ROLE OF TESTOSTERONE BINDING TO THE ANDROGEN RECEPTOR IN MALE SEXUAL DIFFERENTIATION OF PATIENTS WITH 5α-REDUCTASE DEFICIENCY

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SUMMARY

Testosterone (T) and dihydrotestosterone (DHT) were found to bind to a specific protein in genital skin fibroblasts of a patient with 5α-reductase deficiency. While maximum number of binding sites (B_{max}) were similar for both androgens, the apparent dissociation constant (K_D) of the androgen receptor for T was somewhat greater than for DHT. In competition studies of [³H]-T bound to the receptor with unlabeled T or DHT, the inhibitor constant (K_i) for T was two to three fold greater than the K_i for DHT. Also, the dissociation rate constant (k_D) for [³H]-T bound to the receptor was greater than for [³H]-DHT ($t_{1/2}$ for T = 10 h and $t_{1/2}$ for DHT = 74.5 h). These results suggest that T may play a role in the sexual differentiation of male patients with 5α-reductase deficiency during their fetal life and at puberty. The low degree of masculinization during fetal life would be explained by the lower affinity and faster turnover rate of the T–receptor complex relative to the DHT–receptor complex. The relatively greater masculinization observed at puberty would be explained by the higher plasma T levels attained in the adult when compared to the fetus.

INTRODUCTION

In the last 10 years, evidence has been accumulated to indicate that three major hormones control the process of masculinization of the fetus: (1) The Müllerian Inhibiting Factor secreted by the Sertoli cells inhibits the Müllerian anlage [1]. (2) Testosterone (T) produced by the Leydig cells appears to potentiate the development and differentiation of the Wolffian ducts into epididymis, vas deferens and seminal vesicle [2]. (3) Dihydrotestosterone (DHT) is responsible for the masculinization of the urogenital sinus, the genital tubercle and folds. It has been shown that DHT arises from conversion of T by action of an intracellular 5α-reductase enzyme [2]. The mode of action of both T and DHT on their target cells involves binding of the steroid to a cytoplasmic receptor followed by translocation of the complex into the nucleus and its association with specific acceptor sites on the chromatin. This binding in turn, results in gene transcription and synthesis of a specific messenger-RNA. Among the various causes of male pseudohermaphroditism, complete androgen insensitivity (previously called "testicular feminization") and 5α-reductase deficiency have been the object of considerable investigations in the recent past [3–9].

At birth, most patients with 5α-reductase deficiency have minimal masculinization of their external genitalia including a small phallus, partial posterior fusion of the labioscrotal folds and a perineal urogenital sinus [10]. At puberty, the external genitalia of these patients appear to masculinize well with marked phallic enlargement [10], although the penis remains small in size when compared to normal adult males. No data are presently available to explain the minimal masculinization of the external genitalia during fetal life and the apparently appropriate masculinization at puberty. One possibility would be that 5α-reductase deficiency is greater during fetal life than at puberty. Another would be that the masculinization of the external genitalia during fetal life requires preferentially DHT, whereas the secondary sexual characteristics of puberty can be induced by T as well as DHT.

In this study, we have investigated the characteristics of the binding of T and DHT to the androgen receptor in genital skin fibroblasts from a patient with 5α-reductase deficiency. Our results showed that T and DHT have similar maximal binding capacity (B_{max}) for the receptor, but that the apparent dissociation constant (K_D) of the receptor for T was greater than for DHT. These findings along with the fact that androgen levels are higher during puberty than during fetal life could explain the discrepancy in degrees of masculinization observed during these two important periods of sexual development.

The following trivial names and abbreviations have been used: dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; androstanedione, 5α-androstane-3, 17-dione; androstanediol, 5α-androstane-3α, 17β-diol.

MATERIALS AND METHODS

Materials for cell culture and thin layer chromatography have been described previously [11]. [1, 2, 3, 6, 7 ^{3}H]-T (85 Ci and 98.9 Ci/mmol) and [1, 2, 4, 5, 6, 7 ^{3}H]-DHT (123 Ci/mmol) were obtained from New England Nuclear and purified by paper chromatography before use.

Cell culture

Fibroblasts were established from explants of the patient's foreskin obtained at the time of the surgical repair of his hypospadias and from the foreskin of 12 normal subjects. Informed, written consent in each case was obtained. Confluent monolayers of fibroblasts were grown in 100-mm Petri dishes with minimal essential medium and Earle's salts supplemented with nonessential amino acids, 15% fetal-calf serum, 27 mM sodium bicarbonate, penicillin (100 U/ml), amphotericin B (2.5 μg/ml), streptomycin (25 μg/ml) and gentammycin (4 μg/ml). Fibroblasts were stored in liquid nitrogen after the initial passages and were subsequently thawed and used prior to the fifteenth transfer. Cultures were maintained in a humidified incubator at 37°C in the presence of 5% CO_2 and 95% air.

5α-reductase assay

After aspirating the culture medium, confluent monolayers of fibroblasts in 100-mm Petri dishes were washed with 2.5 ml Hank's balanced salt solution and then incubated at 37°C with 4 ml of minimal essential medium without fetal-calf serum containing 1.6 μCi of [^{3}H]-T and 7×10^{-8} M nonradioactive T. Before incubation and after 15, 30 and 60 min, 0.5 ml of the medium was aspirated and saved for extraction and chromatographed. At the end of the incubation the medium was removed and the cell monolayer was washed with Hank's solution and dissolved in 2 ml of 0.1 N NaOH and assayed for DNA content [12].

After addition of [^{14}C]-T, [^{14}C]-androstenedione and [^{14}C]-DHT for calculation of recovery, the 0.5 ml media aliquots were extracted once with 20 volumes of carbon tetrachloride. The extracts were dried and applied to a silica-gel thin-layer-chromatography plate with a small volume of methanol. The plates were developed with two ascents of the solvent system chloroform–ether (90:10, vol/vol) at 25°C. Nonradioactive standards, androstanediol, T, DHT together with androsterone, androstenedione and androstanedione, were visualized using iodine vapor. The corresponding areas of the media extracts were identified, eluted with methanol and counted in 5 ml of 5% methanol in LSC (Yorktown Res.) using a 2-channel liquid scintillation counter. The counter efficiency was 45% for ^{3}H and 75% for ^{14}C. Correction for recoveries was made for T, DHT and androstenedione. Androstanediol recovery was assumed to be similar to that of T and androstenedione recovery similar to androstenedione, since each pair was

handled identically. Determination of ^{3}H and ^{14}C c.p.m. in each sample was obtained using a dual-channel analysis described previously [13]. The fraction of tritium radioactivity recovered in all 5α-reduced steroids (DHT, androsterone, androstanedione and androstanediol) was multiplied by the amount of unlabeled T added to the plate to determine the quantity of 5α-reduced products. The 5α-reductase activity was expressed as picograms of 5α-reduced steroids generated by unit time per microgram of DNA.

Receptor binding assay

After aspirating the medium and washing the Petri dishes with Hank's solution, fibroblasts were incubated for 15 min at 37°C with variable amounts of [^{3}H]-DHT or [^{3}H]-T dissolved in minimal essential medium without fetal-calf serum in presence or absence of a 100-fold excess of nonradioactive DHT or T in order to obtain a saturation curve. Whole cell DHT or T binding was measured by gel-filtration-chromatography (G-25) as previously described [14] but with minor modifications. After the incubation period, the monolayers were washed three times with 5 ml of 0.9% NaCl at 4°C in order to remove most free radioactive steroids. Then the cells were harvested in Tris–KCl buffer (0.02 M Tris–HCl, pH 7.4 at room temperature, 1.5 mM Na$_2$ EDTA, 0.5 M KCl) by scraping with a rubber policeman instead of using trypsin as described before [14]. In several experiments, trypsin was found to increase the rate of degradation of the hormone–receptor complex (unpublished results). In one experiment, half of each fraction of the protein-bound DHT or T peak of the G-25 eluate was pooled for extraction and chromatographed as described below. Specifically bound DHT was calculated by subtracting nonspecific from total protein-bound counts [14]. The maximum binding capacity (B_{max}) and the apparent dissociation constant (K_D) were derived from Scatchard plots using linear regression analysis [15]. Only those saturation curves that fulfilled all the following criteria were considered: a mean DNA content per plate of more than 40 μg per plate, a correlation coefficient greater than -0.70 with a P value less than 0.05 for the estimated regression line. Binding data were expressed in mol $\times 10^{-18}$ per μg of DNA.

Chromatographic analysis of the protein-bound DHT and T

In order to determine the amount of DHT and T present in the peak of radioactive hormone bound to protein, pooled aliquots from the G-25 eluates were extracted with 20 ml of carbon tetrachloride. Before extraction, 4,800 c.p.m. [^{14}C]-DHT and 2,500 c.p.m. [^{14}C]-T were added to correct for procedural losses; 100 μg of nonradioactive DHT and T were also added as carriers. The extracts were dried and chromatographed on methanol (spectroquality) washed Whatman paper No. 2 for 3 h at 37°C using the system

of solvents, benzene–heptane–methanol–water (166:333:400:100, by vol.). T was localized by U.V. light and DHT was visualized by spraying parallel channels with 1% *m*-dinitrobenzene (Sigma) in a saturated solution of potassium hydroxide (1:1, V/V). Areas of the chromatographed samples corresponding to DHT and T were eluted with ethanol in counting vials. After evaporation, the radioactive steroids were counted in 10 ml of 5% methanol in LSC using a Nuclear Chicago liquid scintillation counter.

Competition studies

Confluent monolayers from the same strain of fibroblasts were incubated for 15 min at 37°C with 2×10^{-9} M of [³H]-T and simultaneously with various concentrations of unlabeled T or DHT (0 to 100×10^{-9} M). Each set of T and DHT competitions were carried out on the same day under the same experimental conditions. Specific binding was determined as described in the binding assay. Data were analyzed by Dixon [16] and logit-log [17] plots using linear regression analysis.

Dissociation rates

Fibroblasts monolayers were incubated at 37°C for 15 min with either [³H]-T or [³H]-DHT (10×10^{-9} M). In parallel, plates were incubated with the same concentration of [³H]-androgen and a 100-fold excess of nonradioactive T or DHT for estimation of nonspecific binding. After incubation, the plates were washed three times with 5 ml of 0.9% NaCl at 4°C. The cells were scraped and collected by centrifugation at 800 *g* for 20 min at 4°C. These cells were then suspended in Tris–KCl buffer and sonicated at 4°C with a sonifer cell disruptor (model W185, Heat Systems-Ultra Sonic, Inc.) at 50 watt power for two 5 s periods, and centrifuged at 1600 *g* at 4°C for 20 min. To the supernates, obtained from the fibroblasts previously incubated with [³H]-T or [³H]-DHT, a 1000-fold excess of unlabeled T or DHT was added and the mixtures were maintained at 4°C. At various times, aliquots were obtained for the measurement of total and nonspecific binding using G-25 Sephadex column chromatography [14].

CASE REPORT

The patient was the full-term product of a 24 yr old mother, delivered by repeat caesarean section, after an uncomplicated gestation. At 3.5 yrs of age he was brought to the United States from Pakistan for evaluation of ambiguous genitalia.

At birth, he was noted to have a bifid scrotum, small phallus and one undescended testis. Male gender was assigned on the basis of a buccal smear showing no Barr bodies. The child's subsequent growth and development were normal.

Review of the family history revealed that the paternal great-grandfather and maternal great-great-grandfather were the same person. There was no history of abnormal sexual development in the other members of the family.

Physical examination revealed height and weight at the 75th percentile. Examination was entirely normal except for the external genitalia. The stretched phallus measured 1.9 cm in length and 0.8 cm in diameter. The testes were descended with the right measuring 1.2×1.8 cm and the left 1.0×1.5 cm. The scrotum was bifid with a perineal meatus.

Chromosomal studies were performed on 50 leukocytes and a 46, XY t(11; 20) (q21; p12) karyotype with a presumed balanced translocation of the long arm of chromosome 11 to the short arm of chromosome 20 was identified.

A 5 day hCG stimulation test (1000 IU/day im) resulted in an increase of plasma T levels from 2 to 300 ng/dl. A rise of T of 200 ng/dl or more above the normal basal value is considered as a normal response [18, 19].

Subsequently a 2-stage hypospadias repair was performed. The patient has since returned to Pakistan.

RESULTS

5α-Reductase activity of the patient's foreskin fibroblasts

(Fig. 1). The amounts of 5α-reduced steroids formed at 15, 30 and 60 min were respectively 13.4, 20.0 and 29.4 pg/μg DNA (means of four determinations). These amounts were significantly lower than those obtained at 30 and 60 min with normal foreskins. The patient's values were comparable to those reported for genital fibroblasts from patients with 5α-reductase deficiency described by Walsh *et al.*[7] and Wilson[9]. About 98% of T added at the beginning of the incubation was recovered at the end of the 60 min incubation. By contrast, normal genital skin fibroblasts metabolized 50–75% of the added T after 60 min incubation. Furthermore, chromatographic analysis of the aliquots from the protein-bound DHT
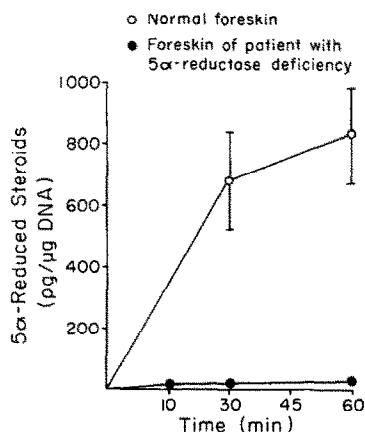

Fig. 1. Time course of formation of 5α-reduced steroids from T by fibroblasts from normal foreskins (O; mean ± 1 SEM) and foreskin from the patient with 5α-reductase deficiency (●, mean of four experiments).

Table 1. Nature of steroids bound to the androgen receptor from the patient's foreskin fibroblasts incubated with either [^{3}H]-T or [^{3}H]-DHT (4×10^{-9} M)

Steroid incubated	Percent of total radioactivity recovered as	
	[^{3}H]-T	[^{3}H]-DHT
[^{3}H]-T	97.9	2.1
[^{3}H]-DHT	5.4	94.6

Aliquots of the respective cell sonicates were chromatographed on G-25 sephadex. Eluates containing the steroid–receptor complex were mixed with known amounts of [^{14}C]-T and [^{14}C]-DHT for recovery purposes and non-radioactive T and DHT, as carriers. The samples were then extracted and subjected to paper chromatography. The areas of the chromatograms corresponding to T and DHT were eluted and assayed for radioactivity.

or T peaks of the G-25 eluate confirmed the low level of 5α-reductase activity in the foreskin fibroblasts of the patient (Table 1).

Receptor binding studies on the patient's foreskin fibroblasts

When [^{3}H]-DHT binding of four different fibroblast subcultures was analyzed by Scatchard plots, the mean B_{max} was 1017×10^{-18} mol/μg DNA (range 698–1336) and the mean K_D was 1.19×10^{-9} M (range 0.26–2.46). In five other subcultures, Scatchard analysis of [^{3}H]-T binding gave a mean B_{max} of 1038×10^{-18} mol/μg DNA (range 633–1479) and a mean K_D of 1.85×10^{-9} M (range 1.21–2.43). Although B_{max} and K_D for DHT and T were not significantly different, the K_D for DHT-binding was on two occasions (0.26 and 0.32×10^{-9} M) well below that for T-binding.

Competition studies

The androgen receptors of the patient were bound

to [^{3}H]-T (1.9×10^{-9} M) and either unlabeled DHT or unlabeled T were used as competitors. By analogy with a Dixon plot (Fig. 2), the intercept on the abscissa represents

$$-K_i\left(1 + \frac{S}{K_S}\right).$$

In our experiments, S is the concentration of [^{3}H]-T and K_S is its K_D [16]. The ratio of the intercepts on the abscissa from plots using either unlabeled T or unlabeled DHT represents the ratio $K_i(T):K_i(DHT)$ since in both cases concentration of [^{3}H]-T and its K_D were the same. The mean ratio $K_i(T):K_i(DHT)$ of three experiments were 3.2 (range 2.1–5.3), indicating a lower affinity of the receptor for T than for DHT. Similarly, the ordinate intercept is known to be

$$\frac{1}{B_{max}}\left(1 + \frac{K_S}{S}\right)$$

and therefore the ratio of the intercepts of the ordinates for T and DHT plots represents the ratio $B_{max}(DHT):B_{max}(T)$. The mean ratio of three experiments was 0.97 (range 0.81–1.2), confirming that B_{max} for T and DHT are similar.

When the same data were used in logit-log plots (Fig. 3), the mean 50% displacement dose (ID_{50}) for T was 7.4×10^{-9} M (range 2.4–12.0) two times greater than the mean ID_{50} for DHT: 3.7×10^{-9} M (range 1.3–6.2). These results further suggest a greater affinity of the androgen receptor in genital skin fibroblasts for DHT than for T.

Dissociation rate constants (K_D)

The rate of dissociation of [^{3}H]-androgen–receptor complex was analyzed after adding a large excess of unlabeled T or DHT to prevent reassociation of the receptor with the [^{3}H]-androgens. The determination of dissociation rates at 0°C was not complicated by
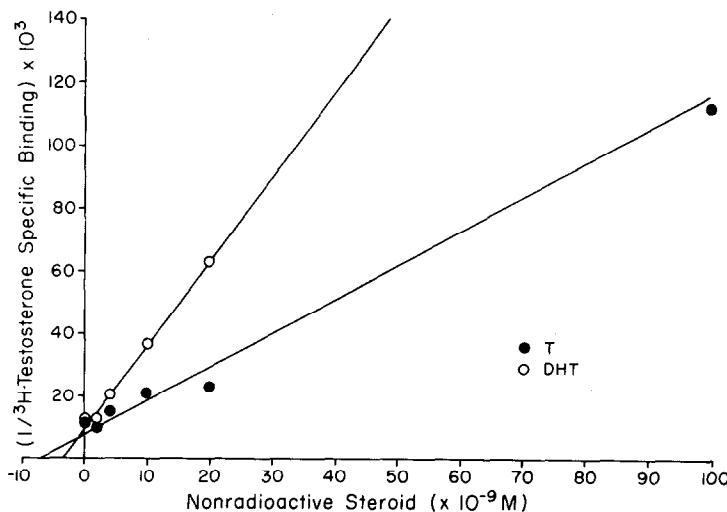

Fig. 2. Dixon plot of competition of [^{3}H]-T binding to the androgen receptor by unlabeled T (●) or DHT (○) in the patient's foreskin fibroblasts. The ratio K_i (T): K_i (DHT), calculated as described in the text, is 2.3.
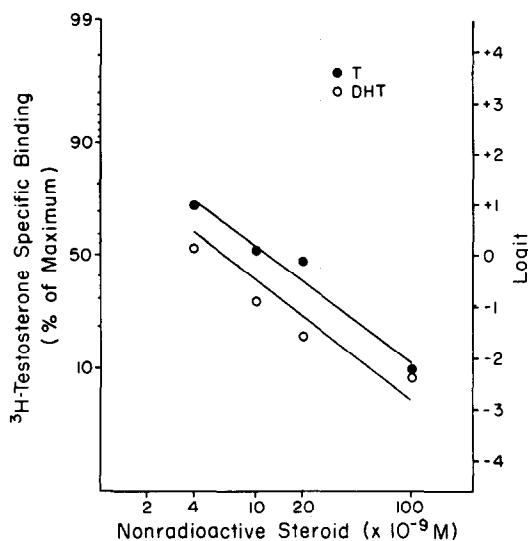
Fig. 3. Logit-log plot of the same competition studies, presented in the Dixon plot of Fig. 2. Only the linear portion of the competition curve is represented and analyzed, both extremes of the plot being non-linear. The dose of unlabeled T (●) required to displace 50% of the maximal [³H]-T specific binding (ID₅₀) is 12 × 10⁻⁹ M while the ID₅₀ for DHT (○) is 6.2 × 10⁻⁹ M.

receptor inactivation since androgen-bound-receptors are stable for at least 48 h (range of inactivation rate constant = 0.0166 h⁻¹ to 0.0211 h⁻¹) (data not shown). [³H]-T and [³H]-DHT dissociated from the receptor with a half-life ($t_{1/2}$) of 10 h for [³H]-T and 74.5 h for [³H]-DHT, yielding respective K_D's of 0.0692 h⁻¹ and 0.0093 h⁻¹ (Fig. 4).

DISCUSSION

Normal human skin fibroblasts metabolize T to 5α-reduced steroids [11], and it is therefore not possible to study T binding to receptors. For this reason, we have used in this study genital skin fibroblasts from a patient with 5α-reductase deficiency.

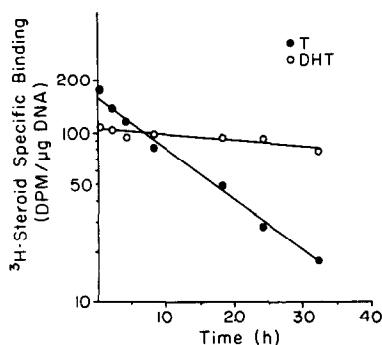We first demonstrated that less than 2% of T in the incubation medium, was converted to 5α-reduced

Fig. 4. Dissociation of [³H]-T (●) and [³H]-DHT (○) from the androgen receptor of skin fibroblasts from the patient with 5α-reductase deficiency. The $t_{1/2}$ for T and DHT are 10 and 74.5 h, respectively.

metabolites. This low conversion rate of T is in agreement with the diagnosis of 5α-reductase deficiency as reported by others [7–9]. Therefore, the binding, when the fibroblast monolayers of the patient were incubated with [³H]-T, was predominantly Y itself, rather than its metabolites. This was confirmed by the results of the paper chromatography of the G-25 Sephadex eluates which contained the androgen-receptor complex.

In our patient with 5α-reductase deficiency, Scatchard's plots indicated the same B_{max} for T and DHT, suggesting that these two steroids share the same receptor. Similar B_{max} for these two androgens has been reported recently by Wilson et al.[20] in normal genital skin fibroblasts and in two patients with partial forms of adrogen resistance. Also in rats, evidence has been presented that androgen responsive tissues contain an identical receptor protein for T and DHT [21]. In two saturation curves, a definitely lower apparent dissociation constant was found for DHT than for T. This finding was confirmed by the competition studies. Either Dixon or logit-log plots gave a K_i of the receptor for T which was two to three fold greater than the K_i for DHT. The dissociation rate constant of T from the androgen-receptor at 0°C was 0.0692 h⁻¹, while that of DHT was 0.0093 h⁻¹, indicating a faster off-rate of T from the receptor. A similar difference in the K_D and dissociation rates for T and DHT from androgen-receptor in rat testis, epididymis and prostate were reported by Wilson and French[21].

Normal DHT-binding to the androgen receptor of skin fibroblasts in a patient with 5α-reductase deficiency agrees with previously reported studies [5, 22]. Therefore, the lack of DHT synthesis does not exclude normal intracellular DHT-binding.

The presence of a common receptor for T and DHT in genital skin may explain the observation that human XY individuals homozygous for an autosomal 5α-reductase deficiency show minimal masculinization of their external genitalia at birth, but apparently appropriate virilization at puberty.

The fact that T has a lower affinity than DHT for the receptor suggests that the T–receptor complex is biologically less effective relative to the DHT–receptor complex. This would explain the partial masculinization of the patients with 5α-reductase deficiency. The discrepancy in relative androgenicity reported in fetal life and at puberty [10], could be explained by the fact that T concentrations are lower in the fetus [23] than in the adult male [24].

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