Maintenance of spermiogenesis by exogenous testosterone in rats treated with a GnRH antagonist: relationship with androgen-binding protein status

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The relationship of the testicular distribution and [³H]dihydrotestosterone-binding capacity of androgen-binding protein (ABP) to the completion of spermiogenesis was examined in mature rats given daily injections of 25 or 250 µg kg⁻¹ body weight of GnRH antagonist (GnRH-A; Ac-D{2}, Nal¹, 4Cl-D, Phe², D-Trp³, D-Arg⁶, D-Ala¹⁰) for two weeks with or without subcutaneous implantation of 10 cm testosterone capsules. GnRH-A administration resulted in a dose-dependent suppression of serum FSH, which was partially prevented in the 250 µg GnRH-A kg⁻¹ group by exogenous testosterone. The total testicular testosterone content and concentration of testosterone in seminiferous tubular fluid were equally suppressed in both groups of rats treated with GnRH-A and receiving the testosterone supplement. ABP concentrations in interstitial and seminiferous tubular fluid were normal in rats given the 25 µg GnRH-A kg⁻¹ dose, and were increased ($P < 0.05$) by concomitant testosterone treatment. In contrast, ABP concentrations in interstitial and seminiferous tubular fluid were increased in rats given the 250 µg GnRH-A kg⁻¹ dose. This effect was attenuated when exogenous testosterone was given. Although binding of [³H]dihydrotestosterone by ABP in seminiferous tubular fluid was not affected by GnRH-A treatment, with or without exogenous testosterone, it was reduced in interstitial fluid by GnRH-A in a dose-dependent manner, and partially restored by testosterone administration. While complete spermatogenesis was maintained in rats given 25 µg GnRH-A kg⁻¹, the number of step 7 and step 19 spermatids were both reduced by 35%, and were not affected by testosterone implants. However, administration of 250 µg GnRH-A kg⁻¹ injections resulted in a 60% and 90% reduction of step 7 and step 19 spermatids, respectively. Administration of exogenous testosterone greatly increased the number of step 19 spermatids. This finding was associated with partial normalization of serum FSH, and [³H]dihydrotestosterone-binding by ABP in interstitial fluid, without changes in testicular testosterone. These results suggest that the bioavailability of ABP within the testis may be critical for the final steps of spermiogenesis.

Introduction

Although the effectiveness of exogenous testosterone in the maintenance of normal spermatogenesis in rats administered GnRH antagonist (GnRH-A) has been reported (Rea *et al.*, 1986; Bhasin *et al.*, 1987), the mechanisms by which androgens exert this effect remain undefined. Suppression of intratesticular testosterone frequently results in the disruption of normal spermiogenesis (Russell and Clermont, 1977; Huang and Boccabella, 1988), but qualitatively normal spermatogenesis can be maintained in the presence of 15–20% of normal testosterone concentration in testes in intact rats (Cunningham and Huckins, 1979; Huang and Nieschlag, 1986; Zirkin *et al.*, 1989), as well as in hypophysectomized animals (Huang *et al.*, 1987; Santulli *et al.*, 1990). These observations suggest that the differentiation of spermatids during the second half of

spermiogenesis may involve specific local mechanisms that are not directly related to testosterone concentration.

We have recently noted that the impairment of spermiogenesis following the administration of a GnRH-antagonist (GnRH-A) was associated with a significant decline in epididymal androgen-binding protein (ABP) content while testicular ABP content and serum ABP concentration increased (Huang *et al.*, 1992). Furthermore, enhancement of the testosterone maintenance of spermatogenesis in hypophysectomized rats by FSH was associated with an increase in testicular testosterone and ABP content, as well as epididymal ABP content (Huang *et al.*, 1991). These results suggest that ABP distribution within the testis, the transport of ABP to the epididymis, or both factors, may be important in the completion of spermiogenesis.

The present study examined ABP distribution and [³H]dihydrotestosterone (DHT)-binding activity in the testis, and its content in the epididymis in rats treated with GnRH-A,

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Table 1. Effects of testosterone implants on the organ weights of rats treated with GnRH-antagonist (GnRH-A)

Treatment	n	Testis (mg)	Epididymis (mg)	Seminal vesicle (mg)	Pituitary (mg)
Control	6	1626 ± 30	498 ± 30	1361 ± 63	11.8 ± 0.5
GnRH-A (µg kg ⁻¹)					
25	6	1486 ± 41	475 ± 12	1097 ± 65 ^a	11.4 ± 0.5
25 plus testosterone	5	1521 ± 50	488 ± 17	2006 ± 98 ^{ab}	11.4 ± 0.4
250	6	1001 ± 128 ^a	204 ± 24 ^a	156 ± 15 ^a	9.8 ± 0.5 ^a
250 plus testosterone	6	1503 ± 46 ^b	469 ± 15 ^b	2155 ± 109 ^{ab}	8.9 ± 0.6 ^a

Values are means ± SEM.

^aSignificantly different from control ($P < 0.05$).

^bEffect of testosterone implant was significant ($P < 0.05$).

Fig. 1. Serum (a) LH and (b) FSH responses in rats receiving daily injections of 25 or 250 µg GnRH-A kg⁻¹ with (+TC) or without (-TC) 10 cm testosterone capsule implants for 2 weeks. Results are expressed as mean ± SEM ng hormone per ml⁻¹ serum. ^aSignificantly different from control ($P < 0.05$); ^beffect of testosterone treatment significant compared with GnRH-A alone ($P < 0.05$). $n = 5$ or 6 rats.

with or without testosterone supplement, and their relationship with the maintenance of spermiogenesis.

Materials and Methods

Treatment of animals

Mature Sprague-Dawley male rats (250–275 g) were randomly assigned to receive a daily subcutaneous injection of

25 or 250 µg GnRH-A kg⁻¹ (Ac-D{2}, Nal¹, 4Cl-D, Phe², D-Trp³, D-Arg⁶, D-Ala¹⁰) for 2 weeks. Half of the animals of each group received s.c. implants of 10 cm testosterone capsules immediately after the first GnRH-A injection. Control animals were given a daily injection of 0.1 ml autoclaved water. Throughout the experiment, animals were in cages in an air-conditioned, light-controlled animal room and were fed Purina rat chow and water *ad libitum*.

At the end of the treatment period, animals were lightly anaesthetized with ether and 3–4 ml of retro-orbital sinus blood was collected from each animal. Animals were subsequently killed by an overdose of ether. One testis from each animal was used immediately for the collection of interstitial fluid and seminiferous tubular fluid as described by Turner *et al.* (1984). The epididymides were stored at -70°C for subsequent measurement of androgen-binding protein. The other testis was fixed in Bouin's solution and processed for histology.

Spermatogenesis

Paraffin sections were stained with periodic acid-Schiff's (PAS) reagent and counterstained with haematoxylin (Pearse, 1968). The stages of the seminiferous epithelium were determined by the development of the PAS-positive acrosome of spermatids (LeBlond and Clermont, 1952). The number of step 7 and 18–19 spermatids was determined in 15–20 cross-sections of stage VII seminiferous tubules and the result was expressed as the number of cells per 100 Sertoli cell nuclei.

Hormone measurement

Serum FSH and LH were determined by double antibody radioimmunoassay as described by Pogach *et al.* (1988). Reagents provided by NIADDK (Bethesda, MD), rat FSH-RP-2, rat FSH-I-5 and anti-rat FSH-S-11, and rat LH-RP-2, rat LH-I-6 and anti-rat LH-S-7 were used for FSH and LH assay, respectively. The sensitivity (90% binding) of the assay was 2.6 and 0.21 ng ml⁻¹ for FSH and LH, respectively. The intra and inter-assay coefficients of variation were approximately 8% and 12%, respectively, for both assays.

Table 2. Distribution of testosterone in serum and testis of rats treated with GnRH-antagonist (GnRH-A)

Treatment	n	Testis		Seminiferous tubular fluid (ng ml ⁻¹)	Serum (ng ml ⁻¹)
		Content (ng per testis)	Concentration (ng g ⁻¹)		
Control	6	347 ± 49	213 ± 28	54 ± 14	3.2 ± 0.3
GnRH-A (µg kg ⁻¹)					
25	6	153 ± 21 ^a	103 ± 14 ^a	39 ± 4	3.0 ± 0.3
25 plus testosterone	5	14 ± 2 ^{ab}	9 ± 1 ^{ab}	8 ± 2 ^{ab}	12.9 ± 2.8 ^{ab}
250	6	8 ± 4 ^a	7 ± 3 ^a	8 ± 2 ^a	0.1 ± 0.1 ^a
250 plus testosterone	6	8 ± 1 ^a	5 ± 0 ^a	7 ± 1 ^a	12.3 ± 1.8 ^{ab}

Values are means ± SEM.

^aSignificantly different from control ($P < 0.05$).

^bEffect of testosterone implants was significant ($P < 0.05$).

Testosterone concentration in serum, testes and seminiferous tubular fluid was determined by radioimmunoassay in respective ether extracts as described by Huang *et al.* (1990) using anti-serum provided by Radioassay System Laboratory (Carson, CA) with approximately 18% crossreactivity with DHT. Testicular testosterone was determined in ether extracts of 60–100 mg of decapsulated testicular tissue homogenized in 1 ml phosphate-buffered saline (pH 7.4) without chromatography. The sensitivity of testosterone assay was 2.25 pg per tube. The intra- and interassay coefficients of variation were 5 and 12%, respectively.

Measurement of androgen-binding protein

Androgen-binding protein (ABP) concentrations in serum, interstitial fluid, seminiferous tubular fluid and epididymal cytosol were determined by radioimmunoassay as described by Pogach *et al.* (1988). The sensitivity of the assay was 30 ng ml⁻¹ with 6 and 11% inter- and intra-assay coefficients of variation, respectively.

[³H]DHT-binding capacity of androgen-binding protein

For the measurement of the binding activity of ABP, 50 µl of interstitial fluid or seminiferous tubular fluid was diluted with 450 µl of PBS (pH 7.2). The endogenous androgen in these fluids was stripped with activated charcoal (20 mg mg⁻¹ protein) in the cold room (4°C) overnight. This procedure removed 90–95% of [³H]testosterone in pilot experiments. After the removal of the charcoal by centrifugation (1500 g for 30 min, three times), the supernatants were dialysed against 0.2 mol NH₄(CO₃)₂ l⁻¹ overnight at 4°C. The dialysed materials were subsequently lyophilized and reconstituted in the appropriate volume of sample buffer (5 mmol Tris l⁻¹, 0.5 mmol EDTA l⁻¹, 10% glycerol and 0.1% bromophenol blue).

The binding capacity of ABP was determined by measuring its binding with [³H]DHT during steady state electrophoresis (Ritzen *et al.*, 1974). The gels were cut into 50 slices, and the radioactivity of each slice was determined by scintillation counting. Two radioactive peaks (slices 12–16, and slices 18–23)

were detected in all samples. The radioactivity of the first peak representing the binding of [³H]DHT by ABP was used for calculation and the results were expressed as d.p.m [³H]DHT per ng of radioimmunoactive ABP.

Statistical analysis

Analysis of variance was used to detect the significance of treatments. When the treatment effects were significant, Scheffe's multiple range test was used to detect the significance among groups.

Results

Organ weights

The weights of the testis and epididymis were unaffected after 2 weeks of daily injections of 25 µg GnRH-A kg⁻¹, with or without subcutaneous testosterone implants (Table 1). Daily injection of 250 µg GnRH-A kg⁻¹ resulted in a 40% and 60% reduction ($P < 0.05$) in the weights of the testis and epididymis, respectively. These changes were prevented by the testosterone implants ($P < 0.05$). The weight of the seminal vesicle was significantly less ($P < 0.05$) in animals receiving either dose of GnRH-A, but was increased relative to controls ($P < 0.05$) in animals receiving a testosterone implant. The weight of the pituitary gland was not affected in animals in the 25 µg GnRH-A kg⁻¹ group, but was significantly less than controls in those receiving 250 µg GnRH-A kg⁻¹ injections ($P < 0.05$).

Hormone concentrations

Daily injections of 25 or 250 µg GnRH-A kg⁻¹ suppressed serum FSH concentration of adult rats to 85 and 20%, respectively, of the control value ($P < 0.05$, Fig. 1). Testosterone implants decreased serum FSH further in rats given 25 µg GnRH-A kg⁻¹ injections ($P < 0.05$) but increased those of rats

Fig. 2. Concentration of androgen-binding protein (ABP) in (a) interstitial fluid and (b) seminiferous tubular fluid of rats receiving injections of 25 or 250 µg of GnRH-antagonist (GnRH-A) kg⁻¹ with (+ TC) or without (-TC) testosterone capsules for 2 weeks. Results are expressed as means ± SEM µg ml⁻¹. ^aSignificantly different from control ($P < 0.05$); ^beffect of testosterone capsule treatment was significant compared with GnRH-A alone ($P < 0.05$). $n = 5-6$ rats.

in the group receiving 250 µg GnRH-A kg⁻¹ ($P < 0.05$), to values comparable to that of the 25 µg kg⁻¹ group (50% of the control value).

Serum LH was depressed by approximately 60% in rats given 25 µg GnRH-A kg⁻¹ injections ($P < 0.05$), and was further decreased when testosterone implants were given ($P < 0.05$). In rats in the 250 µg GnRH-A kg⁻¹ group, with or without testosterone implants, serum LH concentrations were at the detection limit of the assay ($P < 0.05$, Fig. 1).

Injection of GnRH-A resulted in a dose-dependent suppression of testosterone in serum, testis and seminiferous tubular fluid ($P < 0.01$; Table 2). Testosterone implants further decreased testosterone concentrations in rats given 25 µg GnRH-A kg⁻¹ injections but had no additional effect upon the testosterone profile in rats in the 250 µg GnRH-A kg⁻¹ group.

Androgen-binding protein

Androgen-binding protein concentration in interstitial fluid and seminiferous tubular fluid was not affected by daily injections of 25 µg GnRH-A kg⁻¹, but was significantly increased when testosterone implants were also given ($P < 0.05$, Fig. 2). Injections of 250 µg GnRH-A kg⁻¹ resulted in a significant increase in ABP concentration in interstitial and seminiferous tubular fluid ($P < 0.05$), but these increments were less pronounced when animals were given testosterone implants.

Fig. 3. Epididymal content of androgen-binding protein (ABP) (mean ± SEM ng per epididymis) of adult rats given 25 or 250 µg GnRH-antagonist (GnRH-A) with (+ TC) or without (-TC) testosterone capsules. ^aSignificantly different from control ($P < 0.05$); ^beffect of testosterone capsule treatment was significant compared with GnRH-A alone ($P < 0.05$). $n = 5-6$ rats.

Table 3. Effects of GnRH-antagonist (GnRH-A) upon the biological activity of androgen-binding protein in rats

Treatment	<i>n</i>	Interstitial testicular fluid ^a	Seminiferous testicular fluid ^a
Control	4	252 ± 23	10.9 ± 0.9
GnRH-A (µg kg ⁻¹)			
25	4	180 ± 6 ^b	10.6 ± 0.8
25 plus testosterone	4	206 ± 12 ^b	13.3 ± 1.1
250	4	120 ± 8 ^b	11.1 ± 0.9
250 plus testosterone	4	171 ± 12 ^{bc}	13.5 ± 1.1

^aValues expressed as means ± SEM of c.p.m. [³H]-dihydrotestosterone ng⁻¹ androgen-binding protein.

^bSignificantly different from control ($P < 0.05$).

^cEffect of testosterone capsule implant was significant ($P < 0.05$).

Although ABP concentration per gram of tissue was not affected (data not shown), epididymal ABP content was significantly less in rats given 250 µg GnRH-A kg⁻¹ injections ($P < 0.05$), but this suppression was prevented by testosterone implants (Fig. 3).

[³H]DHT-binding activity of androgen-binding protein

The [³H]DHT-binding activity of ABP in seminiferous tubular fluid was not affected by either dose of GnRH-A, with or without testosterone implants, but it was significantly decreased in interstitial fluid following the administration of 25 or 250 µg GnRH-A kg⁻¹ ($P < 0.05$; Table 3). This effect was partially prevented by testosterone implants.

Fig. 4. Photomicrograph of testis of control rat. The presence of step 18–19 spermatids (arrowheads) at the luminal edge of stage VII–VIII epithelium demonstrates the completeness of spermatogenesis. Bar = 10 μ m.

Table 4. The effect of GnRH-antagonist (GnRH-A) on the number of spermatids in intact adult rats

Treatment	n	Step 7 ^a	Step 19 ^a	Step 19/Step 7
Control	5	927 $\pm$ 42	865 $\pm$ 49	0.93
GnRH-A (μ g kg ⁻¹)				
25	3	598 $\pm$ 52 ^b	556 $\pm$ 54 ^b	0.93
25 plus testosterone	3	618 $\pm$ 23 ^b	595 $\pm$ 38 ^b	0.96
250	4	460 $\pm$ 87 ^b	69 $\pm$ 46 ^{cd}	0.15
250 plus testosterone	3	561 $\pm$ 58 ^b	516 $\pm$ 51 ^{be}	0.92

^aValues expressed as means $\pm$ SEM per 100 Sertoli cell nucleoli.

^b $P < 0.05$ versus control.

^c $P < 0.01$ versus control.

^d $P < 0.01$ versus 25 μ g GnRH-A kg⁻¹ dose.

^e $P < 0.01$ versus testosterone without implant.

Spermatogenesis

In control animals, the presence of mature spermatids at the luminal edge of stage VII–VIII epithelium demonstrated the completeness of spermatogenesis (Fig. 4). Although complete spermatogenesis was maintained in rats given 25 μ g GnRH-A kg⁻¹ injections, the number of step 7 and step 18/19 spermatids was reduced by 35% ($P < 0.05$) and was not affected by testosterone implants (Table 4). Daily administration of 250 μ g GnRH-A kg⁻¹ resulted in the arrest of spermatogenesis in the majority (4 of 6) of the animals (Fig. 5). The number of step 7 and step 18/19 spermatids was decreased by 65 and 95%, respectively (Table 4). Testosterone implants resulted in the maintenance of complete spermatogenesis in all animals receiving daily injections of 250 μ g GnRH-A kg⁻¹, and restored the number of spermatids to approximately 60% of controls (Fig. 6).

The efficacy of spermatid differentiation, as indicated by the ratio between step 7 and step 19 spermatids, was normal in rats given 25 μ g GnRH-A kg⁻¹ injections; however, in rats receiving 250 μ g GnRH-A, less than 15% of the step 7 spermatids could differentiate to maturity. Exogenous testosterone administration enabled more than 90% of the step 7 spermatids to develop to maturity in rats receiving the 250 μ g kg⁻¹ dose of GnRH-A.

Discussion

The failure of exogenous testosterone alone to maintain or restore a normal number of spermatids in hypophysectomized rats (Huang *et al.*, 1987; Santulli *et al.*, 1990; Huang *et al.*, 1991) demonstrates the importance of pituitary factors for normal

Fig. 5. Photomicrograph of testis of a rat given 250 µg GnRH-antagonist kg⁻¹ for 2 weeks. Note the disruption of spermatogenesis and absence of elongated spermatids in all tubules. Bar = 10 µm.

Fig. 6. Photomicrograph of testis of a rat given 250 µg GnRH-antagonist kg⁻¹ plus testosterone implant for 2 weeks. Note the presence of elongated spermatids (arrowheads) in all tubules. Bar = 10 µm.

spermiogenesis. The importance of FSH for quantitative spermiogenesis has previously been demonstrated in hypophysectomized rats (Bartlett *et al.*, 1989; Huang *et al.*, 1991), but the mechanism by which FSH affects spermatid differentiation remains unknown.

In the current study, despite the marked reduction in the number of spermatids, complete spermiogenesis was maintained in the group of rats receiving the 25 µg GnRH-A kg⁻¹. However, a drastic reduction of the number of young spermatids was associated with the absence of mature spermatids in stage VII epithelium in rats receiving 250 µg GnRH-A kg⁻¹.

This result demonstrates that the mechanisms regulating the final maturation of spermatids was impaired by high doses of GnRH-A. This impairment was prevented by the administration of exogenous testosterone. The lack of a relationship between the testicular testosterone content or the concentration of testosterone in seminiferous tubular fluid and the status of spermiogenesis is consistent with the hypothesis that the differentiation of spermatids during the second half of spermiogenesis may involve local mechanisms that are not directly related to testicular testosterone concentration (Huang and Boccabella, 1988).

It is generally accepted that FSH and testosterone modulate the differentiation of spermatogenic cells through their actions on Sertoli cells (Roberts and Zirkin, 1991). Despite the lack of an unequivocal demonstration of androgen receptor in spermatogenic cells using nuclear exchange assays (Frankel *et al.*, 1989), the presence of assayable testosterone and the binding of radioactive testosterone in spermatids (Sanborn *et al.*, 1975; Frankel *et al.*, 1989), and the recent demonstration of mRNA transcripts of androgen receptor in germ cells (Huang *et al.*, 1992b) suggests that testosterone may affect the differentiation of spermatids directly. However, since spermatids are separated from the androgens in interstitial fluid by Sertoli cells, spermatids must acquire testosterone through Sertoli cells. The presence of immunoactive ABP in Sertoli cell processes surrounding spermatids (Attramadal *et al.*, 1981; Pelliniemi *et al.*, 1981) suggests that Sertoli cell ABP may provide a vehicle for delivering testosterone to spermatids.

The possible importance of ABP in spermiogenesis is suggested by the findings of Huang and Boccabella (1988), who showed that the maintenance of spermiogenesis in intact rats given various doses of exogenous testosterone correlated with the ABP content in testes. In hypophysectomized rats, FSH enhancement of maintenance of spermiogenesis by testosterone was associated with an increase in testicular ABP and testosterone content, and epididymal ABP content (Huang *et al.*, 1991). It was postulated that FSH facilitates the accumulation or distribution of testosterone in the testis and the transport of ABP to epididymis through the modification of the biochemical properties of ABP (Huang *et al.*, 1991).

An increase in the ABP concentration in seminiferous tubular fluid and interstitial fluid and a decrease in epididymal ABP following high doses of GnRH-A have been noted in a previous study (Huang *et al.*, 1992a). It was postulated that a failure in the transport of seminiferous tubular fluid to the epididymis may account for the decrease in epididymal ABP and the accumulation of testicular ABP. This may in turn cause an increased ABP release through the basement membrane of Sertoli cell resulting in the higher ABP concentration in interstitial fluid and serum. These changes may result from the suppression of endogenous testosterone production and serum FSH since both hormones are implicated in the regulation of the bidirectional release of ABP, and FSH may be particularly important in the luminal release of ABP (Mather *et al.*, 1983; Sharpe, 1988; Danzo *et al.*, 1990). The concomitant restoration of serum FSH and epididymal ABP in rats receiving the 250 µg GnRH-A kg⁻¹ injections and testosterone implants supports the above notion.

Although ABP in seminiferous tubular and interstitial fluid are extracellular products, changes in the concentration or binding capacity of ABP in these fluids undoubtedly reflect alterations in the ABP status inside Sertoli cells. A decrease in the [³H]DHT-binding capacity of ABP in interstitial fluid tubular fluid suggests that GnRH-A treatment results in the modification of the ABP molecule (Cheng *et al.*, 1986). Such a change could affect the testosterone distribution within Sertoli cells and perhaps its delivery to spermatids, resulting in impaired spermiogenesis. This contention is corroborated by the concomitant restoration of the [³H]DHT-binding capacity of ABP in interstitial fluid and the efficacy of spermatid differentiation in rats given the high dose of GnRH-A and testosterone implants. This effect was correlated with the partial maintenance

of serum FSH, rather than with the status of testicular testosterone content or concentration of testosterone in seminiferous tubular fluid. Although not determined in this study, maintenance of the bioactive as well as the immunoreactive FSH in the rats receiving GnRH-A and testosterone is likely (Sharma *et al.*, 1990). These results suggest that FSH modulates the final differentiation of spermatids through modification of the biochemical properties of ABP, although effects on other Sertoli cell proteins certainly cannot be excluded.

In conclusion, the present study demonstrated that the preservation of complete spermatogenesis by exogenous testosterone in rats treated with GnRH-A was associated with the maintenance of serum FSH and restoration of the [³H]DHT binding activity of ABP in interstitial fluid, rather than with testicular testosterone distribution. Further work, including the immunocytochemical localization of ABP in the seminiferous epithelium under various experimental conditions, and the hormonal regulation of the post-translational modification of ABP molecules is needed so that the significance of this protein in the endocrine control of spermiogenesis can be understood.

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