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CONCENTRATIONS OF ANDROGENS IN NUCLEI OF SEMINIFEROUS TUBULES IN DEVELOPING AND MATURE RATS

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SUMMARY

Homogenates of seminiferous tubules obtained from 26-, 32- and 60-day-old rats were fractionated by ultracentrifugation and the concentrations of testosterone, dihydrotestosterone and 5α-androstane-3α,17β-diol were determined by radioimmunoassay in unpurified subcellular fractions and purifed nuclei. The three androgens were detected in cytosol at all ages. Similar to what has been described for whole tissue homogenates, testosterone was the main steroid present in the cytosol of mature rats while reduced androgens were predominant in young animals.

5α-androstane-3α,17β-diol was present in high concentrations in the cytosol fraction of 26-day-old rats but was undetectable in nuclei at all ages. In the nuclei of young rats the levels of androgens were higher than in 60-day-old rats, particularly the concentrations of dihydrotestosterone. The absence of 5α-androstan-3α,17β-diol in nuclei suggests that this steroid does not stimulate transcription in this tissue.

INTRODUCTION

The metabolism of testosterone (T) in rat testis during maturation has been extensively studied using labeled substrates and short-time *in vitro* incubations [1]. Very active 5α-reductase and 3α-hydroxy-steroid oxido-reductase enzymatic activities have been found to be present in the interstitial tissue [2] and in the seminiferous tubules [3].

The major event taking place in the testes of these rats is the maturation of the germinal epithelium and, in this respect, we had reported that maximal reducing activity was detected at the time of the first meiotic division which takes place in the young testis. T, dihydrotestosterone (DHT) and particularly 5α-androstan-3α,17β-diol (DIOL) were the main 5α-reduced androgens measured in homogenates of rat seminiferous tubules at this age.

The role of these three androgens on the stimulation of the germinal epithelium is not understood. Based on the temporal correlation between the initiation of meiosis and the detection of the highest concentrations of DIOL, we have suggested that DIOL might stimulate initiation of this cellular division. This was supported by the fact that DIOL, as well as DHT and T, was able to prevent estrogen-induced inhibition of development of spermatogenesis in maturing rats [4]. However, DIOL does not bind with high affinity to the cytosol androgen receptor, and it has been suggested that conversion to it might be a mechanism of T and DHT inactivation [5].

It is generally accepted that, upon entering the androgen-target cell, testosterone is bound to a cytosol receptor as such, or after conversion to DHT, and the steroid–receptor complex migrates inside the nucleus.

Therefore, the active androgens should be concentrated in the nucleus. With this reasoning in mind, we have measured by radioimmunoassay (RIA), T, DHT and DIOL concentrations in subcellular fractions of seminiferous tubules of rats at different stages of sexual development.

EXPERIMENTAL

Materials and methods. Non radioactive steroids were purchased from Steraloids, U.S.A., and tritium-labeled steroids from New England Nuclear, U.S.A. Specific activities for T, DHT and DIOL were 85, 40 and 40 Ci/mmol respectively.

Radioactive steroids were purified by silica-gel thin layer chromatography before use. Anti-testosterone serum, was generously provided by Dr. E. Nieschlag (Düsseldorf, F.R.G.), was used for RIA of T and DHT, anti-DIOL serum was a gift of Dr. D. Loriaux (NIH, U.S.A.).

Experimental animals. Twenty six-, 32- and 60-day-old rats were sacrificed by cervical dislocation. Testes were weighed, decapsulated and seminiferous tubules separated from interstitial tissue by teasing in an appropriate buffer at 4°C [6].

Between 4 and 5 g of tissue was homogenized in 4 volumes of cold 50 mM Tris–Hl, 3 mM $MgCl_2$, 0.32 M sucrose buffer, using an Ultraturrax mixer.

Subcellular fractions. Homogenates were centrifuged at 800 *g* for 10 min in a refrigerated centrifuge. Purification of the nuclear fraction was carried out as published by R. S. Calandra *et al.*[7]: the 800 *g*

pellet was resuspended in a 2 M sucrose buffer and centrifuged at 60,000 *g* for 60 min in a Beckman L5 65 ultracentrifuge. The pellet was washed with 0.32 M sucrose buffer containing 0.3% Triton X-100 and centrifuged at 800 *g*. Two additional washings were carried out with the 0.32 M sucrose buffer in absence of Triton X-100. The final pellet (purified nuclear fraction) was resuspended in 1 ml of buffer. The supernatant from the first 800 *g* spin was centrifuged at 15,000 *g* for 30 min to obtain a fraction enriched in mitochondria. The supernatant was then centrifuged at 100,000 *g* for 60 min to obtain a fraction enriched in microsomes. Electron microscope observations of these fractions have already been published [8].

Purity of the nuclear fractions was also checked by measurement of its protein/DNA ratios. A ratio of 2 or less was considered to be acceptable. DNA was determined by the method of Fleck[9] and proteins by the method of Lowry[10].

RIA of T, DHT and DIOL in homogenates or subcellular fractions. Samples were processed in duplicate. Radioactive tracers were added and, after standing for 10 min in the cold, the steroids were extracted with 5 ml acetone. The solvent was evaporated to dryness and the residue dissolved in 1 ml distilled water. Acetone precipitation was omitted for the cytosol fractions. Samples were submitted to several solvent partitions: water–ether (1:10, V/V) (1:4 in the case of cytosol), 70% methanol–hexane (1:1, V/V) and 70% methanol–dichloromethane (1:4, V/V). The dichloromethane was then evaporated to dryness.

Micro glass columns were packed with celite coated with ethylene glycol and propylene glycol 1:(2:1). Ten ml of iso-octane, run under positive pressure (N2), was discarded. Samples were applied in 1 ml of iso-octane and after washing with 10 ml of iso-octane, DHT was eluted with 4 ml of iso-octane–toluene (70:30, V/V) T with 4 ml of iso-octane–toluene (60:40, V/V) and DIOL with 6 ml of cyclohexane–ethyl acetate (85:15, V/V). One tenth of the sample was used for the assay and $\frac{1}{4}$ for estimation of recovery.

Phosphate buffer 0.01 M, pH 7, with 0.1% w/v sodium azide, 0.9% w/v NaCl and 0.1% w/v gelatine was used for the assay incubation. Antibody, tracer and standards or unknowns were incubated during 15 h at 4°C in a final volume of 0.3 ml. Bound and free fractions were separated with 0.2 ml of charcoal, 1.25 g/100 ml buffer.

Logit-log transformation of data was carried out for evaluation of standard curves and calculation of unknowns. Linear regression was calculated after truncation. Mean overall recovery was influenced by the amount of tissue processed. Water blanks were made non-significant by taking $\frac{1}{10}$ of column eluates for the assay. Test of parallelism were carried out for T, DHT and DIOL in homogenates of seminiferous tubules. No significant difference between slopes for standard curves and tissues was found in every case.

RESULTS

The concentrations of proteins and DNA in subcellular fractions of seminiferous tubules are shown in Table 1. DNA was determined in the four subcellular fractions and in the whole tissue homogenate. Losses taking place during purification of the nuclear fraction were corrected according to recovery of DNA. DNA levels in the other fractions were negligible. The correction for the losses of the nuclear fraction was applied to the calculation of the concentrations of proteins and androgens. Proteins/DNA ratios in the nuclear fraction greater than 2 were considered unsatisfactory and experiments were discarded.

Contents of T, DHT and DIOL in nuclei and cytosol are shown in Fig. 1.

In 26-day-old rats, DHT and DIOL were the main androgens detected in the cytosol while T was present in small amounts. By contrast, no DIOL was detected in the nuclear fraction which concentrated DHT as well as T. In 32-day-old rats DHT was the main androgen present in cytosol nuclei. Finally, T was the main androgen present in the cytosol of the 60-day-old rat with small amounts of DHT (T/DHT ratio 5.64) and DIOL. In the nuclear fraction, however, T and DHT were present in similar amounts (T/DHT ratio 1.19). DIOL could not be detected in the nucleus at any age.

Contents of T, DHT and DIOL in the 15,000 *g* and 100,000 *g* pellets are shown in Fig. 2. DHT and

Table 1. Proteins and DNA measurements in subcellular fractions of rat seminiferous tubules.

	Age of rats		
	26	32	60
Proteins (mg/g testis)			
Nuclei	10.2	7.5	5.5
15,000 *g* pellet	2.9	5.0	6.2
100,000 *g* pellet	1.2	1.9	2.3
Cytosol	4.6	12.5	8.2
Protein/DNA ratio			
Tissue homogenate	6.1	3.4	14.9
Nuclear fraction	2.0	1.2	1.6
Nuclear DNA recovery (%)	80.0	45.0	25.0

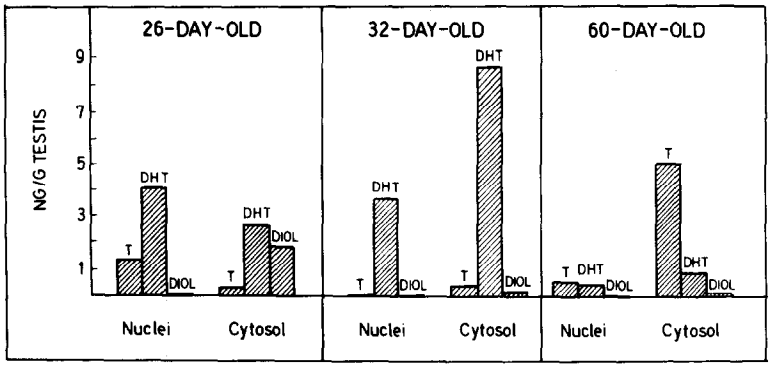

Fig. 1. Concentrations of T, DHT and DIOL in purified nuclei and cytosol of rat seminiferous tubules at different stages of spermatogenic maturation.

DIOL were the main androgens present in the two fractions in 26-day-old rats. In 32-day-old animals, the reduced androgens were present in much higher concentrations than T except for DIOL in the 100,000 *g* pellet.

These values, expressed in ng/100 mg proteins are shown in Table 2.

DISCUSSION

The measurement of steroids in tissue homogenates presents several difficult technical problems. Low endogenous levels along with considerable non-specific interferences require special care in the validation of results. This is particularly true for target organs. Higher levels are present in endocrine secreting tissues but measurements might not be as informative. In the testis there is an unique situation, where the endocrine secreting tissue is in close association with one of its targets, the seminiferous tubules. For this reason it was of major interest to separate interstitial and tubular compartments. Inevitably this procedure introduced two artifacts: metabolization of endogenous steroids by the active tissue enzymes and transfer of the easily diffusible steroids from one compartment to another. Therefore, values detected do not necessarily represent actual endogenous levels in a particular compartment.

Furthermore, the same two phenomena can occur during homogenization and differential centrifugation to separate subcellular fractions. Steroids bound to macromolecules probably remain unmetabolized and within a particular compartment but those unbound, or released from their binding, might end up in a different fraction and converted to a metabolic product. This criticism is valid for all determinations of tissue steroid levels which have been published in the literature unless special care was taken to stop metabolization immediately after organ removal. In this case no compartmentalization studies are possible. Therefore, the levels of T, DHT and DIOL in subcellular fractions of seminiferous tubules that we are presenting in this paper represent endogenous levels modified by changes of unknown magnitude which might occur during the isolation procedure and have the value of an *in vitro* study.

Possible contamination of tubules with Leydig cells was checked by microscopic examination. Occasional small clumps of interstitial cells were observed in the sinus of folded tubules. The number of contaminating cells was negligible.

Extensive purification of the samples was critical

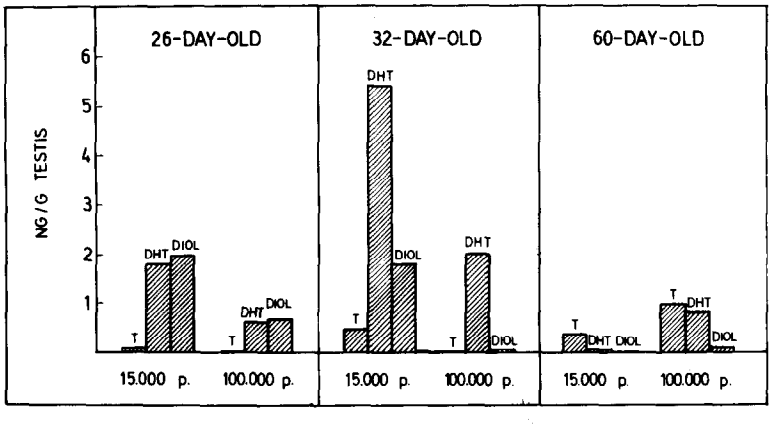

Fig. 2. Concentrations of T, DHT and DIOL in the 15,000 and 100,000 *g* pellets of rat seminiferous tubules at different stages of spermatogenic maturation.

Table 2. Concentrations of androgens in ng/100 mg proteins in subcellular fractions of rat seminiferous tubules

	Age of rats		
	26	32	60
T			
Nuclei	13.4	N.D.	9.2
15,000 *g* pellet	3.83	8.30	6.3
100,000 *g* pellet	N.D.	N.D.	43.7
Cytosol	5.25	2.04	62.6
DHT			
Nuclei	40.1	49.1	9.1
15,000 *g* pellet	62.4	109.0	3.0
100,000 *g* pellet	56.5	107.4	36.9
Cytosol	57.6	70.0	11.1
DIOL			
Nuclei	N.D.	N.D.	N.D.
15,000 *g* pellet	67.6	36.6	N.D.
100,000 *g* pellet	59.2	5.94	N.D.
Cytosol	40.6	1.48	N.D.

N.D.: Non-detectable

for reproducible assays. Efforts were conducted to get rid of heavy tissue contaminants with minimal steroid losses. The combination of solvent partitions and celite column chromatography gave satisfactory recovery and adequate blanks. Test for parallelism of regression between standards curves and unknowns were taken as evidence for absence of interferences in the assays.

The predominance of T over the 5α-reduced metabolites DHT and DIOL previously reported in the testis of the sexually mature 60-day-old rat was reflected in the levels of the cytosol fraction. Seminiferous tubule cytosol is a mixture of inter and intracellular as well as intraluminal fluids. Concentrations of DHT and particularly DIOL were much lower than of T. By contrast, in the nuclear fraction, T and DHT were detected in similar amounts suggesting a preferential transfer of DHT over T inside the nucleus. In the 32-day-old rat, DHT was the main androgen present in all fractions and the only one detected in the nuclei. Of particular interest is the comparison of the distribution of the three androgens in the cytosol and nuclear fractions of the 26-day-old rat. The active 5α-reductase and 3α-hydroxysteroid dehydrogenase present at this age results in the accumulation of DIOL in the testis [11]. Indeed, DIOL was detected in cytosol, 15,000 *g* and 100,000 *g* pellets but was absent in the nuclear fraction. Smith *et al.*[12] had found that only [^{3}H]-T and [^{3}H]-DHT, but no [^{3}H]-DIOL, were present in nuclei of testis after *in vivo* adminstration of [^{3}H]-T to hypophysectomized rats. This lack of penetrance or retention of DIOL in the nuclei is probably related to the low affinity of this steroid for the androgen receptor [13]. This finding does not support our previous suggestion [11] that the increment of the concentration of DIOL in the testis during the initiation of spermatogenesis, particularly the meiotic division, implicated a specific stimulatory effect of DIOL upon this process. A remaining possibility for this hypothesis is that DIOL does not act through a mechanism of action which involves the androgen receptor. Evidences for alternative mechanisms have been published in the activation of kidney β-glucuronidase by androgens [14], and high-affinity, low-capacity binding sites specific for estradiol-17β have been found in hepatocyte plasma membrane subfractions [15]. Furthermore, it has been proposed that progresterone stimulates meiotic division in amphibian oocytes by a mechanism which does not require transcriptional events and the hypothesis was proposed that the first interaction of progesterone is located at the membrane level [16].

It is difficult to speculate on the meaning of the levels of T, DHT and DIOL in the 15,000 *g* pellet, enriched in mitochondria and in the 100,000 *g* pellet, enriched in microsomes. In general, values in these fractions reflected those of cytosol.

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