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ANDROGEN BINDING IN PERIPHERAL TISSUES OF FETAL RHESUS MACAQUES: EFFECTS OF ANDROGEN METABOLISM IN LIVER*

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Summary—In rhesus monkeys sexual differentiation of the brain and reproductive tract (RT) is androgen-dependent. Presumably these effects are mediated through the androgen receptor (AR). The AR has not been characterized in fetal tissues such as liver, kidney, heart, spinal cord and RT in this species. We characterized AR binding using [^{3}H]R1881 as the ligand in cytosols from tissues obtained on days 100–138 of gestation. Scatchard analyses revealed a single, saturable, high affinity AR in liver, kidney, heart, spinal cord and RT. The apparent dissociation constant (K_d) ranged from 0.52 to 0.85 nM with no significant tissue differences. The number of AR (B_{max}; fmol/mg protein) differed significantly ($P < 0.01$) between tissues (liver > RT ≫ kidney ≥ heart ≥ spinal cord).

Radioinert testosterone (T) and 5α-dihydrotestosterone (DHT) but not androstenedione, progesterone, estradiol-17β, estrone or cortisol in a 50-fold molar excess inhibited [^{3}H]R1881 binding to the AR in spinal cord, heart, kidney and RT. However, in liver only DHT competed significantly ($P < 0.01$) for binding. This difference in binding of DHT vs T in the liver was further investigated by incubating liver and kidney cytosols with [^{3}H]DHT and [^{3}H]T at 4°C. We identified the metabolic products by mobility on Sephadex LH-20 columns and reverse isotope dilution. Liver cytosols metabolized [^{3}H]DHT to 5α-androstane-3α,17β-diol (5α-diol) and [^{3}H]T to 5β-androstane-3α,17β-diol (5β-diol) at 4°C. In contrast, kidney cytosols metabolized [^{3}H]DHT while [^{3}H]T remained unchanged. Further studies indicated that a 50-fold molar excess of 5α-diol inhibited the binding of [^{3}H]R1881 in liver cytosols by about 50% whereas the same molar concentration of 5β-diol had no effect.

These data demonstrate the presence of AR in peripheral tissues of fetal rhesus monkeys and suggest that androgens through their receptors may affect development of these tissues. Liver cytosols are capable of metabolizing T and DHT at 4°C at conditions similar to those used for measuring cytosolic AR. However, T and DHT are metabolized differently, generating different isomers which have different affinities for hepatic AR.

INTRODUCTION

In rhesus monkeys testicular androgens regulate prenatal development of tissues such as male genital tract, accessory organs of reproduction, the scrotum and external genitalia [1, 2]. In non-human primates, androgens also masculinize those brain areas that control sexual behavior [2, 3], but appear to have little or no permanent organizing effects on those areas of the nervous system that mediate pituitary gonadotropin secretion [3–5].

The liver is also a sexually-differentiated organ since in the rat androgens imprint hepatic function which includes synthesis of a male-specific estrogen binding protein [6, 7] and the maintenance of sexually dimorphic steroid-metabolizing enzymes [7–9]. Androgens stimulate, in an irreversible and permanent fashion, the 17α-hydroxysteroid and 3β-hydroxysteroid reductases but exert suppressive effects on the 5α-reductases in the male rat liver [10, 11]. Some tissues such as the heart of the adult rhesus monkey contain specific androgen receptors (AR; [12]); therefore we assume them to be androgen-dependent. Data on fetal tissues, however, are not available to compare with the adult.

The presence or function of AR in peripheral tissues of fetal rhesus monkey have not been widely studied. We addressed this deficit by

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studying the kinetics and specificity of AR in liver, heart, kidney, spinal cord and reproductive tract (RT). These studies led us to investigate the ability of high-speed supernatants obtained from fetal monkey hepatic tissue to metabolize androgens at incubation conditions that mimic those used to measure cytosolic androgen receptors.

MATERIALS AND METHODS

Animals and tissue preparation

Five male and 6 female rhesus monkey fetuses (*Macaca mulatta*) of known gestational ages were used in this study. Fetuses were delivered by Caesarean section between days 100–138 of gestation. In all cases, samples of umbilical artery blood were taken to determine steroid hormone concentrations in serum. Immediately after delivery, fetuses were killed by decapitation, and their peripheral organs [liver, heart, kidney, spinal cord and RT (the entire reproductive tract minus the gonads)] were removed and immersed in ice-cold TEGMD buffer (10 mM Tris, 10 mM EDTA, 10% glycerol, 25 mM molybdate and 10 mM dithiothreitol, pH 7.4). The organs were dissected and homogenized in 5–10 vol ice-cold TEGMD buffer using a polytron (Brinkman, setting of 6, 2 × 10 s) and centrifuged at 1000 *g* for 10 min at 4°C. The resulting nuclear pellet was processed in the manner described below and used for nuclear AR assay. The supernatant was centrifuged at 106,000 *g* for 10 min at 4°C and the resulting purified cytosol was subsequently used for cytosolic AR assay and, in the cases of the liver and kidney, to study testosterone (T) and 5α-dihydrotestosterone (DHT) metabolism.

The crude nuclear pellet from the 1000 *g* centrifugation was washed in buffer (pH 6.8) containing 1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 1 mM dithiothreitol, 10% (v/v) glycerol and suspended in 15 μl of the same buffer containing 5 mg Cellex 410 (Bio-Rad Laboratories, Richmond, Calif.). Subsequently 200 μl of buffer (pH 6.8) containing 1 mM KH_2PO_4, 2.4 M sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% (v/v) glycerol were added, mixed and centrifuged at 57,750 *g* at 4°C. Nuclear AR were then extracted from the resulting pellet and assayed according to methods described previously [13, 14].

Cytosolic AR assay

Cytosolic AR was assayed by *in vitro* binding of [^{3}H]R1881 ([17α-methyl-^{3}H]methyltrienolone; 87.4 Ci/mmol; New England Nuclear, Boston, Mass) to cytosolic preparations according to previously described methods [13]. Triamcinolone acetonide (10 μM) was added to all incubation tubes to prevent binding of the radiolabeled ligand to progestin and corticoid receptors. Incubation of cytosolic fractions from liver and RT with 3 nM [^{3}H]R1881 for 2, 4, 8, 20 and 40 h at 0–4°C determined that maximum binding occurred at 20 h (Fig. 1). Thereafter all incubations were performed at 0–4°C for 20 h. To estimate the apparent dissociation constants (K_d) of R1881, aliquots of cytosolic preparations (100 μl) from different tissues were incubated with increasing concentrations of [^{3}H]R1881 (0.07–3.0 nM) at a total incubation volume of 150 μl. Parallel incubation tubes that contained a 200-fold molar excess of radioinert R1881 were used to determine nonspecific binding. Bound and free ligand were separated by Sephadex LH-20 column chromatography. Specific binding was calculated by subtracting nonspecific from total binding. In all tissues nonspecific binding remained very low and even in heart and spinal cord, the tissues with the lowest binding capacity, specific binding exceeded 50% of total binding. Data were analyzed according to the method of Scatchard [15]. Cytosolic androgen receptors were expressed as fmoles of [^{3}H]R1881 bound per milligram protein. The soluble protein content of cytosols was determined by the method of Lowry *et al.* [16].

To determine the specificity of [^{3}H]R1881 binding, increasing amounts (10- and 50-fold excess) of radioinert T, DHT, androstenedione, estradiol-17β, estrone, progesterone and cortisol were added to the total and nonspecific binding incubation tubes. The capacity of these steroids to inhibit [^{3}H]R1881 binding was determined by comparison to binding without competing steroid.

Metabolism of T and DHT by liver and kidney tissues in vitro

The metabolism of T and DHT by cytosols of fetal kidney and liver was investigated. Incubation conditions paralleled those used for the AR assay. Purified cytosol (100 μl) of liver and kidney were incubated with 3.0 nM of either [1, 2, 6, 7-^{3}H]T (99.1 Ci/mmol) or [1, 2, 4, 5, 6, 7-^{3}H]DHT (148.1 Ci/mmol; New England Nuclear, Boston, Mass) in a total incubation volume of 150 μl of TEGMD buffer at 4°C for 18 h. The incubation was terminated by

addition of 850 μl distilled H_2O and extracted twice with 6 ml of distilled ether. The ether extracts were combined, dried under a gentle stream of air and dissolved in 1 ml of ethanol. A 100 μl aliquot of the ether extract was chromatographed in Sephadex LH-20 columns using the solvents hexane:benzene:methanol (85:15:5; by vol) as previously described from this laboratory [17]. Fractions were collected, dried and dissolved in 1 ml ethanol after which a 100 μl aliquot was counted in 10 ml scintillation fluid. The radioactive metabolites obtained from T and DHT as substrates exhibited the mobility of androstane-3α,17β-diol. The metabolites from the liver were then transferred to 10 ml glass-stoppered conical centrifuge tubes to which were added 20 mg of either 5α-androstane-3α,17β-diol (5α-diol) or 5β-androstane-3α,17β-diol (5β-diol) and subjected to reverse isotope dilution using the solvents ethanol, ethyl acetate and methanol as previously described [18].

The difference in metabolism of T and DHT by liver cytosols led us to test the specificity of 5α-diol and 5β-diol for AR binding in this tissue.

Use of animals and statistical analyses

Data were analyzed by one-way analysis of variance and differences between means were assessed by the Newman–Keuls multiple range test [19]. Eleven fetal rhesus monkeys (5 males and 6 females) ranging in age from 100 to 138 days of gestation were used in this study. Sex differences were not found; therefore, data obtained from males and females were combined. The data presented in Figs 1–3 and Table 1 were obtained from untreated fetuses whereas those presented in Fig. 4 and Table 2 from fetuses whose mothers received DHT. In these latter cases the DHT concentrations were not elevated in the fetal circulation. The data presented in Fig. 5 were obtained from fetuses injected i.m. with 500 μg of DHT but the nature of the experiment performed on these tissues preclude bias by the treatment regimen.

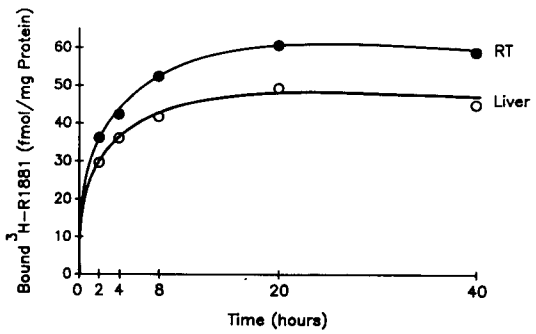

Fig. 1. Time-course of [^{3}H]R1881 binding to purified cytosol of liver and reproductive tract (RT) from a male fetal monkey on day 136 of gestation showing maximum binding and equilibrium after 20 h of incubation at 4°C.

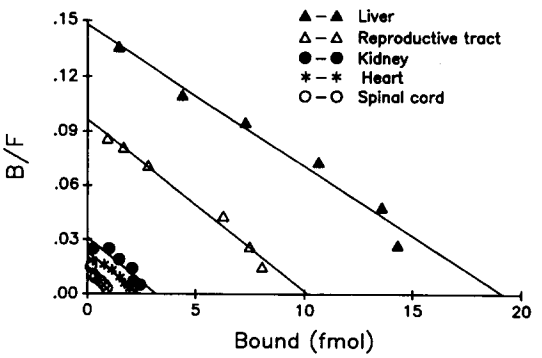

Fig. 2. Representative Scatchard analyses of saturation curves of cytosolic AR obtained from peripheral tissues of a female fetal monkey on day 125 of gestation. Androgen receptor binding affinities and capacities in these tissues are listed in Table 1.

RESULTS

Cytosolic AR binding kinetics

Saturation analyses of cytosolic AR in spinal cord, heart, kidney, RT and liver showed a single, saturable, high affinity binding site for [^{3}H]R1881 (Fig. 2). The apparent K_d and B_{max} values for cytosolic AR in these tissues are listed in Table 1. No significant differences were observed in binding affinities (K_d) between tissues. Affinities ranged from 0.52 ± 0.11 nM (SEM; $n = 4$) in the kidney to 0.85 ± 0.37 nM (SEM; $n = 4$) in the heart. Although AR binding affinity in these tissues did not differ significantly, the estimated number of AR (B_{max}; fmol/mg protein) differed significantly between tissues (Table 1). The number of AR in the liver (42.2 ± 1.1) was significantly different ($P < 0.01$) than that in the RT (30.7 ± 5.5). Moreover, AR content in the liver and RT were significantly greater ($P < 0.01$) than those in the

Table 1. The binding affinities and maximum binding capacities of AR in peripheral tissues obtained from fetal monkeys between days 125–138 of gestation (mean ± SEM; $n = 4$)

	K_d (nM)	B_{max} (fmol/mg protein)
Spinal cord	0.68 ± 0.27	$3.0 \pm 0.4^{*,a}$
Heart	0.85 ± 0.37	3.5 ± 0.3^{a}
Kidney	0.52 ± 0.11	4.3 ± 1.5^{a}
RT†	0.54 ± 0.10	30.7 ± 5.5^{b}
Liver	0.70 ± 0.22	42.2 ± 1.1^{c}

*Means with dissimilar superscripts are significantly different ($P < 0.01$).
†Reproductive tract.

kidney, 4.3 ± 1.5; heart, 3.5 ± 0.3 and spinal cord, 3.0 ± 0.4 ($n = 4$ in each tissue). However, the number of AR was not significantly different between kidney, heart and spinal cord.

Competition studies showed that T and DHT were efficient competitors for the radiolabeled ligand for AR in spinal cord, heart, kidney and RT (Fig. 3). However, in the liver only DHT showed significant competition ($P < 0.01$), whereas, T did not compete for hepatic AR. In both sexes in all tissues studied, estradiol-17β, estrone, progesterone, cortisol and androstenedione were poor competitors (data not shown).

Nuclear AR

Our attempts to demonstrate nuclear AR in peripheral tissues of fetal monkeys were not successful. We measured them only in RT, but even in this tissue, they were not always detectable.

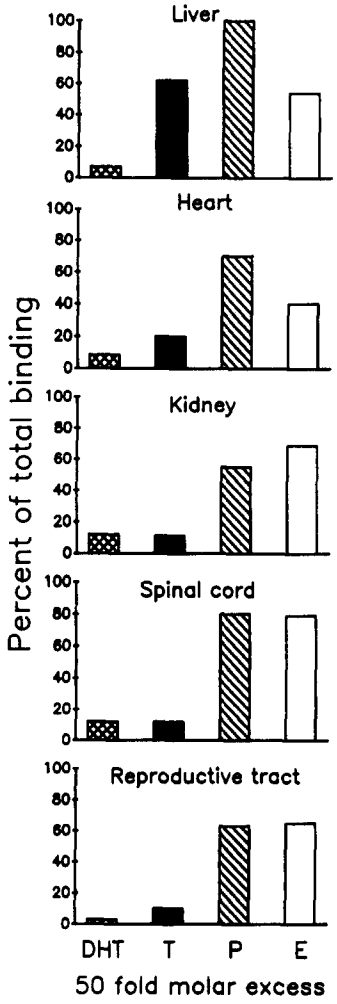

Fig. 3. Competition studies showing the relative binding of [^{3}H]R1881 to cytosol obtained from peripheral tissues of a male fetus on day 136 of gestation in the presence of 50-fold molar excess of different competitors (DHT = 5α-dihydrotestosterone; T = testosterone; P = progesterone; E = estradiol-17β).

Metabolism of T and DHT by liver and kidney tissues in vitro

The elution profile of radioactive metabolites produced by liver and kidney cytosols after incubation with [^{3}H]T is depicted in Fig. 4 (panel A). Almost all of [^{3}H]T (fraction 6) was converted to its 5-diol metabolite (fraction 8) in the liver. This fraction was determined to be the 5β-diol after recrystallization due to the fact that the specific activity of the crystals did not vary more than 10% from the mean (Table 2). However, in the case of kidney, none of [^{3}H]T was metabolized. In contrast, both liver and kidney cytosols converted [^{3}H]DHT (fraction 4; Fig. 4, panel B) into a metabolite that eluted in fraction 8. Recrystallization of the metabolite from the liver showed it to be 5α-diol (Table 2).

Using the cytosolic AR binding assay, 5α-diol was a more effective competitor for liver AR than T (Fig. 5). A 50-fold molar excess of 5α-diol resulted in more than 50% inhibition of the binding of [^{3}H]R1881 to AR in liver, whereas the same concentration of T displaced only 30% of [^{3}H]R1881. In contrast, 5β-diol

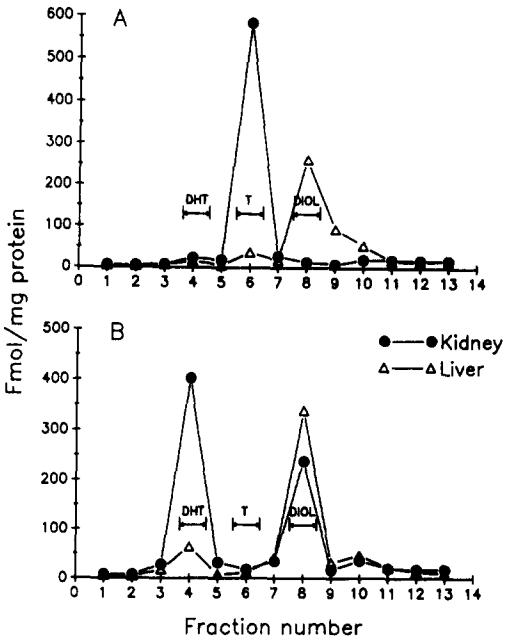

Fig. 4. Elution profile of radioactive metabolites produced by liver and kidney cytosols at 4°C after 20 h of incubation with [^{3}H]T (panel A) and [^{3}H]DHT (panel B). Standard DHT, T and a mixture of 5α-androstane-3α-17β-diol and 5β-androstane-3α-17β-diol eluted in fractions 4, 6 and 8, respectively. Samples were chromatographed on Sephadex LH-20 columns in the solvent system (hexane:benzene:methanol, 85:15:5, by vol).

had little or no capacity for binding to AR in liver.

DISCUSSION

This study demonstrates for the first time the presence of a single, saturable, high affinity AR in liver, RT, kidney, heart and spinal cord of fetal rhesus monkeys. The binding kinetics of AR in these tissues are well within the physiological range (nM) and comparable to those reported for AR in other tissues; primate and rat hearts [12, 20]; cynomolgus [21], human [22, 23] and rabbit [24] livers; rat prostate [25] and spinal cord [26]; mouse kidney [27]; as well as primate [13, 28, 29] and rat [14, 30] brains and pituitaries.

Although the binding affinity of [^{3}H]R1881 for AR was similar in all tissues studied in this research, the estimated number of AR varied significantly among tissues. The liver contained the greater number of AR followed by RT, kidney, heart and spinal cord. This indicates that these tissues are potential target organs for androgen action during fetal development in rhesus monkey. Sexual organization of the developing primate nervous system and regulation of the prenatal development of the primate genital tract are achieved by androgens [1–4]. The organizing effects of androgens on sexual differentiation of the rat liver are well documented [6–11]. This results in an irreversible stimulation of the 17α- and 3β-hydroxysteroid reductases and inhibition of the 5α-reductases as well as the synthesis of a male-specific estrogen binding protein during adulthood. It is, therefore, possible that androgens may have similar organizing and regulatory effects on the development of peripheral tissues of the rhesus monkey.

Table 2. Recrystallization of radiolabeled androgen metabolites formed by liver cytosols obtained from fetal monkeys (days 100–138 of gestation)

Radioinert steroid added[a]	Crystals	Solvent[b]	Specific activity (dpm/mg) [^{3}H]T[c]	Specific activity (dpm/mg) [^{3}H]DHT[c]
5α-diol	Initial[d]		1480	4170
	CX_1[d]	A	1500 (50.5)[e]	3950 (9.3)
	CX_2	B	940 (5.7)	3460 (4.2)
	CX_3	C	550 (44.8)	3430 (5.1)
5β-diol	Initial		1310	3920
	CX_1	A	1110 (8.3)	2620 (49.2)
	CX_2	C	1320 (9.1)	1600 (8.9)
	CX_3	A	1200 (0.8)	1050 (40.2)

[a]20 mg of crystalline 5α-androstane-3α,17β-diol (5α-diol) or 20 mg of crystalline 5β-androstane-3α,17β-diol (5β-diol) were added to the pool of radioactivity obtained after Sephadex LH-20 chromatography.
[b]A = ethanol; B = ethyl acetate; C = methanol.
[c]Substrate used for incubation.
[d]Initial = specific activity before crystallization. CX = crystals; subscript represents consecutive crystallizations.
[e]Numbers in parentheses represent percent deviation of specific activity in CX from mean specific activity of all three crystallizations.

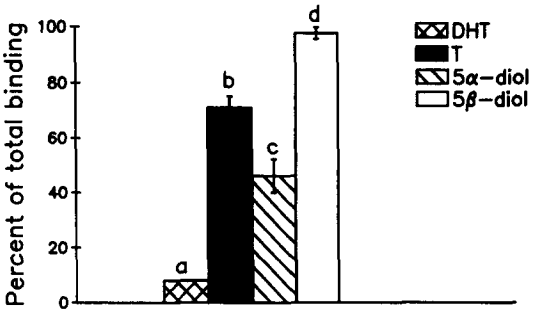

Fig. 5. Competition studies showing the specificity of DHT, T, 5α-androstane-3α,17β-diol (5α-diol) and 5β-androstane-3α,17β-diol (5β-diol) for AR in liver cytosols ($n = 4$; mean $\pm$ SEM). Means with dissimilar superscripts are significantly different ($P < 0.01$).

The specificity of AR was measured by testing the ability of different radioinert steroids to displace [^{3}H]R1881 binding. Because R1881 is specific for AR and does not bind to serum steroid binding proteins [13, 31, 32], this competition represents binding to AR alone. Nonspecific binding of [^{3}H]R1881 to progesterone and glucocorticoid receptors was minimized by the addition of triamcinolone acetonide to the assay buffer [13, 33, 34].

Binding of [^{3}H]R1881 was androgen-specific in all tissues studied. Dihydrotestosterone inhibited the binding of [^{3}H]R1881 by >90% in all tissues in both male and female. Testosterone was equally effective in inhibiting the binding of [^{3}H]R1881 except in the liver where it was a weak competitor <40% in both sexes. The possible effect of fetal sex on metabolism and binding of androgen by hepatic tissue in the current study is not clear due to the limited number of fetuses used. Estradiol-17β showed some ability to displace [^{3}H]R1881 at high concentration (50-fold excess) while all other steroids tested showed minimal affinity for AR.

The inability of T to compete for the AR in hepatic tissue of the fetal monkey appears to be related to the metabolism of T under the conditions of our AR assays. Our data showed that liver cytosols metabolize both T and DHT at 0–4°C. However, the metabolic products generated from these two androgens are quite different. Although DHT was metabolized to 5α-diol, T was converted to the inactive isomer, 5β-diol. These two isomers are different in their specificity for hepatic AR. A 50-fold molar excess of 5α-diol inhibited the binding of

[^{3}H]R1881 by about 50% whereas the same molar concentration of 5β-diol had no effect. Similar differences in specificity of DHT vs T for AR were observed in liver cytosols from adult male cynomolgus monkeys (*M. fasicularis*; data not shown). In the same species, DHT is highly specific for the hepatic AR but the specificity of T was not tested [21]. In addition, human [23], rabbit [24] and rat [35] hepatic AR are specific for both T and DHT. In the rat, however, the specificity of DHT is detected only if steroid metabolizing enzymes are eliminated to prevent the conversion of DHT to 5α-diol [35]. In the present study we found that nonhuman primate liver cytosols metabolize both DHT as well as T. However, while DHT is converted to 5α-diol which binds well to hepatic AR, T was converted to the inactive isomer 5β-diol which did not compete with [^{3}H]R1881 for liver AR. This difference in competition between 5α-diol and 5β-diol does not represent binding to plasma binding protein because R1881 does not bind to these proteins [13, 31, 32]. The discrepancy between rat and rhesus monkey may be due to species differences in steroid metabolism and the ability of AR to bind 5α-diol in primate liver.

These data demonstrate high affinity, low capacity specific AR in liver, RT, kidney, heart and spinal cord of fetal rhesus monkeys and imply that androgens through their receptors may exert an important role in the fetal development of these tissues. The liver cytosols metabolized T and DHT differently, thus generating the isomers, 5β-diol and 5α-diol, respectively, which have different affinities for hepatic AR. Because of this metabolism, DHT but not T appears to be the active androgen in the monkey liver.

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