Evaluation of the Pituitary-adrenal axis in hyperandrogenic women with polycystic ovary syndrome

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Abstract

OBJECTIVES: Impaired adrenal function is common in patients with polycystic ovary syndrome (PCOS). Abnormal regulation of cytochrome P450 17 alpha is believed to cause the exaggerated 17-hydroxyprogesterone (17OHP) response to ACTH stimulation. The aim of the study was to evaluate cortisol and 17OHP response to low dose (1µg) ACTH test and to compare it with the standard ACTH (250µg) test in hyperandrogenic women with PCOS.

DESIGN AND MEASUREMENTS: We studied 27 PCOS and 22 control women. All participants were examined for mutations of the CYP21 gene, Cortisol and 17OHP levels before, 30 and 60 minutes after the IV injection of 250µg ACTH (SDT) and after 1µg ACTH (LDT). Fasting serum levels of LH, FSH, testosterone, DHEAS were determined in all participants.

RESULTS: Basal and ACTH stimulated Cortisol during the SDT (470±138 nmol/L and 761±143, respectively) were significantly higher in PCOS vs. controls (232±124 and 670±130, respectively) (p<0.03, p<0.02, respectively). Basal 17OHP (6.1±2.1 nmol/L) and the peak response to SDT (14.2±3.6 nmol/L) were significantly higher in PCOS vs. controls (4.2±2.1, 10.9±3.0, respectively) (p<0.003, p<0.004, respectively). Abnormally elevated 17OHP response to SDT was detected in 6/27 PCOS women (22%). No statistically significant difference between the PCOS and control groups were noted during the LDT in both cortisol and 17OHP levels.

CONCLUSIONS: These data suggest that the exaggerated 17-hydroxyprogesterone (17OHP) response to ACTH stimulation in PCOS is revealed by stimulation at a pharmacological dose (250µg) but not by a physiological dose (1µg).
Introduction

The nature of adrenal abnormalities observed in women with polycystic ovary syndrome (PCOS) is still unclear. An increase in basal and ACTH stimulated 17 hydroxyprogesterone (17OHP) levels were repeatedly reported in PCOS women [1]. Adrenal overactivity in PCOS was attributed to altered cortisol metabolism [2], increased ACTH secretion resulting from cortisol-dependent negative feedback [3] or to subtle increases in plasma cortisol response to ACTH [4]. Alternatively, it was related to an exaggerated secretory response of the adrenal cortex for DHA and Δ4, but not to an altered pituitary responsivity to CRH or to increased sensitivity of these adrenal androgens to ACTH stimulation [5]. The standard ACTH test (250 µg) provides pharmacological dose of the hormone resulting in over stimulation of the adrenal glands that might lead to false-positive Cortisol response. The 30 min Cortisol response to low dose (1 µg) ACTH has become increasingly accepted as the best criterion for evaluation assessment of the Pituitary-adrenal axis [6–10]. Since plasma ACTH levels after injection of 1 µg ACTH are comparable to plasma ACTH diurnal changes [11] and the peak plasma ACTH levels produced in the low dose test are of the same order of magnitude as those obtained during insulin-induced hypoglycemia, the test was suggested to investigate the physiological adrenal sensitivity [9].

We hypothesized that in PCOS women the exaggerated 17OHP response to 250 µg ACTH may be due to the non-physiological stimulation of the adrenal glands.

To examine this hypothesis we determined the Cortisol and the androgen precursor 17-hydroxyprogesterone responses during 1 µg and 250 µg ACTH tests in a group of patients with PCOS and in normal cycling non-hirsute women.

Material and Methods

Participants and Protocol

We studied 27 PCOS, and 22 control women aged 18–30 years. The institutional review board (Helsinki committee) approved the study and all of the participants gave their informed consent before the start of the study.

The diagnosis of PCOS was based on the findings of three or more of the following criteria: oligomenorrhea and/or amenorrhea, hirsutism and/or acne, serum LH/FSH > 1, hyperandrogenemia, and polycystic ovaries on pelvic ultrasound examination (presence of 10 or more cysts, 2–8 mm in diameter, arranged around the ovarian periphery with increased echo dense stroma). Anovulation was diagnosed using a basal body temperature (BBT) chart. Other known causes of hyperandrogenism were excluded: Cushing’s syndrome, late onset adrenal hyperplasia (LOCAH), androgen secreting tumor and hyperprolactinemia.

All the control women were eumenorrheic, non-hirsute subjects with normal serum androgen levels and LH/FSH ratio < 1. We determined serum total testosterone and DHEAS levels to establish hyperandrogenemia.

Genetic Analysis of the CYP21 Gene

DNA was isolated from peripheral blood leukocytes. For mutation analysis of CYP21, we employed PCR amplification of CYP21 specific fragments and restriction enzymes analysis [12]. The following mutations in CYP21 were analyzed: P30L, I2 splice, I172L, exon 6/E6 cluster, V281L, Q318X, and the homozygous state of the exon 3 del. 8 BP [13].

ACTH tests protocol

A standard dose ACTH test was performed in all participants between 08:00 and 09:00h. An indwelling venous canula was inserted into an antecubital vein. After a resting time of about one hour between the vein puncture and the basal blood sampling, a 250 µg tetracosactin (tetracosactide esacetate) (Synacthen, Novartis Pharma AG, Basle, Switzerland) was injected IV as a bolus, after a basal blood sample had been withdrawn for Cortisol and 17OHP measurements. Additional blood samples were taken 30 and 60 minutes after injection. A low dose ACTH test was performed in all subjects three-four days after the standard dose ACTH test. The content of a 250-µg ampule of tetracosactin (Synaethen) was diluted into a bottle containing 250 ml sterile saline solution and thoroughly mixed. A venous canula was inserted and after a basal blood sample had been drawn, 1 ml of the freshly prepared solution (1 µg tetracosactin) was injected IV as a bolus. Additional blood samples for the determination of Cortisol and 17OHP were obtained 30 and 60 minutes after injection. The ACTH tests were performed in the early follicular phase of the menstrual cycle in the eumenorrheic subjects.

Hormone measurements

Blood samples were centrifuged, then immediately separated and stored at –20 °C until assayed. Commercial kits were used to determine the concentrations of serum LH, FSH, testosterone, Cortisol, 17 OHP and DHEAS. Serum LH and FSH were determined by the immunometric technique (Biodata Diagnostics, Rome, Italy). Normal levels are LH: 10–18 IU/L and FSH: 4–13 IU/L (follicular phase of the menstrual cycle). DHEAS and 17-hydroxyprogesterone were determined by radioimmunoassay methods (Diagnostic Products Corporation, Los Angeles, CA). The normal levels of these hormones are: DHEAS: 0.1–1.2 µmol/L 170HP: less than 15.1 nmol/L. Serum total testosterone and Cortisol
levels were determined by competitive immunoassay using the Immulite analyzer (Diagnostic Products Corporation, Los Angeles, CA). The normal testosterone levels are 0.7–2.8 nmol/L during the follicular phase of the menstrual cycle.

The intra-assay and interassay coefficients of variation (CVs) for Cortisol ranged from 6.8% to 9.0% and from 9.9% to 10.3%, respectively. The intra-assay and interassay CVs for 17OHP ranged from 3.5% to 7.1% and from 5.05 to 8.5%, respectively.

The LH intraassay CVs were 2.1% and 3.2% for low (2.2–3.3 IU/liter) and high (27–41 IU/liter) concentrations, respectively. The interassay CVs were 3.7% and 0.8%, respectively. The sensitivity of the assay was 0.15 IU/liter. The testosterone intraassay CVs were 0.3% and 0.0% for low (2.2–4.0 nmol/liter) and high (29.4–62.0 nmol/liter) concentrations, respectively. The interassay CVs were 1.9% and 1.6%, respectively. The sensitivity of the assay was 0.15 nmol/liter.

**Statistical analysis**

Results are expressed as the mean±SD. The responses to ACTH stimulation were calculated as follows: 1) the net maximal response (Δ), determined as the difference from baseline to maximum [peak] hormonal value; and 2) the overall response, determined by the area under the curve [AUC] for the response to ACTH for Cortisol and for 17OHP. Statistical significance was taken as p<0.05. The two groups were compared by t-tests or by Wilcoxon 2 sample tests if the variable was not normally distributed. The variations of Cortisol levels in the low dose and standard dose tests were compared by repeated measures ANOVA after logarithmic transformation of the Cortisol data. The variations of 17OHP levels in low dose and standard dose tests were analyzed by ANOVA. Spearman rank correlations were performed on all the variables in each group separately and then together. Abnormal 17OHP values in PCOS patients were considered when levels exceeded the mean in controls by 2SD.

**Results**

The clinical and laboratory data of the subjects studied are given in Table I. None of the 27 PCOS and 22 control women was carrying mutations of the CYP21 gene. The PCOS women had higher BMI, testosterone and LH values compared with the control group. Basal and stimulated Cortisol and 17OHP levels in response to the two doses of ACTH are shown in Figure 1. Repeated measures ANOVA of the transformed Cortisol data revealed a time and dose effects (p<0.0001;p<0.0006,respectively), and a time by dose interaction.

Peak Cortisol levels during the SDT (250µg) were higher than during the LDT (1µg) (Tables II–III). Post hoc testing of the interaction revealed that there was a difference in groups in the SDT (p<0.04) but not in the LDT. Moreover, at 60 minutes, Cortisol levels differed between the SDT and LDT (p<0.0001). Basal and peak

![Figure 1. Serum Cortisol and 17-hydroxyprogesterone (17OHP) levels during a 1-µg and 250-µg ACTH tests in PCOS (n=27) and control (n=22) women. Values are mean± SD. See text for significant differences between groups during the ACTH tests.](image-url)
cortisol levels during the SDT were significantly higher in PCOS compared with controls. However, the net maximal cortisol response in PCOS was significantly lower than the ∆Cortisol in controls (p<0.03). Cortisol levels were significantly higher in PCOS compared with controls after 30 minutes in the SDT (p<0.02).

Repeated measures ANOVA of the 17OHP data revealed that there was a statistically significant difference between the PCOS and control women (p<0.001), the 2 doses and a time effect (p<0.0001). The PCOS group had higher basal and peak 17OHP levels than the control women during the SDT (p<0.003; p<0.004, respectively).

However, the net maximal 17OHP responses were similar in PCOS and control women (Table III). It is apparent that in both groups of subjects, peak Cortisol and peak 17OHP were observed 30 minutes after injection of 1µg ACTH.

Correlation analysis revealed that for all subjects (PCOS and controls), testosterone was correlated with DHEAS (r=0.45, p<0.03), ACTH stimulated 17OHP (r=0.62, p<0.002) and with Cortisol (r=0.42, p<0.05). LH was significantly correlated with basal and ACTH stimulated 17OHP (r=0.54, p<0.003; r=0.51, p<0.006). Basal Cortisol levels were correlated with basal 17OHP (r=0.54, p<0.003). Peak Cortisol levels in the SDT were correlated with 17OHP levels (r=0.59, p<0.003).

Abnormal 17OHP response to ACTH stimulation (>17.0 nmol/L), was detected in 6/27 PCOS women (22.2%) during the SDT.

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**Table I. Clinical and laboratory data in PCOS and control women (data are given as mean ± SD)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCOS</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [yr]</td>
<td>23.3±4.0</td>
<td>21.7±4.2</td>
<td>NS</td>
</tr>
<tr>
<td>BMI [kg/m2]</td>
<td>27.2±6.8</td>
<td>23.7±4.8</td>
<td>0.07</td>
</tr>
<tr>
<td>Testosterone [nmol/L]</td>
<td>1.7±0.7</td>
<td>1.0±0.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>DHEAS [mmol/L]</td>
<td>0.7±0.4</td>
<td>0.5±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>LH [IU/L]</td>
<td>14.0±2.1</td>
<td>6.3±2.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>FSH [IU/L]</td>
<td>5.7±1.4</td>
<td>4.8±1.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS- not significant.

**Table II. Cortisol and 17OHP responses to low dose (1 µg) ACTH test in PCOS and control women.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCOS</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal [nmol/L]</td>
<td>414±146</td>
<td>414±132</td>
<td>NS</td>
</tr>
<tr>
<td>Peak [nmol/L]</td>
<td>681±130</td>
<td>711±163</td>
<td>NS</td>
</tr>
<tr>
<td>∆Cortisol [nmol/L]</td>
<td>267±138</td>
<td>300±148</td>
<td>NS</td>
</tr>
<tr>
<td>AUC [nmol/Lx 60 min]</td>
<td>36810±4095</td>
<td>39690±4845</td>
<td>NS</td>
</tr>
<tr>
<td>17OHP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal [nmol/L]</td>
<td>6.1±2.1</td>
<td>5.4±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Peak [nmol/L]</td>
<td>10.7±3.4</td>
<td>9.7±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>∆17OHP [nmol/L]</td>
<td>4.6±2.8</td>
<td>4.3±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>AUC [nmol/Lx 60 min]</td>
<td>540±84</td>
<td>480±90</td>
<td>NS</td>
</tr>
</tbody>
</table>

∆= net maximal hormonal response, AUC= area under the curve, NS= not significant.

**Table III. Cortisol and 17OHP responses to standard dose (250 µg) ACTH test in PCOS and control women.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCOS</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Basal [nmol/L]</td>
<td>470±138</td>
<td>232±124</td>
<td>0.03</td>
</tr>
<tr>
<td>Peak [nmol/L]</td>
<td>847±143</td>
<td>798±141</td>
<td>0.1</td>
</tr>
<tr>
<td>D Cortisol [nmol/L]</td>
<td>377±141</td>
<td>566±133</td>
<td>0.03</td>
</tr>
<tr>
<td>AUC [nmol/Lx 60 min]</td>
<td>48240±4290</td>
<td>43770±4065</td>
<td>0.1</td>
</tr>
<tr>
<td>17OHP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal [nmol/L]</td>
<td>6.4±2.1</td>
<td>4.2±2.1</td>
<td>0.003</td>
</tr>
<tr>
<td>Peak [nmol/L]</td>
<td>14.2±3.6</td>
<td>10.9±3.0</td>
<td>0.004</td>
</tr>
<tr>
<td>D 17OHP [nmol/L]</td>
<td>7.8±2.9</td>
<td>6.7±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>AUC [nmol/Lx 60 min]</td>
<td>798±108</td>
<td>636±84</td>
<td>0.01</td>
</tr>
</tbody>
</table>

∆= net maximal hormonal response, AUC= area under the curve, NS= not significant.
Discussion

In this study we have demonstrated that basal and maximal levels of 17OHP after SDT were significantly higher in PCOS compared with control women. This group difference was not observed after LDT. Compared with controls, abnormal elevated 17OHP levels were observed in 22% of PCOS women. Basal cortisol levels in PCOS were higher than the values in controls, but the $\Delta$ cortisol response to 250 µg ACTH stimulation was significantly lower in PCOS compared with control women. Basal testosterone and LH levels were significantly elevated in PCOS while DHEAS concentrations were similar in PCOS and control women.

Adrenal hyperandrogenism is observed in 40–70% of women with PCOS [2,14]. Elevated basal serum levels of DHEAS and 17OHP in PCOS were demonstrated in several studies [15–17] but others reported that mean basal 17OHP [18] or DHEAS [19] levels were similar in PCOS and control women. The most common adrenal abnormality in PCOS is hyper responsiveness to ACTH of DHEAS, 17OHP, androstenedione and 17-hydroxyprogrenolone. Cortisol responses to ACTH were also increased in PCOS women [20]. The mechanisms underlying the exaggerated response to ACTH in PCOS are still uncertain. Several explanations were suggested. Exaggerated or blunted responses of ACTH to CRH were demonstrated in PCOS patients [21] while in another report, plasma ACTH and cortisol responses to CRH were normal in PCOS women [22]. Normal Cortisol response to ACTH was shown in hyperinsulinemic and normoinsulinemic PCOS women [5]. Although the data suggested that accelerated turnover of Cortisol and resetting of ACTH secretion resulting in generalized adrenocortical over activity, hyper secretion of Cortisol in absolute terms was not documented in PCOS women [2]. Altered hypothalamic-pituitary-adrenal function was suggested to operate in PCOS women exposed to insulin hypoglycemia. In these patients, the ratio between maximal increments of Cortisol and ACTH were higher, suggesting an adaptation to increased responsiveness [20]. Accelerated turnover of Cortisol, resulting in ACTH over activity [14] or deregulation of 17-hydroxylase and 17, 20 lyase activities, in both ovaries and adrenal glands, were also suggested [2].

In our study, we used the low dose ACTH test to simulate the physiological stimulation of the adrenal to assess the cortisol pathway and the androgen precursor 17OHP Gonzalez et al [23] have also studied adrenal cytochrome P450c17α enzyme activity after physiologic vs. pharmacological ACTH stimulation in PCOS women. The authors suggested that there is an increase in adrenal androgen secretion and an increase in the activity of cytochrome P450c17α enzyme after physiologic ACTH stimulation. However, in contrast with our tests protocols, they used two 0.2 µg i.v. ACTH injection (at 2-h interval) with the SDT performed 2h after the last dose of 0.2 µg i.v. ACTH injection. Colak et al [24], investigated adrenal cytochrome P450c17α enzyme activity in PCOS women using the 1 µg ACTH stimulation test. In agreement with our results, the authors found that cortisol and 17OHP responses to LDT were similar in PCOS and control women. Measuring androstenedione and DHEAS during these tests, the authors showed that the responses of these androgens to LDT were higher in PCOS women.

Our results suggest that a subset of PCOS women have elevated basal and ACTH stimulated 17OHP levels. Hyper responsiveness to ACTH of 17OHP and cortisol is probably due to the use of supraphysiological dose of exogenous ACTH in the standard test.

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