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METABOLISM OF [^{3}H]-TESTOSTERONE AND [1,2-^{3}H]-DIHYDROTESTOSTERONE BY PROLACTIN SECRETING RAT PITUITARY TUMOUR CELLS IN CULTURE

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SUMMARY

Rat pituitary tumour cells in monolayer culture (GH_3 cells) respond to oestradiol-17β and testosterone with increased synthesis of prolactin. The present study was undertaken to establish whether this effect of testosterone could be mediated via conversion to oestradiol-17β. Cell cultures were incubated with 400 μCi of [7α-^{3}H]-testosterone at a concentration of 10^{-6} M for 96 h. The metabolites were isolated and identified by repeated paper chromatography, derivative formation and final crystallization to constant specific activity from different solvents.

Testosterone was metabolized to a great extent, 88–96%, but no conversion (<0.0001%) of testosterone to oestradiol-17β or oestrone could be detected. 5α-Dihydrotestosterone and 5α-androstane-3β,17β-diol were found as the major metabolites, isolated in a radiochemically pure form. 5α-Androstane-3α,17β-diol and 4-androstene-3,17-dione were also isolated and identified, and they constituted a minor fraction, 5 and 0.2%, respectively.

The kinetics of the metabolism of [^{3}H]-testosterone were studied by harvesting the cell cultures after 6, 12, 24, 48, and 96 h. The data suggested that 5α-androstane-3β,17β-diol was formed via 5α-dihydrotestosterone, that was subsequently shown to be a good substrate for the formation of the former.

It is concluded that the stimulatory effect of testosterone on prolactin secretion by these cells is probably not mediated via conversion to oestradiol-17β. The extensive metabolism of testosterone opens the possibility that the effect of testosterone is in some way related to the formation of a metabolite.

INTRODUCTION

In a previous publication on the regulation of prolactin (PRL) secretion by rat pituitary cells in culture, it was demonstrated that testosterone stimulated the secretion in a dose-dependent way [1]. Other investigators have shown that androgen administration to male rats gave rise to increased serum levels of PRL [2, 3] and caused morphological changes in the PRL secreting cells indicative of increased secretory activity [3]. The latter investigators attributed this androgen effect to aromatization to oestrogens [3] which are known to be potent stimulators of PRL secretion [1].

The possibility does exist, however, that testosterone exerts its effect as such in the pituitary, since testosterone in this organ is metabolized only to a minor extent to 5α-dihydrotestosterone (DHT) [4].

The present study was undertaken to establish whether the effect of testosterone on PRL secretion by the rat pituitary cells in culture could be mediated via conversion to oestrogen. Because of the marked difference in dose-response curves for oestradiol-17β and testosterone on PRL secretion by these cells [1], only a minute conversion of testosterone to oestradiol-17β might explain the effect of the former. The androgen metabolizing characteristics of these cells was furthermore studied.

MATERIALS AND METHODS

Chemicals

[7α-^{3}H]-Testosterone, S.A. 5.7 Ci/mmol, [1,2-^{3}H]-testosterone, S.A. 45 Ci/mmol, [1,2-^{3}H]-DHT, S.A. 44 Ci/mmol, [4-^{14}C]-oestradiol-17β, S.A. 53 mCi/mmol, [4-^{14}C]-DHT, S.A. 59 mCi/mmol, were all purchased from The Radiochemical Center, Amersham, U.K. The purity was checked by paper chromatography in appropriate systems, and purified whenever necessary.

Non-radioactive steroids were purchased from Steraloids Inc., Wilton, U.S.A.

Bovine serum albumin (BSA) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., Ham's F-10 media, amphotericin B (250 μg/ml) sterile horse serum and sterile foetal calf serum from Flow Laboratories, Irvine, U.K.

Cell culture

Plastic tissue culture dishes (large, 145 × 20 mm and small, 60 × 15 mm) were purchased from C.A. Greiner und Söhne, Nürtingen, Germany.

Monolayer cultures of PRL secreting pituitary tumour cells (GH_3 cells), established by Tashjian[5], were used in these studies. Details of the procedure have been described previously [1]. Briefly, the cells were grown in plastic tissue culture dishes containing

Ham's F-10 medium supplemented with 15% (V/V) horse serum and 2.5% (V/V) foetal calf serum. Penicillin, streptomycin and amphotericin B were added to the culture medium, and did not at these concentrations influence cell growth or hormone production. The cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2, with change of culture medium every 2–3 days.

Incubations with [^{3}H]-androgens

A. *Cell culture with [7α-^{3}H]-testosterone.* The cells were grown in large culture dishes for 7 days prior to the experiment, reaching a cell concentration of 32 × 10^6 cells per dish. Then fresh medium (18 ml) containing 400 μCi [7α-^{3}H]-testosterone at a concentration of 10^{-6} M was added, and 96 h later the cells and medium were harvested separately. The cells were sonicated in 0.15 M NaCl and both cells and medium immediately stored at −70°C until analysis. This experiment was carried out on two separate occasions.

B. *Cell culture with [1,2-^{3}H]-testosterone and [1,2-^{3}H]-DHT.* Cells were grown in small culture dishes for 4 days with either testosterone or DHT at a concentration of 10^{-6} M prior to the experiment, reaching a cell concentration of 3.4 × 10^6 cells per dish. At the change of medium after 4 days [^{3}H]-testosterone, 9.6 μCi, or [^{3}H]-DHT, 13.8 μCi, were added and the concentrations were maintained at 10^{-6} M. Duplicate culture dishes were harvested 6, 12, 24, 48, and 96 h after the addition of the radioactive androgens. The cells were sonicated in the medium and the samples stored at −70°C until assayed for radioactive metabolites.

Isolation of radioactive steroid metabolites

In experiment A [4-^{14}C]-oestradiol, 4500 d.p.m., and [4-^{14}C]-DHT, 5500 d.p.m., were added to the samples for recovery correction, and 100 μg of each substance was added as carrier.

In experiment B non-radioactive steroids were added to the samples in order to correct for losses, 50 μg of each: DHT, 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol), and 4-androstene-3,17-dione (androstenedione).

The samples were extracted three times with three volumes of diethylether. The ether phase was evaporated to dryness under nitrogen and the residue chromatographed on Whatman No. 1 paper with benzene as mobile phase and formamide as stationary phase [6]. The strips were examined over U.V. light and subsequently scanned for radioactivity in a windowless Actigraph III scanner (Nuclear Chicago). In experiment A four areas of radioactivity were eluted: Area A, R_F = 0.18, corresponding to oestradiol, area B, a peak with R_F = 0.45, area C, a peak corresponding to testosterone, R_F = 0.65, and area D, corresponding to androstenedione, R_F = 0.83. The radioactivity was measured in an aliquot of each fraction.

In experiment B the the same areas were eluted except area A.

The subsequent isolation and identification of the various metabolites were based upon repeated chromatography, and derivative formation. The final identification was based upon recrystallization to constant specific activity from different solvents after the addition of about 30 mg of the respective steroid added as carrier substance.

Measurement of radioactivity was carried out by liquid scintillation spectrometry (Nuclear Chicago Mark I) calibrated for double isotope counting. Instagel (Packard) was used as scintillation solvent. When correction for losses was based upon the addition of non-radioactive steroids, gas-liquid chromatography was employed on an aliquot (1/20) of the sample after the addition of 2.5 μg of cholesterol as internal standard. The chromatography was carried out on a Hewlett-Packard Model 402 instrument with a 12 feet column of OV-17, 1% at 275°C with a flame ionization detector.

RESULTS

Identification of oestradiol-17β

The total material of area A was rechromatographed in the same system and then subjected to methylation [7] and rechromatographed on paper with hexane as mobile phase and formamide as stationary phase. The radioactive peak corresponding to oestradiol-3-methylether was eluted, an aliquot taken for measurement of radioactivity and the remaining sample used for crystallization with authentic oestradiol-3-methylether.

The recovery of [4-^{14}C]-oestradiol-17β varied between 69.0 and 78.7%. Before crystallization approximately 0.01% of the tritium from the substrate was found associated with [4-^{14}C]-oestradiol-3-methylether, but all tritium disappeared during recrystallization, demonstrating a conversion of testosterone to oestradiol of less than 0.0001%.

Identification of 3β-diol

Following elution of area B the material was rechromatographed on paper in the system hexane–benzene (1:1, V:V)/formamide. On scanning for radioactivity one peak was observed, R_F = 0.22, corresponding to 3β-diol and 5β-androstane-3α,17β-diol. Following acetylation with 0.5 ml acetic anhydride: pyridine (1:5, v:v) overnight at room temperature the radioactivity material moved very close to the front (R_F = 0.90) in the system hexane/formamide. After having taken an aliquot for measurement of radioactivity, two more aliquots were taken, each recrystallized with authentic 3β-diol diacetate and 5β-androstane-3α,17β-diol diacetate. The recrystallization data for the former are presented in Table 1, whereas all radioactivity disappeared from the crystals when crystallizing with 5β-androstane-3α,17β-diol diacetate.

There was no drop in specific activity of the crystals during the recrystallization, indicating that very close to 100% of the radioactivity in peak B was 3β-diol.

Table 1. Recrystallization of [^{3}H]5α-androstane-3β,17β-diol diacetate isolated as described following incubation of rat pituitary tumour cells in culture with [7α-^{3}H]-testosterone; Authentic 5α-androstane-3β,17β-diol diacetate (appr. 30 mg) was used as 'carrier'

Cryst.	Solvent system	d.p.m./mg
Before		23,600
1st	Methanol/water	23,900
2nd	Hexane	24,100
3rd	Ethanol/water	24,050

Table 2. Recrystallization of [^{3}H]5α-androstane-3α,17β-diol diacetate isolated as described following incubation of rat pituitary tumour cells in culture with [7α-^{3}H]-testosterone; authentic 5α-androstane-3α,17β-diol diacetate (appr. 30 mg) was used as 'carrier'

Cryst.	Solvent system	d.p.m./mg
Before		131,000
1st	Methanol/water	126,000
2nd	Hexane	122,000
3rd	Ethanol/water	125,000
4th	Acetone/water	124,000

Identification of 3α-diol

Area C which was made up by a double peak, corresponded to 3α-diol, testosterone and oestrone. A similar peak was again observed on rechromatography in the system hexane:benzene (1:1, V:V)/formamide, whereas acetylation and chromatography in the system hexane/formamide gave two peaks, one with $R_F = 0.48$ corresponding to testosterone acetate and the other near the front corresponding to 3α-diol diacetate. After having taken aliquots for measurement of radioactivity, the latter was crystallized with authentic carrier (Table 2).

The area corresponding to oestrone acetate, $R_F = 0.33$, was eluted and an aliquot taken for counting, but no radioactivity could be detected in this fraction, indicating a conversion to oestrone from testosterone of less than 0.0001%.

Identification of DHT and androstenedione

Following rechromatography of the material in area D in the system hexane/formamide one peak was observed, $R_F = 0.11$. After acetylation and rechromatography in the same system, two peaks were observed: One with $R_F = 0.11$ corresponding to androstenedione and the other with $R_F = 0.73$ corresponding to DHT acetate. Both compounds were recrystallized after having taken aliquots for measurement of radioactivity (Tables 3 and 4).

The distribution of radioactivity among the isolated compounds can be seen in Table 5. Unmetabolized testosterone accounted for 12.1 and 4.0% of the radioactivity in the two experiments, and DHT and 3β-diol were the most abundant metabolites. Most of the radioactive metabolites were found in the medium, less than 1% of the total activity was detected in the

Table 3. Recrystallization of [^{3}H]5α-DHT acetate isolated as described following incubation of rat pituitary tumour cells in culture with [7α-^{3}H]testosterone; authentic DHT acetate (appr. 30 mg) was used as 'carrier'

Cryst.	Solvent system	d.p.m./mg
Before		275,000
1st	Methanol/water	268,000
2nd	Hexane	251,000
3rd	Acetone/water	256,000

Table 4. Recrystallization of [^{3}H]androstenedione isolated as described following incubation of rat pituitary tumour cells in culture with [7α-^{3}H]testosterone; authentic androstenedione (appr. 30 mg) was used as 'carrier'

Cryst.	Solvent system	d.p.m./mg
Before		19,000
1st	Methanol/water	15,200
2nd	Hexane	10,700
3rd	Ethanol/water	10,300
4th	Acetone/water	10,200

Table 5. Distribution of radioactivity among the isolated steroids following a 96 h cell culture (GH_3 cells) with 400 μCi of [7α-^{3}H]testosterone

	% Of isolated radioactivity			
	Cell culture 1		Cell culture 2	
Steroid	Cells	Medium	Cells	Medium
Testosterone	0.1	12.1	0.1	4.0
Dihydrotestosterone	0.6	59.8	0.3	50.3
5α-Androstane-3α,17β-diol	0.1	5.1	<0.1	4.8
5α-Androstane-3β,17β-diol	0.1	22.0	<0.1	40.4
Androstenedione	<0.1	0.2	<0.1	0.1
Oestrone	ND*	ND	ND	ND
Oestradiol	ND	ND	ND	ND

* Less than 0.0004 μCi, indicating a conversion of less than 0.0001%

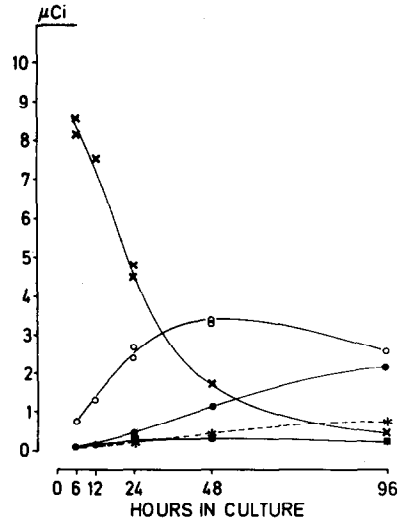

Fig. 1. Metabolism of 9.6 μCi [1,2-^{3}H]-testosterone, 1 × 10^{-6} M, by 3.4 × 10^6 rat pituitary tumour cells (GH_3 cells) in culture. Single points represent duplicate cultures too close for separate presentation. Symbols: ×——×, testosterone; ○——○, dihydrotestosterone; ●——●, 5α-androstane-3β,17β-diol; ■——■, 5α-androstane-3α,17β-diol; *——*, ether nonsoluble material. For further details, see the experimental section.

cell fraction, and here DHT was the major metabolite.

The kinetics of the metabolism of [^{3}H]-testosterone and [^{3}H]-DHT can be seen on Figs 1 and 2. Between 93 and 99% of the radioactivity in the ether phase could be accounted for by the compounds isolated (data not shown). The same metabolites were found with both substrates, except testosterone and androstenedione that were isolated only in the cultures with testosterone. The amount of androstenedione never exceeded 0.1 μCi (data not shown).

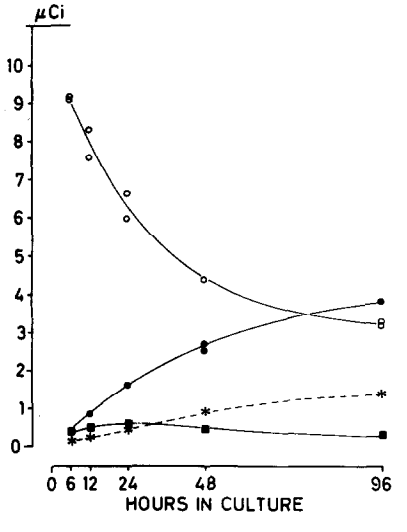

Fig. 2. Metabolism of 13.8 μCi [1,2-^{3}H]-dihydrotestosterone, 1 × 10^{-6} M, by 3.4 × 10^{-6} rat pituitary cells (GH_3 cells) in culture. For explanation see legend to Fig. 1.

DISCUSSION

It is evident from these data that the PRL secreting GH_3 cells did not metabolize testosterone to oestradiol-17β or oestrone. Haug and Gautvik[1] found that an oestradiol concentration in the medium of 10^{-11} M gave approximately the same stimulation (to 129% of controls) of PRL secretion by these cells as 10^{-6} M of testosterone (to 131% of controls). The present experimental design made it possible to exclude a conversion of testosterone to oestradiol-17β of 0.0001% or greater, which is more than one order of magnitude less than might be expected if a conversion to oestradiol-17β were the underlying mechanism for the effect of testosterone on PRL secretion. It is therefore highly unlikely that the stimulatory effect of testosterone on these cells can be mediated by a conversion to oestrogens.

Extensive metabolism of testosterone took place during the 96 h incubation period, the main metabolites being DHT and 3β-diol. In the kinetic study there was a continuous and gradual increase in 3β-diol, whereas DHT reached a maximum at 48 h, which was followed by a moderate drop. This suggests that DHT was an intermediate in the formation of 3β-diol from testosterone, a conclusion that is further supported by the finding of steadily increasing 3β-diol from [^{3}H]-DHT (Fig. 2). 3α-Diol was a minor metabolite throughout the culture period in all experiments.

The extensive metabolism of testosterone to 3β-diol compared with the 3α-epimer by the GH_3 cells is remarkable. In most androgen metabolizing organs testosterone metabolism seems to favor 3α-diol over 3β-diol. In the rat epididymis Djøseland *et al.* found 3β-diol to be a minor metabolite of testosterone both *in vivo* and *in vitro* compared to 3α-diol [8, 9]. Stenstad *et al.* demonstrated a similar relationship in a cell culture of a uterine cell line [10], and Ofner *et al.*[11] reported that both rat and human prostate *in vitro* preferentially formed the 3α-epimer.

No compounds with a 5β-configuration could be detected, and unknown steroid metabolites must constitute a small fraction, since 93–99% of the radioactivity in the ether extract could be accounted for by the isolated steroid.

The nature of the radioactive compounds in the water phase after ether extraction was not studied, but it may be suggested that the fraction represents conjugated steroid metabolites. The greatest amount was found in the DHT experiment.

A stimulatory effect of large doses of testosterone on pituitary PRL secretion has been demonstrated *in vivo* in the male rat [2, 3], in which case the effect on the pituitary via peripheral conversion to oestrogens cannot be excluded, *in vitro* in whole terrapin pituitary tissue [12] and in these pituitary tumour cells [1]. In these investigations no attempt has been done to study the effect of testosterone metabolites, like the androstanediols on PRL secretion. Therefore, an open question remains that the effect of testosterone may

possibly be mediated via a metabolite. It should be pointed out that DHT has been found to possess 4–5 times the activity of testosterone in suppressing LH and FSH in castrated male rats [13], suggesting that DHT may be the important intracellular androgen in the pituitary.

Magrine *et al.*[14] have presented evidence that PRL may inhibit the conversion of testosterone to DHT in man. One may therefore speculate that a feed-back system can exist for the conversion of testosterone to DHT via PRL, and thus the activation of testosterone in target tissues [15, 16].

The great conversion of testosterone to 3β-diol is interesting in light of the effect of this substance of the binding of oestradiol-17β to its receptor in the rat pituitary [17]. One might therefore foresee a possible effect as an anti-oestrogen of this testosterone metabolite, or it might have an androgen effect of its own since a receptor for 3β-diol has been suggested in the anterior pituitary of the rat [18], or it could be without any effect, as is the case in the ventral prostate in castrated male rats [19].

The rat pituitary tumour cells used is derived from a monoclonal cell line [5], responsive to a number of hormonal stimuli [1, 20, 21, 22]. Since they do respond to androgens, and do not convert testosterone to oestradiol-17β, they may be considered androgen target cells. With these cells we thus have a model system for studying androgen metabolism and effect in homogeneous cell culture, with the advantages over the use of whole tissue or a dispersed cell system, where a number of cell types will be present.

Using this experimental model it may be possible to assign the androgen effect on PRL secretion to one particular compound, and also study the possible interaction between different steroids. Such an interaction could possibly exist for 3β-diol, that will bind to the oestradiol-17β receptor [17] and it might therefore inhibit the effect of oestrogens.

A study on the effect of these testosterone metabolites on the PRL secretion by rat pituitary tumour cells is in progress, and will be the subject of another publication.

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