Cyproterone acetate-ethinyl estradiol treatment alters urinary 6-sulfatoxymelatonin excretion in hyperandrogenic women

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ORIGINAL ARTICLE

Abstract

OBJECTIVES: To investigate melatonin production in hyperandrogenic women before and during treatment with cyproterone acetate and ethinyl estradiol (Diane 35).

MATERIAL AND METHODS: We studied 10 women with late onset adrenal hyperplasia due to 21-hydroxylase deficiency (LOCAH) and 10 women with idiopathic hirsutism (IH). Patients were treated with Diane 35 for four months. Fasting blood samples for the determination of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone and dihydroepiandrosterone sulfate (DHEAS) and 24-hour urine collections for the determination of 6-sulfatoxymelatonin (aMT6s) excretion were obtained from all patients at baseline and after 4 months of treatment. Results were compared with those obtained in 15 control women.

RESULTS: At baseline, women with LOCAH had significantly higher serum testosterone, 17-hydroxyprogesterone (17OHP) and ACTH stimulated 17OHP values than IH and control women. Their aMT6s values (51.0 ± 20.5 µg/24h) were significantly higher than the values in IH (34.3 ± 7.1) and control women (30.5 ± 6.5) ($p < 0.001$). Diane 35 treatment significantly decreased serum LH, FSH and testosterone levels and aMT6s values in LOCAH patients (29.8 ± 16.6 µg/24h) ($p < 0.0001$) in LOCAH patients.

CONCLUSIONS: These results indicate that hyperandrogenic women with LOCAH have increased melatonin production. The normalization of aMT6s and testosterone values during cyproterone acetate-ethinyl estradiol treatment, suggest that sex steroids either directly or through the suppression of gonadotropin, modulate melatonin secretion in these patients.

ABBREVIATIONS
1. ACTH   Adrenocorticotropic hormone
2. aMT6s   6-sulfatoxymelatonin
3. BMI   Body mass index
4. DHEAS   dihydroepiandrosterone sulfate
5. DIANE 35 cyproterone acetate-ethinyl estradiol
6. FSH   follicle-stimulating hormone
7. IH   idiopathic hirsutism
8. LH   luteinizing hormone
9. LOCAH   late onset adrenal hyperplasia due to 21-hydroxylase deficiency
10. 17OHP   17-hydroxyprogesterone

Introduction

The role of melatonin in human reproduction is still enigmatic at present. A large body of information suggests that melatonin and the reproductive hormones are inter-related under both physiological and pathological conditions [1]. This concept is based on observations of increased melatonin levels in hypogonadal patients with GnRH deficiency [2–5], decreased melatonin concentrations in precocious puberty [6] and in primary hypogonadism [7–8]. Testosterone or estradiol (E2) replacement therapy given to hypogonadal patients normalized their melatonin levels [3–4,8]. Long-term melatonin administration given to men decreased sperm density and motility as well as seminal plasma E2 levels [9].

Melatonin secretion profiles in hyperandrogenic women were examined in only few studies. In children with congenital adrenal hyperplasia, plasma melatonin levels were normal and replacement therapy with glucocorticoids did not modify their melatonin levels [10]. In hyperandrogenic non-obese women, urinary sulfatoxymelatonin concentrations were higher compared to matched controls [11]. Recently, we have demonstrated that women with late onset adrenal hyperplasia due to 21-hydroxylase deficiency (LOCAH) had elevated melatonin production that was associated with their increased androgen levels [12]. Compared with LOCAH, idiopathic hirsutism (IH), a form of hyperandrogenism found in women with regular ovulatory cycles and normal androgen levels, results from hyperactivity of 5α-reductase in peripheral tissue [13]. Cyproterone acetate is the most widely used drug for the treatment of hirsutism [14]. Treatment of polycystic ovary syndrome and idiopathic hirsutism patients with cyproterone acetate-ethinyl estradiol (Diane 35) effectively decreased the hirsutism score and reduced androgen levels in these patients [15–16].

The measurement of urinary 6-sulfatoxymelatonin (aMT6s), the major metabolite of melatonin, was shown to correlate with plasma hormone levels and is considered to be a good indicator of pineal melatonin secretion [17–19].

To further delineate possible relations between melatonin and the reproductive hormones, we studied melatonin secretion, as determined by the 24-hour urinary aMT6s excretion, in hyperandrogenic women with LOCAH and IH before and after 4 months of antiandrogen treatment with Diane 35.

Subjects and methods

Participants and Protocol

We studied 10 LOCAH, 10 IH and 15 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 21-hydroxylase deficiency (LOCAH) was made on the basis of genetic analysis and a serum 17-hydroxyprogesterone (17OHP) level after ACTH (250 μg IV) more than 2SD above the normal mean [20]. The diagnosis of IH was made by the findings of hirsutism and/or acne in patients who had regular menstrual cycles, normal androgen levels, LH/FSH ratio < 1, normal ovarian ultrasound patterns, and normal 17OHP response to ACTH. All 20 hyperandrogenic women were treated with Diane 35 (Schering, Berlin, Germany) from day 1 to day 21. Fasting serum FSH, LH, testosterone and DHEAS and the 24-hour urinary aMT6s excretion were determined before and after 4 months of treatment.

Hormone Measurements

Melatonin was determined as urinary aMT6s. Urine was collected over a 24-hour period; the volume of each collection was recorded and aliquots were frozen at –20°C until assayed. Creatinine concentration was determined in each sample to verify a complete 24-hour collection as previously described [12]. The concentrations of aMT6s in urine samples were determined by an enzyme immunoassay method (ELISA). The melatonin sulfates ELISA kits were provided by immunobiological laboratories, Hamburg, Germany. The assay sensitivity was 0.3 ng/ml. The intra-assay and interassay coefficients of variation (CV) were 10% and 16%, respectively. The cross-reactivity of the anti-melatonin sulfate antiserum is 0.0002% for melatonin, 0.001% for 6-hydroxymelatonin, 0.0005% for N-acetyl-L-hydroxytryptophan and less than 0.0001% for N-acetyl-L-tryptophan.

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, 17 OHP and DHEAS. Serum LH and FSH were determined by the immunometric technique (Biodata Diagnostics, Rome, Italy). Normal levels are LH: 10–18 mIU/ml and FSH: 4–13 mIU/ml (follicular phase of the menstrual cycle). DHEA-S and 17-hydroxyprogesterone were determined by radioimmunoassay methods (Diagnostic Products Corporation, Los Angeles, CA). The normal levels of these hormones are: DHEAS: 35–430 ng/ml and 17OHP: less than 5.0 pg/ml. Serum total testosterone levels were determined by competitive immunoassay using the Immulite analyzer (Diagnostic Products Corporation. Los Angeles, CA). The normal levels are 0.2–0.8 ng/ml during the follicular phase of the menstrual cycle.

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 [21]. 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 (E3 del8) [22].

Statistical Analysis

We have examined urinary aMT6s (expressed as total per 24-hour, per BMI, per body weight, and as the rate of excretion), serum LH, FSH, testosterone and DHEAS in-patients with LOCAH and IH, and in control women. Data are given as mean ± SD. Comparison among aMT6s, LH, FSH, testosterone and DHEAS values before and during Diane 35 treatment were performed using the Wilcoxon matched pair's rank test. Comparison between the different groups (PCOS vs. control, IH vs. control and LOCAH vs. controls; IH vs. controls) was conducted using the Mann Whitney test. Spearman rank correlation was computed for the entire population and for each group separately.

Results

The clinical and endocrine characteristics of the study subjects are given in table 1. The LOCAH women had hirsutism score (13.6±3.7), which was similar to the index observed in IH (15.1±3.6) as compared with a score of <8 in controls (p<0.0001). The three groups had similar BMI values. All 10 patients with LOCAH were carrying mutations of the CYP21 gene. Six women carried the Q318X mutation and four women carried the V281L mutation. Their serum testosterone, basal and ACTH stimulated 17OHP levels were significantly higher than the values in IH and control women (table 1). The three groups did not statistically significantly differ in DHEAS levels.

The results of urinary aMT6s excretion values in the women studied before and during treatment are given in table 2. At baseline, LOCAH patients had statistically significantly higher aMT6s values than did the IH and control women. Analysis of the pretreatment and during treatment variables revealed that testosterone levels during treatment differed from pretreatment levels only in LOCAH (p<0.02), whereas DHEAS during treatment differed from the pretreatment levels in LOCAH and IH (p<0.02) (table 1). Serum gonadotropin levels were significantly suppressed during Diane 35 treatment as compared to baseline values in LOCAH and IH women. The aMT6s values during treatment were statistically significantly different from the pretreatment values in LOCAH (table 2, *figure 1*). Spearman correlation analysis revealed that in LOCAH patients aMT6s values positively correlated with DHEAS(r=0.41;p=0.02) and with ACTH stimulated 17OHP (r=0.36;p=0.04) but not with testosterone (r=0.04;p=not significant). In control women aMT6s values were positively correlated with testosterone (r=0.51;p=0.05 for aMT6s per body weight and r=0.53;p= 0.04 for rate of aMT6s excretion).

Discussion

In this study, we have evaluated the daily melatonin excretion in hyperandrogenic women and studied the effect of Diane 35 treatment on aMT6s excretion. In LOCAH patients, testosterone and aMT6s values were increased in comparison to IH and control women. Treatment with Diane 35 was associated with a significant decrease in serum testosterone and urinary

Table 1. Clinical and hormonal data for hyperandrogenic women before and during Diane 35 treatment (Data are mean ± SD).

Parameter	LOCAH (n=10)		IH (n=10)		Control (n=15)
	Baseline	4 months	Baseline	4 months	
Age (years)	21.4±2.7		21.6±2.8		23.7±2.2
BMI (kg/m2)	24.9±3.3	24.9±3.3	23.7±3.0	23.7±3.0	24.0±5.2
LH (mIU/ml)	5.3±2.3	2.3±1.2xx	4.9±2.3	2.7±1.2xx	4.6±2.2
FSH (mIU/ml)	5.6±1.8	2.7±1.1xx	5.1±1.9	2.8±1.1xx	6.5±1.4
Testosterone (ng/ml)	0.5±0.1xxx	0.3±0.1	0.3 ±0.1	0.3±0.2	0.3±0.1
DHEAS (ng/ml)	314±101	194±103xx	321±207	214±100xx	197±84

xx p<0.02-compared with pretreatment values; **xxx** p<0.05 LOCAH vs. Control

Table 2. Urinary 6-sulfatoxymelatonin (aMT6s) excretion values in hyperandrogenic women before and during Diane 35 treatment (Data are mean ± SD).

aMT6s	LOCAH		IH		Control
	Before	During	Before	During	
Total (ng/24h)	51.0±20.5x	29.8±16.6xx	34.3±7.1	28.8±12.3	30.5±6.5
Rate of excretion (ng/h/kg)	32.7±17.2x	18.9±9.2xx	19.4±7.6	16.4±10.8	21.4±7.2
Per body weight (ng/kg/24h)	788±411x	455±219xx	555±202	392±259	530±186
Per BMI (ng/kg/m2)	2037±1071x	1191±605xx	1347±524	1031±727	1330±429

x p<0.001 LOCAH vs.controls; **xx** p<0.0001,compared with pretreatment values.
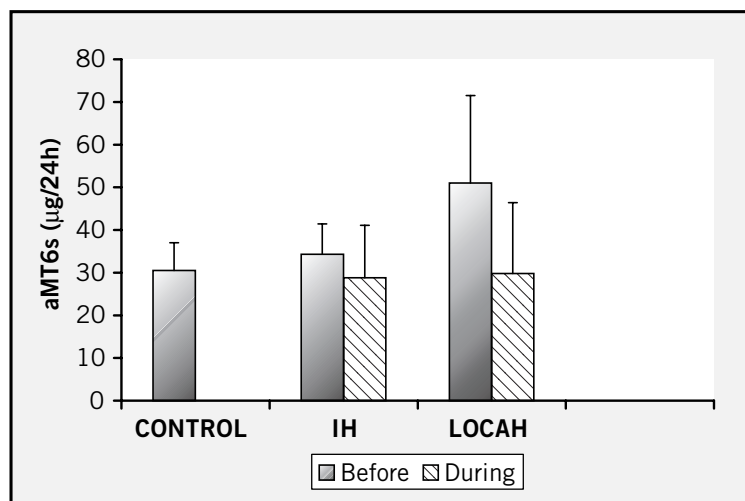
Figure 1. Urinary 6-sulfatoxymelatonin (aMT6s) excretion data in hyperandrogenic and control women (**LOCAH**: Late onset adrenal hyperplasia due to 21-hydroxylase deficiency; **IH**: idiopathic hirsutism) before and after four months of treatment with Diane 35(ethinyl estradiol-cyproterone acetate). At baseline, LOCAH patients had significantly higher aMT6s values than IH and control women ($p < 0.001$). During treatment aMT6s values were significantly lower than the pretreatment values in these patients ($p < 0.0001$).

aMT6s values in LOCAH patients. The efficacy of Diane 35 on androgen levels in hyperandrogenic women was previously described in numerous studies [15–16,13]. During Diane 35 treatment, gonadotropin, testosterone, dihydrotestosterone and DHEAS were suppressed whereas sex hormone-binding globulin (SHBG) levels increased [14,16]. The increase in SHBG levels resulted in decreased free testosterone fraction [23]. In the present study, correlation analysis revealed that in LOCAH patients, aMT6s values positively correlated with DHEAS and 17OHP levels, suggesting that increased melatonin secretion is associated with their androgen levels.

Several reports imply that gonadal steroids may modulate melatonin secretion. This hypothesis is supported by the current findings of decreased aMT6s values during Diane 35 treatment with concomitant decline in androgen levels in the present study, presence of androgen and estrogen receptors in the rat and human pineal glands [24,25], and decreased melatonin content during the addition of testosterone to perfused rat pineals [26]. The diminution of melatonin levels after estrogen replacement in women with GnRH deficiency also suggest that sex steroids, through the activation of specific receptors in the pineal gland, modulate melatonin secretion. Yet the efficacy of estradiol in modulating melatonin secretion was not confirmed by others [27], nor was suppression of estradiol levels during GnRH agonist therapy given to female patients with precocious puberty, associated with normalization of melatonin levels [6]. The possibility that androgens, either directly or indirectly through the suppression of gonadotropin, may modulate melatonin secretion is suggested by several observations. In GnRH deficient men and in patients with Klinefelter's syndrome, testosterone replacement successfully normalized melatonin concentrations [4,8,28]. On the other hand, in

men with leuprolide-induced hypogonadism, testosterone treatment did not change melatonin levels [29]. Similarly, in primary hypogonadism and in adult-onset hypogonadotropic hypogonadism, testosterone replacement therapy did not alter blood melatonin levels [17,30]. These data indicate that the pathophysiological role of melatonin in human reproduction is far from being clarified. It is clear though, that there is no simple classic feedback regulation between the pineal and the gonads.

In conclusion, LOCAH women have increased melatonin production, which is normalized during cyproterone acetate-ethinyl estradiol treatment. In hyperandrogenic women due to idiopathic hirsutism, melatonin secretion is normal and not altered during Diane 35 treatment.

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