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CLINICAL ORIGINAL ARTICLE

Increased Urinary 6-sulfatoxymelatonin Excretion in Women with Non-classical Steroid 21-hydroxylase Deficiency

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Abstract

OBJECTIVES: To determine melatonin production in hyperandrogenic women.

MATERIAL AND METHODS: Seventeen women with late onset adrenal hyperplasia due to 21-hydroxylase deficiency (LOCAH) and 15 control women were studied in early follicular phase of the menstrual cycle. Fasting serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E_2), testosterone, dihydroepiandrosterone sulfate (DHEA-S), 17-hydroxyprogesterone (17-OHP) as well as the peak 17-OHP response to ACTH (250 μg IV) and 24h urinary 6-sulfatoxymelatonin (aMT6s) were determined in all participants.

RESULTS: All 17 hyperandrogenic women were carrying mutations of the CYP21 gene. Women with LOCAH had significantly higher serum testosterone, DHEA-S, 17-OHP and ACTH stimulated 17-OHP values compared with controls. Their aMT6s values (44.6±20.3 μg/24h) were significantly higher than the values in control women (31.5±20.3) ($p<0.03$). The urinary aMT6s values were positively correlated with testosterone ($p<0.04$), DHEA-S ($p<0.02$) and peak 17-OHP ($p<0.04$).

CONCLUSIONS: Women with LOCAH have increased melatonin production. There is a relationship between adrenal androgens and melatonin in these women.

Introduction

The significance of melatonin in human reproduction is not established at present. However, a large body of information suggests that melatonin and the reproductive hormones are inter-related. This concept is based on observations of elevated melatonin levels in hypogonadal patients due to GnRH deficiency [1-4], decreased melatonin concentrations in primary hypogonadism [5] and in precocious puberty [6]. Testosterone or E_2 replacement therapy given to hypogonadal patients, normalized their melatonin levels [2, 7]. 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 [8–10].

Non-classical adrenal hyperplasia due to 21-hydroxylase deficiency (LOCAH) is the most frequent autosomal-recessive genetic disorder in man. LOCAH is a mild disorder of cortisol biosynthesis characterized clinically in syptomatic females by manifestations of androgen excess as hirsutism, acne, menstrual disorders or unexplained sterility [11, 12]. In most patients, serum 17OHP levels are elevated after stimulation with ACTH [13]. On occasion, blood DHEA-S levels are increased after ACTH [11]. Levels of 3α-androstanedione glucoronide, an androgen metabolite, are elevated in LOCAH patients and highly correlated with levels of androstanedione and testosterone [14].

So far, melatonin secretory profiles in women who are hypoandrogenic as a result of adrenal hyperplasia have not been studied. Based on the literature cited, we hypothesized that hyperandrogenic state would cause lower melatonin production. To examine this hypothesis, we determined 24h urinary aMT6s excretion in hyperandrogenic women with LOCAH and in normal cycling nonhirsute women.

Material and methods

Subjects and Protocol

The study was approved by the Institutional Review Board (Helsinki Committee) and all participants gave their informed consent before the start of the study. The control group for this study consisted of 15 normal women aged 23.7±5.2 years with regular menses and no signs of virilization. None were taking medications including oral contraceptives. The patient population consisted of 17 women aged 21.9±5.0 years, all carrying mutations for the CYP21 gene. Patients were referred for investigation of hirsutim, acne or menstrual disorders. Hirsutism was defined as a score ≥8 by the Ferriman-Gallwey index [15]. Oligomenorrhea was defined as menstrual cycles ≥35 days [16]. Obesity was assessed by estimating body mass index (BMI) with normal values ranging between 17.0–25.9 kg/m^2 [17]. Blood samples and urine collection were performed during early follicular phase of the spontaneous menses when present or otherwise in amenorrhea, for the determination of serum LH, FSH, prolactin, E_2, testosterone and DHEA-S. In all participants we determined serum 17OHP at base-line and in response to 250 μg IV ACTH at 30 and 60 mins.

The diagnosis of non-classical steroid 21-hydroxylase deficiency was made on the basis of genetic analysis and a serum 17OHP level after ACTH more than 2SD above the normal mean [18].

Melatonin production was assessed as the 24h urinary 6-sulfatoxymelatonin (aMT6s) excretion. All studies were performed between January and May of the same year.

Genetic Analysis of the CYP21 Gene

DNA was isolated from peripheral blood leucocytes. For mutation analysis of CYP21, we employed PCR amplification of CYP21 specific fragments and restriction enzymes analysis [19]. The following mutations in CYP21 were analysed: P30L, I2 splice, I172L, exon 6(E6) cluster, V281L, Q318X, and the homozygous state of the exon 3 del 8 bp (E3 del8) [20].

Hormone Measurements

Blood samples were centrifuged, immediately separated and stored at -20°C until assayed. The concentrations of serum LH, FSH, E_2, testosterone, cortisol, DHEA-S and 17OHP were determined by commercial kits. 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). Serum cortisol, DHEA-S and 17-hydroxyprogesterone were determined by radioimmunoassay methods (Diagnostic Products Corporation, Los Angeles, CA). The normal levels of these hormones are as follows: cortisol – 5–25 μg/dl, DHEA-S – 35–430 ng/ml and 17OHP – less than 5.0 pg/ml. Serum total testosterone and 17β estradiol were determined by competitive immunoassay using the Immulite analyzer (Diagnostic Products Corporation. Los Angeles, CA). The normal levels are: testosterone – 0.2–0.8 ng/ml and E_2 – 7–178 pg/ml during the follicular phase of the menstrual cycle. Melatonin was measured as urinary 6-sulfatoxymelatonin (aMT6s). Urine was collected over a 24h period, the volume of each collection was recorded and aliquotes were frozen at –20°C until assayed. Creatinine concentration was determined in each sample to verify complete 24h collection with values of 18mg/kg/24h taken for normal females [21].

The concentrations of aMT6s in urine samples were determined by an enzyme-immunoassay method (ELISA) as previously described [22]. The melatonin-sulfate ELISA kits were provided by Immunobiological Laboratories, Hamburg, Germany. The assay sensitivity was 0.3 ng/ml. The intra-assay and inter-assay coefficients of variation (CV) were 10% and 16%, respectively.

The assay sensitivity was 0.3 ng/ml. 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.

Statistical Analysis

We examined urinary aMT6s values expressed as total μg per 24h, serum testosterone, DHEA-S, 17OHP (basal and peak) as well as LH, FSH and E_2 levels in LOCAH patients and controls. Data are given as mean±SD. Differences between groups were tested by Wilcoxon 2 sample test. Spearman rank correlation were computed for the entire population and for each group separately.

Results

The clinical and endocrine characteristics of the study subjects are shown in Table 1. All 17 patients with LOCAH had hirsutism, four had acne (23.5%), three had oligomenorrhea (17.6%) and two patients were amenorrheic (11.6%). Genetic analysis revealed that nine women carried the Q318X mutation and eight women carried the V281L mutation. Serum testosterone, basal and ACTH stimulated 17OHP levels were significantly higher in LOCAH patients than in controls. Their cortisol levels at baseline and in response to ACTH stimulation were not different from values in controls. Serum DHEA-S levels in LOCAH patients (236±90 ng/ml) were higher than the values in controls (196±84 ng/ml) although not statistically significantly different.

Results of urinary aMT6s excretion are shown in Table 2. Patients had significantly higher total aMT6s values (44.6±20.3 μg/24h vs. 31.5±7.5 in controls, $p<0.03$). When aMT6s were expressed per body weight, per BMI or as the rate of excretion, values in patients were statistically not significantly different from the values in controls.

Spearman correlation analysis revealed that aMT6s values were positively correlated with DHEA-S levels ($r=0.41$; $p=0.02$) and with peak 17OHP levels ($r=0.36$; $p=0.04$) but not with testosterone levels ($r=0.04$; p=NS). A significant positive correlation between aMT6s (expressed as rate of excretion) and testosterone was observed in controls ($r=0.53$; $p=0.04$) or when expressed per body weight ($r=0.51$; $p=0.05$). In LOCAH patients, these correlations were of borderline significance ($r=-0.42$; $p=0.09$, $r=-0.44$; $p=0.07$, respectively). Plot of BMI vs. aMT6s (Fig. 1) revealed a weak correlation between these two variables. Regression analysis indicated no linear relationship between aMT6s and BMI in either group.

Table 1. Clinical and Endocrine Characteristics in Hyperandrogenic Women with Late-Onset Adrenal Hyperplasia due to 21-Hydroxylase Deficiency (Data are the mean±SD)

Group	Age (years)	BMI (kg/m^2)	Hirsutism Score*	Testosterone (ng/ml)	DHEA-S (ng/ml)	E_2 (pg/ml)	LH (mIU/ml)	FSH (mIU/ml)	ACTH Stimulation Test 17OHP (pg/ml) Basal	17OHP (pg/ml) Peak	Cortisol (μg/dl) Basal	Cortisol (μg/dl) Peak
Patients (n=17)	21.9±5.0	26.4±4.9	13.6±3.7	0.5±0.2	236±89	57.6±25.9	5.3±2.3	5.6±1.8	3.6±0.7	15.7±3.8	13.3±3.9	30.3±6.1
Controls (n=15)	23.7±5.2	24.0±5.2	<8	0.3±0.1	196±84	50.9±21.3	4.6±2.2	6.5±1.4	1.3±0.7	3.5±0.9	14.1±5.6	29.5±5.3
P value	N.S.	0.05	0.0001	0.03	N.S.	N.S.	N.S.	N.S.	0.02	0.0001	N.S.	N.S.

* Ferriman-Gallwey Index N.S.: Not significant

Table 2. Urinary 6-Sulfatoxymelatonin (aMT6s) Excretion in Hyperandrogenic Women with Late-Onset Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. (Data are the Mean±SD)

Group	AMT6s-total (μg/24h)	AMT6s per BMI (ng/kg/m^2)	AMT6s – Rate of excretion (ng/h/kg)	AMT6s – Per Body weight (ng/kg/24h)
Patients (n=17)	44.6±20.3	1757±905	28.1±14.9	675±357
Controls (n=15)	31.5±7.5	1375±429	22.4±7.8	537±186
P value	0.03	N.S.	N.S.	N.S.

N.S.: not significant

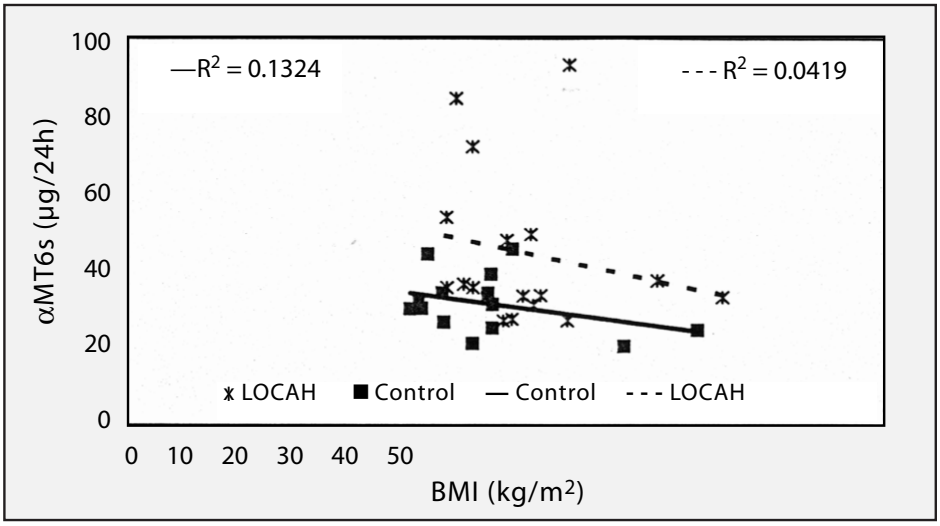

Fig. 1. Urinary aMT6s excretion in hyperandrogenic women with late-onset adrenal hyperplasia (LOCAH) and controls revealed a weak correlation with BMI. Regression analysis indicates no linear relationship between melatonin and BMI in patients and controls.

Discussion

In the current study we have demonstrated that total aMT6s values were significantly higher in hyperandrogenic women with late-onset adrenal hyperplasia compared with controls. Total aMT6s values positively and significantly correlated with DHEA-S and peak 17OHP levels. Also, a positive correlation was observed between testosterone and aMT6s expressed as rate of excretion. Our findings first demonstrate that LOCAH patients have increased melatonin secretion which is associated with their androgen levels.

How the reproductive hormones modulate melatonin secretion in humans is still largely unknown. Several candidates are possible: androgens (either directly or through conversion to estradiol), gonadotropins, adrenal steroids, mainly cortisol or a combination of all. In children with congenital adrenal hyperplasia who had low cortisol levels, plasma melatonin levels were normal. Replacement therapy with glucocorticoids did not modify their melatonin levels [23]. In patients with Cushing's syndrome, melatonin secretion was intact [24]. In our study, all patients had intact basal and ACTH stimulated cortisol levels, suggesting that cortisol does not participate in the modulation of melatonin secretion.

A possible relationship between melatonin and GnRH or gonadotropins was suggested by the findings of increased melatonin secretion in hypogonadal patients [3,4], decreased melatonin levels in conditions of gonadotroipins excess as precocious puberty [6] and Klinefelter's syndrome [5], the demonstration of melatonin receptors in the hypothalamic suprachiastmatic nuclei [25] and by the enhancing effect of melatonin on the gonadotropins response to GnRH in normal women [26].

However, the lack of correlation between serum gonadotropin levels and aMT6s in the present study and in a previous report [27] suggest that the possibility of gonadotropins or GnRH modulate melatonin secretion is unlikely.

In favor of estrogens affecting melatonin secretion in our patients are the findings of elevated melatonin levels in estrogen deficient women with endometriosis receiving GnRH analogue therapy and the diminution of melatonin after E_2 replacement in women with GnRH deficiency [2]. Yet the efficacy of E_2 in modulating melatonin secretion was not confirmed by others [28], nor was suppression of E_2 levels during GnRH agonist therapy given to patients with precocious puberty, associated with normalization of melatonin levels [6]. In the current study, E_2 serum levels were normal in LOCAH patients and were not correlated with aMT6s values suggesting that estrogens probably do not participate in the modulation of melatonin secretion.

A more plausible explanation is that androgens modulate melatonin secretion in our patients. This hypothesis is supported by the findings of significant positive correlations between aMT6s and serum androgen levels in the current study, increased aMT6s values in hyperandrogenic women with polycystic ovary syndrome which was also correlated with their testosterone levels [29], the normalization of melatonin levels during testosterone treatment given to hypogonadal men [3,7] and by the demonstration of androgen and estrogen receptors in rat and human pineal glands [30].

On the other hand, in men with leuprolide-induced hypogonadism, testosterone treatment did not change melatonin levels [31]. Similarly, in primary hypogonadism or in adult-onset hypogonadism, testosterone replacement therapy did not alter plasma melatonin levels [22, 32]. Caglayan et al [27] have recently demonstrated that in patients with Klinefelter's syndrome, plasma melatonin levels tended to be higher than levels in normal controls, whereas those of

aMT6s were lower. Testosterone replacement was associated with a fall in plasma melatonin levels and urinary aMT6s values increased. The authors suggested that alterations in melatonin metabolism rather than any effect on net sympathetic activity were responsible for their findings. These data suggest that the pathophysiological role of melatonin in human reproduction is far from being clarified. It is clear though that there is no simple classic feed-back regulation between the pineal and the gonads.

In conclusion, women with adrenal hyperandrogenism due to 21-hydroxylase deficiency have increased melatonin production which is associated with their increased androgen secretion.

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