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ORIGINAL ARTICLE

Endocrinological and genetic studies in patients with Polycystic Ovary Syndrome (PCOS)

Zahra Ghanaati,[1] Hartmut Peters,[1] Susanne Müller,[3] Manfred Ventz,[2] Bettina Pfüller,[3] Zend-Ajusch Enchshargal,[1] Wolfgang Rohde[4] & Günter Dörner[4]

1. Humboldt University, Medical School Charité, Institute of Medical Genetics,
2. Clinic for Internal Medicine,
3. Clinic for Obstetrics and Gynaecology,
4. Institute of Experimental Endocrinology, Schumannstr. 20/21,D-10098 Berlin, Germany

Correspondence to: Hartmut Peters, Ph.D., Institute of Medical Genetics, Humboldt University, Charité; 10098 Berlin, Germany
TEL: 049-030-28025681; FAX: 030-28021286
E-mail: hartmut.peters@charite.de

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Abstract Our studies involved 21 women of Caucasian descent with PCOS, as diagnosed by ultrasound, clinical and hormonal findings. We determined 17α-OHP, 21-DOF and cortisol levels both just before and one hour after intravenous ACTH administration (0.25 mg Synacthen) in addition to the basal plasma levels of DHEA-S and free testosterone. We screened the CYP21 gene for 12 common-point mutations, finding five heterozygous mutations in 4 out of 21 cases (19%): a splice-site mutation in intron 2 in three cases, and a I172N mutation in exon 4 in two cases. These patients also displayed significantly higher 21-DOF or 17α-OHP plasma levels after ACTH administration, suggesting a partial 21-hydroxylase deficiency. Furthermore, we found the basal plasma DHEA-S levels or DHEA-S/F ratios in 11 of 21 patients (52%) to be higher than the means + 2SD of control females, indicating partial 3β-HSD deficiency or 17,20-LHA. According to the literature, mutations could not be found in the genes of PCO cases with hormonal activity changes in the last two enzymes. The DDT metabolite o,p'-DDD is a strong inhibitor of 3β-HSD, and DDT can induce 17,20-LHA, implying a possible connection between cases of PCOS women born in East Germany after 1955 and their prenatal DDT uptake. The approximately fourfold higher prevalence of PCO and significantly increased frequency of 3β-HSD-deficiency and 17,20-LHA found in women with PCOS born since 1955–the time of massive application of DDT–give weight to the thesis that DDT, rather than genetic factors, has played a part in this upsurge.

Abbreviations

ACTH	Adrenocorticotrope Hormone
BMI	Body Mass Index
CYP	Cytochrom P
DHEA-S	Dehydroepiandrosterone-Sulfate
DDT	Dichlordiphenyltrichlorethane
o,p'-DDD	Dichlordiphenyldichlorethane (DDT-Metabolite)
ELISA	Enzyme-Linked Immuno Sorbent Assay
F	Cortisol
FSH	Follicle Stimulating Hormone
LH	Luteinizing Hormone
RIA	Radioimmunoassay
PCOS	Polycystic Ovary Syndrome
PCO	Polycystic Ovaries
3β-HSD	3β-Hydroxysteroid Dehydrogenase
17,20-LHA	17,20 Lyase Hyperactivity
17α-OHP	17α-Hydroxyprogesterone
21-DOF	21-Deoxycortisol
LO-CAH	late-onset congenital adrenal hyperplasia

Introduction

Polycystic Ovary Syndrome is a common multifactorial endocrine disorder of unknown etiology, presenting with symptoms including polycystic ovaries, menstrual disturbances, anovulatory infertility, hirsutism, hyperandrogenemia, adipositas, hyperinsulinism and an increased LH/FSH ratio [1, 2]. 25% of secondary amenorrhea cases and 50% of those with oligomenorrhea or hirsutism suffer from polycystic ovaries, defined as 10 or more follicles of 2–8 mm diameter and hyperplastic stroma [3]. The most consistent biochemical finding is an elevation in serum androgens [4]. Insulin resistance seems inconsistent [5]. Meirow et al. [6] have suggested a division into insulin resistant and non-insulin resistant groups. Three genetic models for PCOS have been proposed: I. the "single-gene Mendelian" model, where most defects are unique; II. the "multifactorial" model, where the defects are a conglomeration of widely distributed abnormalities; and III. the "variable expression single-gene" model, a synthesis of both [7]. Several studies point to familial clustering, suggesting a genetic component [8, 3]. An association with both the P450 cholesterol side chain cleavage gene (CYP11a) and an insulin gene minisatellite has been demonstrated [9]. No mutation involving CYP11A, CYP17, the insulin receptor gene [10, 5], the glycogen synthetase gene or IGF-II has yet been identified [9, 3]. Polson et al. [11] detected polycystic ovaries in about 25% of the hyperandrogenemia cases they screened. The groups of Polson and Talbot found more than 90% of women with a sonographic diagnosis of PCO had hormonal disturbances typical of this syndrome [11, 10]. Various authors [12, 13, 14] found evidence of heterozygous CYP21 mutations in hyperandrogenic women, though not screening for PCO. Our ACTH tests, suggesting a partial 21-hydroxylase deficiency, led us to perform a CYP21 screening on a group of PCOS patients.

In contrast, Dörner [15,16] observed an approximately fourfold increased prevalence of PCO in women born since 1955 in East Germany, following the massive application of the insecticide DDT, known to have a strong bioaccumulation and some estrogenic activity. Also, in rats, perinatal low estrogen doses produce a PCOS-like syndrome in adulthood [17]. The DDT-metabolite o,p'-DDD is a strong inhibitor of the 3β-hydroxysteroid dehydrogenase/ Δ5-Δ4-isomerase [18], and chlorinated compounds like DDT and its metabolites may also activate cytochrome P450 enzymes, like the 17,20 lyase, leading to 17,20 lyase hyperactivity [19]. These would lead to an increase in plasma levels of DHEA-S or the DHEA-S/F ratio, which were therefore assayed in our study.

Material and Methods

Patients

A group of 21 Caucasian unrelated female patients were diagnosed with PCOS after extensive clinical and ultrasound examinations. BMI was also determined. PCO was diagnosed on the unambiguous identification of 10 or more follicles (2 to 8 mm in size) and enhanced ultrasound stroma reflection.

Hormone assays

Plasma cortisol was estimated with the Synchron ELISA kit (ELIAS Medizintechnik GmbH, Freiburg, Germany) or RIA (IMMUNOTECH-Coulter Comp., Marseille, France). The intra-assay and inter-assay coefficients of variation were 4–6% and 7–9% for the ELISA, and between 3.1% and 5.8% and between 5.3% and 9.2%, respectively, for the RIA. Cortisol values from 50 samples in both assays revealed no significant differences ($p>0.05$).

21-DOF was estimated with an RIA (tracer from Amersham-Buchler GmbH, Braunschweig, antibody gift of Dr. Paul Vecsei. Heidelberg). The elute was dried, redissolved in diluted anti-21-DOF, incubated for 30 mins. at room temperature and 20 mins. in ice, then overnight with 200 μl of tracer at 4°C. The free hormone was separated with dextran-charcoal and centrifuged for 10 mins. at 2000 g. The supernatants were measured in a Wallac 1410 beta-ray counter (Pharmacia-LKB, Freiburg). The intra-assay coefficient of variation was 12%; the inter-assay coefficient of variation was 16%.

DHEA-S was estimated using a competitive enzyme immunassay (Biochem Immuno Systems GmbH, Freiburg, Germany), with intra-assay and inter-assay coefficients of variation between 6.4% and 7.05% and between 10.8% and 16.6%.

The levels of free plasma testosterone and androstenedione were determined with solid phase radioimmunoassay kits (Diagnostic Systems Laboratories Deutschland GmbH, Sinsheim), with intra-assay and inter-assay coefficients of variation for free testosterone of between 3.7% and 6.2% and 7.3% and 9.7%, respectively, and between 2.8% and 5.6% and 6.0% and 9.8%, respectively, for androstenedione. All samples were duplicated and values were calculated using the RIA calculation program of Pharmacia-LKB. 17α-hydroxyprogesterone was directly estimated using a coated tube radioimmunoassay from ICT Biomedicals GmbH (Eschwege, Germany), with intra-assay and inter-assay coefficients of variation between 7.8% and 12.3% (0.114 and 4.36 μg/L) and 9.8% and 12.9% (0.97–7.53 μg/L), respectively. Normal values for premenopausal women in the follicular phase lay at 0.10–0.16 μg/L, in the luteal phase at 0.27–2.90 μg/L and after ACTH-stimulation at under 3.2 μg/L. LH and FSH were estimated using a double sandwich enzyme immunoassay (Biochem Immuno Systems GmbH, Freiburg, Germany). The inter-assay and intra-assay coefficients of variation for LH lay between 4.8% and 7.5% and 4.0% and 8.4%, respectively, for values between 3.2 and 40.4 mIU/ml, and those for FSH between 5.2% and 7.5% and 5.3% and 6.4%, respectively, for values between 4.2 and 35.1 mIU/ml.

Mutation analysis

Genomic DNA was extracted from peripheral leukocytes using standard procedures. We screened for 12 small mutations (P30L, spl intron2, I172N, cluster in exon 6, V281L, G292S, 1760insT, Q318X, R339H, R356W, P453S, 2675G>C) within the CYP21 gene using PCR and sequencing as described by Kapelari et al. [20].

Results

Patients and hormone investigations

The main clinical features and results are shown in **Tables 1** and **2**. The mean BMI (25.7) corresponds to grade I overweight according to the WHO classification (Bray et al. 1998). Ratios of LH/FSH were elevated (>2) in 9 out of 21 patients (42.9%). Levels of F, 21-DOF, 17α-OHP, DHEA-S and free testosterone are summarized in Table II. In 4 cases out of 21 we found a strong indication of partial 21-hydroxylase deficiency, since

Patient	Age	BMI	Major findings	Ratio of LH/FSH	Heterozygous pointmutation
1	32	23	Oligomenorrhea, secondary sterility	0.8	none
2	35	25	Secondary sterility, hirsutism	2.4	none
3	35	26	Hyperandrogenemia, oligoamenorrhea	2.3	none
4	18	24	Hyperandrogenemia, secondary amenorrhea, hirsutism	0.8	none
5	39	29	Secondary amenorrhea, hirsutism	1.7	none
6	21	31	Hyperandrogenemia, secondary sterility	2.1	Spl Int 2, I172N
7	34	27	Secondary amenorrhea, hirsutism	1.3	none
8	36	24	Hyperandrogenemia, secondary amenorrhea, hirsutism	1.3	none
9	39	21	Hyperandrogenemia, secondary sterility	2.3	none
10	28	33	Oligoamenorrhea	2.5	I172N
11	39	26	Hyperandrogenemia, secondary sterility	2.1	Spl Int 2
12	33	22	Hyperandrogenemia, secondary sterility, hirsutism	0.8	none
13	30	21	Hyperandrogenemia, oligoamenorrhea, hirsutism	2.3	none
14	38	27	Hyperandrogenemia, secondary amenorrhea	0.6	none
15	22	24	Secondary amenorrhea, acne, hirsutism	1.4	none
16	28	24	Secondary amenorrhea, hyperandrogenemia	2.5	none
17	29	29	Primary sterility hyperandrogenemia	1.7	none
18	30	23	Oligoamenorrhea, acne, hirsutism, hyperandrogenemia	3.0	none
19	21	29	Secondary sterility, hyperandrogenemia	1.6	none
20	34	27	Secondary amenorrhea, hyperandrogenemia	1.3	Spl Int 2
21	22	24	Secondary amenorrhea, hirsutism, hyperandrogenemia	0.7	none

Table 1. Major findings of patients with PCOS
BMI = Body Mass Index; LH / FSH: ratio of Luteinizing Hormone and Follicle Stimulating Hormone.
Mutations: Spl Int 2 (splice site mutation intron 2), I172N (isoleucine to asparagine exchange in exon 4)

Patient	Cortisol (F) *	21-DOF *	17 α OHP*	DHEA/S	DHEAS: F (basal)	Free testostrone
No	nmol/l	nmol/l	nmol/l	µmol/l		pmol
1	242/477	0.1/0.59	1.7/3.9	3.36	13.9	6.2
2	402/639	0.22/0.89	9.1/13.0	12.90	32.2	12.8
3	448/786	0.38/1.12	5.2/8.4	6.51	14.5	14.1
4	204/686	0.33/0.53	8.1/14.9	8.10	37.3	22.4
5	412/715	0.14/0.96	3.2/8.1	6.50	15.5	5.7
6	475/1306	0.19/1.09	7.1/21.5	9.20	20.4	20.5
7	230/820	0.21/0.26	2.5/6.7	3.39	14.7	11.8
8	742/1278	0.13/0.41	7.7/8.8	11.90	16	27.1
9	773/1380	0.38/0.82	6.3/10.4	5.50	6	13.7
10	493/856	0.10/3.12	1.9/7.5	5.10	10.3	5.0
11	340/848	0.17/1.68	4.4/5.9	12.8	37.6	12.5
12	169/951	0.13/0.53	4.0/9.7	12.75	74	14.8
13	354/599	0.13/0.39	2.5/3.5	16.25	45	9.35
14	181/881	0.01/0.37	2.3/8.5	2.68	15	12.3
15	214/1200	0.01/0.37	1.3/6.0	5.26	24.5	10
16	230/670	0.16/0.40	2.9/6.3	10.10	44	8.7
17	452/984	0.12/0.40	8.1/14.6	3.91	8.6	17
18	388/769	0.14/0.32	4.7/6.6	11.90	30.7	8.8
19	395/649	0.5/0.7	7.0/9.0	7.35	18.6	12.35
20	415/1358	0.69/2.5	3.5/18.2	9.82	20.8	16.5
21	189/904	0.03/0.56	2.3/8.7	23.80	62.40	4.5
Means +2SD of controls	**447/1130**	**0.46/1.51**	**9.7/16.2**	**9.0**	**26.9**	**12.4**

Table 1. Plasma steroid hormone levels of patients with PCO.
* Before and after ACTH (0.25 mg Synacthen)

21-DOF and /or 17α-OHP plasma levels one hour after ACTH stimulation (0.25mg Synacthen) were higher than the means +2SD of control females. In addition, we found the DHEA-S plasma levels and/or the ratios of DHEAS/F to be higher than the means +2SD of the control females in 11 out of 21 patients with PCOS (52.4%), indicating a partial 3-β-hydroxylase deficiency and/or 17,20-lyase-hyperactivity.

Sequencing

Parts of the CYP21-gene were amplified using gene specific primers and sequenced at 12 positions described as prone to mutation. Two different types of heterozygous single base pair mutations were detected (Table I). In three unrelated patients, we found an identical heterozygous splice site mutation in intron 2 which activates a cryptic splice acceptor site 19 bp from the end of intron 2 and thus leads to a frame shift [21]. A heterozygous isoleucine to asparagine exchange (I172N) in exon 4 was identified in two patients (one of these also had a splice mutation on the same allele).

Discussion

This paper describes a mutational and hormonal screening in a cohort of 21 patients ultrasonically diagnosed with PCO. Our data show single heterozygous base pair CYP21 mutations in 4 patients. The four women with PCOS and CYP21 mutations also displayed clear signs of partial 21-hydroxylase deficiency through a significant rise in 21DOF or 17α-OHP plasma levels after ACTH stimulation. Azziz et al. [22] have reported several heterozygous mutations in hyperandrogenic women with LO-CAH. Other studies report several heterozygous point mutations in hyperandrogenic woman [13, 14] who, however, were not examined for polycystic ovaries. The correlation between the hormone profiles and genetic screening results found with our patients underscores the latter's usefulness with PCOS patients. In contrast to the hormone profile, genetic screening is not influenced by external factors. The frequency of heterozygous CYP21 mutations is higher (19%) than in the normal population (5-8%) [21], suggesting a link with PCOS in some cases.

The ratio of LH/FSH was significantly raised in 43% of the cases. Most importantly, basal plasma

DHEA-S levels and DHEA-S/F ratios were clearly increased, higher than the means +2 SD in controls. This suggests a partial 3β-hydroxysteroid dehydrogenase deficiency or 17,20 lyase hyperactivity. Other authors, however, were not able to find mutations in the corresponding genes [23]. This could be explained by the fact that the DDT metabolite o,p'DDD is a strong inhibitor of 3β-HSD [18], and that DDT and its metabolites may be able to activate the 17,20 lyase, a cytochrome P450 enzyme. Furthermore, DDT has some oestrogen activity, and its perinatal administration can produce a PCOS-like syndrome in rats [24].

Very significantly, there has not only been an approximately fourfold increased prevalence of PCO in women born since 1955 in East Germany, following a massive prenatal exposure to DDT, but also a notable shift in the hormone profiles of those affected. A predominance of 3β-HSD deficiencies and 17,20 lyase hyperactivity (70%) vs. 21-hydroxylase deficiency (23%) has emerged, in contrast with 21-hydroxylase deficiencies in 70% vs. 3β-HSD deficiencies or 17,20 lyase hyperactivity in 14% for those born earlier than 1955 [16]. Similar results were obtained in this study for women with PCOS born since 1955, suggesting that the prenatal exposure of high amounts of DDT and its metabolites indeed appear to be responsible—at least in part—for the major increase in PCO and PCOS.

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