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TESTOSTERONE MICROMILIEU IN STAGED RAT SEMINIFEROUS TUBULES

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Summary—Endogenous testosterone concentrations in rat seminiferous tubules were measured in relation to different stages of the cycle of the seminiferous epithelium. For this purpose, the seminiferous tubules were mechanically separated from the interstitial tissue on a cooled (1°C) petri dish under a stereomicroscope without added medium. After recognition of the stages of the cycle by transillumination, the specimens were rapidly transferred by dry forceps into test tubes for testosterone radioimmunoassay. The results of the dry dissection method were compared with measurements on tubules that were kept after separation in phosphate buffered saline (PBS, pH 7.4), in order to reveal the possible leakage of testosterone from the tubules. The maximal concentration of testosterone per unit length of seminiferous tubule was found in stages VII and VIII of the cycle (288 ± 60 fmol/cm, mean ± SEM, $n = 12$), and the minimal in stages IX–XII (219 ± 57 fmol/cm, $P < 0.01$). If the levels were correlated with unit volumes of the seminiferous tubules, identical concentrations of testosterone (521–542 fmol/mm^3, approx. 500 nmol/l) were found in the different stages of the cycle. Despite the similarity of testosterone concentrations in the different parts of the seminiferous tubules the local concentrations of biologically active (i.e. free) testosterone may be modulated by extracellular and intracellular androgen binding components.

INTRODUCTION

Although the dependence of spermatogenesis on androgen stimulation is well documented, very little is known about the molecular mechanisms of androgen action in the seminiferous epithelium compared with other androgen-dependent tissues [1]. Also the amount of androgen actually needed for spermatogenesis is a matter of controversy [2, 3]. One approach to elucidate this matter is the measurement of actual local concentration of androgens in the seminiferous tubules and to correlate it with different stages of the cycle of the seminiferous epithelium with specific cellular events. Several observations suggest that androgens have a specific effect in stages VII and VIII of the cycle of rat seminiferous epithelium, the site of specific early degeneration of cells after hypophysectomy [4] or during low intratesticular testosterone levels after selective destruction of Leydig cells [5]. Analyses of seminiferous tubule segments obtained by transillumination-assisted microdissection indicate that the endogenous concentration of testosterone, secretion of androgen-binding protein and local concentration of nuclear androgen receptors are high in the same or adjacent stages [6, 7]. However, the concentration of endogenous testosterone may have been affected by the dissection conditions used in the past; diffusion of testosterone into the dissection medium has been demonstrated in a temperature-dependent fashion [8]. The present study was undertaken to find out the actual levels of testosterone within the seminiferous tubules and the rate of its diffusion into the dissection medium. A mediumless transillumination-assisted microdissection of seminiferous tubules was performed for measurement of testosterone concentrations in the different stages of the epithelial cycle in conditions as close to the *in vivo* situation as possible.

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EXPERIMENTAL

Seminiferous tubule microdissection without medium

Testes of 17 young adult (3–5 months) rats of the Sprague–Dawley strain were analyzed. After killing by cervical dislocation, the testes were rapidly removed and decapsulated. The seminiferous tubules were separated under a stereomicroscope in a petri dish placed on a transparent plate from an electrophoresis apparatus (LKB 2117 Multiphor, LKB Bromma, Sweden), which was adjusted to maintain a dissection temperature of 1°C by circulating −6°C ethanol cooled by an electronic thermoregulator (MGW Lauda K4R; Dr R. Wobser, KG, Lauda/Tauber, West Germany). The dissection was performed without medium, and the characteristic

transillumination pattern of the seminiferous tubules [9] was photographed.

For diffusion experiments, quadruplicate segments of 1 cm from stages VII and VIII of the cycle were separated from two rats and incubated for 0, 5, 15, 30 and 60 min in 100 μl of 0.01 mol/l phosphate buffered 0.9% saline (PBS, pH 7.4) solution. Thereafter, the medium was removed under stereomicroscopic control with a micropipette and testosterone was measured separately in the medium and in the tubules. In another diffusion experiment, the middle 1 cm of approx. 3-cm segments of seminiferous tubules were immersed in 100 μl of PBS in a shallow beaker at 2°C. Both ends of the tubular segment were kept outside the medium, to find out the diffusion rate of testosterone through the tubular wall. From the 100μl amount of the medium, 20 μl samples were removed and replaced with fresh PBS at 0, 5, 15, 30 and 60 min after onset of the incubation. Further incubations were carried out to estimate the possible influence of length of the seminiferous tubule segments on leakage of testosterone. Quadruplicate samples with 2, 5, 10 and 20 mm in length were isolated from stages VII and VIII of the cycle in dry conditions and analyzed for testosterone.

To estimate the effect of temperature on testosterone diffusion, quadruplicate segments of 5 mm in length from two rats at stages VII and VIII of the cycle were incubated in 100 μl of PBS for 1 h at 4, 21 and 32°C, and testosterone concentrations in the media and tubules were measured.

Finally, to compare endogenous testosterone contents in different stages of the cycle, quadruplicate 5 mm samples of stages II–VI, VII–VIII, IX–XII and XIII–I were dissected in cold without medium from 12 rats, and directly transferred to test tubes for testosterone measurement.

Radioimmunoassay of testosterone

Testosterone was measured by radioimmunoassay, using ^{125}I-labelled testosterone as the tracer, essentially as described before [10]. The sensitivity of the method was about 1.8 fmol/tube and the intra- and interassay coefficients of variation were below 5 and 10%, respectively.

RESULTS

In a cooled Petri-dish without medium, the transillumination pattern of the seminiferous tubules was clearly identifiable permitting recognition of pale (stages IX–XII), weak spot (XIII–I), strong spot (II–VI) and dark homogeneous absorption zones (VII–VIII) (Fig. 1). By careful dissection, it was

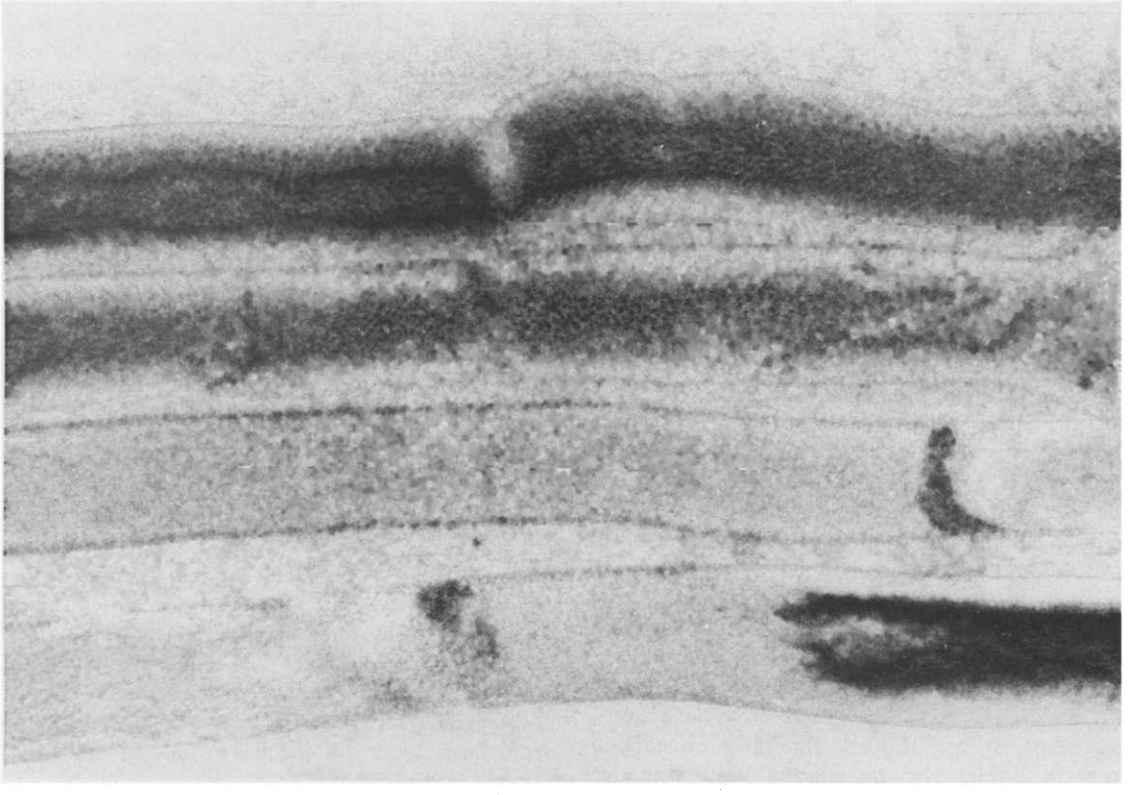

Fig. 1. The appearance of freshly isolated, unstained rat seminiferous tubules in transillumination without medium on a cooled petri-dish under a stereomicroscope. The four characteristic absorption zones can be distinguished: The uppermost tubule is from the strong spot zone (approximate stage V), the next is from approximate stage I–early II (weak spot), the next one is from the pale zone (stages IX–XII) and the lowermost tubule shows the spermiation point at stage VIII (at right). The left side of this tubule shows a damage caused by dissection. Magnification: 60×.

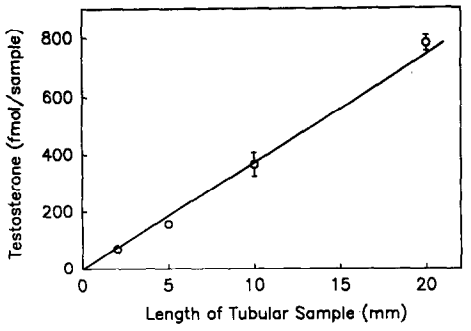

Fig. 2. Testosterone content in "dry-dissected" seminiferous tubules from stages VII and VIII of the cycle. Each point is the mean ± SEM of 4 measurements. 1 mm of tubule is equivalent of approx. 0.05 mm^3 or 0.05 mg wet weight of tissue (see text).

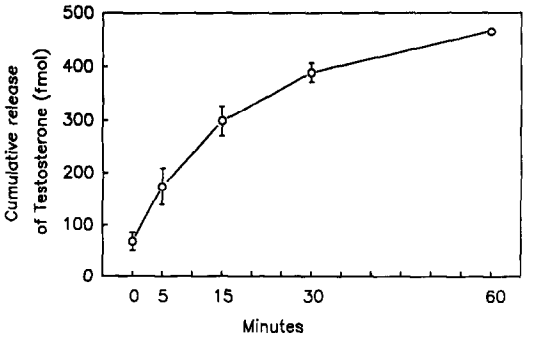

Fig. 4. Diffusion rate of testosterone through tubular wall as a function of time. The middle 1 cm of a approx. 3 cm segment of seminiferous tubule was immersed in PBS in a shallow beaker at 2°C with both ends outside the medium. Each point is the mean ± SEM of quadruplicate measurements.

possible to separate 5 and 10 mm segments from each of the four transillumination types, measuring them by a ruler placed beneath the petri dish.

The amount of testosterone in different lengths of stages VII and VIII tubule segments shows linearity (Fig. 2). We next measured the diffusion speed of testosterone from 1 cm tubule segments at stages VII and VIII of the cycle. A plateau in diffusion was reached in 15 min when more than half of testosterone originally present in tubules had leaked out (Fig. 3). The sum of testosterone present in the tubules and media was the same at each time point. To find out whether testosterone leaks out through the cut ends of the tubules or through the tubular wall, the cumulative amount of testosterone was measured in the medium where a seminiferous tubular segment was incubated with both ends outside the medium. A virtually identical pattern of testosterone diffusion was found when compared to incubations where the whole segments of seminiferous tubules were immersed in the medium (Fig. 4, compare with Fig. 3). The effect of temperature on testosterone diffusion was next examined. Figure 5 shows that almost identical amounts were found in the tubules and in the medium after a 1-h incubation at 4°C, whereas the diffusion speed was significantly increased at 21 and 32°C. In all incubation conditions, the total amount of testosterone found in the medium and in the tubules, was very similar.

When testosterone contents of seminiferous tubule segments from various stages of the cycle were compared, relatively small differences were found. Tubule segments from stages VII and VIII of the cycle showed constantly the highest testosterone concentration, significantly higher than in stages IX–XII ($P < 0.01$) and XIII–I ($P < 0.05$). Likewise, testosterone concentration of stages II–VI was significantly ($P < 0.05$) higher than that of stages IX–XII (Fig. 6).

DISCUSSION

The actual androgen concentrations inside the seminiferous tubules and in the seminiferous epithelium, needed for normal spermatogenesis, are not known. Qualitatively—though not quantitatively—normal spermatogenesis has been maintained in hypophysectomized animals with intratesticular testosterone levels that are 10–20% of normal [2]. Likewise, reduction of human testicular testosterone to 2.5–5% of normal is not sufficient to completely

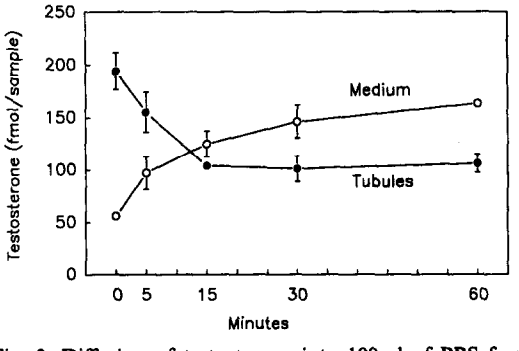

Fig. 3. Diffusion of testosterone into 100 µl of PBS from 1-cm tubule segments at stages VII and VIII of the cycle as a function of time. Testosterone released into the medium (open circles) and remaining in the tissue (solid circles) represent means ± SEM of 4 measurements.

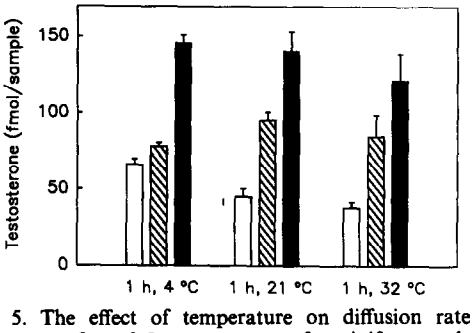

Fig. 5. The effect of temperature on diffusion rate of testosterone from 0.5 mm segments of seminiferous tubules at stages VII and VIII of the cycle during 1-h incubation at 4, 21 and 32°C. Testosterone after the incubation period in the tubular tissue (open bars), medium (hatched bars) and their sum (solid bars) was measured from quadruplicate samples (means ± SEM).

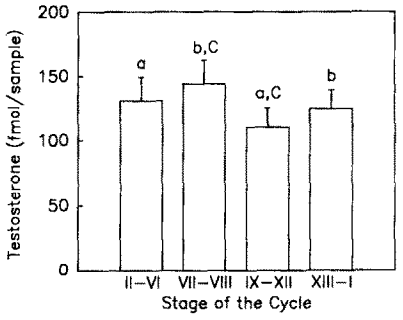

Fig. 6. Testosterone contents of 5 mm segments of seminiferous tubules at different stages of the cycle. Each bar is the mean ± SEM of quadruplicate measurements of 12 animals. The same letter above the bars indicates statistically significant differences between the stages (small letters, $P < 0.05$, capital letter, $P < 0.01$; Duncan's new multiple range test).

block spermatogenesis in prostatic cancer patients treated with a GnRH agonist [11]. The present study was designed to find out optimal conditions to measure the actual physiological concentration of testosterone within the seminiferous tubules.

In wet dissection of the tubules according to the method described by Christensen and Mason[12], testosterone rapidly diffuses out of the tubules, particularly when dissection is performed at room temperature [8]. However, the physiological androgen microenvironment in the seminiferous tubules is obviously critical for development of the spermatogenic cells. This has been measured in dissected seminiferous tubules by several investigators, but with rather varying results. Cooke *et al.*[13] found a testosterone concentration of 80 ± 42 SD nmol/l and de Jong *et al.*[14] 74 ± 29 SD nmol/l in testis tissue, whereas Podesta and Rivarola[15] found 32.6 pmol/g protein. Probably more accurate estimations have been made when testosterone concentrations were measured in tubular fluid collected through micropuncture of seminiferous tubules. Comhaire and Vermeulen[16] found 318 ± 49 nmol/l with a method approaching its limit of sensitivity. Recently, Turner *et al.*[17] found a level of 174 ± 7.8 nmol/l in the seminiferous tubule fluid.

According to morphometric analysis [18], the rat seminiferous tubule diameter shows a significant variation in different stages of the epithelial cycle. The volume of 1 cm of seminiferous tubule can be calculated using the formula $\pi r^2 l$ where r is the seminiferous tubular radius and $l = 1$ cm. The volume that is interchangeable into milligrams since the specific gravity of fresh testis tissue is near unity, 1.040 ± 0.004 SEM [19], is in different stages as follows: II–VI: 0.50 mm^3, VII–VIII 0.54, IX–XII 0.42 and in stages XIII–XIV 0.45 mm^3/1 cm of tubule. When the values presented in Fig. 6 are converted to testosterone levels per unit volume of seminiferous tubules at different stages, the following values are found: II–VI 521 nmol/l, VII–VIII 531, IX–XII 521 and XIII–I 542 nmol/l.

After corrections for tubular volumes, the testosterone concentration per unit volume in freshly isolated "dry-dissected" seminiferous tubules seems to be identical in all stages of the epithelial cycle. This is in agreement with a recent observation by Bartlett *et al.*[20]. No stage-dependent differences were found in intratesticular testosterone concentration in rats with spermatogenic synchronization achieved by depletion and restoration of vitamin A. These observations are different from what has been found earlier in "wet-dissected" tubules [8] or in the secretion of androgen binding protein [21] that show a peak at stage VIII of the cycle. The androgen receptor concentration showed maximal values at stages IX–XII of the cycle [22]. Although the movement of androgens through the seminiferous tubular wall seems to be restricted [23–25], these hormones obviously are available for all parts of the seminiferous tubule in similar concentrations. The local cellular availability of androgens in different stages of the cycle may be modulated by extracellular binding to ABP, nuclear binding to androgen receptor or cytoplasmic binding to components such as described by Schmidt and Danzo[26]. All these observations together may indicate that the action of androgens in the seminiferous epithelium is regulated by the binding proteins that regulate the local extracellular, intracellular and nuclear levels of active androgens.

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