperandrogenism might be a dysregulated ovarian cytochrome P450c17 α enzyme complex (17 α -hydroxylase plus 17,20 lyase activity) (16). Based on this hypothesis, a specific biochemical diagnostic test for this disorder has been established by analyzing the specific stimulation of the pituitary-ovarian axis by GnRH agonists (GnRHa). Barnes *et al.* (6) and Ehrmann *et al.* (7) first used the GnRHa [6-D-(2-naphthyl)alanine]GnRH (nafarelin) to stimulate pituitary and gonadal secretion in PCOS patients. This agonist is 200-fold more effective in stimulating the pituitary-gonadal axis than endogenous GnRH (17, 18). Compared with those in healthy individuals, women suffering from this disorder display patterns of basal and stimulated levels of serum LH, FSH, and 17 α -hydroxyprogesterone (17HPROG) similar to those in men. A masculinized pituitary and ovarian response to a GnRHa stimulus, therefore, implies that the cytochrome P450c17 α enzyme complex within the ovarian thecal-interstitial cells has become hyperfunctional.

In addition to the pathological response to nafarelin and the related GnRHa buserelin (19), biochemical findings in PCOS patients normally include either an increased concentration of serum LH or an increased LH/FSH ratio (5, 20). Hyperandrogenemia is characteristic in the syndrome and is often reflected clinically as hirsutism. The basal serum levels of the androgens T, A, and dehydroepiandrosterone sulfate (DHEAS; suppressible with dexamethasone) are elevated (21–25). Furthermore, ADT and androstenediol glucuronides and sulfates (metabolites of dihydrotestosterone) are often found to be elevated (34–36). Hyperinsulinemia and insulin resistance are also found in both the lean and, more pronouncedly, the obese PCOS patients (26–30). This together

with the increased androgen levels leads to a suppression of sex hormone-binding globulin (SHBG) in serum (31, 32). PCOS patients have also been reported to have increased urinary free F or F metabolite levels (14, 15, 33).

As the value of the diagnostic test presented by Barnes *et al.* (6) has yet to be evaluated, the question arises of whether additional urinary steroid metabolite determinations during the course of the stimulation would be helpful to increase the diagnostic efficiency of the test. Regarding the high resolution gas chromatography analyses of unstimulated steroid concentrations in urine of PCOS patients reported by Rodin *et al.* (15), it would seem worthwhile to apply this technique in patients stimulated with the nafarelin test protocol. The aim of our study, therefore, is to evaluate the nafarelin test as a diagnostic tool for PCOS by measuring a subset of stimulated steroid metabolites in both the serum and urine of affected patients. In contrast to the original protocol using 100 μ g sc administered nafarelin (6), an intranasal application of 1.20 mg nafarelin was chosen. Additionally, we investigated the metabolic changes in serum and urinary steroids involved in adrenal and gonadal steroidogenesis. Besides the well known alterations in serum hormone levels, we document here significant changes in urinary steroid levels, especially with regard to androgens, gestagens, and corticoids, unmasked by the ovarian stimulation test.

Subjects and Methods

Subjects

Nineteen women with PCOS and 15 healthy women with regular menses every 26–32 days were recruited for this study. All subjects were assumed to be free of acute or chronic disease based on a medical examination and clinical chemistry. Informed consent was obtained from all subjects. The study was approved by the ethical committees of the Ludwig-Maximilians University and the Technical University of Munich. Women with PCOS were patients admitted to the gynecological departments of the university hospitals of Grosshadern and Rechts der Isar; the clinical data of all subjects are given in Table 1.

The patients were diagnosed as having PCOS on the basis of the typical ultrasound features combined with two or more of the following clinical and biochemical criteria: 1) hirsutism (Ferriman-Gallwey score, >6), 2) infertility (duration of 2 yr minimum, after exclusion of other causes), 3) oligo/amenorrhea, and 4) free androgen index (FAI/T/SHBG \times 100) greater than 7. Typical sonographic features included multiple cysts (0.5–0.8 cm in diameter), arranged either peripherally or scattered throughout the ovarian stroma of the facultatively enlarged ovaries (34). Pelvic ultrasound examinations were performed with a 5-megahertz 240-degree phased array vaginal endoprobe (Combison 310, Kretz, Zipf, Austria) by two investigators (T.S. and C.H.). Exclusion criteria for both the PCOS patients and the controls were 1) thyroid dysfunction, 2) hyperprolactinemia (PRL, >570 mIU/L; third international standard 84/500), 3) late-onset congenital adrenal hyperplasia (17HPROG in-

TABLE 1. Clinical characteristics of PCOS patients and controls

| | PCOS patients | Controls |
|---|----------------|----------------|
| n | 19 | 15 |
| Age (a) | 28.1 \pm 1.1 | 29.5 \pm 1.5 |
| BMI | 26.4 \pm 1.5 | 21.1 \pm 0.4 |
| Hirsutism grading (Ferriman-Gallwey) | 7.5 \pm 1.1 | 2.0 \pm 0.4 |
| Menstrual pattern | | |
| Regular | 3 | 15 |
| Oligomenorrhea | 5 | |
| Amenorrhea | 11 | |

crease, >45 nmol/L after adrenocortical stimulation with 0.25 mg ACTH), 4) endocrine-active tumors, 5) hormonal replacement therapy or oral contraceptives within the last 3 months, and 6) controls were excluded if they had polycystic ovaries on ultrasound.

Nafarelin test

The nafarelin test was performed in a modification of the original protocol (6); GnRHa was administered intranasally instead of sc (37). To each study subject, six sniffs (three sniffs in each nostril) of 0.23 mg nafarelin acetate (Synarel, Syntex Pharma, Aachen, Germany), equivalent to a total of 1.20 mg nafarelin, were administered. The bioavailability of intranasally applied nafarelin is 3% (17, 18), and the serum half-life is 4.4 h. All subjects were admitted to the ward on the morning of the test between 0800–1000 h between the third to sixth day of the menstrual cycle, if present. All subjects collected a pretest 24-h urine sample. Blood samples were drawn 15 min before and 0, 2, 4, and 24 h after nafarelin administration, and a second 24-h urine sample was collected with the time set from time zero. All blood and urine specimens were centrifuged at $2000 \times g$ and frozen at -20 C until analyzed. No serious side-effects were observed during our study.

Hormonal analysis in serum

Steroid measurements were performed in duplicate, using the following assays [in parentheses are indicated the manufacturer, interassay coefficient of variation (CV), mean, and lower detection limit (DL)]: T, RIA [Immuchem, Carson, CA; CV, 9.7%; mean, 25.3 nmol/L ($n = 34$); DL, 0.52 nmol/L]; A, RIA [Diagnostic Systems Laboratories, Webster, TX; CV, 18.6% ($n = 10$); mean, 5.6 nmol/L ($n = 10$); CV, 9.0%; mean, 30.1 nmol/L ($n = 10$); DL, 0.18 nmol/L]; E_2 , enzyme immunoassay [Boehringer Mannheim, Mannheim, Germany; CV, 12.1%; mean, 118 pmol/L ($n = 6$); CV, 9.9%; mean, 2364 pmol/L ($n = 6$); DL, 20 pmol/L]; DHEAS, RIA [Diagnostic Products Corp., Los Angeles, CA; CV, 10.2%; mean, 4.9 $\mu\text{mol/L}$ ($n = 13$); CV, 5.0%; mean, 15.9 $\mu\text{mol/L}$ ($n = 13$); DL, 0.13 $\mu\text{mol/L}$]; 17HPROG, RIA [Diagnostic Products; CV, 16.3%; mean, 12.1 nmol/L ($n = 13$); DL, 0.30 nmol/L]; SHBG, immunoradiometric assay [Farnos Group, Oulunsalo, Finland; CV, 6.8%; mean, 40 nmol/L ($n = 9$); DL, 2.5 nmol/L]; LH, chemiluminescence immunoassay [CLIA; Kodak Clinical Diagnostics, Amersham, United Kingdom; CV, 4.0%; mean, 18.8 mIU/mL ($n = 12$); DL, 0.5 mIU/mL]; FSH, CLIA [Kodak; CV, 2.6%; mean, 9.0 mIU/mL ($n = 12$); DL, 0.9 mIU/mL]; androstenediol glucuronide (ADG), RIA [Diagnostic Systems Laboratories; CV, 5.6%; mean, 16.6 nmol/L ($n = 10$); DL, 0.6 nmol/L]; F (in serum), CLIA [Kodak; CV, 3.8%; mean, 574 nmol/L ($n = 8$); DL, 13 nmol/L]; F (free in urine), fluorescence polarization immunoassay without sample extraction [Abbott Laboratories, Irving, TX; CV, 6.1%; mean, 397 nmol/L ($n = 26$); DL, 25 nmol/L]; the cross-reactivity of the specific anti-F antibody to E is 1.1%; and progesterone (PROG), CLIA [Kodak; CV, 5.3%; mean, 6.0 nmol/L ($n = 12$); DL, 0.5 nmol/L]. ADT glucuronide (ADTG) was determined according to the method previously described (35).

Steroid analysis in urine

Apparatus. For capillary gas chromatographic (GC) analyses, a model 3700 GC (Varian, Darmstadt, Germany) was used. Separations were obtained on a 30-m DB-1 fused-silica capillary column (internal diameter, 0.32 mm; J & W Scientific, Folsom, CA). The carrier gas was helium at a flow rate of 2.2 mL/min. The injector temperature was set at 290 C; the column temperature isothermal was 150 C for 5 min, then 150–300 C with a heating rate of 2 C/min; the flame ionization detector temperature was 320 C; the splitting ratio was 1:20. A Mega-Integrator D-2500 (Merck, Darmstadt, Germany) was used as system controller. The reagents β -glucuronidase and arylsulfatase (type H-2, crude solution from *Helix pomatia*), as well as the reference steroid compounds, and the hydrocarbon calibrators C_{22} (*n*-docosane), C_{24} (*n*-tetracosane), and C_{32} (*n*-dotriacontane) were obtained from Sigma (Deisenhofen, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Fluka (Buchs, Switzerland), and cortolone (CORT) from Makor (Jerusalem, Israel). All other laboratory chemicals were purchased from Merck. Sep-Pak cartridges with C_{18} and NH_2 materials were

obtained from Millipore-Waters (Eschborn, Germany). For sample preparation, 1/100th of the 24-h urine was used for analysis. After the addition of 50 μg $3\beta\text{ET}$ (3β -hydroxy- 5β -androstan-17-one) as an internal standard, the pH was adjusted to 4.6 with 3 mol/L boric acid-acetate buffer. The mixture was adsorbed on a Sep-Pak C_{18} cartridge (38), which was primed twice with 5 mL methanol and twice with 5 mL water. The cartridge was eluted twice with 1-mL portions of methanol, the eluate was evaporated at 50 C under vacuum, and the residue was dissolved in 100 μL methanol, 10 mL water, 1 mL 0.2 mol/L sodium acetate buffer (pH 5.2), and 100 μL β -glucuronidase/arylsulfatase solution (100,000 U/mL glucuronidase and 5,000 U/mL sulfatase; U = modified Fishman unit). Hydrolysis was then performed at 55 C for 2 h (39). The hydrolyzed residue was adsorbed again on a Sep-Pak C_{18} cartridge. One gram of anhydrous sodium sulfate was placed on the top of a NH_2 cartridge, and the C_{18} cartridge was connected on the top of this (40). The cartridges were eluted with two portions of 1.5 mL ethyl acetate-methanol (9:1, vol/vol). Twenty micrograms each of C_{22} , C_{24} , and C_{32} hydrocarbons dissolved in 100 μL *n*-hexane were added as calibrators for the retention indices to the eluate, and the solvents were then evaporated. The derivatization was performed with 50 μL MSTFA and 100 μL pyridine containing 10 mg sodium acetate at 70 C for 24 h (41). Two microliters were used for GC analysis. This procedure of derivatization fully converts 17-keto groups into enol-trimethylsilyl ethers and hydroxy groups into trimethylsilyl derivatives, with the exception of 11-hydroxy and 11-keto groups.

Peak identification: By coinjection of authentic steroid compounds (extracted in parallel with the urine samples), the methylene units of the relevant steroids were calculated and used to identify the peaks. Confirmation of steroid identity, when necessary, was assessed by GC/mass spectrometry techniques.

Peak quantification: The steroid concentrations found in urine were calculated from peak area ratios related to the internal standard $3\beta\text{ET}$ and expressed as micromoles of steroid per g creatinine in the respective urines.

A typical urinary steroid profile of a PCOS patient performed using the GC technique applied is shown in Fig. 1. The overall interassay CV for all steroids measured was 4.5% ($n = 6$).

Statistical analysis

Statistical analyses were calculated using the BMDP386 package (version 7.0, BMDP Statistical Software, Los Angeles, CA). Initial plots of several steroid parameters showed skewed distributions, precluding the assumption of normal data distributions, and therefore, we used a non-parametric test panel in an exploratory evaluation. Hormone variables between the groups were compared by use of the Mann-Whitney rank sum test; the intragroup changes during the test were compared by use of either the Wilcoxon test (applied for urinary analytes) or the Friedman test (applied for serum analytes). Optimal cut-off values for the distinction of the steroid analytes 17HPROG, 17α -hydroxypregnanolone

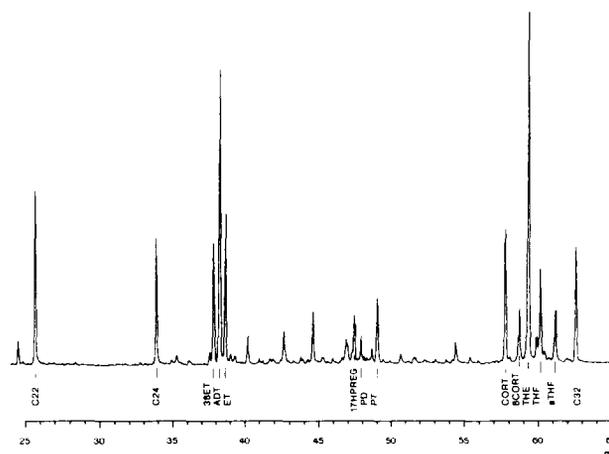


FIG. 1. Typical urinary steroid profile from a PCOS patient, performed by use of the GC technique.

(17HPREG), and allo-tetrahydrocortisol (aTHF) between PCOS patients and controls were derived from receiver-operating characteristic plots. All results are presented as the mean \pm SEM.

Results

Serum levels of gonadotropins and steroids

The intranasal application of nafarelin resulted in a significant rise in serum LH and FSH levels ($P < 0.001$) in all women (PCOS patients and controls). For both gonadotropins, peak levels occurred after 4 h. Two patients showed increasing concentrations of LH or FSH even up to the last measurement at 24 h. In all women tested with nafarelin, the levels of LH and FSH at 24 h were higher than the respective basal pretest values. As shown in Table 2, there were no significant differences between controls and PCOS patients in basal or stimulated LH or FSH levels. The LH/FSH ratio, however, was significantly higher in PCOS patients than in controls 24 h after GnRHa application (2.6 ± 0.4 vs. 1.6 ± 0.1 ; $P < 0.05$).

Serum 17HPROG levels were elevated at all time points in PCOS patients compared with those in control subjects ($P < 0.001$). The baseline level of 17HPROG in PCOS patients was 3.5 ± 0.2 compared to 1.8 ± 0.1 nmol/L in the controls. In both groups, 17HPROG and its precursor, PROG, rose significantly during the test ($P < 0.001$; Table 2 and Fig. 2); the rates of increase in 17HPROG were similar in the two groups. At baseline, no difference was seen in the serum levels of PROG between the groups.

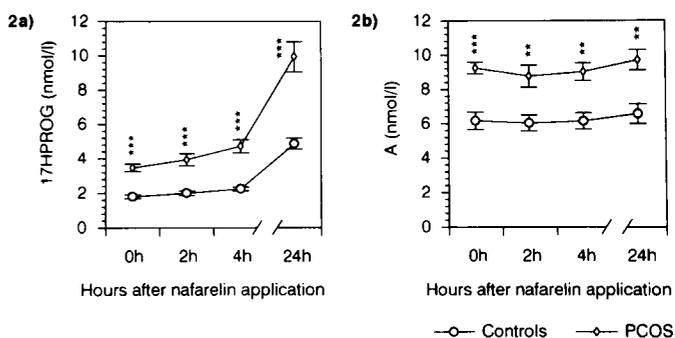


FIG. 2. Responses of 17HPROG (a) and A (b) to nafarelin in sera of PCOS patients and controls. Serum concentrations are given as the mean \pm SEM. Significant differences between PCOS and controls are indicated: **, $P < 0.01$; ***, $P < 0.001$.

Basal serum A levels were higher in PCOS patients than in controls (9.2 ± 0.3 vs. 6.2 ± 0.5 nmol/L; $P < 0.001$). Neither the PCOS patients nor the controls exhibited a significant change in A levels during the test (Table 2). Similar to those of A, the basal serum levels of T were elevated in PCOS patients compared to the control values (2.2 ± 0.2 vs. 1.5 ± 0.1 nmol/L; $P < 0.05$). Only in the PCOS patients was a significant rise in T observed during the test (at 24 h, 3.2 ± 0.4 vs. 1.8 ± 0.2 nmol/L; $P < 0.01$). SHBG levels were lower in the PCOS patients than in the controls (data not shown). Therefore, the FAI levels were significantly higher in the PCOS patients, both basally and during the course of the

TABLE 2. Mean values and ranges (\pm SEM) for serum parameters in the course of the nafarelin test for PCOS patients and controls after intranasal nafarelin application

| Group | Parameter | Basal 0 h | Post 2 h | Post 4 h | Post 24 h |
|-------------------------|-------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------|
| Controls | LH (IU/L) | 4.1 \pm 0.5 | 35.5 \pm 4.1 | 62.2 \pm 6.5 | 22.1 \pm 2.6 ^a |
| | FSH (IU/L) | 5.5 \pm 0.5 | 13.9 \pm 1.1 | 23.5 \pm 2.0 | 13.7 \pm 1.4 ^a |
| | LH/FSH ratio | 0.8 \pm 0.1 | 2.6 \pm 0.3 | 2.7 \pm 0.3 | 1.6 \pm 0.1 ^a |
| | A (nmol/L) | 6.2 \pm 0.5 | 6.0 \pm 0.5 | 6.1 \pm 0.5 | 6.6 \pm 0.6 |
| | T (nmol/L) | 1.5 \pm 0.1 | 1.4 \pm 0.1 | 1.4 \pm 0.1 | 1.8 \pm 0.2 |
| | FAI (%) | 3.0 \pm 0.4 | 2.7 \pm 0.3 | 2.7 \pm 0.3 | 3.6 \pm 0.5 ^b |
| | DHEAS (μ mol/L) | 6.1 \pm 0.5 | 6.2 \pm 0.6 | 6.3 \pm 0.6 | 6.1 \pm 0.5 |
| | PROG (nmol/L) | 1.6 \pm 0.2 | 1.6 \pm 0.2 | 1.7 \pm 0.2 | 2.8 \pm 0.5 ^a |
| | 17HPROG (nmol/L) | 1.8 \pm 0.1 | 2.0 \pm 0.1 | 2.2 \pm 0.1 | 4.9 \pm 0.3 ^a |
| | ADG (nmol/L) | 9.0 \pm 1.2 | 9.0 \pm 1.1 | 9.4 \pm 1.1 | 9.5 \pm 1.2 |
| | ADTG (nmol/L) | 162 \pm 26.9 | | | 166 \pm 30.3 |
| | E ₂ (pmol/L) | 111 \pm 18 | | | 591 \pm 67 ^a |
| | F (nmol/L) | 257 \pm 27 | 209 \pm 23 | 199 \pm 32 | 218 \pm 17 |
| | PCOS patients | LH (IU/L) | 5.6 \pm 0.8 | 37.7 \pm 4.2 | 62.2 \pm 7.2 |
| FSH (IU/L) | | 6.0 \pm 0.4 | 13.3 \pm 1.1 | 20.5 \pm 1.6 | 12.3 \pm 1.2 ^a |
| LH/FSH ratio | | 1.0 \pm 0.1 | 3.0 \pm 0.3 | 3.3 \pm 0.4 | 2.6 \pm 0.4 ^{a,c} |
| A (nmol/L) | | 9.2 \pm 0.3 ^d | 8.8 \pm 0.6 ^e | 9.0 \pm 0.5 ^e | 9.7 \pm 0.6 ^e |
| T (nmol/L) | | 2.2 \pm 0.2 ^e | 2.2 \pm 0.2 ^c | 2.1 \pm 0.2 ^c | 3.2 \pm 0.4 ^{c,f} |
| FAI (%) | | 12.8 \pm 3.6 ^e | 12.5 \pm 3.8 ^e | 13.8 \pm 4.5 ^e | 18.1 \pm 5.5 ^{e,f} |
| DHEAS (μ mol/L) | | 6.3 \pm 0.7 | 6.4 \pm 0.7 | 6.6 \pm 0.7 | 6.4 \pm 0.8 |
| PROG (nmol/L) | | 1.7 \pm 0.3 | 1.8 \pm 0.4 | 2.3 \pm 0.5 | 3.4 \pm 0.8 ^a |
| 17HPROG (nmol/L) | | 3.5 \pm 0.2 ^d | 3.9 \pm 0.3 ^d | 4.7 \pm 0.4 ^d | 9.9 \pm 0.9 ^{a,d} |
| ADG (nmol/L) | | 8.9 \pm 1.5 | 8.5 \pm 1.4 | 8.4 \pm 1.4 | 9.8 \pm 1.6 ^b |
| ADTG (nmol/L) | | 216 \pm 25.6 | | | 231 \pm 27.2 |
| E ₂ (pmol/L) | | 132 \pm 11 | | | 950 \pm 123 ^{a,c} |
| F (nmol/L) | | 378 \pm 27 ^e | 283 \pm 21 ^c | 281 \pm 23 ^c | 360 \pm 38 ^e |

^a $P < 0.001$, significant changes in the course of the nafarelin test (Friedman test).

^b $P < 0.05$, significant changes in the course of the nafarelin test (Friedman test).

^c $P < 0.05$, PCOS vs. controls (Mann-Whitney test).

^d $P < 0.001$, PCOS vs. controls (Mann-Whitney test).

^e $P < 0.01$, PCOS vs. controls (Mann-Whitney test).

^f $P < 0.01$, significant changes in the course of the nafarelin test (Friedman test).

test [at baseline, $12.8 \pm 3.6\%$ vs. $3.0 \pm 0.4\%$ ($P < 0.01$); at 24 h, $18.1 \pm 5.5\%$ vs. $3.6 \pm 0.5\%$ ($P < 0.01$)]. E_2 levels rose significantly ($P < 0.001$) in both groups during the test; the posttest levels were significantly higher in PCOS than in controls (950 ± 123 vs. 591 ± 67 pmol/L; $P < 0.05$).

Neither serum DHEAS, ADG, nor ADTG concentrations showed any significant differences when comparing PCOS and controls. Although ADG levels increased as a result of nafarelin stimulation ($P < 0.05$), no changes in the serum concentrations of ADTG or DHEAS were observed (Table 2).

Serum F levels were higher in PCOS patients than controls at all time points ($P < 0.01$ at 0 and 24 h; $P < 0.05$ at 2 and 4 h). Due to the dynamic fluctuations in serum F during the day, the Friedman test was not applied to the different values of the two groups.

Levels of steroids in the 24-h urine samples

The results of the steroid profiles for both the PCOS patients and the controls during the nafarelin test are shown in Table 3. In the controls, only 17HPREG levels were signifi-

cantly stimulated ($P < 0.05$) by nafarelin; all other steroid levels remained unchanged. In PCOS patients, however, there were significant increases in urinary androgen derivatives (ADT and ET), gestagens [17HPREG and pregnanetriol (PT)] and glucocorticoids [tetrahydrocortisone (THE), THF, CORT, and β -cortolone (β CORT)] including free F (see Table 3 for significances). With respect to the differences observed between the patients and controls, the following parameters were higher at baseline: THE ($P < 0.05$), aTHF ($P < 0.5$), ADT/ET ratio ($P < 0.05$), and aTHF/THF ratio ($P < 0.01$). Ratios of ADT/ET and aTHF/THF may be considered to be indirect measures of 5 α -reductase activity. After stimulation, the following parameters were higher in PCOS patients: ADT ($P < 0.01$), 17HPREG ($P < 0.05$), PT ($P < 0.05$), THE ($P < 0.05$), THF ($P < 0.05$), aTHF ($P < 0.001$), ADT/ET ratio ($P < 0.05$), corticoid sum (= CORT + β CORT + THE + THF + aTHF) ($P < 0.05$), and free F ($P < 0.05$). Significant stronger increases over 24 h were found in urine from PCOS patients for ADT, ET, 17HPREG, PT, CORT, THF, and free F

TABLE 3. Mean values and ranges (\pm SEM) for urine parameters pre- and postnafarelin testing for PCOS patients and controls

| Group | Parameter | Pretest | Posttest | Difference |
|---------------|----------------|-------------------|------------------|------------------------|
| Controls | ADT | 7.4 ± 0.8 | 7.2 ± 0.8 | -0.2 ± 0.3 |
| | ET | 11.5 ± 1.3 | 11.3 ± 1.2 | -0.2 ± 0.6 |
| | DHEA | 2.9 ± 0.8 | 2.2 ± 0.4 | -0.8 ± 0.7 |
| | 17HPREG | 1.3 ± 0.3 | 1.6 ± 0.3 | 0.3 ± 0.1^a |
| | PD | 10.4 ± 2.2 | 9.4 ± 2.0 | -1.0 ± 1.0 |
| | PT | 3.5 ± 0.3 | 3.6 ± 0.3 | 0.1 ± 0.2 |
| | THS | 0.8 ± 0.1 | 0.7 ± 0.1 | -0.2 ± 0.1 |
| | CORT | 4.1 ± 0.3 | 3.9 ± 0.3 | -0.2 ± 0.3 |
| | β CORT | 2.9 ± 0.2 | 2.7 ± 0.2 | -0.2 ± 0.2 |
| | THE | 9.5 ± 0.7 | 9.9 ± 0.8 | 0.4 ± 0.4 |
| | THF | 5.3 ± 0.5 | 5.4 ± 0.4 | 0.1 ± 0.4 |
| | aTHF | 1.8 ± 0.1 | 1.8 ± 0.1 | 0.0 ± 0.1 |
| | ADT/ET ratio | 0.7 ± 0.1 | 0.7 ± 0.1 | |
| | THE/THF ratio | 1.91 ± 0.14 | 1.86 ± 0.09 | -0.05 ± 0.14 |
| | aTHF/THF ratio | 0.37 ± 0.04 | 0.34 ± 0.02 | -0.03 ± 0.04 |
| | Cort.-sum | 23.6 ± 1.6 | 23.7 ± 1.6 | 0.1 ± 1.1 |
| | F | 256 ± 19 | 261 ± 17 | 5 ± 19 |
| PCOS patients | ADT | 10.0 ± 0.9 | 13.4 ± 1.5^b | $3.3 \pm 1.0^{b,c}$ |
| | ET | 10.7 ± 0.7 | 14.9 ± 1.8 | $4.3 \pm 1.5^{c,d}$ |
| | DHEA | 3.9 ± 1.3 | 3.5 ± 1.0 | 0.1 ± 0.9 |
| | 17HPREG | 1.3 ± 0.2 | 2.6 ± 0.3^d | $1.4 \pm 0.2^{c,f}$ |
| | PD | 6.4 ± 1.2 | 8.2 ± 1.5 | 1.7 ± 1.3 |
| | PT | 4.1 ± 0.4 | 6.1 ± 0.7^d | $1.9 \pm 0.5^{b,f}$ |
| | THS | 0.6 ± 0.1 | 0.8 ± 0.1 | 0.3 ± 0.2 |
| | CORT | 4.5 ± 0.6 | 5.6 ± 0.8 | $1.1 \pm 0.5^{a,d}$ |
| | β CORT | 2.5 ± 0.3 | 3.6 ± 0.5 | 1.1 ± 0.4^a |
| | THE | 12.1 ± 0.9^d | 16.4 ± 2.1^d | 4.2 ± 1.8^a |
| | THF | 5.7 ± 0.5 | 8.1 ± 0.9^d | $2.4 \pm 0.9^{c,d}$ |
| | aTHF | 2.8 ± 0.3^d | 3.4 ± 0.4^e | 0.6 ± 0.3 |
| | ADT/ET ratio | 1.0 ± 0.1^d | 1.0 ± 0.1^d | |
| | THE/THF ratio | 2.23 ± 0.12^d | 2.03 ± 0.10 | -0.24 ± 0.10^a |
| | aTHF/THF ratio | 0.52 ± 0.05^b | 0.45 ± 0.05 | $-0.08 \pm 0.03^{c,d}$ |
| | Cort.-sum | 27.6 ± 2.1 | 37.2 ± 4.3^d | 9.4 ± 3.7^a |
| | F | 279 ± 19 | 350 ± 25^d | $72 \pm 25^{a,d}$ |

All parameters, except ratios and F, are given as micromoles per g creatinine; F is given as nanomoles per g creatinine. Cort.-sum, CORT + β CORT + THE + THF + aTHF.

^a $P < 0.05$, pre- vs. postnafarelin (Wilcoxon test).

^b $P < 0.01$, PCOS vs. controls (Mann-Whitney test).

^c $P < 0.01$, pre- vs. postnafarelin (Wilcoxon test).

^d $P < 0.05$, PCOS vs. controls (Mann-Whitney test).

^e $P < 0.001$, PCOS vs. controls (Mann-Whitney test).

^f $P < 0.001$, pre- vs. postnafarelin (Wilcoxon test).

compared to those in the healthy controls. The most prominent differences between the two groups were found for the increase in 17HPREG levels (1.4 ± 0.2 vs. 0.3 ± 0.1 $\mu\text{mol/g}$ creatinine; $P < 0.001$) and the poststimulation value of aTHF (3.4 ± 0.4 vs. 1.8 ± 0.1 $\mu\text{mol/g}$ creatinine; $P < 0.001$), as shown in Fig. 3. Tetrahydro-11-deoxycortisol (THS), a derivative of the F precursor 11-deoxycortisol, was found only in low concentrations in both patients and controls, and significant differences or changes could not be determined. In contrast to PT (a derivative of 17HPROG), no significant differences or changes were seen in pregnanediol (PD) levels, which is a product of PROG.

The ratio of THE/THF, an indicator of the 11 β HSDH activity and similar to the ratio of the 11-oxo/11-hydroxy metabolites of F was significantly different ($P < 0.05$) in PCOS patients vs. controls at baseline, whereas in the course of the test, the PCOS patients showed a significant decrease ($P < 0.05$).

Calculation of sensitivities and specificities of several analytes in the course of the test revealed that, based on 93% specificity, the posttest 17HPROG level in serum has a sensitivity of 89% (cut-off value, 6.4 nmol/L), whereas in urine, the posttest aTHF level shows a sensitivity of 78% (cut-off value, 2.26 $\mu\text{mol/g}$ creatinine), and the difference value of 17HPREG shows a sensitivity of 72% (cut-off value 0.8 $\mu\text{mol/g}$ creatinine). The sum of both urinary parameters resulted in a sensitivity of 89% (cut-off value, 2.8 $\mu\text{mol/g}$ creatinine).

Additionally, correlations between the urinary steroid metabolites were determined. In the patient group, there were significant positive correlations between the following post-test parameters: 17HPREG correlates with THE, THF, CORT, β CORT, ADT, ET, and PT; and PT correlates with CORT, β CORT, THE, THF, ET, and ADT. The respective P values varied between less than 0.001 (17HPREG vs. PT or PT vs. ET) and 0.013 (17HPREG vs. CORT). No significant correlations were observed in the control group.

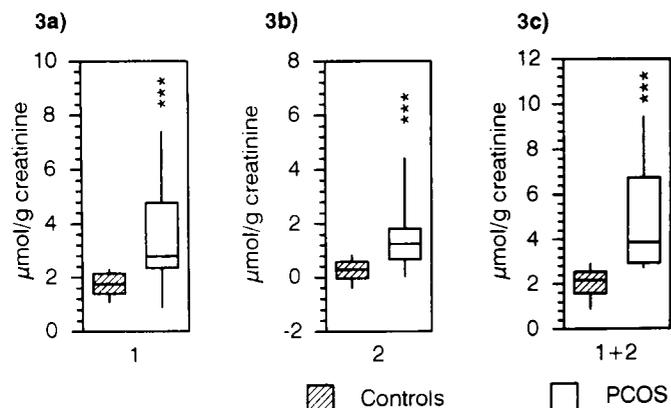


FIG. 3. Box and whisker plots (graphical displays of the data distributions) of the posttest levels of aTHF (a; 1), the difference values of 17HPREG (b; 2), and the combination of both (c; 1 + 2) in urine of PCOS patients and controls undergoing nafarelin testing. The box and whisker plot presentation shows the median, 25–75% range, and extremes (without outliers). Significant differences between PCOS and controls are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The serum and urine test parameters described above were not significantly different in lean and obese PCOS subjects.

Discussion

The use of basal hormone levels, LH/FSH ratios, elevated androgen levels, or pelvic ultrasound examinations often fail to give reliable diagnostic indications for PCOS. The nafarelin test, developed by Barnes *et al.* (6), was the first test to assess suitable biochemical criteria for the diagnosis. The GnRHa nafarelin is a potent specific stimulator of the pituitary-gonadal axis; given as a single dose, it is much more potent than the commonly used GnRH. A short term stimulation test using this drug appears to be a useful tool in distinguishing PCOS from other causes of hyperandrogenism in women (7). Our study, using an intranasal application with pharmacodynamically equivalent doses, has confirmed these data concerning levels of 17HPROG and the gonadotropins in serum. In addition, we were able to document significant changes in urinary steroid metabolite levels, especially in those of androgens, gestagens, and corticoids.

In accordance with the data described by Barnes *et al.* (6), we found that the maximal gonadotropin responses in serum occurred after 4 h, whereas the ovarian steroid responses continued to rise during the entire 24-h test period. The LH/FSH ratio was clearly elevated in the patients at 24 h, as expected for this disorder. The most distinctive increase was that found for 17HPROG. In contrast to the findings of Barnes *et al.* (6), we observed no significant change in A in either the PCOS patients or controls, although the respective levels were higher in the patient group throughout. In contrast T together with A, a product of the P450c17 α enzyme complex, was found to be elevated in the PCOS patients compared to control levels and underwent a significant increase during the test in the patients. Of the T metabolites ADG and ADTG, only ADG significantly increased during the test in the PCOS patients. The observation that the concentration of ADTG remained unchanged in PCOS patients together with a marginally elevated T level can be explained by the fact that in our patient group the androgen excess was only moderate and the mean Ferriman-Gallwey index for the group of 19 patients was 7.5. Serum DHEAS, the main adrenal androgen parameter, showed no changes during the test in either group. The E₂ response to nafarelin was exaggerated in PCOS patients compared to the controls.

These data give rise to the suggestion that cytochrome P450c17 α may be dysregulated in the PCOS patients. Nevertheless, with regard to the serum T concentrations of the affected patients, increased activities of both the ovarian 17 α -hydroxylase and 17,20-lyase enzymes in the Δ^4 -steroid pathway cannot be ruled out. In addition to the diagnostic discrimination afforded by baseline parameters such as 17HPROG (with a sensitivity of 78% at 93% specificity; cut-off value, 2.5 nmol/L), only the posttest serum levels of 17HPROG allow a further distinction to be drawn between PCOS patients and controls with a sensitivity of 89% at 93% specificity (cut-off value, 6.4 nmol/L).

Applying the high resolution GC technique using the MSTFA derivatization method to the urine samples, we were

able to quantify the abnormal steroidogenic response to nafarelin in the affected patients. The examination of the respective steroid profiles in the test subjects revealed a quite different pattern in the PCOS patients compared to the controls. In healthy women, only 17HPREG, the urinary derivative of 17HPROG, showed a significant increase during the test. In patients, however, there was a significant increase in the urinary metabolites of androgens (ADT and ET), gestagens (17HPREG, PD, and PT), and glucocorticoids (THE, THF, aTHF, CORT, β CORT, and F).

In our study, the urinary steroid profiles revealed greater 5α THF/ 5β THF and ADT/ET ratios in PCOS patients (Fig. 4). This is in accordance with the report of Stewart *et al.* (14), who demonstrated increased 5α -reductase activity in the liver and

skin of patients suffering from this endocrine disorder. In the course of the nafarelin test, however, only the aTHF/THF-ratio is altered, due to an increase in aTHF levels; the origin of this increased 5α -reductase activity level in PCOS is still under debate. According to Rosenfield (42), the overproduction of ovarian androgens seems to result in a stimulation of this enzyme in skin and liver (12, 13). The borderline change in aTHF secretion in urine that was observed ($P = 0.07$) also suggests that the enhanced 5α -reductase activity may be the result of androgen excess and not due to a primary mechanism, as reported by Stewart *et al.* (14).

According to Giagulli *et al.* (12), ADG appears to be a better parameter for determining peripheral 5α -reductase activity than ADT. Therefore, it would be necessary to determine the levels of urinary androstanediol, rather than ADT, to confirm our model. This, however, was not possible with the GC technique applied in this study due to peak interferences.

It can be stated that the basal serum F level and basal free F level in urine are slightly elevated in PCOS patients compared with those in controls. This may be due to a preexisting alteration in adrenal corticoid secretion. Invitti *et al.* (33) documented an increased urinary free F level in PCOS patients and suggested that an overactive hypothalamic-pituitary-adrenal axis might be responsible. Rodin *et al.* (15) reported increased adrenal secretion of F due to an augmented adrenocortical activity in women with PCOS, but decreased urinary free F levels. The exaggerated glucocorticoid excretion in PCOS patients after nafarelin treatment, however, is surprising and could be explained by an increased oxidation of F to E due to a dysregulated 11β HSDH, which is not accounted for by obesity. As a result of the enhanced F clearance rate, the synthesis of adrenal androgens is augmented. In our study, the THE/THF ratio was significantly higher in PCOS patients *vs.* controls at baseline ($P < 0.05$), which is in agreement with the ratio of the 11-oxo/11-hydroxy metabolites of F reported by Rodin *et al.* (15). In the course of the test, however, the PCOS patients showed a decrease in this ratio, which results in a loss of significance in the differences between the ratios in PCOS and control subjects. Therefore, it can be suggested that the abnormal 11β HSDH activity is attenuated in the course of the GnRH α test. Due to a reduced conversion of F to E, the excretion of the respective F and corticosterone metabolites increases acutely over the 24-h test period. Our hypothesis is that the excess production of E_2 released by the gonadotropin surge in PCOS patients *vs.* controls is able to attenuate the 11β HSDH activity. Together with an increased metabolism of 17HPROG toward PT, this would explain the increased corticoid excretion in urine and the absence of a parallel increase in A and T in relation to 17HPROG in serum during the GnRH α test. Another possible explanation for the enhanced glucocorticoid excretion in PCOS patients after nafarelin application is that the gestagen surge that results from GnRH α stimulation displaces serum F from corticosteroid-binding globulin and leads to an augmented F metabolism. It has previously been described that serum corticosteroid-binding globulin levels are significantly decreased in PCOS patients (33).

Therefore, our data suggest that an altered interconversion of F and E is able to play a role in PCOS, as suggested for the

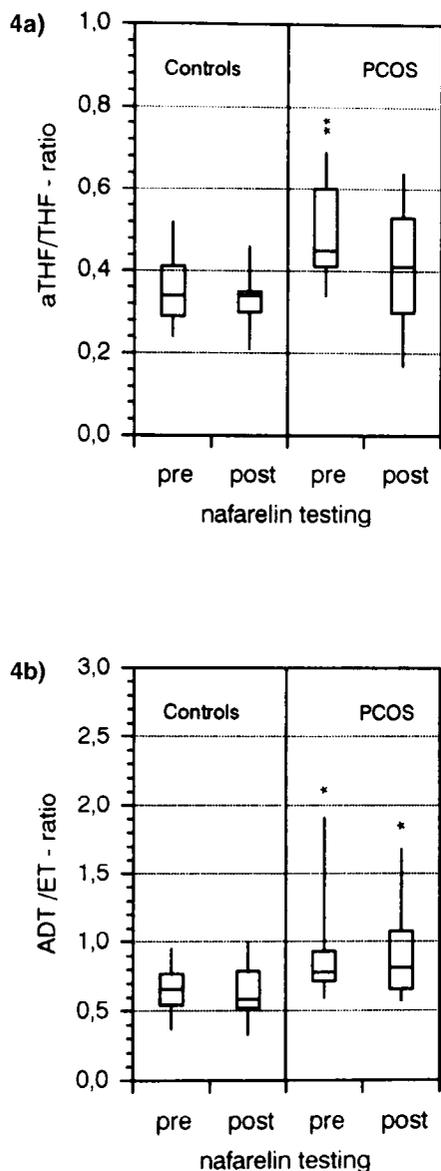


FIG. 4. Box and whisker plots of the 5α -reductase ratios of aTHF/THF (a) and ADT/ET (b) in urine of PCOS patients and controls before and after nafarelin testing. The box and whisker plot presentation shows the median, 25–75% range, and extremes (without outliers). Significant differences between PCOS and controls are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

augmented/dysregulated ovarian cytochrome P450c17 α activity. The proportion and importance of the proposed enzyme dysregulation in the pathogenesis of PCOS have yet to be proven, because measurements of basal serum levels of 11-hydroxyandrostenedione, a marker of adrenal androgen secretion, support the idea that the ovaries and not the adrenals are the major source of androgens in PCOS (5). Additionally, divergent results concerning the elevation of DHEAS levels in PCOS patients have previously been described (22, 23, 43, 44) and argue against a pivotal role of the adrenal glands in the etiology of this disorder. However, an altered sensitivity of the adrenal cortex to corticotropin, evidenced by higher 11-hydroxyandrostenedione levels after stimulation with corticotropin-releasing factor and an increased metabolic clearance of F and corticosterone, appears to be a supplementary symptom of the pathophysiological changes in PCOS.

One additional observation from our study is that, interestingly, in the PCOS patients, no significant differences in urinary steroid concentrations between the lean and obese patients could be found during the test. This would appear to rule out the idea that increased F production rates in obesity cause higher secretion rates of E in the adipose tissue of PCOS patients.

This study, undertaken with 19 PCOS patient and 15 controls, documents the efficiency of the nafarelin test in confirming the ovarian abnormality in PCOS suspected by the clinical diagnosis. The more practical intranasal application mode together with the measurements of urinary steroids gives an effective diagnostic tool for investigating this endocrine disorder. The study suggests that 17HPROG levels in serum together with those of aTHF and 17HPREG in urine are the best discriminatory parameters of the GnRH α stimulation test. An additional investigation concerning these three parameters should confirm our results by the application of confirmatory statistical analysis.

The sensitivity of the test, as defined by the ability to detect an ovarian abnormality, was 89% for both the posttest level of 17HPROG in serum and the combination of the difference value of 17HPREG and the posttest value of aTHF in urine (all at a 93% specificity). Further studies are necessary to determine whether a combination of the nafarelin stimulation test with, for example, short term dexamethasone suppression, is capable of distinguishing between the different forms of hyperandrogenism in women.

Due to the variability of symptoms displayed by patients suffering from this endocrine disorder, additional investigation is required to confirm whether the suggested dysregulated enzyme systems, such as 11 β HSDH or cytochrome P450c17 α , are alone sufficient to cause PCOS or whether other ovarian and/or adrenal factors play a role in the pathogenesis. In addition, the reason for the exaggerated corticoid excretion in the course of the nafarelin stimulation is in need of clarification.

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