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Steroidogenic pathways involved in androgen biosynthesis in eumenorrheic women and patients with polycystic ovary syndrome

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ABSTRACT

The conventional Δ5 and Δ4 steroidogenic pathways mediate androgen production in females. While multiple non-conventional pathways to dihydrotestosterone (DHT) have recently been postulated in humans, the functional significance of these pathways remains to be elucidated. The aim of this study was to clarify the origin of androgens in healthy women and in patients with polycystic ovary syndrome (PCOS), a multifactorial disorder characterized by androgen overproduction. We measured 13 steroids in blood samples of 31 eumenorrheic females and 28 PCOS patients using liquid chromatography-tandem mass spectrometry and chemiluminescent enzyme immunoassay. We found that 17-hydroxy (17-OH) progesterone (17-OHP), androstenedione (Δ4A), testosterone, androstanedione, androsterone, and androstanediol levels were higher in the patient group than in the eumenorrheic group, while levels of other steroids were comparable between the two groups. In the eumenorrheic group, DHT levels were correlated with testosterone, androstanedione, and androstanediol. Quantitative correlations were also observed among 17-OH allopregnanolone, androsterone, androstanediol, and DHT, and among Δ4A, androstanedione, androsterone, and androstanediol. In the patient group, DHT levels were correlated with testosterone levels, but not with androstanedione or androstanediol levels. Δ4A and testosterone paralleled 17-OHP. Androstanedione, androsterone, androstanediol, and 17-OH allopregnanolone were quantitatively correlated. In both groups, multivariable linear regression analyses suggested relationships between androsterone and androstanedione, as well as between androsterone and 17-OH allopregnanolone. These results indicate that multiple androgen biosynthesis pathways are operating in eumenorrheic females and PCOS patients. In PCOS patients, excessive androgens are produced primarily via the conventional pathways, while two alternative pathways; *i.e.,* an androstanedione-mediated pathway and a so-called backdoor pathway, likely serve as sources of a weak androgen and potential precursors of DHT.

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Abbreviations: BMI, body mass index; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate; DHT, dihydrotestosterone; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PCOS, polycystic ovary syndrome; 17-OH, 17-hydroxy; 17-OHP, 17-hydroxyprogesterone; Δ4A, androstenedione.

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1. Introduction

Healthy women secrete larger amounts of androgens than estrogens [1]. Androgen biosynthesis in females is mediated by two conventional pathways; namely, the Δ5 and Δ4 pathways (Graphical abstract) [2–4]. These pathways operate in the ovaries and adrenals [1]. Testosterone has the capacity to be metabolized by peripheral 5α-reductase into the most potent androgen, dihydrotestosterone (DHT) [1,2]. Recent studies have postulated a "backdoor pathway" to DHT that starts with 5α,3α-reduction of progesterone or 17-OHP (Graphical abstract) [2,5]. However, the functional significance of this backdoor pathway remains to be elucidated.

Androgen excess is one of the key clinical features in patients with polycystic ovary syndrome (PCOS), a multifactorial disorder that affects 5–10% of reproductive age females [6–8]. In PCOS patients, large amounts of dehydroepiandrosterone (DHEA), androstenedione (Δ4A), and testosterone are produced in the ovaries through the Δ5 and Δ4 pathways [2,9–11]. The adrenal glands are also likely to be sites of these pathways, because blood levels of DHEA-sulfate (DHEA-S), the marker of adrenal androgen precursors, are elevated in a substantial percentage of PCOS patients [8,12,13]. Activity of steroidogenic enzymes in the conventional pathways is often dysregulated in patients with PCOS [2–4,10,11,14]. However, it is currently unknown whether androgens in PCOS are exclusively derived from these conventional pathways. The backdoor pathway may provide an alternative route from 17-OHP to DHT in patients with PCOS, because blood 17-OHP levels are usually increased in these individuals [11]. Indeed, the backdoor pathway has been implicated in DHT overproduction in genetic females with steroid 21-hydroxylase deficiency and cytochrome P450 oxidoreductase deficiency [15,16]. In addition, Fassnacht et al. have raised the possibility that an alternative non-conventional pathway from DHEA to DHT is activated in PCOS patients [17]. This assumption is primarily based on the observation that oral DHEA administration during dexamethasone-induced adrenal suppression led to a greater increase in blood DHT levels in PCOS patients than in control individuals, while the treatment caused a similar increase in testosterone levels in both groups [17]. The authors proposed that the non-conventional pathway is driven by increased activity of peripheral 5α-reductase, which converts Δ4A into androstanedione (5α-androstane-3,17-dione). However, since no other studies have validated these results, the presence of this androstanedione-mediated pathway remains rather speculative.

The aim of this study was to determine the steroidogenic pathways involved in androgen production in eumenorrheic females and PCOS patients. To this end, we measured several steroids in blood samples using the liquid chromatography-tandem mass spectrometry (LC–MS/MS), which is a more sensitive and accurate method in steroid measurements than standard immunoassays [11].

2. Materials and methods

2.1. Ethical approval

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development.

2.2. Subjects

The study group consisted of 31 Japanese females with regular menstrual cycles ("the eumenorrheic group") and 28 Japanese patients with PCOS ("the patient group") (Table 1). The eumenorrheic group included healthy women aged 20–46 years who showed regular menses and no clinical signs of hyperandrogenism (Supplementary Table 1). The PCOS group comprised all patients who satisfied the following conditions: i) visited our clinics between May 2014 and January 2015; ii) diagnosed with PCOS based on the Rotterdam criteria [6]; and iii) gave written informed consent to participate in this study. Specifically, all patients showed oligomenorrhea (menstrual intervals of >35 days) or anovulation (Supplementary Table 2). All but one patient had ultrasonography-diagnosed polycystic ovaries (≥ 12 follicles with a diameter of 2–9 mm). Most patients had clinical hyperandrogenism, *i.e.*, hirsutism, acne, and/or male-type alopecia; and/or biochemical hyperandrogenism, *i.e.*, high serum levels of testosterone and Δ4A indicated by routine laboratory examinations in the clinic. Patients with Cushing syndrome, hypothalamic amenorrhea, or hyperprolactinemia were excluded from this study. The mean age and body mass index (BMI) were similar between the eumenorrheic and patient groups (Table 1).

2.3. Laboratory methods

2.3.1. Steroid measurements

Blood samples of the eumenorrheic females were collected during the mid-follicular phase. Samples of PCOS patients with oligo/amenorrhea were obtained during the course of diagnostic evaluations after confirmation of the absence of follicles with a diameter of >9 mm. The serum specimens were frozen immediately after sampling.

Blood levels of 12 steroids, *i.e.*, pregnenolone, progesterone, 17-OH pregnenolone, 17-OHP, allopregnanolone, 17-OH allopregnanolone, Δ4A, testosterone, DHT, androstanedione, androsterone, and androstanediol were measured with LC–MS/MS (ASKA Pharmaceutical Medical Corporation, Kanagawa, Japan). DHEA-S was measured by chemiluminescent enzyme immunoassay (LSI Medience Corporation, Tokyo, Japan).

2.3.2. LC–MS/MS procedures

2.3.2.1. Sample extraction. Serum samples (0.2 ml) were diluted with 1.0 ml of 0.1 M KH_2PO_4 solution, and internal standards were added to the samples. Steroids were extracted from each matrix with 4.0 ml methyl *tert*-butyl ether. After the organic layer evaporated to dryness, each extract was dissolved in 0.5 ml methanol and diluted with 1.0 ml distilled water. The samples were applied to the Adande:l PAX cartridge (Shiseido Company, Tokyo, Japan) and steroids were eluted with 1.0 ml of a methanol/pyridine mixture (100:1, v/v). After evaporation, the residues were subjected to derivatization.

2.3.2.2. Derivatization. Each sample was treated with 50 μl of the derivatizing reagent A (2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, and picolinic acid in acetonitrile) and 10 μl triethylamine for 30 min at room temperature. After the

Table 1
Characteristics of the participants.

	Eumenorrheic women	PCOS patients	*p*-value
Number	31	28	
Age (years)	24.0 (23.0–37.0)	29.5 (26.5–33.8)	0.114
Body mass index (kg/m^2)	21.0 (20.3–25.4)	23.0 (19.8–28.4)	0.387
Waist (cm)	70.0 (67.0–79.0)	80.1 (66.8–91.8)	0.060
Hip (cm)	95.0 (93.0–99.0)	94.5 (88.1–102.4)	0.802
Waist/hip ratio	0.75 (0.71–0.79)	0.84 (0.76–0.91)	0.001

PCOS, polycystic ovary syndrome.
Data are expressed as the median (25–75 percentile).

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