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GREATER CONVERSION OF TESTOSTERONE TO 5α-DIHYDROTESTOSTERONE, REFLECTING INCREASED PERIPHERAL 5α-REDUCTASE ACTIVITY IN NUDE MICE TREATED WITH HIGH DOSES OF CYCLOSPORINE A

PHILIPPE BOUDOU,[1]* JEAN FIET,[1] PATRICK VEXIAU,[2] JEAN-MARIE VILLETTE,[1] NOAH HARDY[1] and CLAUDE DREUX[3]

[1]Hormone Biology Laboratory, [2]Diabetology and Endocrinology Service and [3]Biochemistry and Neuroendocrinology Laboratory, Hôpital Saint-Louis, 75010 Paris, France

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Summary—Following cyclosporine A (CsA) immunosuppressive therapy in kidney grafts, increased body hair growth (hypertrichosis and/or hirsutism) without significant variation in normal circulating plasma androgen levels (as observed in idiopathic hirsutism) has been reported by several authors. Other authors have described increased hair growth in nude mice treated with CsA. In order to evaluate the action of this drug in target tissues, using dorsal skin homogenates from nude mice treated with various doses of CsA, we measured the metabolic conversion of testosterone (T) to its 5α-reduced products, reflecting 5α-reductase activity (5α-RA). Three groups of 5 female nude mice were treated with an oral suspension containing CsA 5 mg/kg (group 1), 25 mg/kg (group 2) and 100 mg/kg (group 3), respectively, and the results, including 5α-DHT and Adiol formation, were compared with those obtained in a control group ($n = 5$) receiving only the olive oil vehicle. Cutaneous metabolic conversion of T was determined using tritiated T as substrate. After 1 h of incubation, 5α-DHT and other 5α-reduced products formed were separated and quantified using a reverse-phase chromatography column fitted to a flow-through radioactivity detector. Mean ± SD 5α-DHT formation (expressed as pmol per 100 mg of protein per h) was found to be increased in the treated groups (group 1: 3.17 ± 0.37, group 2: 3.10 ± 0.13, group 3: 4.26 ± 0.20), respectively 7.5% (NS), 5.10% (NS) and 44.4% ($P = 0.01$) higher than in the control group (2.95 ± 0.13). In addition to 5α-DHT, enhanced formation of Δ4-androstenedione (Δ4), 5α-androstan-3β,17β-diol (3β-diol) and 5α-androstan-3α,17β-diol (3α-diol) were also observed in the treated groups. These results show a significantly increased formation of 5α-DHT (and Adiol) in nude mice treated with high dose-levels of CsA.

INTRODUCTION

Several authors have described increased hair growth in the mouse [1] and increased body hair growth in man [2–4] after cyclosporine A (CsA) administration, and hypertrichosis or hirsutism is frequently observed in renal transplant patients treated with this immunosuppressive substance [2, 5].

However, these and other authors [2, 5, 6] did not report on variations in plasma androgen levels in treated subjects, which is not surprising, since it is often difficult, as studies of idiopathic hirsutism have shown, to demonstrate modifications in androgen metabolism on the sole basis of their circulating levels [7–9]. For this reason, studies of the metabolism of these steroids in their target tissues have been undertaken by several authors, demonstrating the importance of measuring cutaneous 5α-reductase (5α-RA) activity in subjects with acne or hirsutism [7, 10–14]. As has already been noted in idiopathic hirsutism, the stimulation of body hair growth observed with CsA administration could be due to increase in 5α-RA activity in peripheral tissues.

Several authors have shown that circulating levels of 5α-androstan-3α,17β-diol glucuronide (Adiol-G), a steroid implicated in the peripheral metabolism of androgens, is well-correlated with hirsutism [15] and 5α-RA activity [16]. We previously reported an increase in plasma Adiol-G concentrations in diabetics treated with CsA [17]. It therefore appeared interesting to study the effects of this immunosuppressor on the metabolism of androgens in skin. The present study reports on 5α-DHT formation, reflecting 5α-RA activity in dorsal skin homogenates obtained from cyclosporine-treated and untreated groups of female nude mice.

MATERIALS AND METHODS

Experimental protocol

20 female nude Swiss mice aged 40 days and weighing approximately 20 g were divided into the

*Author to whom correspondence should be addressed.

following three treatment and control groups:

—group 1: cyclosporine oral suspension† 5 mg/kg;
—group 2: cyclosporine oral suspension† 25 mg/kg;
—group 3: cyclosporine oral suspension† 100 mg/kg;
—group 4: olive oil vehicle only (control).

100 μl volumes of the various suspensions were administered daily to the experimental and control groups for 23 days by means of an insulin syringe attached to a 3.5 cm long, curved 30-gauge canula introduced *per os*, the tip of which was verified to be in the animal's gastric cavity.

Skin samples and tissue preparation

The animals were killed by cervical dislocation on the 24th day and fragments of skin surgically removed from the dorsal region. After dissection of subcutaneous fat, the skin was minced with a scalpel and preserved in liquid nitrogen until the time of homogenization, prior to enzyme assay.

The frozen minced skin samples were powdered prior to carrying out the assay, using a tissue grinder (Touzard et Matignon) previously submerged in liquid nitrogen. The powder was then successively homogenized at 4°C using Teflon and glass pestles in 4 vols of a 0.05 M monosodium phosphate buffer (pH 7.4).

Reagents

[1,2,6,7-^{3}H]testosterone, specific activity (SA): 105 Ci/mmol and 5α-dihydro-4-[^{14}C]testosterone, SA: 58 mCi/mmol were obtained from Amersham, France. NADPH, in the form of the anhydrous tetrasodium salt, used as a cofactor, was from Sigma, St Louis, Mo. The ethyl acetate, cyclohexane and acetonitrile used were all spectroscopic grade. [1,2,6,7-^{3}H]testosterone was purified prior to use according to the technique of Manlimos and Abraham[18].

Metabolic conversion of testosterone in a broken-cell system

The incubation mixture contained of 100 μl of the homogenate in a total volume of 1 ml which consisted of pH 6.9 phosphate buffer (0.05 M) containing Ca^{2+} (10^{-6} M), Mg^{2+} (10^{-4} M), K^+ (10^{-3} M), NADPH cofactor (0.5 mM) and a 20 nM solution of the labeled testosterone, and was incubated for 1 h at 37°C under constant agitation. Immediately after incubation, 5α-dihydro-4[^{14}C] testosterone (5000 dpm) was added in order to evaluate the extraction ratio and the reaction medium was extracted using an ethyl-acetate/cyclohexane mixture (50/50, v/v). The organic phase was evaporated to dryness and the dry extract redissolved in 1 ml of the mobile phase. 200 μl of this solution were then introduced into the HPLC system for assay.

†Sandimmune (Sandoz, France) in olive oil.

Separation and quantification of steroid metabolites

Separation of the substrate (T) from 5α-DHT and the other radioactive metabolites formed was accomplished by high-performance liquid chromatography (HPLC) with an LKB Spherisorb ODS2 RP18 reverse-phase column (250 × 4 mm), using an acetonitrile/water (57/43, v/v) solvent mixture. Radioactivity was continuously measured by means of a Flo-One Beta liquid scintillation counter (Radiomatic Instruments, La-Queue-lez-Yvelines, France) connected directly to the column [19–21]. The counting efficiency of the detector was 27% for tritium and 44% for carbon-14. In order to eliminate any non-specific radiodegradation in the labeled steroids, blank reactions (without tissue homogenate) were incubated and analyzed under the same conditions as those employed for the skin samples. The intra- and inter-assay precision of the method were 6.7 and 8.9%, respectively ($n = 10$). Each sample was run in duplicate and the coefficient of variation between samples found to be 7.3 ± 1.4%. The mean recovery rate for the reaction products was 94 ± 2%. Total tissue homogenate protein content was measured according to the method of Bradford[22]. Formation of 5α-DHT was expressed in pmol per 100 mg of protein per hour.

Statistical analysis

Data were analyzed according to the non-paired Wilcoxon test.

RESULTS

The above-described chromatography conditions allowed separation of labeled testosterone from 5α-DHT and other 5α-reduced metabolites, comparing their elution profiles with those of tritiated reference steroids: testosterone (T), Δ4-androstenedione (Δ4), 5α-androstan-3β,17β-diol (3β-diol), 5α-androstan-3α,17β-diol (3α-diol) and 5α-dihydrotestosterone (5α-DHT). The retention times of these labeled androgens were respectively 13.4, 15.7, 15.7, 17.6 and 19.5 min (Fig. 1).

The optimal rate of 5α-DHT formation was obtained at pH 6.9 after 1 h of incubation at 37°C (Fig. 2). Under these conditions, the quantity of 5α-DHT formed per unit of time was linear with a 20 nM testosterone concentration (Fig. 3), and after 1 h there was little increase in the production of the steroid (Fig. 4).

The 5α-DHT formed is reported (Fig. 5) in the form of a mean ± SD. The values found in treated groups 1, 2 and 3 were respectively 3.17 ± 0.37, 3.10 ± 0.13 and 4.26 ± 0.20, corresponding to an increase of 7.50 (NS), 5.10 (NS) and 44.4% ($P = 0.01$), compared with the control group, in which mean 5α-DHT formation was 2.95 ± 0.13. Moreover, mean 5α-DHT formation observed in group 3 was higher than in groups 1 and 2 (34.4 and

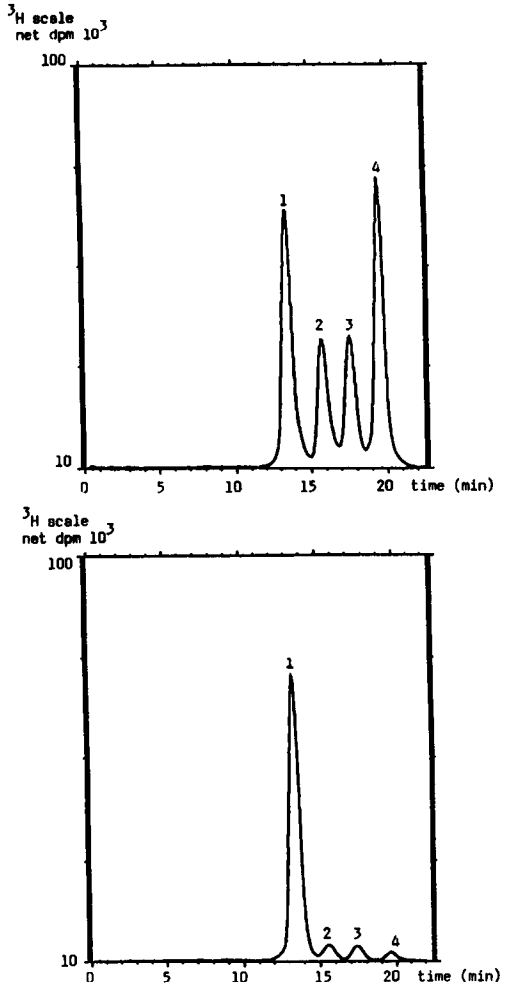

Fig. 1. The upper and lower plots refer to the direct analysis of labeled steroids using a Flo-one HP radioactivity flow detector. The upper panel shows the results obtained with reference-labeled steroids: testosterone (T), Δ4-androstenedione (Δ4), 5α-androstan-3α,17β-diol (3α-diol), 5α-androstan-3β,17β-diol (3β-diol) and 5α-dihydrotestosterone (5α-DHT). The lower panel shows the results obtained from mouse skin homogenates following *in vitro* incubation with labeled testosterone [^{3}H]T. Separation and detection of labeled substrate [^{3}H]T and metabolites (Δ4,3α-diol, 3β-diol, 5α-DHT) were carried out using a 5 μm C18 reverse-phase column. The number above each peak refers to the identity of the corresponding steroid (1: T, 2: Δ4 + 3β-diol, 3: 3α-diol, 4: 5α-DHT). The HPLC eluant was mixed with a non-gelling scintillation fluid for a total flow rate of 1.95 ml/min and counted directly. An isocratic elution using acetonitrile/water 57/43 (v/v) was employed.

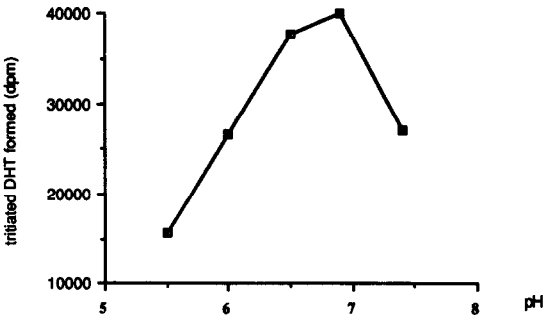

Fig. 2. pH-related activity profile of 5α-reductase in homogenized dorsal skin samples from female nude mice. Incubation carried out at 37°C in phosphate buffer containing NADPH cofactor 0.5 mM and [^{3}H]testosterone 20 nM under constant agitation.

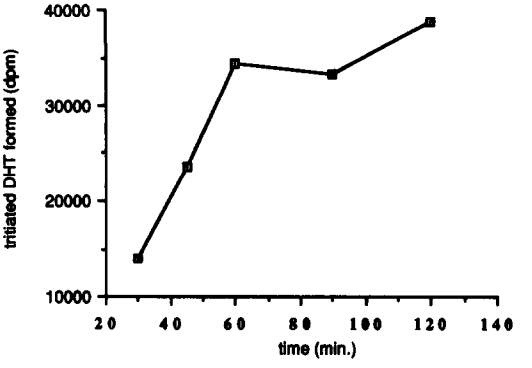

Fig. 3. Evolution of 5α-reductase activity in mouse dorsal skin homogenates over a 2-h period. The initial [^{3}H]testosterone concentration was 20 nM.

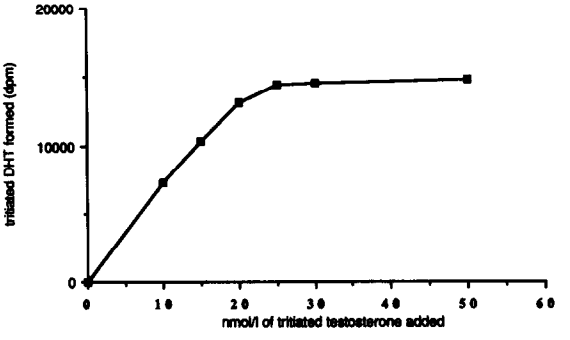

Fig. 4. 5α-reductase activity in nude mouse dorsal skin homogenates as a function of substrate ([^{3}H]testosterone) concentration. Samples were incubated in phosphate buffer containing NADPH cofactor (0.5 mM) and with varying concentrations of [^{3}H]testosterone (10–50 nM) for 1 h at 37°C under constant agitation.

37.4%, $P = 0.01$, respectively). Under the same above-defined experimental conditions for determination of 5α-DHT formation, we also observed the formation of 3α-diol and Δ4 + 3β-diol. In groups 1, 2 and 3, and in the control group (group 4), the quantities of product formed per 100 mg of protein per hour in the Δ4 + 3β-diol fraction were respectively 2.60 ± 0.24, 2.60 ± 0.17, 2.70 ± 0.28 and 2.10 ± 0.20. These values are significantly ($P = 0.01$) higher in all the treated groups compared with the controls, although no significant differences were found among the treated groups themselves. In addition, the formation of 3α-diol found in the three treated groups were respectively 6.15 ± 0.29, 6.50 ± 0.16 and 7.45 ± 0.77 and in the control group 4.80 ± 0.10 pmol of 3α-diol per 100 mg of protein per hour. The analysis of these latter results indicates a significant increase ($P = 0.01$) in the formation of 3α-diol in all treated animals compared with controls. In contrast, the only significant difference appearing

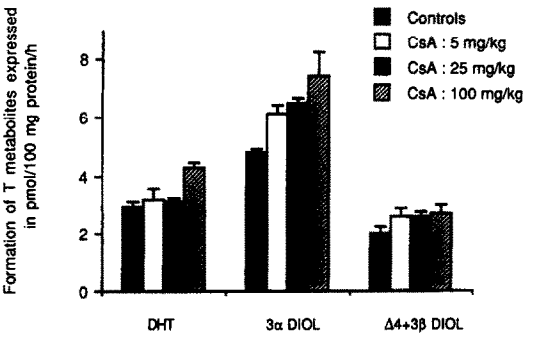

Fig. 5. [^{3}H]testosterone metabolism in homogenized dorsal skin samples from female nude mice after oral CsA administration (5, 25 and 100 mg/kg) daily for 23 days. Results ($\bar{X} \pm SD$, $n = 5$) obtained from each group, expressed as 5α-dihydrotestosterone formation (indicating 5α-reductase activity), as well as the quantities of 3α-diol and Δ4 + 3β-diol produced, were compared. (For statistical comparisons, see results.)

among the various treated groups was between groups 1 and 3 ($P = 0.05$) (Fig. 5).

DISCUSSION

In its target tissues, and specially in the skin, testosterone (T) is converted by 5α-reductase into its active metabolites, including 5α-dihydrotestosterone (5α-DHT). This latter steroid is then metabolized by 3-ketoreductases into 5α-androstanediols. The final product of testosterone catabolism via 5α-DHT is the glucuroconjugated form of the 5α-androstanediols, mainly of the 5α-androstan-3α,17β-diol (3α-adiol).

One of the side effects of cyclosporine treatment in man is increased hair growth [2–5], which in female diabetic patients may attain the levels found in hirsutism [17]. In spite of the observed secondary effects (hypertrichosis, hirsutism and/or acne) associated with cyclosporine treatment, most authors do not describe changes in plasma androgen levels, except for one recent study reporting on Adiol-G [17], whose level seems to be a good marker of cutaneous 5α-reductase activity [16]. For this reason, we studied the action of CsA on the cutaneous conversion of testosterone into its 5α-reduced metabolites in the nude mice. We observed that the skin of female nude mice possessed the enzymatic mechanisms necessary to transform T into 5α-DHT and secondarily into adiols. T could be also transformed into Δ4-androstenedione (Δ4), although our chromatographic system did not enable us to separate Δ4 from 3β-diol.

The *in vitro* cutaneous catabolism of testosterone to its 5α-reduced metabolites has been studied by numerous authors [10–12, 14, 23] in order to evaluate 5α-reductase activity. Most of them measured 5α-DHT and Adiol formation from tritiated testosterone under experimental conditions presumed to ensure maximum 5α-reductase activity, separating 5α-reduced products from testosterone by means of chromatographic techniques such as thin-layer chromatography, paper chromatography or a combination of these two methods. These techniques are so demanding and time-consuming that we preferred to use a liquid elution chromatography technique (HPLC) associated with an on-line radiodetector, which allowed direct separation and quantification of the labelled substrate and of its 5α-reduced metabolites. Until recently this method has seldom been used because there were no detectors of sufficient sensitivity available. Some studies have extensively demonstrated the possibilities and advantages provided by this technique in the investigation of the enzymatic conversion of steroids [19–21].

As described by Le Goff *et al.*[21] in the prostate, it is difficult to determine the activity of a single enzyme *in vitro* when several enzyme complexes are present in the same sample. However, the activity of other enzymes can be minimized by selecting analytical conditions which allow optimal measurement of the enzyme under study. For this reason, and since no selective inhibitors of the interfering enzyme were available, we found that the optimal rate of 5α-DHT formation for our experimental material was obtained at pH 6.9 after 1 h of incubation at 37°C. Our study of T metabolism as a function of time indicated that the quantity of 5α-DHT formed per unit of time increased little or not at all after 1 h of incubation, as had already been observed by several authors [16, 23]. Under these conditions, with respect to testosterone, the apparent K_m-value for the system was 12 nM. K_ms of such order have already been found in various tissues by different authors [24–27].

At the end of our experimentation, we observed that the CsA-treated nude mice had increased hair growth, confirming the preliminary work of Sawada *et al.*[1]. Furthermore, we found higher 3α-diol formation in the treated groups than among the control animals, indicating increased 5α-DHT formation. This 3α-diol formation was found to be higher in CsA-treated animals than was Δ4 + 3β-diol formation, which could be explained by preferential orientation toward the formation of 3α-diol from 5α-DHT. Mowszowicz *et al.*[28] and Pasupuleti *et al.*[29] have previously described such phenomena in mouse kidney and in rat skin, respectively.

Moreover, formation of 5α-DHT, which reflects 5α-RA activity, increased in a dose-dependent manner in our treated animals compared with the control group. This increase, which became significant in group 3, might reflect a greater number of, or increased activity of sebaceous glands in treated animals. Preliminary studies [1] have not reported any differences in skin or hair follicle structure between treated and control animals (nude mice). For Flanagan[30], hairlessness in the nude mouse is due to abnormal keratinization of hair in the follicle. Sawada *et al.*[1] have speculated that temporary keratinization might occur with CsA administration. This hypothesis might explain the observed increase

in 5α-DHT formation since keratinocytes are an abundant source of 5α-RA activity [31]. These *in vitro* results with CsA-treated animals support clinical data obtained from CsA-treated diabetic patients [17], in whom an increase in plasma Adiol-G concentration has been noted, reflecting increased cutaneous 5α-RA activity.

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