Temporal relationship between androgen-dependent changes in the volume of seminiferous tubule fluid, lumen size and seminiferous tubule protein secretion in rats

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This study assessed whether changes in production of seminiferous tubule fluid underlie the previously described androgen-dependent changes in protein secretion by seminiferous tubules at stages VI–VIII of the spermatogenic cycle. Testosterone withdrawal was induced in adult rats by administration of ethane dimethane sulfonate (EDS) and temporal changes in lumen area, the volume of seminiferous tubule fluid and testicular interstitial fluid were assessed and compared with the changes in secretion of [³⁵S]methionine-labelled proteins *in vitro* by isolated seminiferous tubules at stages VI–VIII. Testicular interstitial fluid was reduced by about 50% by day 4 and later after EDS treatment when compared with controls. In contrast, the volume of seminiferous tubule fluid was unaffected at days 3 and 4 but was reduced by about 50% at days 6 and 8 after EDS treatment. In perfusion-fixed control testes, the lumen area of seminiferous tubules at stages VII–VIII was significantly greater than at stages I–VI and IX–XIV. This difference was also evident at 4 days after EDS treatment, but was abolished at 6 and 8 days after treatment. The volume of testicular interstitial fluid was reduced significantly at 3 and 4 days after EDS treatment, but was further reduced (about 50%) at 6 and 8 days. Administration of 25 mg testosterone esters every 3 days to EDS-treated rats prevented all of the changes described above. As the decrease in protein secretion by seminiferous tubules at stages VI–VIII following androgen withdrawal precedes the changes in lumen area and volume of seminiferous tubule fluid, it is concluded that there is differential control of these two androgen-dependent processes. The present data add to the evidence showing that testosterone plays a central role in regulating the production and movement of fluids (and therefore of nutrients) to and within the testis.

Introduction

It is still unknown how testosterone controls spermatogenesis in mammals (Sharpe, 1994), although we have recently taken the first steps towards better understanding of this area by showing that, in adult rats, testosterone exerts both general and specific effects on protein secretion by seminiferous tubules at stages VI–VIII of the cycle of the seminiferous epithelium (Sharpe *et al.*, 1992, 1993; McKinnell and Sharpe, 1992). These observations are particularly intriguing because the stage-dependent increase in protein secretion at stages VI–VIII coincides with a substantial increase in lumen size at stages VII–VIII (Wing and Christensen, 1982; Kerr, 1988; Sharpe, 1989). In turn, this increase is thought to reflect increased production of seminiferous tubule fluid by the Sertoli cells at stages VII–VIII, based on the observation that at 6–7 days after experimental testosterone withdrawal in adult rats lumen size at stage VII is reduced by nearly half (Ghosh *et al.*, 1992),

coincident with a 50% reduction in production of seminiferous tubule fluid per testis (O'Leary *et al.*, 1987). Other evidence clearly documents the androgen dependence of production of seminiferous tubule fluid in the adult (Free *et al.*, 1980; Jégou *et al.*, 1983; Au *et al.*, 1986), but not in the immature (Jégou *et al.*, 1982), rat. Moreover, selective depletion of elongate spermatids from the seminiferous epithelium results in comparable reductions in production of seminiferous tubule fluid (Jégou *et al.*, 1984), in lumen area at stages VII–VIII (Sharpe, 1989) and in total protein secretion by isolated seminiferous tubules at stages VI–VIII (McKinnell and Sharpe, 1992).

Because of the occluding tight junctions between adjacent Sertoli cells, all meiotic and post-meiotic germ cells are absolutely dependent on the Sertoli cells for their nutritional and energy support (Sharpe, 1994). The primary route via which this support is delivered will be via the secretion of these nutrients by the Sertoli cells, presumably into the seminiferous tubule fluid surrounding the germ cells. The halving in protein secretion by seminiferous tubules at stages VI–VIII which occurs within 4 days of testosterone withdrawal (Sharpe *et al.*,

1992) could therefore be a manifestation of reduced production of seminiferous tubule fluid. The present studies were designed to test this possibility by detailing changes in lumen area and the volume of seminiferous tubule fluid at different times after testosterone withdrawal induced by ethane dimethane sulfonate (Kerr *et al.*, 1985; Sharpe *et al.*, 1990), and comparing this to the temporal changes in protein secretion by isolated seminiferous tubules at stages VI–VIII. Moreover, as testicular blood flow, vasomotion and testicular interstitial fluid formation are also known to be androgen-dependent (Maddocks and Sharpe, 1989; Sharpe *et al.*, 1990; Damber *et al.*, 1992; Collin *et al.*, 1993), changes in the volume of interstitial fluid were also evaluated for comparison.

Materials and Methods

Animals and treatments

Wistar rats, 90–100 days of age, from our own colony were used for all of the experiments. Animals were maintained under standard conditions and had free access to food and water. Complete testosterone withdrawal was induced via the selective destruction of all Leydig cells by administering 75 mg ethane dimethane sulfonate (EDS) kg^{-1} body weight in 2 ml kg^{-1} 1:3 (v:v) dimethylsulfoxide:water by i.p. injection of groups of rats. Other EDS-treated rats were supplemented with testosterone esters (25 mg; Sustanon, Organon Labs Ltd, Cambridge) administered by s.c. injection every third day, beginning at the time of EDS injection. This dose of testosterone esters has been shown to maintain quantitatively normal spermatogenesis and fertility for up to 10 weeks (Sharpe *et al.*, 1988, 1990). Control rats were given the injection vehicle only. Animals from these three treatment groups (control: EDS:EDS + testosterone esters) were killed by inhalation of CO_2 and cervical dislocation at intervals ranging from 3 to 8 days after treatment and subjected to the procedures described below. It is emphasized that, in the first 4 days following EDS-induced testosterone withdrawal, the only abnormal changes to the seminiferous epithelium are the appearance of occasional degenerating germ cells at stages VII–VIII (< 0.5% of germ cells are affected). More extensive germ cell degeneration occurs between days 6 and 8, primarily at stages IX–XII (for details see Kerr *et al.*, 1993a; Sharpe, 1994).

Assessment of the volume of seminiferous tubule fluid and testicular interstitial fluid

Groups ($n = 5$ or 10) of rats from one or more of the above three treatment groups were killed at 3, 4, 6 and 8 days after treatment and the paired testes dissected out rapidly and cooled (4°C) and then weighed. Interstitial fluid was collected from the left testis overnight at 4°C using the 'drip collection' method as detailed and validated elsewhere (Sharpe and Cooper, 1983; Maddocks and Sharpe, 1989). Seminiferous tubule fluid was collected from the contralateral testis using the methods validated by Turner *et al.* (1984, 1985) and its volume assessed. Neither of these methods recovers all of the relevant fluid from the testis, but the volume recovered varies in proportion to the total fluid volume present.

Measurement of lumen area

Groups of three EDS-treated rats were fixed at 4, 6 and 8 days after treatment by vascular perfusion with a combination fixative containing 3% glutaraldehyde, as described by Kerr and Sharpe (1985) and Kerr *et al.* (1993a). A group of three control rats and three animals treated with EDS plus testosterone esters for 6 days were also perfusion-fixed. Tissue blocks were processed into plastic using standard procedures and semi-thin ($0.75 \mu\text{m}$) sections were then cut and stained with toluidine blue. Blocks were oriented for cutting such that the sections contained predominantly seminiferous tubules cut in transverse section. For each rat, a total of 25 round cross-sections of seminiferous tubules in each of the following three stage-groupings, I–VI, VII–VIII and IX–XIV, were then subjected to image analysis (Cue-2, Olympus) to delineate and measure the cross-sectional area of the tubule lumen; data were derived from at least three blocks per rat. The mean lumen area ($n = 25$) for each of the three stage groupings per rat was then determined and the mean $\pm$ SD for each treatment group ($n = 3$) calculated. The selection of stage groupings for analysis was based on previous data showing that lumen areas at stages VII–VIII were substantially larger than those at earlier or later stages (see Wing and Christensen, 1982; Kerr, 1988; Sharpe, 1989).

Protein secretion by isolated seminiferous tubules

Long lengths (≥ 1 cm) of seminiferous tubules at stages VI–VIII were isolated using transillumination from five or six rats in each of the three treatment groups at 3, 4 and 6 days after treatment and a total of 10 cm cultured *in vitro* for 22 h in the presence of [^{35}S]methionine. Full details of the isolation and culture conditions were described by Sharpe *et al.* (1992). Incorporation of [^{35}S]methionine into total secreted protein was assessed following precipitation of proteins using trichloroacetic acid (Sharpe *et al.*, 1992), and used as an index of the level of overall protein secretion. Previous studies have shown that protein secretion by seminiferous tubules at stages VI–VIII is approximately twice that at earlier and later stages and that this difference is androgen dependent (Sharpe *et al.*, 1992, 1993; McKinnell and Sharpe, 1992).

Statistical analysis

Data were analysed first by analysis of variance (ANOVA). Where significant differences between groups were indicated, further between-group comparisons were made using Student's *t* test but using the overall variance from ANOVA as the best estimate of error.

Results

Testicular weight and general morphology

The temporal pattern of germ cell degeneration observed after EDS treatment was comparable to that described by Sharpe *et al.* (1990) and Kerr *et al.* (1993a) and these changes were prevented completely by the administration of 25 mg testosterone esters every 3 days. No Leydig cells were

Table 1. Testicular weight of rats at various times after administration of ethane dimethane sulfonate (EDS) alone or EDS + testosterone esters (TE)

Days after treatment	n	EDS	EDS + TE
0 (control)	10	(1870 ± 84)	(1870 ± 84)
3	5	1846 ± 69	—
4	10	1741 ± 87**	1931 ± 116
6	5	1553 ± 100***	1732 ± 122
8	5	1501 ± 144***	1831 ± 85

Values are means ± SD. n = Number of animals. Significantly different (**P < 0.01, ***P < 0.001) from the control group.

identified in any of the EDS-treated rats. Testicular weight in EDS-treated rats was not different from that of controls on day 3, was reduced marginally (93% of control) on day 4 and more substantially on days 6 (83% of control) and 8 (80% of control) after treatment (Table 1). These changes were prevented by the administration of testosterone esters.

Protein secretion by isolated seminiferous tubules

After EDS-induced testosterone withdrawal, total protein secretion by seminiferous tubules at stages VI–VIII was reduced by nearly 40% compared with that of controls at 3 days after treatment and by 50% or more at days 4 and 6. The latter decrease was prevented completely by the administration of testosterone esters (Fig. 1).

Lumen areas

As reported previously, the lumen area of seminiferous tubules at stages VII–VIII was approximately 60% greater than that in seminiferous tubules at stages I–VI or IX–XIV. At 4 days after EDS treatment, when protein secretion by isolated seminiferous tubules was reduced maximally, there was no change in the stage-dependent differences in lumen area. In contrast, by day 6 after EDS treatment, the normal stage-dependent changes in lumen area were abolished completely and remained so at day 8 (Fig. 1). Administration of testosterone esters to EDS-treated rats could maintain the normal stage-dependent changes in lumen area (Fig. 1). An unexpected finding was that, at days 4 and 6 (but not at day 8) after treatment with EDS alone, the lumen area of seminiferous tubules at stages I–VI was reduced to a small but significant extent when compared with that of controls (Fig. 2).

Volume of seminiferous tubule fluid

As measured using the method of Turner *et al.* (1984), the recovered volume of seminiferous tubule fluid was unaffected by EDS treatment at days 3 and 4 but was reduced by nearly half at days 6 and 8, compared with that of controls. Administration of testosterone esters prevented the latter changes completely and, indeed, at day 8 after EDS plus testosterone esters the volume of seminiferous tubule fluid was increased significantly compared with that of the control group (Fig. 2).

Fig. 1. Temporal changes in (a) secretion of radiolabelled proteins by isolated seminiferous tubules at stages VI–VIII and (b) lumen area of seminiferous tubules at different stages of the spermatogenic cycle. Data for isolated seminiferous tubules (a) is from five rats per group and has been expressed as a percentage (mean ± SD) of the mean value for the respective control. Lumen areas (b) are the mean ± SD for three perfusion-fixed rats per group. Significantly different (*P < 0.05; **P < 0.01, ***P < 0.001) from respective control group. Significantly different (^aP < 0.05, ^bP < 0.01) from stages I–VI and IX–XIV of the same group. EDS: ethane dimethane sulfonate; TE: testosterone ester.

Volume of interstitial fluid

In contrast to the data obtained for seminiferous tubule fluid, the volume of interstitial fluid was found to be significantly lower at days 3 and 4 after EDS treatment, although this decrease was far more marked at days 6 and 8 when compared with controls (Fig. 2). Administration of testosterone esters to EDS-treated rats prevented these decreases in volume of interstitial fluid.

Discussion

The primary objective of the present study was to assess whether the androgen-dependent increase in total protein secretion by isolated seminiferous tubules which occurs at stages VI–VIII in rats is a consequence of increased production

Fig. 2. Temporal changes in the recovered volume of (a) seminiferous tubule fluid (STF) and (b) testicular interstitial fluid (IF) in rats: (□) control; (▨) ethane dimethane sulfonate (EDS); (■) EDS plus testosterone esters. Data are the means $\pm$ SD for either five (EDS days 3, 6 and 8) or ten (control, EDS day 4) animals per group. Note that data for the control group are derived from two groups of five animals killed on days 3 and 6 of treatment; data from these two groups did not differ significantly and were thus pooled to improve clarity of presentation. Significantly different (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) from respective control group. Significantly different ($^bP < 0.01$, $^cP < 0.001$) from EDS-treated group at the same time.

of seminiferous tubule fluid at these stages. The results reported here show that this is probably not the case, as after EDS-induced testosterone withdrawal, protein secretion by isolated seminiferous tubules at stages VI–VIII was reduced maximally by day 4 after treatment, whereas lumen area at stages VII–VIII and the volume of seminiferous tubule fluid recovered from the whole testis were unchanged at this time; however, both parameters were reduced maximally by day 6 after treatment. It is therefore more likely that the latter reduction is a consequence rather than a cause of the altered protein secretion by seminiferous tubules at stages VI–VIII after the withdrawal of testosterone support.

At present, the production of seminiferous tubule fluid by Sertoli cells cannot be assessed at the different stages of the spermatogenic cycle and, although changes in lumen area at the different stages may reflect altered production of seminiferous tubule fluid, this cannot be proved definitively (see Sharpe, 1989; Ghosh *et al.*, 1992; for discussion). However, in other

serous-secreting epithelial tissues, such as the parotid gland, experimental induction of secretory activity is accompanied by a significant enlargement of luminal area of acini, which is reversed dramatically upon cessation of secretion (Amsterdam *et al.*, 1969). In the study reported here, a combination of approaches was used to ascertain the likely temporal and stage-dependent changes in volume or production of seminiferous tubule fluid in relation to changes in protein secretion by isolated seminiferous tubules. The findings concur with those of Ghosh *et al.* (1992) in showing that lumen area at stages VII–VIII is reduced by about 50% at day 6 and also at day 8 after testosterone withdrawal, and this coincided with a fall of similar magnitude in the volume of seminiferous tubule fluid recovered using the method of Turner *et al.* (1984). Direct measurement of the rate of production of seminiferous tubule fluid at day 7 after testosterone withdrawal, using the efferent duct ligation method, gave a similar 50% decrease in production (O'Leary *et al.*, 1987). These comparisons indicate that lumen area at stages VII–VIII and measurement of the volume of seminiferous tubule fluid recovered by the method of Turner *et al.* (1984) probably provide accurate reflections of the volume and rate of production of seminiferous tubule fluid. The absence of any change in these two parameters at day 4 after EDS-induced testosterone withdrawal thus suggests that production of seminiferous tubule fluid is unaffected at this time. The only qualification to this conclusion was the finding of a small, but significant, reduction in lumen area at stages I–VI at days 4 and 6 after EDS treatment. However, as the volume of seminiferous tubule fluid recovered from the whole testis was unchanged at these times and lumen area at stages I–VI was not different from that of controls at day 8 after EDS treatment, this may be a chance finding and of no physiological significance.

Maximal suppression of protein secretion by isolated seminiferous tubules at stages VI–VIII was evident at day 4 after EDS-induced testosterone withdrawal, as reported by Sharpe *et al.* (1992). At this time, no change was evident in lumen area at stages VII–VIII or in the volume of seminiferous tubule fluid per testis, as measured by the method of Turner *et al.* (1984). Decreased production of seminiferous tubule fluid is therefore unlikely to explain the marked and stage-specific fall in protein secretion by isolated seminiferous tubules following testosterone withdrawal *in vivo*. It is therefore presumed that the subsequent 50% fall in volume of seminiferous tubule fluid at day 6 after EDS treatment is probably secondary to the altered protein secretion by the Sertoli cells or germ cells in the seminiferous tubules or to unknown alterations in function of the Sertoli or germ cells which underlie both changes. A parallel example of dissociation between fluid and protein secretion by epithelial cells is the exocrine pancreas in which fluid secretion (water, electrolytes) and enzyme discharge are induced separately by secretin or cholecystokinin, respectively (Gorelick and Jamieson, 1987; Schulz, 1987). With regard to fluid secretion, Kerr *et al.* (1993b) identified that at days 6–8 after testosterone withdrawal, focal dilatations of the intercellular spaces associated with inter-Sertoli cell junctions occur specifically at stages VII–VIII, a possible cause being the inappropriate secretion of seminiferous tubule fluid into the lateral intercellular spaces between adjacent Sertoli cells. Studies have shown that normal volume of seminiferous tubule

fluid (Jégou *et al.*, 1984) and lumen size at stages VII–VIII (Sharpe, 1989) depend on the presence of elongate spermatids. However, the decreases in volume of seminiferous tubule fluid and lumen size observed in the present studies at days 6 and 8 after EDS treatment are not associated with marked loss of elongate spermatids or other germ cells (Kerr *et al.*, 1993a).

In the present, as in previous (Maddocks and Sharpe, 1989; Sharpe *et al.*, 1990) studies, the volume of testicular interstitial fluid was found to decrease rapidly after EDS-induced testosterone withdrawal, although the decrease observed at days 3–4 was markedly less than that at days 6–8. At the latter times, the volume of interstitial fluid was reduced to a comparable degree to the reduction in volume of seminiferous tubule fluid. Testosterone is now known to regulate testicular blood flow and vasomotion (Damber *et al.*, 1992; Collin *et al.*, 1993); these effects are probably mediated via androgen receptors in small arterioles of the testis (Bergh and Damber, 1992). Vasomotion is thought to be responsible for the formation of testicular interstitial fluid (Damber *et al.*, 1992), and the observed reductions in volume of interstitial fluid after EDS treatment are consistent with this contention. However, because EDS treatment involves destruction and phagocytosis of Leydig cells by macrophages and mild inflammation (Kerr *et al.*, 1985), some of the early changes in volume of interstitial fluid may be related to these phenomena rather than to the loss of testosterone and hence of vasomotion. This might provide an explanation for the 'two-step' reduction in volume of interstitial fluid observed after EDS treatment (small reduction at days 3 and 4; large reduction at days 6 and 8).

In conclusion, the present study showed that changes in the production of seminiferous tubule fluid are probably not responsible for the marked changes in protein secretion by seminiferous tubules that occur in a stage-dependent manner after testosterone withdrawal. However, the present data add to the evidence showing that testosterone plays a vital role in controlling the fluid dynamics of the testis, starting with blood flow and vasomotion and ending with the production of seminiferous tubule fluid. As this represents the only pathway via which substances such as nutrients are delivered to, and distributed within, the testis, it is likely that its regulation by testosterone is of fundamental importance in the control of normal testicular function.

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