Intratesticular Androgen Levels, Androgen Receptor Localization, and Androgen Receptor Expression in Adult Rat Sertoli Cells

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ABSTRACT

In the rat, quantitatively normal spermatogenesis is maintained only when intratesticular testosterone (ITT) levels greatly exceed the peripheral T concentration. When ITT concentrations fall below a threshold, germ cells are lost at specific stages of the seminiferous cycle. Germ cells can be restored by high doses of T that binds to androgen receptors (AR) in Sertoli cells. However, the relationships between germ cell dynamics, AR-mediated molecular events, and ITT concentrations are not established. ITT levels may regulate germ cell life and death through an effect on AR localization and AR mRNA or protein levels within Sertoli cells at specific stages of the cycle. We determined AR localization and mRNA and protein expression in adult rat Sertoli cells in relation to reduced and then restored ITT concentrations in vivo. ITT levels were reduced by implanting rats with T- and estradiol (E)-filled capsules for 7–28 days and subsequently restored with large T-filled capsules. AR is normally localized within Sertoli cell nuclei at stages VII–VIII of the seminiferous epithelium. After T/E treatment, AR immunostaining in Sertoli cell nuclei became nondetectable by 14–28 days but was restored 6 h following T restoration. The loss of Sertoli cell nuclear AR localization correlated with increasing numbers of apoptotic germ cells. AR mRNA levels in isolated Sertoli cells did not change through 14 days of T/E treatment, increased significantly by Day 28, and remained elevated 24 h after T restoration. AR mRNA levels in microdissected tubules at stages II–IV, VI–VIII, and IX–XII did not decrease through 14 days of T/E treatment. In contrast, AR protein levels were reduced in seminiferous tubules by Day 14 and in testes at Day 28 post-T/E treatment but were restored within 24 h by T repletion. Therefore, the reduction of ITT concentration results in a time-dependent redistribution of AR and reduced AR protein but not AR mRNA levels in Sertoli cells. Repletion of T restored AR protein and it relocated to Sertoli cell nuclei. By an unknown mechanism, T regulates AR localization within Sertoli cells to determine germ cell life or death.

INTRODUCTION

Although it is well established that testosterone (T) is required for spermatogenesis, the biologic mechanism(s) that underlies the androgen dependency of spermatogenesis is poorly understood. Sertoli cells are considered to be the target cells for androgen action; testosterone elicits its action through androgen receptor (AR) binding and translocation into the nucleus, where it initiates androgen-dependent gene transcription [1–6]. In untreated rats, AR nuclear localization in Sertoli cells occurs at specific stages of the cycle of the seminiferous epithelium, with the highest level being during stages VII–VIII [2–6], the so-called androgen-dependent stages of the cycle [7, 8].

The intratesticular concentration of T in the rat is approximately 40–50 times higher than the physiologic T concentration in serum of 1–2 ng/ml [9]. Interestingly, quantitatively normal spermatogenesis can be maintained in the rat only when intratesticular T (ITT) levels are above 20 ng/ml, a level that is 10-fold greater than in the peripheral circulation [10–12]. When T levels within the testis fall below the threshold of 20 ng/ml, step 19 spermatids fail to be released, pachytyene spermatocytes undergo apoptosis, and round spermatids are sloughed from the seminiferous epithelium [7–12]. These events are initiated specifically at stages VII–VIII and presumably occur because of reduced androgen-dependent gene transcription mediated via the AR in Sertoli cells associated with these stages of germ cell development [2, 3, 5, 7, 8]. Germ cells that are lost as a consequence of reduced ITT can be restored to the testis by the administration of high doses of exogenous T that are sufficient to reestablish ITT levels that exceed the critical threshold of 20 ng/ml [9–11].

The relationships among germ cell dynamics, AR-mediated molecular events, and ITT concentrations have not been established. Immunohistochemical studies have shown a dramatic loss of AR from Sertoli cell nuclei at stages VII–VIII of the seminiferous epithelium, when the concentration of T is profoundly decreased by the administration of a GnRH antagonist, Azaline B [5], or the Leydig cell toxicant ethane dimethanesulfonate (EDS) [13]. This suggests that AR redistribution might be an important consequence of the effect of reduced ITT on germ cell apoptosis. However, the regulation of AR in Sertoli cells by T, involving transcriptional, translational, and posttranslational control mechanisms, also may play important roles. For example, T was reported to decrease the AR mRNA level in Sertoli cells on Day 20 of life [14] but to have stimulatory effects on the level of Sertoli cell AR protein [15, 16]. In studies of the effects of EDS treatment, an alkylating agent that kills Leydig cells, total testis AR mRNA and protein levels were not affected by reduced ITT, but ligand binding assays revealed lower AR protein levels in the nuclear frac-
tion isolated from whole testes of EDS-treated rats [17, 18]. A testable, unifying hypothesis that might explain the relationship of ITT concentration to AR-mediated molecular events that lead to germ cell survival or death is that ITT concentration has effects on intracellular AR localization, AR mRNA, and/or AR protein levels within Sertoli cells at specific stages of the cycle. Perturbations of AR localization and/or protein expression may determine whether germ cells live or die.

The study reported herein was designed to address the previously mentioned hypothesis. We focused on the in vivo effects of changes in T concentration within the Sertoli cell microenvironment, that is, within the seminiferous tubule fluid of the testis, on AR localization, and on AR mRNA and protein levels within Sertoli cells, in relationship to germ cell death. For this reason, rats were implanted with T- and estradiol (E)-filled capsules to reduce ITT secondary to a reduction in serum LH but without a confounding effect on serum FSH levels [9, 10]. Our results show a time-dependent loss of AR nuclear localization in Sertoli cells at stages VI–VIII of the seminiferous epithelium and of germ cell apoptosis that accompany the reduction of ITT levels following the administration of contraceptive doses of T and E, with AR protein levels also decreasing. In contrast, AR mRNA levels remained unchanged. Within 24 h following the administration of exogenous T to the rats that had received T/E implants, AR protein was restored, and AR was again localized to Sertoli cell nuclei. These results suggest that, by an as-yet-unknown mechanism, T regulation of AR localization within Sertoli cells and of AR level in turn regulates germ cell life or death.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, 8–12 wk of age, were purchased from Charles River Laboratories (Kingston, MA). All rats were housed in a vivarium under a 14:10 light:dark cycle and provided water and rat chow ad libitum. To experimentally suppress LH-stimulated T production from the peripheral circulation. Seminiferous tubule fluid (STF) was collected from rats by centrifugation according to the method previously described [19, 20]. All samples were stored at −80°C. T concentrations in serum and STF were assayed in duplicate by RIA using a testosterone antibody purchased from ICN (Costa Mesa, CA) and 3H-T (New England Nuclear, Boston, MA) as previously described [12, 21]. The sensitivity of the assay was 10 pg/tube.

Radioimmunoassays

Trunk blood was collected and serum was obtained to measure T in the peripheral circulation. Seminiferous tubule fluid (STF) was collected from rats by centrifugation according to the method previously described by Turner et al. [21]. All samples were stored at −80°C. T concentrations in serum and STF were assayed by a testosterone radioimmunoassay, which labels fragmented DNA with digoxigenin-deoxy-UTP using terminal deoxynucleotidyl transferase as detected by the anti-digoxigenin-conjugated reporter system [Chemicon/Serologicals, Norcross, GA]. Briefly, serial sections (6 μm) from the same samples used for AR immunohistochemistry were deparaffinized, rehydrated through a series of graded alcohols and PBS, digested with 20 μg/ml proteinase K for 15 min at room temperature, and washed with water. Endogenous peroxidase activity was quenched in 3% H2O2 in PBS for 5 min, and tissue sections were incubated sequentially in a humidified chamber with equilibration buffer for 10 min at room temperature, terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled deoxy-UTP for 1 h at 37°C, and anti-digoxigenin peroxidase conjugate for 30 min at room temperature. Sections were then stained with 3,3′-diaminobenzidine peroxidase (DAB) substrate solution for 2 min and counterstained with 1% methyl green. The presence of TUNEL-positive germ cells was observed by light microscopy with a Plan Apo 20× objective as described previously for androgen receptor immunohistochemistry. The numbers of apoptotic germ cells detected by TUNEL staining were counted in tubules corresponding to specific stages of the cycle of the seminiferous epithelium. Random areas were viewed on testis tissue sections from three animals within each treatment group until a total of 50 tubules corresponding to each grouping of stages (II–IV, VII–VIII, or IX–XII) were examined.

Semeniferous Tubule Microdissection

Semeniferous tubule segments were isolated from rat testes by transillumination-assisted microdissection as previously described [22]. For RNA isolation, approximately 60 cm of seminiferous tubules at stages II–IV, VI–VIII, and IX–XII were dissected from two testes (one rat, 30 cm per testis) from control rats and from rats implanted with T/E capsules for 7, 10, or 14 days. For protein analyses, approximately 15–20 cm of seminiferous tubules from the same stages were dissected from testes of control rats and rats implanted with T/E capsules for 7 or 14 days. Dihydrotestosterone (1 nM) was added to the dissection medium and to the tissue lysate buffer to stabilize the AR protein prior to Western blot analyses [23]. Testes from different rats (n = 3) were included as independent samples within each experimental group for isolation of RNA or protein.

Sertoli Cell Isolation

Sertoli cells were isolated from whole testes as described by Anway [24], except the 10-min trypsin digestion step was omitted. Briefly, two decapsulated testes were incubated in 0.5 mg/ml collagenase in Hanks buffered salt solution (HBSS), pH 7.4, at 34°C with shaking for 15 min and then washed three times to eliminate the interstitial cells. To separate Sertoli and germ cells, the tubules were incubated in a mixture of enzymes, 0.1% collagenase (C2674; Sigma, St. Louis, MO), 0.2% hyaluronidase
T/E-treated rats and 28-day T/E-treated rats with testosterone (T) replace-
ment consistently contained pan blue exclusion.

Preparations of seminiferous tubules from testes of 120-day-old rats by unit gravity sedimentation (Staput) according to methods previously described [25]. The purity of the pachytene spermatocyte and round spermatid fractions was estimated to be 90% for each 

**Western Blot Analysis**

Seminiferous tubule segments, testis, pachytene spermatocytes, round spermatids, spleen, kidney, or liver were homogenized in RIPA buffer (1% Triton X-100, 15 mM Hepes pH 7.5, 0.15 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM EDTA, 1 mM DHT, and 0.5% protease inhibitor cocktail). Protein concentration was determined by the BCA method (Pierce, Rockford, IL). The protein samples were added to an equal volume of 2X loading buffer (100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, and 2% glycerol). The samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel system and transferred to a nitrocellulose membrane using a Trans-Blot SD Semi Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) according to the manufacturer’s specifications. AR protein was detected by Western blot analyses using anti-AR antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) as previously described [23]. Briefly, membranes were blocked for 30 min with 10% nonfat dry milk in PBS plus 0.2% Tween 20 (PBS + T) and then incu-

**Relative Levels of mRNA Determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA (1.5 μg) from microdissected seminiferous tubules and freshly isolated Sertoli cells was reverse transcribed in a 20-μl reaction at 46°C for 60 min using 0.2 units of Superscript II (Invitrogen) and 50 ng of oligo-dT primer in first-strand synthesis buffer according to the manufac-

trurer’s specifications. PCR was performed in a reaction volume of 25 μl containing 0.5 μl of the RT reaction, 400 nM sense primer, and 400 nM antisense primer using the QuantiTech SYBR green PCR kit (Qiagen, Valencia, CA). Each reaction was spiked with 0.05 units of Taq polymer-

ase (Invitrogen) to amplify cDNA products greater than 250 bp. Gene-
specific primers were as follows: for AR, 5’ GGGGCAATTCGACCA-

TATCT (sense) and 5’ CCCTTTGGGCTTAAACTC (antisense) to amplify a 277-bp fragment corresponding to the region 1661–1938 of the AR cDNA GenBank accession number M20133 [30]; for ADR, 5’ CAGGAACCATCCTTTC (sense) and 5’ TTCATCCTACCATGAGCAG (antisense) to amplify a 516-bp fragment corresponding to the region 2038–

2554 of the ABP cDNA GenBank accession number M20199 [31]; and for ribosomal L19, 5’ CTGAAGGTCAAAGGGAATGTG (sense) and 5’ GGACAGACTTCTGTGTATCTC (antisense) to amplify a 195-bp fragment corresponding to the region 401–595 of the L19 cDNA GenBank accession number NM031103. The PCR conditions were 26 cycles at 94°C for 20 sec, 56°C for 20 sec, and 72°C for 45 sec, with a final extension at 72°C for 1 min. PCR products (10 μl) were separated on a 1.5% agarose gel, and the fluorescent signal was detected using a Typhoon 9200 Imaging System and quantified using ImageQuant software (Amersham Biosciences). PCR products were cloned into p-GemT Easy Vector (Promega, Madison, WI) and sequenced to validate the cDNA insert against the GenBank database. Signal intensities were normalized to L19 expression with the ratio of 1.0 assigned to the control group, and all treatment groups are presented relative to the control.
FIG. 2. In situ localization of fragmented DNA (TUNEL) in germ cells of testicular tissue sections following T/E treatment. (A) Control; (B) 7 days; (C) 10 days; (D) 14 days; and (E) 21 days of T/E treatment. TUNEL-positive germ cells are indicated by the arrows. Sections were counterstained with methyl green. Similar results were obtained in tissue sections from at least three rats in each group. Magnification ×200.

bated overnight at room temperature in 1% nonfat milk in PBS + T and anti-AR antibody (1:500). The next day, the membranes were washed in PBS + T and incubated in a secondary anti-rabbit HRP-linked IgG (1:3000) in PBS + T for 1 h at room temperature. The chemiluminescent signal was detected on film using the SuperSignal WestPico Chemiluminescent kit (Pierce) according to the manufacturer’s specifications. Membranes were then stripped using Restore Western Blot Stripping Solution (Pierce) according to the manufacturer’s instructions. Membranes were re-blocked for 1 h in 5% nonfat dry milk in TBSS (25 mM Tris, 137 mM NaCl, 3 mM KCl) and 0.1% Tween 20 (blocking solution) at room temperature, followed by anti-beta actin antibody (1:1000, A5441; Sigma) and/or anti-tyrosine tubulin (1:1000, T9028; Sigma) for 3 h in blocking solution followed by anti-mouse HRP-linked IgG (1:3000) for 1 h at room temperature. The chemiluminescent signal for each protein was detected as described previously. All films were scanned and intensities quantified by MacBAS software version 2.2 (Fuji Photo Film, Edison, NJ). Signal intensities were normalized to Sertoli cell tyrosine tubulin expression with a ratio of 1.0 assigned to the control groups and the treatment groups represented relative to the control. Equivalent amounts of total protein in samples were verified by detection of actin expression.

Statistical Analysis

Data are expressed as the mean ± SEM for three to five animals per group. Statistical differences involving multiple group comparisons were determined by one-way ANOVA followed by a multiple-range test according to the Scheffe F-test (P < 0.05).

RESULTS

Testis Weights and T Levels

As shown in Figure 1A, testis weights decreased significantly by 7 days of T/E administration, with further reductions seen through 28 days. These results coincide with the progressive loss of germ cells as previously reported [8, 9, 12]. As expected, testis weights did not change at 6 or 24 h of T replacement.

Serum T concentrations did not change over the course of T/E administration for 28 days (Fig. 1B). However, as seen in Figure 1C, the concentration of T in STF was significantly reduced from 160 ng/ml in control rats to approximately 20 ng/ml within 7 days after T/E administration. At subsequent times following implantation of T/E capsules, the T concentration was further reduced, reaching approximately 9 ng/ml by 28 days. When the T/E capsules were removed and replaced with 24 cm T-filled capsules, serum T concentrations increased significantly at 6 and 24 h (Fig. 1B), and the T concentration in the STF increased to over 30 ng/ml (Fig. 1C).

Germ Cell Apoptosis Fragmented DNA (TUNEL)

The decrease in testis weights in response to T/E administration coincided with increased apoptosis of germ cells, as detected by the TUNEL assay (Fig. 2). Only a few apoptotic germ cells typically were observed in testes from control (Fig. 2A) and 7-day T/E-treated (Fig. 2B) rats. An obvious increase in the number of apoptotic germ cells was seen in tubules at stages VI–VIII (Fig. 2C) but not other stages by 10 days of T/E treatment (Table 1). More apoptotic germ cells began to appear in stages II–IV and IX–XII, as well as stages VI–VIII, by 14 days of T/E treatment.
and (dicated by roman numerals for testicular sections shown at lower (epithelium as determined by light and phase contrast microscopy are in-
T/E prior to removal of the T/E capsules and their replacement with 24 h following restoration of T in rats treated for the previous 28 days with (100
viewed in longitudinal cross section at higher magnification. Sections were counterstained with methyl green. The photomicrographs shown are representative of testic-
AR Immunohistochemistry

In the testes of control animals (Fig. 3, A and B), nuclear AR immunostaining was prominent in Sertoli cells at stages VII–VIII of the cycle of the seminiferous epithelium and not at other stages and also in the nuclei of peritubular myoid and Leydig cells throughout the tests. In testes from rats treated with T/E for 7 (Fig. 3, C and D) and 10 (Fig. 3, E and F) days, AR staining intensity became notably reduced in stage VII–VIII Sertoli cell nuclei. By Day 14 of T/E treatment (Fig. 3, G and H), Sertoli cell nuclear AR immunostaining was absent and remained so through 21 (Fig. 3, I and J) and 28 days (Fig. 3, K and L). By contrast, AR immunostaining was still visible in the nuclei of peritubular myoid and Leydig cells throughout the course of T/E treatment (Fig. 3, A–L). The apparent immunostaining present within the tubules of T/E-treated animals resembled that ascribed to 11–19 elongated spermatids, including the residual bodies, by Vornberger et al. [2]. The failure of Sertoli cells to release step 19 spermatids following T/E treatment may account for the persistence of immuno-

![Image](https://academic.oup.com/biolreprod/article-abstract/71/4/1348/2667378)

FIG. 3. Immunostaining of androgen receptor (AR) in testes from control and T/E-treated rats and following testosterone (T) replacement. The different panels correspond to in vivo treatment of rats: (A and B) Control; (C and D) 7 days; (E and F) 10 days; (G and H) 14 days; (I and J) 21 days; and (K and L) 28 days of T/E treatment. (M and N) 6 h and (O and P) 24 h following restoration of T in rats treated for the previous 28 days with T/E prior to removal of the T/E capsules and their replacement with 24 cm T-filled capsules. The specific stages of the cycle of the seminiferous epithelium as determined by light and phase contrast microscopy are indicated by roman numerals for testicular sections shown at lower magnification. Magnification: ×200. (A) Sertoli cells (S), peritubular myoid (PM), and Leydig (L) cells; (B) tubular myoid and Leydig cells throughout the testis. In testes from (100

![Image](https://academic.oup.com/biolreprod/article-abstract/71/4/1348/2667378)

FIG. 4. RT-PCR analyses of the mRNA levels of androgen receptor (AR) and androgen binding protein (ABP) in Sertoli cells isolated from control (Con) rats and 7-, 14-, and 28-day T/E-treated rats and 28-day T/E-treated rats with T replacement for 24 h (T24). A) Amplified products for AR, ABP, and ribosomal L19 mRNA visualized on agarose gels following incorpo-

observed with the PG-21 and N-20 AR antisera (data not shown).

**TABLE 1. Numbers of apoptotic germ cells at specific stages of the seminiferous cycle following T/E treatment.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stages</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>II–IV</td>
<td>0.3 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>T/E 7 days</td>
<td>VI–VIII</td>
<td>0.7 ± 0.3</td>
<td>2.0 ± 0.6</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>T/E 10 days</td>
<td>8.7 ± 1.2</td>
<td>2.3 ± 0.9</td>
<td></td>
<td></td>
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<tr>
<td>T/E 14 days</td>
<td>57 ± 2*</td>
<td>0.0 ± 0.6*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/E 21 days</td>
<td>111 ± 3*</td>
<td>47 ± 2*</td>
<td></td>
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* The values shown represent the mean number (±SEM) of TUNEL-positive germ cells per 10 tubules within the designated stages.
* Different from control, P ≤ 0.05.

RT-PCR Analyses of AR and ABP mRNA

We asked whether the diminished AR protein in Sertoli cell nuclei at 7 and 10 days of T/E treatment and its absence by 14 days were related to changes in the steady-state mRNA levels of AR. Additionally, because ABP plays a role in determining the bioavailability of T within the seminiferous tubules and the expression of ABP by Sertoli cells was shown in some studies to be under T regulation, we also measured ABP mRNA levels in concert with the levels of AR mRNA. Relative mRNA levels were analyzed ex vivo by RT-PCR in Sertoli cells freshly isolated from testes of control and 7-, 14-, and 28-day T/E-treated rats and 28-day T/E-treated rats in which T was replaced for 24 h. Figure 4A illustrates the amplified products for AR and ABP mRNAs and Figure 4B the relative intensities of the amplified products. AR mRNA levels remained constant through 14 days of T/E treatment but increased significantly by 28 days of T/E treatment compared to control levels (Fig. 4B). ABP mRNA levels were unchanged throughout
FIG. 5. Steady-state levels of androgen receptor (AR) and androgen binding protein (ABP) mRNAs in microdissected tubules from stages II–IV, VI–VIII, and IX–XII of control and T/E-treated rats. A) Northern blot analyses showing expression of cathepsin L, clusterin, and ChoB mRNAs in microdissected tubules from stages II–IV (II), VI–VIII (VII), and IX–XII (IX) in testes of control rats and 7- (T/E 7) and 14- (T/E 14) day T/E-treated rats. Each lane was loaded with 8 μg of total RNA. B–D) Semiquantitative RT-PCR analyses of mRNA levels for AR (solid bar) and ABP (hashed bar) in microdissected tubules from stages II–IV (B), VI–VIII (C), and IX–XII (D) in testes of control and 7- (T/E 7), 10- (T/E 10), and 14- (T/E 14) day T/E-treated rats. The relative mRNA levels for AR and ABP were normalized to ribosomal L19 mRNA levels. The relative intensities obtained for AR and ABP mRNA levels in samples from control animals within a given stage were assigned the value of 1.0, and all treatment groups were compared relative to the control. Data represent the mean ± SEM for three rats (two testes per sample) for each group. a: Different from control, P ≤ 0.05.
Fig. 6. Western blot analyses of androgen receptor (AR) protein in testis, pachytene spermatocytes (pach), round spermatids (round), spleen, kidney, and liver. Equal amounts of protein (50 μg) were loaded in each lane. Beta-actin was used as a control.

Fig. 7. Western blot analyses of androgen receptor (AR) protein expression in microdissected tubules from stages II–IV, VI–VIII, and IX–XII in testes of control (Con) and 7- and 14-day T/E-treated rats. A) Representative images from Western blots for AR, tyrosine tubulin (T-tub), and beta-actin. B) Graphical representation of the relative level of AR protein expression normalized to tyrosine tubulin expression. Tyrosine tubulin expression is specific to Sertoli cells in testis. The relative intensities obtained for AR protein expression in samples from control animals within a given stage was assigned the value of 1.0, and the treatment groups are compared relative to the control. Beta-actin was used to verify that equivalent amounts of total protein (25 μg) were loaded in each lane. Data represent the mean ± SEM for three rats (two testes per sample) for each group. a: Different from control, P ≤ 0.05; b: different from 7-day T/E treated, P ≤ 0.05.

Fig. 8. Western blot analyses of androgen receptor (AR) protein expression in total testis homogenates from control (Con) and 28-day T/E-treated (T/E 28) and 28-day T/E-treated rats that received T replacement for 24 h (T24). A) Representative images from Western blots for AR, tyrosine tubulin (T-tub), and beta-actin. B) Graphical representation of the relative level of AR protein expression normalized to tyrosine tubulin expression. Tyrosine tubulin expression is specific to Sertoli cells in testis. The relative intensities obtained for AR protein expression in samples from control animals were assigned the value of 1.0, and the treatment groups were compared relative to the control. Beta-actin was used to verify that equivalent amounts of total protein (40 μg) were loaded in each lane. Data represent the mean ± SEM for three rats (one testis per sample) for each group. a: Different from control, P ≤ 0.05.

Discussion

In the present study, we show that in vivo reduction of ITT concentration in adult rats results in the loss of Sertoli...
cell AR protein and in the stage-specific loss of AR nuclear localization but that steady-state levels of AR mRNA in Sertoli cells remain unchanged or increase. As observed in previous studies [2–6], AR was detected in the nuclei of Sertoli, peritubular myoid, and Leydig cells. In Sertoli cells, the intensity of nuclear immunostaining in untreated rats was greatest in stages VII–VIII of the cycle, as expected from previous reports [2, 3]. AR immunolocalization in Sertoli cell nuclei was notably reduced by 7 days following the reduction of ITT levels by the subcutaneous implantation of capsules containing T and E and approached undetectable levels by 14 days. Implantation of T/E capsules does not affect serum FSH levels but inhibits LH secretion and endogenous Leydig cell testosterone biosynthesis [9, 10]. Within 7 days, T/E treatment effectively reduces ITT below the critical threshold of approximately 20 ng/ml that is necessary to maintain spermatogenesis [9–12]. FSH levels are not affected, and previous studies have shown that FSH is not necessary to maintain or restore quantitative spermatogenesis in the adult rat (reviewed in Zirkin et al. [36]).

We show herein that the increased number of apoptotic germ cells observed by TUNEL staining after 10–14 days of T/E treatment was specific to spermatocytes in seminiferous tubules at stages VII–VIII. AR immunolocalization in Sertoli cell nuclei in stage VII–VIII tubules was no longer observed once the ITT concentration fell below its critical threshold for maintenance of spermatogenesis. The correlation between loss of Sertoli cell nuclear AR localization and apoptosis of germ cells suggests a functional disruption of AR-mediated androgen action in Sertoli cells specific to stages VII–VIII of the seminiferous epithelium. We did, however, observe an unexplained compensatory increase in AR mRNA level in stage II–IV seminiferous tubules after 10 and 14 days of T/E treatment that coincided with the critical decrease in ITT concentration.

Our studies further examined the stage-specific loss of Sertoli cell nuclear AR localization and the apparent disruption of AR-mediated androgen action in Sertoli cells at the levels of AR mRNA and protein expression at various stages of the seminiferous epithelium. We used RT-PCR of RNA from isolated Sertoli cells and seminiferous tubule segments to show that despite the dramatic decrease in Sertoli cell nuclear AR immunolocalization following the diminution of ITT levels, steady-state measurements of AR mRNA in microdissected stage-specific seminiferous tubule segments and in isolated Sertoli cells were not significantly affected. If the concentration of ITT regulated AR mRNA levels, we would have expected a dramatic change in steady-state AR mRNA levels following the significant decrease in ITT concentration provoked by implantation of T/E capsules. Rather, we observed a significant increase in the relative level of AR mRNA in seminiferous tubules from stages II–IV on Days 10 and 14 following T/E treatment but no change in AR mRNA levels in tubules from stages VI–VIII or IX–XII through 14 days of T/E treatment. Similarly, we did not observe changes in AR mRNA levels of Sertoli cells isolated from whole testes during the period up to 14 days of T/E treatment, but AR mRNA levels increased by 28 days of T/E treatment in isolated Sertoli cells.

Interestingly, Shan et al. [35] previously concluded that AR protein levels in Sertoli cells were regulated primarily at the level of AR gene transcription. Their quantitative in situ hybridization assays showed that AR mRNA levels in Sertoli cells increase during stages IV–V and peak during stages VII–VIII of the seminiferous epithelium, thus correlating with the maximal immunocytochemical localization of AR protein in Sertoli cell nuclei at stages VII–VIII. However, similar to our findings, Blok et al. [17] reported that AR mRNA levels as measured by Northern blots with total testis RNA were not affected following treatment of rats with the Leydig cell toxicant EDS, which also reduces ITT levels. Taken together, we conclude that the steady-state levels of AR mRNA in Sertoli cells are not regulated by the concentration of ITT.

We subsequently used Western blots to show that the levels of AR protein were significantly decreased in microdissected stage-specific seminiferous tubule segments and in whole testis homogenates following T/E treatment. Interestingly, AR protein was detected in seminiferous tubule segments from control rats at stages II–IV, VI–VIII, and IX–XII despite the observation that AR was localized to Sertoli cell nuclei at stages VI–VIII but not in stages II–IV or IX–XII. We cannot, however, exclude the possibility that the AR protein and mRNA measured in seminiferous tubule segments from stages II–IV and IX–XII is derived from peritubular myoid cells rather than Sertoli cells. Perhaps AR protein redistributes to the Sertoli cell cytoplasm, although significant reductions in total AR protein levels were observed at 7 and 14 days of T/E treatment in tubule segments from each of the different stages of the seminiferous epithelium. This decrease in AR protein levels following reduction of the ITT concentration was also confirmed in whole testis homogenates from rats treated with T/E for 28 days from which staged tubule segments could not be microdissected by transillumination-assisted microscopy.

A critical factor in our ability to detect AR protein on Western blot analyses was the inclusion of 1 nM DHT in the buffers used to microdissect the seminiferous tubules and prepare the protein extracts [23]. In the absence of DHT during the tissue preparative phase, we were unable to detect AR protein by Western blot utilizing several different AR-specific antisera. This technical observation may explain results obtained by Blok et al. [17, 18], who reported the complete loss of immunoassayable AR in testicular cells following administration of EDS. They were unable to detect AR protein in total testis homogenates from EDS-treated rats by immunoprecipitation or Western blot, an effect that was prevented if EDS-treated rats were immediately given implants containing T. They supposed that this was due to a structural modification of AR that prevented its detection by specific AR antisera following prolonged absence of androgen rather than a decrease in the amount of AR protein. On the basis of our work, however, we conclude that absolute levels of AR protein in testicular cells, specifically Sertoli cells, are decreased following T/E treatment.

Of particular interest is the rapid return of AR protein levels within 24 h following replacement of T to restore the ITT concentration that follows the significant decrease in testicular AR protein levels of 28-day T/E-treated rats. Sertoli cell AR mRNA levels were increased after T/E treatment for 28 days and remained elevated 24 h following T replacement. Within 6 h of T replacement, AR nuclear localization in Sertoli cells reappeared in a subset of seminiferous tubules at specific stages. By 24 h following restoration of ITT levels in the range of 30–35 ng/ml, AR protein levels in the testis measured on Western blots matched the levels observed in control rats. The acute response to testosterone further suggests that additional paracrine factors within the testis are not required for the re-
localization of AR within nuclei of Sertoli cells. These results agree with previous reports in which the nuclear localization of AR in Sertoli cells was maintained or restored by more long-term replacement of androgen subsequent to the reduction of ITT concentration by treatment with EDS [13] or a gonadotropin antagonist [5].

In summary, we propose that the intratesticular level of T has a primary role to regulate the translation and/or post-translational stability of the AR protein and to promote its nuclear translocation for transactivation of androgen-regulated genes. This conclusion is based on our present in vivo experiments that demonstrated a reduction in AR protein levels in Sertoli cells following T deprivation but no effect or an increase in AR mRNA levels. Our conclusion regarding posttranslational stability of AR is supported by previous in vitro studies showing that AR stability is increased in the presence of its ligands, T or DHT, part because of interactions between the amino- and carboxy-terminal domains of the receptor [37]. Caspase 3 can cleave the AR amino-terminal transactivation domain and remove the epitope recognized by the PG-21 and N-20 AR antibodies used in our study [38]. Alternatively, proteolysis of the carboxy-terminal ligand-binding domain would disrupt testosterone binding, androgen-dependent nuclear localization, and transcriptional activation of AR in Sertoli cells [38].

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