Journal of Andrology, Vol. 22, No. 2, March/April 2001

Suppression of Androgen Action and the Induction of Gross Abnormalities of the Reproductive Tract in Male Rats Treated Neonatally With Diethylstilbestrol

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ABSTRACT: This study evaluated whether androgen action is altered in rats treated neonatally with diethylstilbestrol (DES) at a dose that induced reproductive tract abnormalities. Rats were treated on alternate days 2–12 with 10 μg DES and studied on Day 18. DES-induced abnormalities included a 70% reduction in testis weight, distension and overgrowth of the rete, distension and reduction in epithelial height of the efferent ducts, underdevelopment of the epididymal duct epithelium, reduction in epithelial height in the vas deferens, and convolution of the extra-epididymal vas. In DES-treated rats, androgen receptor (AR) immunoexpression was virtually absent from all affected tissues and the testis, whereas AR expression in controls was intense in epithelial and stromal cells. The DES-induced change in AR immunoexpression was confirmed by Western analysis for the testis. In rats treated neonatally with 1 μg DES, reproductive abnormalities were absent or minor, except for a 38% reduction in testis weight; loss of AR immunoexpression also did not occur in these rats. Treatment-induced changes in AR expression were paralleled by changes in Leydig cell volume per testis (91% reduction in the 10-μg DES group; no change in the 1-μg DES group). To test whether suppression of androgen production or action alone could induce comparable reproductive abnormalities to 10 μg DES, rats were treated neonatally with either a potent gonadotropin-releasing hormone antagonist (GnRHa) or with flutamide (50 mg/kg/day). These treatments reduced testis weight (68% for GnRHa, 40% for flutamide), and generally retarded development of the reproductive tract but failed to induce the abnormalities induced by 10 μg DES. GnRHa and flutamide caused no detectable change in AR immunoexpression in target tissues, with the exception of minor changes in the testes of flutamide-treated males. GnRHa treatment caused a reduction (83%) in Leydig cell volume comparable to that caused by 10 μg DES. Immunoexpression of estrogen receptor alpha (ERα) in the efferent ducts and of ERβ in all tissues studied were unaffected by any of the above treatments. Neonatal coadministration of testosterone esters (TE; 200 μg) with 10 μg DES prevented most of the morphological abnormalities induced by 10 μg DES treatment alone, though testis weight was still subnormal (46% reduction in DES + TE vs 72% in DES alone and 49% with TE alone) and some lumenal distension was still evident in the efferent ducts. Coadministration of TE with DES prevented DES-induced loss of AR immunoexpression (confirmed for testis by Western blot analysis). It is concluded that 1) reproductive tract abnormalities induced in the neonatal male rat by a high (10 μg) dose of DES are associated with reduced AR expression and Leydig cell volume; 2) these changes are largely absent with a lower dose of DES (1 μg); 3) treatments that interfere with androgen production (GnRHa) or action (flutamide) alone failed to induce reproductive tract abnormalities or alter AR expression as did 10 μg DES; 4) a grossly altered androgen:estrogen balance (low androgen + high estrogen) may underlie the reproductive tract abnormalities, other than reduced testis weight, induced by high doses of DES.

Key Words: Testis, rete testis, efferent ducts, epididymis, vas deferens, androgen receptor.

J Androl 2001;22:323–338

There is substantial evidence in the literature that exposure of male rodents to high levels of exogenous estrogens such as diethylstilbestrol (DES), either in utero or neonatally, results in major morphological and functional abnormalities of the testis and reproductive tract (Arai et al, 1983; Newbold and McLachlan, 1985; Fisher et al, 1998; Khan et al, 1998; Sharpe et al, 1998). Many of these effects can have life-long consequences (Khan et al, 1998; Atanassova et al, 1999, 2000). Furthermore, studies on the sons of women who were exposed to DES during pregnancy show that a similar range of abnormalities can also be induced in human males exposed to high levels of exogenous estrogens in utero (Stillman, 1982; Toppari et al, 1996).

At present, the exact mechanisms underlying the induction of these abnormalities are unknown. Past consensus has been that estrogen-induced effects on the testis are indirect, and result from suppression of gonadotropin

Supported in part by the European Centre for the Ecotoxicology of Chemicals (ECETOC) and by a Strategic Research Fund Award from AstraZeneca plc. N.A. received a Royal Society/NATO fellowship.

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Received for publication August 21, 2000; accepted for publication September 28, 2000.

secretion by the pituitary gland during estrogen treatment (Brown-Grant et al, 1975; Bellido et al, 1990). However, this conclusion is difficult to reconcile with evidence from our own recent studies comparing the effects of neonatal DES treatment and those of gonadotropin suppression using a potent gonadotropin-releasing hormone (GnRH) antagonist (GnRHa; Sharpe et al, 1998; Atanassova et al, 1999). On the other hand, DES-induced effects such as cryptorchidism, hypospadias, reduced sperm production in adulthood, and abnormal development of the epididymis, bear striking similarities to those induced by in utero exposure to antiandrogens (Imperato-McGinley et al, 1992; van der Schoot, 1992; Silversides et al, 1995; Sharpe et al, 2000), or in which fetal Leydig cell function is compromised (Mylchreest et al, 1999, 2000).

The similarity between the phenotypes of males exposed developmentally to either estrogens or to antiandrogens suggested to us the possibility that both phenotypes might be the result, at least in part, of a similar underlying mechanism. This hypothesis is supported by evidence that in utero exposure of male rats to exogenous estrogens reduces expression of the messenger RNA for 17α-hydroxylase/C17-20 lyase, a key enzyme in testosterone production (Majdic et al, 1996), while neonatal administration of DES reduces both testicular and plasma androgen levels in 12-day-old rats (Keel and Abney, 1985). Furthermore, neonatal treatment with DES can cause life-long suppression of plasma testosterone levels (Atanassova et al, 1999), and postpubertal DES treatment clearly results in reduced androgen production (Cigorraga et al, 1980; Abney and Keel, 1986). At the very least, these various findings suggest an inter-relationship between raised estrogen exposure and reduced androgen production. The primary purpose of the present study was therefore to assess whether the effects of neonatal administration of DES to rats, at a dose that induces widespread reproductive tract abnormalities, was associated with altered androgen action. In planning and undertaking these studies, we also became aware that in our own and most (possibly all) published studies that have described DES-induced abnormalities in males during perinatal life, extremely high doses have to be administered to induce such effects—generally equivalent to, or in excess of, 100 μg/kg. In view of the extremely high affinity of estrogen receptors (ERs) for potent estrogens such as DES (Kuiper et al, 1997), it seemed incongruous that such high doses were necessary if the induction of reproductive abnormalities was mediated solely via an ER receptor-mediated mechanism. Therefore, another aspect of the present studies was to evaluate whether only an extremely high dose of DES could induce reproductive tract abnormalities and, if so, whether this dose-dependence coincided with alterations in androgen action.

Materials and Methods

Animals, Treatments, Sample Collection, and Processing

Wistar rats bred in our own animal house were maintained under standard conditions and diet (rat and mouse breeding diet no. 3; SDS, Dundee, Scotland) that contains 15.5% soy-meal. All-male litters of 8–12 pups were generated by cross-fostering pups on day 1 (= day of birth). Rats were subjected to one of the following treatments:

1) Subcutaneous injection of DES (Sigma Chemical Company, Poole, Dorset, United Kingdom) at a dose of 10 μg or 1 μg in 20 μL corn oil on days 2, 4, 6, 8, 10, and 12.

2) Subcutaneous injection of 10 μg DES plus 200 μg testosterone esthers (TE; Sustanon; Organon Labs, Cambridge, United Kingdom) in 20 μL corn oil on days 2, 4, 6, 8, 10, and 12. The dose of TE administered was based on 3 considerations. First, our previous experience in restoring intratesticular and blood testosterone levels in adult rats by injection of TE (Sharpe et al, 1990), scaled down appropriately. Second, the aim of restoring a balance between androgen and estrogen levels (ie, the dose of TE administered had to considerably exceed the dose of DES that was coadministered). Third, avoidance of doses of TE that have been shown to cause severe impairment when administered neonatally to male rats (see Kincl et al, 1965). The dose of TE was a compromise, taking account of these factors.

3) Injection as in (2) but with 200 μg TE alone.

4) Subcutaneous injection of 10 mg/kg of a long-acting GnRHa (Antarelix; Europeptides, Argenteuil, France) in 20 μL corn oil on days 2 and 5 alone. This treatment regimen has been shown previously (Sharpe et al, 1998; Atanassova et al, 1999) to effectively abolish gonadotropin secretion until days 15–20 postnatal, and to induce a similar reduction in testis weight at days 18–25 to that induced by treatment with 10 μg DES as in (1).

5) Subcutaneous injection of 50 mg/kg of the androgen receptor (AR) antagonist flutamide (Sigma) in 20 μL corn oil on days 2,

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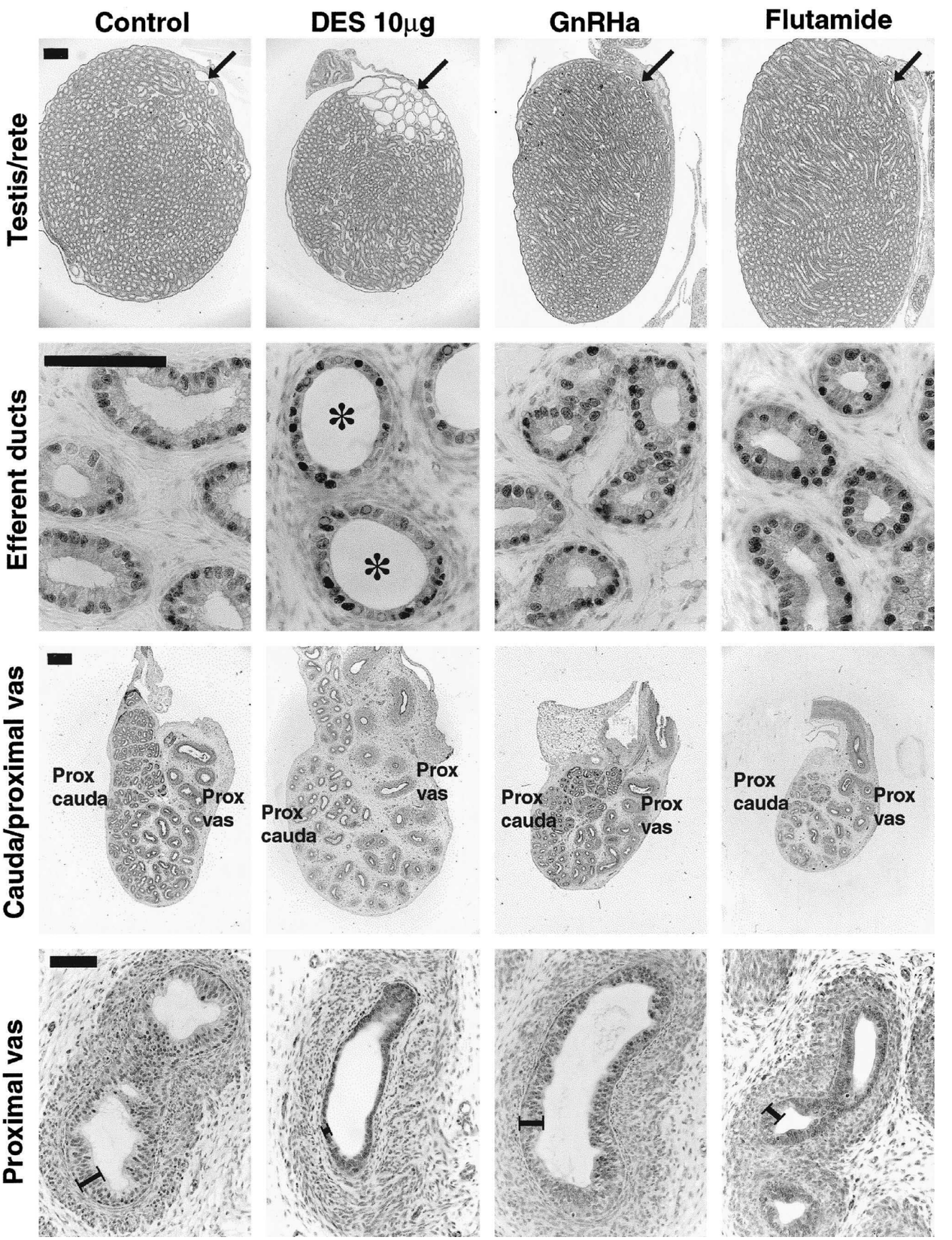
Figure 1. Effect of neonatal treatment with vehicle (control), DES (10 μg), GnRHa, or flutamide on gross morphology of the testis and reproductive tract at Day 18. Note that only DES treatment results in overgrowth and distension of the rete (arrows; top row); distension (asterisks) and reduction in epithelial cell height of the efferent ducts (note loss of apical cytoplasm from epithelial cells; 2nd row); relative undergrowth of the epididymal duct with relative overgrowth of the stroma (note bulb shape of cauda; 3rd row); underdevelopment of the epithelium in the vas deferens (black lines show epithelial height; bottom row). Further examples of these abnormalities, at varying magnification, are shown in other figures. In contrast to the DES-treated group, note that both GnRHa and flutamide treatments caused retardation of development of the efferent ducts (smaller cross-sectional diameter than in controls) and epididymis (proportionately normal but smaller than in 18-day controls) without causing the changes observed in the DES-treated animals. Note that efferent ducts have been immunostained for ERα to highlight the cell nuclei and to show that the levels of immunoexpression are unchanged. Scale bar = 500 μm (top and 3rd rows) or 100 μm (2nd and bottom rows).


Control
DES 10μg
GnRHa
Flutamide
Testis/rete
Efferent ducts
Cauda/proximal vas
Proximal vas
Prox cauda
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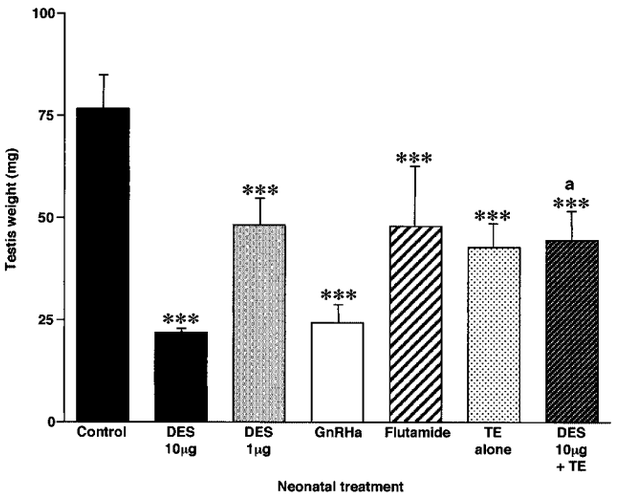

Figure 2. Effect of neonatal treatment with vehicle (control), DES (10 or 1 μg), GnRHa, flutamide, TE alone (200 μg), or 10 μg DES + 200 μg TE on testis weight at day 18. Results are the mean ± standard deviation for 8–23 rats per group. ***$P < .001$, in comparison with control group. [a]$P < .001$, in comparison with DES-alone (10 μg) group.

4, 6, 8, 10, and 12; this dose was shown by Imperato-McGinley et al (1992) to cause major reproductive tract abnormalities in male offspring when administered to pregnant rats.

6) Subcutaneous injection of 20 μL corn oil alone (control).

Rats from the various treatment groups described above were sampled on Day 18, a time at which we have shown that major abnormalities of the excurrent ducts (Fisher et al, 1998, 1999) and reproductive tract (unpublished data) are evident in DES-treated rats. Animals were anesthetized with flurothane and the right testis was dissected out, weighed, and fixed for ~5 hours in Bouins. The left testis was removed with the epididymis and proximal vas deferens still attached and similarly fixed. From some animals, the right testis was not fixed, but was used instead for protein extraction as described below.

After fixation, tissue was transferred into 70% ethanol before being processed for 17.5 hours in an automated Shandon processor and embedded in paraffin wax. Sections (5 μm thickness) were cut and floated onto slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma) and dried at 50°C overnight before being used for immunohistochemistry as described below. All of the studies of the rete testis and reproductive tract described below utilized tissue sections of the left testis with the epididymis attached, in order that minimal artefactual distortion was caused to the excurrent duct system.

For protein extraction, small pieces of unfixed testis tissue were snap-frozen in liquid nitrogen and stored at −70°C. The tissue was subsequently ground with a pestle in a mortar under liquid nitrogen, and then resuspended in ice cold buffer consisting of 10 mM HEPES pH 7.8, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitiol (all from Sigma) and a protease inhibitor cocktail (Complete; Roche, Lewes, United Kingdom). Protein concentration was measured by absorbance at 280 nm and the protein extracts were aliquoted and stored at −70°C. At least 2 separate experiments were performed for each of the treatments specified; comparable results were obtained in each experiment.

Antibodies used for Immunohistochemistry

Immunolocalization of AR was performed with the use of a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) raised against an epitope at the *N*-terminus of human AR, and was used at a dilution of 1:200. ERα was immunolocalized using a mouse monoclonal antibody raised against a full-length human ERα recombinant protein (Novocastra, Newcastle Upon Tyne, United Kingdom), and used at a dilution of 1:20. ERβ was immunolocalized using an affinity-purified, polyclonal antipeptide immunoglobulin G (IgG) raised in sheep against a specific peptide in the hinge (D) domain of human ERβ, as previously described in detail (Saunders et al, 2000); it was used at a dilution of 1:1000. The specificity of the ER antibodies has been detailed in our previous studies (Saunders et al, 2000; Williams et al, 2000).

Immunohistochemistry

Unless otherwise stated, all incubations were performed at room temperature. Sections were deparaffinized in Histoclear (National Diagnostics, Hull, United Kingdom), rehydrated in graded ethanols, and washed in water. At this stage, sections were subjected to a temperature-induced antigen retrieval step (Norton et al, 1994) in either 0.01 M citrate buffer pH 6.0 (for AR and ERα), or 0.05 M glycine pH 3.5, and 0.01% EDTA (for ERβ). After pressure-cooking for 5 minutes at full pressure, sections were left to stand, undisturbed, for 20 minutes, then cooled under running tap water before being washed twice (5 minutes each) in Tris-buffered saline (TBS; 0.05 M Tris-HCl pH 7.4, 0.85% NaCl). Endogenous peroxidase activity was blocked by immersing all sections in 3% (v/v) H_2O_2 in methanol (both from BDH Laboratory Supplies, Poole, Dorset, United Kingdom) for 30 minutes, which was followed by two 5-minute washes in TBS. To block nonspecific binding sites, sections were incubated for 30 minutes with the appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (BSA; Sigma). For AR, normal swine serum (NSW) was used and for ERα and ERβ, normal rabbit serum (NRS) was used (both from Scottish Antibody Production Unit, Carluke, Scotland). Primary antibodies were added to the sections at the appropriate dilution in either NSW/TBS/BSA (for AR) or NRS/TBS/BSA (for ERα and ERβ) and incubated overnight at 4°C in a humidified chamber. After

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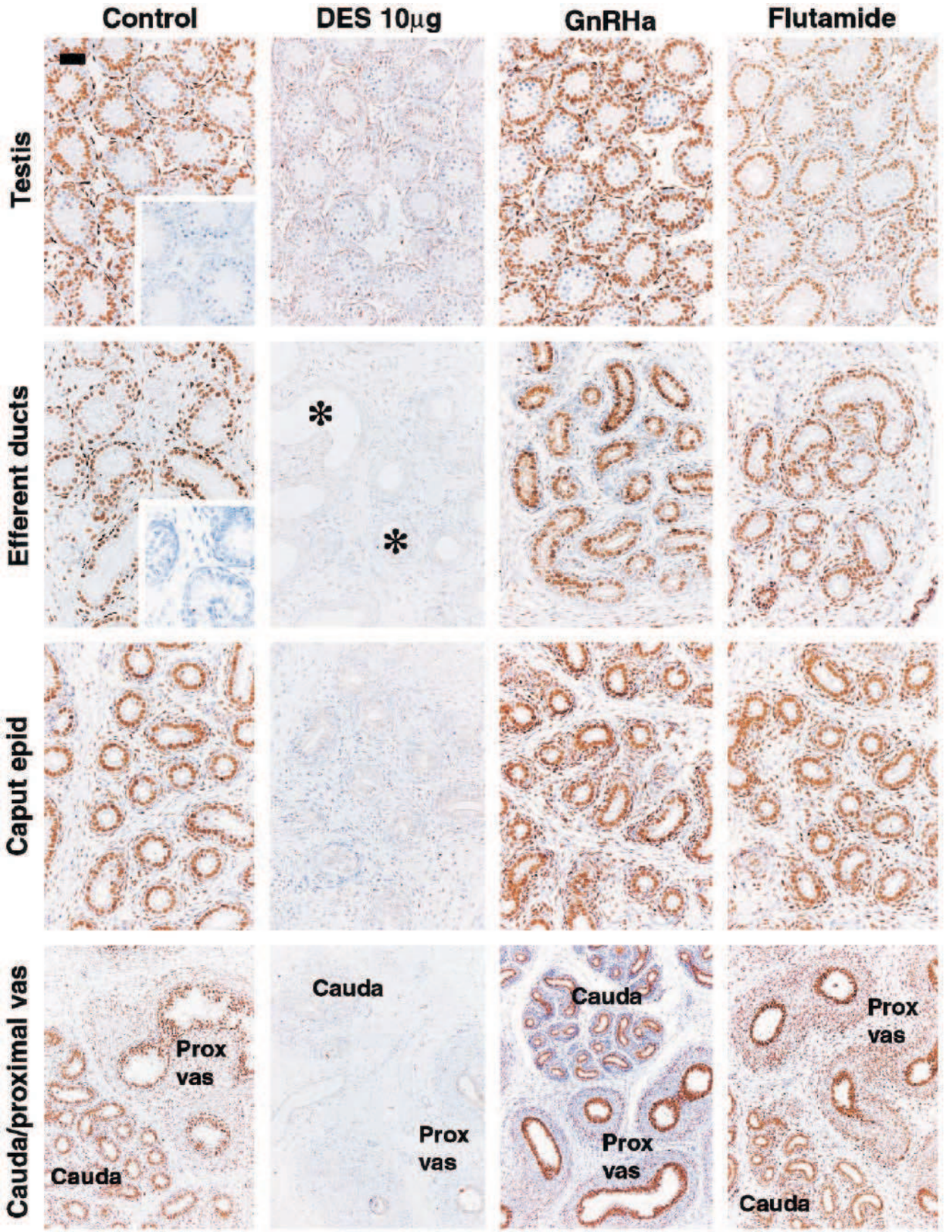
Figure 3. Effect of neonatal treatment with vehicle (control), DES (10 μg), GnRHa, or flutamide on immunoexpression of the AR in the testis (top row), efferent ducts (2nd row), caput epididymis (3rd row), and cauda epididymis/proximal vas deferens (bottom row) on day 18. Note the virtually complete loss of AR immunoexpression in the testis (particularly in Sertoli cells), and in both epithelial and stromal cells of the efferent ducts, caput epididymis, and cauda epididymis/proximal vas deferens of animals treated with DES. In contrast, note that treatment with GnRHa or flutamide causes little if any loss of immunoexpression of AR in these tissues, with the exception of the testis after flutamide treatment. Insets show negative controls in which the antiserum was substituted by (normal) blocking serum. Asterisks indicate lumenal distension of the efferent ducts. Scale bar = 100 μm.


Control
DES 10μg
GnRHa
Flutamide
Testis
Efferent ducts
Caput epid
Cauda/proximal vas
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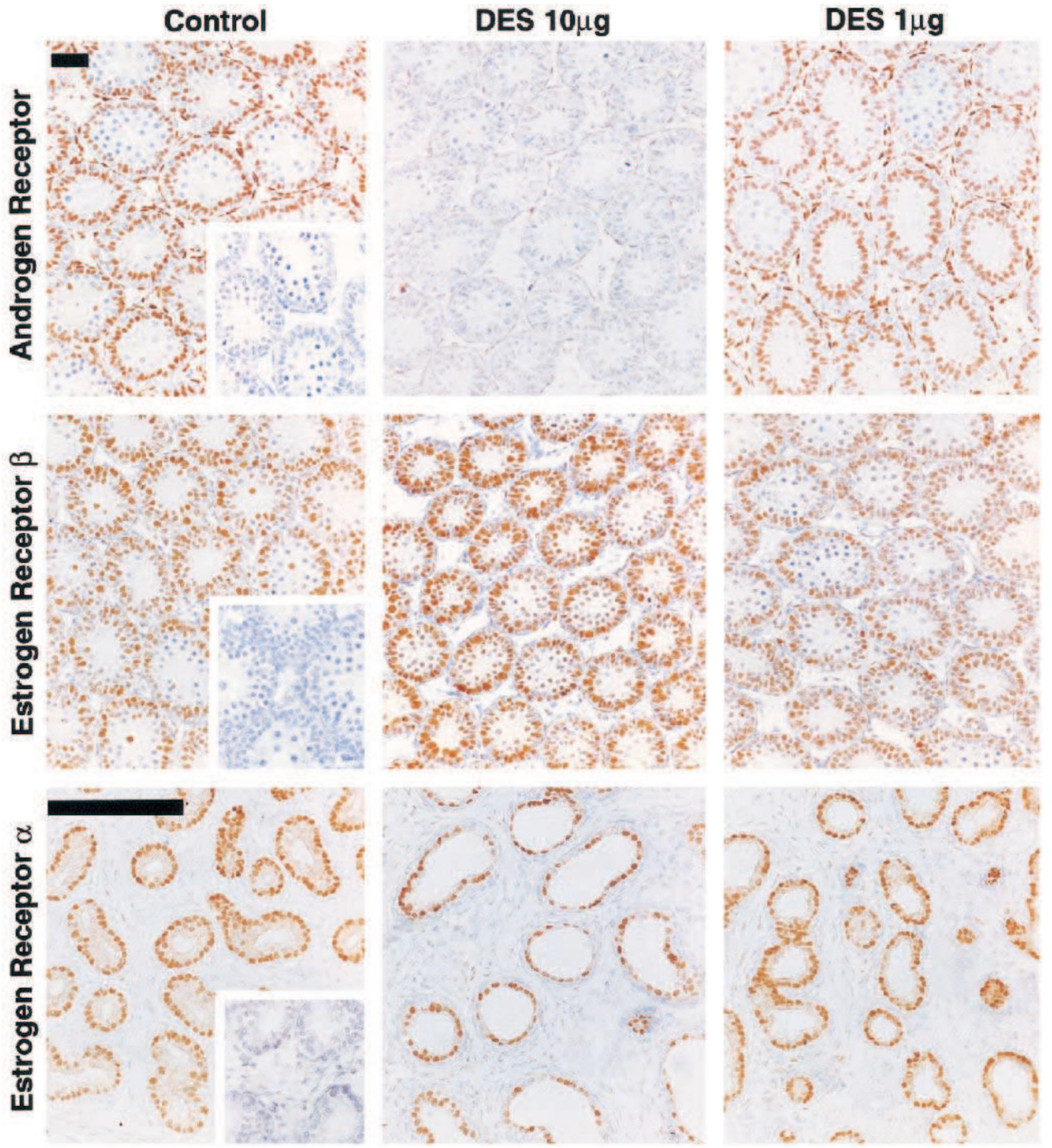

Figure 4. Comparative effect of neonatal treatment with vehicle (control) or with DES at a dose of 10 or 1 μg on immunoexpression of AR (top row) and ERβ (middle row) in the testis, and of ERα (bottom row) in the efferent ducts, on day 18. Note the lack of effect of treatment with the lower dose of DES on AR immunoexpression and the lack of effect of either DES dose on ERβ or ERα immunoexpression. Insets show negative controls in which the antiserum was substituted by (normal) blocking serum (AR) or in which the antiserum was preabsorbed with recombinant peptide (ERα and ERβ). Scale bars = 100 μm.

two 5-minute washes in TBS, sections were incubated with a secondary antibody; namely, a 1:500 dilution in the appropriate blocking serum of biotinylated swine anti-rabbit IgG for AR (DAKO, High Wycombe, United Kingdom), biotinylated rabbit anti-mouse IgG for ERα (DAKO), or biotinylated rabbit anti-sheep IgG for ERβ. After two additional 5-minute washes in TBS, all sections were incubated for 30 minutes with avidin-biotin conjugated to horseradish peroxidase (DAKO) diluted in

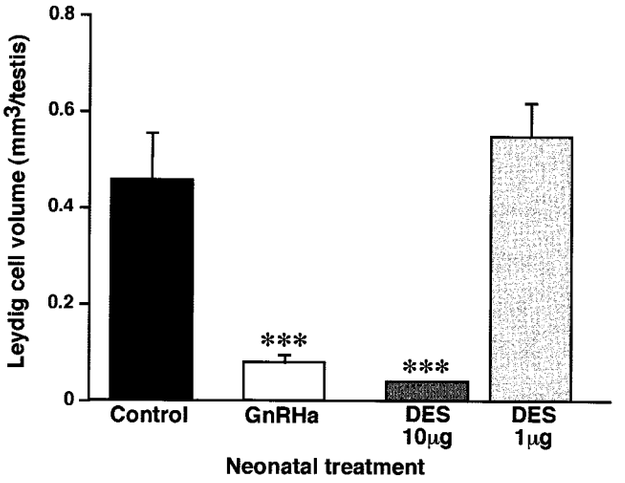

Figure 5. Effect of neonatal treatment with vehicle (control), GnRH antagonist (GnRHa), 10 μg DES, or 1 μg DES on Leydig cell volume per testis at day 18. Values shown are the means + standard deviations for 6 rats per group. ***$P < .001$, in comparison with control group.

0.05 M Tris-HCl pH 7.4, according to the manufacturer's instructions. Sections were washed twice (5 minutes each) in TBS and immunostains were developed using 0.05% 3,3′-diaminobenzidine (Sigma) in 0.05 M Tris-HCl pH 7.4, containing 0.01% (v/v) H_2O_2, until staining in controls was optimal, when the reaction was stopped by immersing all sections in distilled water. All sections were then lightly counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene, and coverslips placed on them using Pertex mounting medium (CellPath plc, Hemel Hempstead, United Kingdom).

To ensure the reproducibility of findings, tissue sections from a minimum of 3–6 animals in each treatment group were evaluated, and this was performed for at least 2 separate experiments. Further confirmation was obtained by undertaking immunohistochemistry with tissue sections from control and treated animals on the same slide. For AR, changes in immunoexpression in treated animals compared with control animals were also confirmed by Western blotting as described below. Specificity of immunostaining was checked for each antibody using previously established procedures. For AR, this involved substitution of the primary antibody by normal (blocking) serum, and for ERα and ERβ, it involved preabsorption of the primary antibody with the respective recombinant protein (Saunders et al, 2000; Williams et al, 2000).

Immunostained sections were examined and photographed using an Olympus Provis microscope (Olympus Optical, Honduras Street, London, United Kingdom) fitted with a Kodak DCS330 camera (Eastman Kodak, Rochester, NY). Captured images were stored on a G4 computer (Apple Macintosh) and compiled using Photoshop 5.0 software before being printed using an Epson Stylus 750 color printer (Seiko Epson Corp, Nagano, Japan).

Western Blotting

Testis protein extracts from control and treated animals were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Gels contained 6% acrylamide and comprised 4 mL 30% acrylamide/bis (Anachem, Luton, United Kingdom), 5 mL 1.5 M Tris-HCl pH 8.8, 10.9 mL distilled water, and 0.1% SDS (Sigma). The gel mixture was degassed and polymerized by addition of 100 μL 10% ammonium persulphate and 5 μL TEMED (both from Sigma). Gels were loaded with 100 μg protein per lane, in denaturing/loading buffer comprising 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol (Sigma), 2% SDS (Sigma), 10% glycerol (BDH, Poole, United Kingdom), and 0.002% bromophenol blue (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom). One lane was loaded with prestained molecular weight markers (Bio-Rad). The gels were run for approximately 1 hour at a constant current of 120 mA. After SDS-PAGE, gels were transferred to blotting buffer comprising 12 mM Tris base, 96 mM glycine, and 20% methanol (NuPAGE buffer; Novex, San Diego, Calif) and blotted onto a PVDF membrane (Immobilon-P; Millipore, Watford, United Kingdom) using the XCell-II system (Novex) run at 100 mA for 90 minutes. Membranes were blocked for 2–3 hours in NRS/TBS containing 0.05% (v/v) Tween-20 (Sigma), and 5% (w/v) skim milk powder (Marvel; Premier Brands Ltd, Moreton, United Kingdom). The AR antibody was diluted 1:200 in TBS and incubated with the membranes overnight at 4°C. To confirm the specificity of detection, AR antibody was preabsorbed overnight with a 10-fold excess of the AR *N*-20 peptide (Santa Cruz). The membranes were washed twice (15 minutes each) and then 4 times (5 minutes each) in TBS, and then incubated for 1 hour at room temperature with peroxidase-conjugated donkey anti-rabbit IgGs (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) diluted 1:4000 in TBS. The membranes were again washed twice (15 minutes each) then 4 times (5 minutes each) in TBS, and bound antibodies were detected using an ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Measurement of Leydig (3β-Hydroxysteroid Dehydrogenase–Positive) Cell Volume per Testis

To evaluate whether neonatal treatment with DES or GnRHa altered Leydig cell volume in 18-day-old animals, sections from Bouins-fixed testes were immunostained for 3β-hydroxysteroid dehydrogenase (3β-HSD) as described previously (Majdic et al, 1996; Sharpe et al, 1998) and the volume of 3β-HSD-positive cells per testis was determined using a point-counting method previously described in detail (Atanassova et al, 1999). One section from each of 3 blocks per animal was examined, and all points falling over 3β-HSD-positive cytoplasm or over the nuclei of cells with 3β-HSD-positive cytoplasm were scored and expressed as percent volume per testis. These data were then converted to absolute volume per testis by multiplication by testis weight (= volume).

Statistics

Comparison of the different parameters for the various treatment groups was made using analysis of variance, as all data were normally distributed. Where significant differences between groups were indicated, subgroup comparisons also utilized analysis of variance but used the variance from the experiment as a whole (for that parameter) as the measure of error.

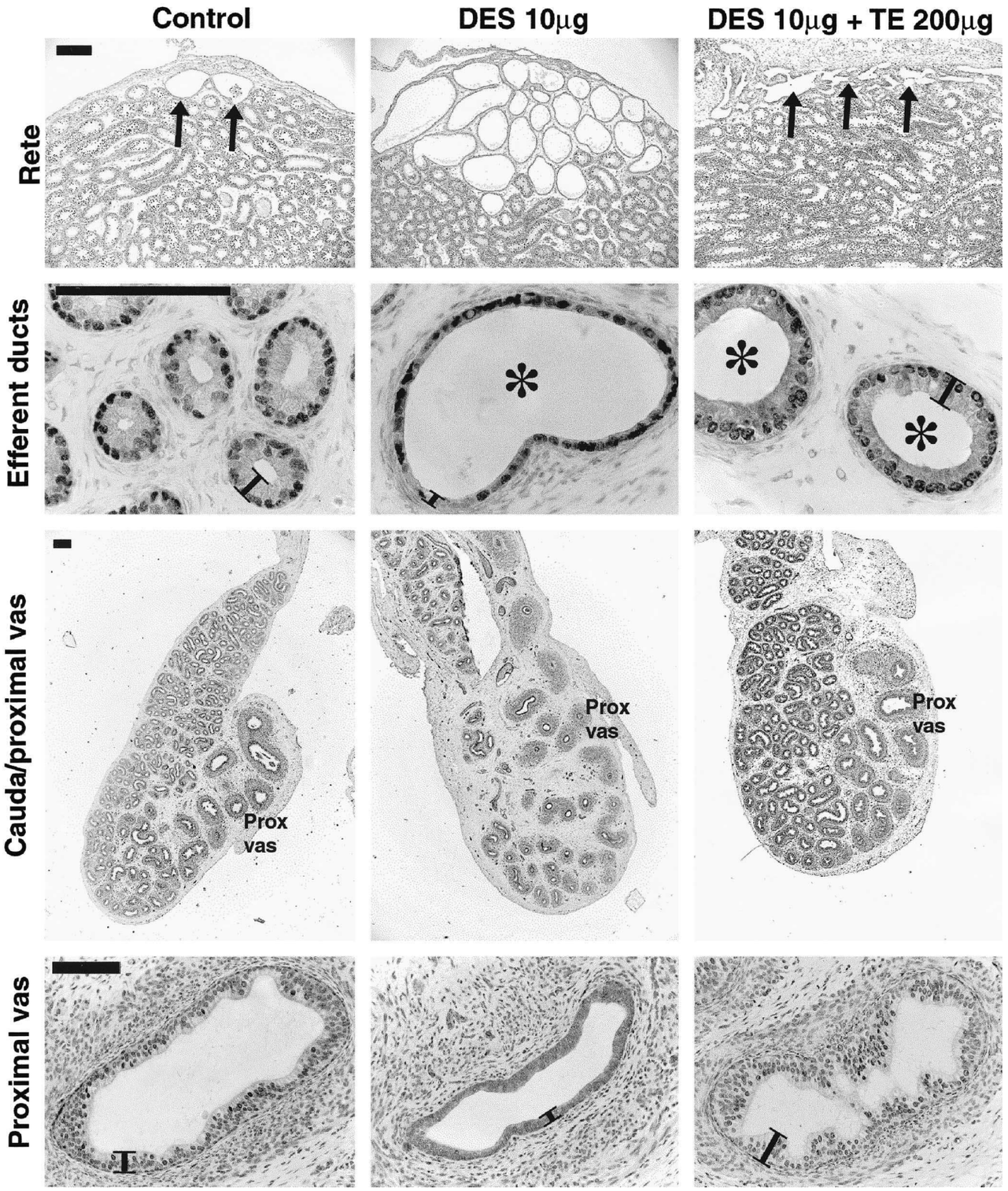

Figure 6. Effect of neonatal treatment with vehicle (control), 10 μg DES or cotreatment with 10 μg DES + 200 μg testosterone (DES + TE) on gross morphology of the rete testis (arrows, top row), efferent ducts (2nd row), gross morphology of the cauda epididymis and proximal vas deferens (3rd row), and epithelial height (indicated by black lines) in the proximal vas deferens (bottom row) at day 18. Note that coadministration of DES + TE was able to prevent the overgrowth and distension of the rete (top row) and the relative underdevelopment of the epithelium and relative overgrowth of the stroma in the cauda and proximal vas (bottom two rows) when compared with animals treated with DES alone. Note also that coadministration

Results

Induction of Gross Abnormalities of the Rete Testis and Reproductive Tract

Observations on DES-induced changes in gross morphology are summarized in the Table. The rete testis of all animals treated with 10 μg DES exhibited considerable overgrowth, with gross distention of the rete lumen and apparent invasion of the rete into the testicular parenchyma (Figure 1). The epithelium of the efferent ducts in all animals treated with 10 μg DES was reduced substantially in height compared with the control group, whereas the duct lumens were highly distended compared with controls (Figure 1); these findings confirm those we described previously (Fisher et al, 1999). Similar, though less pronounced, distension and reduction in epithelial height was also found in the caput epididymal duct of some animals (not shown). The cauda epididymis of rats treated neonatally with 10 μg DES exhibited pronounced underdevelopment of the epididymal duct (ie, fewer duct cross-sections), reduced height of the epithelium, and relative overgrowth of the periductal stroma (Figure 1). The latter changes also extended out into the vas deferens and, in addition, in ~70% of animals the initial region of the vas outside of the epididymis was transformed from a straight to a convoluted duct structure more reminiscent of the epididymal duct and the region of proximal vas present within the caudal bulb (not shown, but see Table).

In contrast to the gross morphological changes induced by neonatal injection of 10 μg DES, administration of a 10-fold lower dose of DES (1 μg) caused only minor changes to the rete and efferent ducts and had no discernible effect on the caput or cauda epididymis and vas deferens (not shown, but summarized in Table). Neonatal treatment with either GnRHa or flutamide did not cause any changes to the gross morphology of the rete, efferent ducts, caput or cauda epididymis, or vas that were comparable to those induced by treatment with 10 μg DES, though it was noticeable that the reproductive tract structures in both of these groups were smaller when compared with controls, indicating that development was retarded (Figure 1, Table).

Testicular Morphology and Weight

Neonatal treatment with 10 μg DES resulted in an average 72% reduction in testicular weight on day 18 (Figure 2, Table) and, at the microscopic level, there was clear retardation of seminiferous cord/tubule development, as has been detailed elsewhere (Sharpe et al, 1998; Atanassova et al, 2000). However, comparable retardation of development and reduction in testis weight was induced by neonatal treatment with GnRHa (Figure 2, Table), as reported previously (Sharpe et al, 1998). Treatment with the lower dose of DES (1 μg) caused only minor retardation of testis development and a correspondingly smaller reduction (38%) in testis weight, whereas neonatal treatment with flutamide caused a similar reduction (Figure 2, Table).

Immunoexpression of AR and ERs in the Testis and Reproductive Tract

Based on gross morphology and testis weight, it was concluded that interference with androgen production (GnRHa) or action (flutamide) alone in neonatal rats simply retarded normal development, whereas exposure to a high dose (10 μg) of DES induced unique morphological abnormalities to the reproductive tract not evident in these other 2 treatment groups. It was next examined whether the 10 μg DES treatment interfered with androgen action.

AR immunoexpression changes in treated animals are summarized in the Table and illustrated in Figure 3. In control animals, strong immunoexpression of AR was evident in interstitial cells, peritubular myoid cells, and Sertoli cells of the testis (Figure 3), and in epithelial cell nuclei of the rete testis (not shown), the efferent ducts, the caput and cauda epididymis, and the vas deferens (Figure 3). Immunoexpression of AR was also found in the nuclei of periductal stromal cells throughout the excurrent ducts (Figure 3). Treatment with 10 μg DES resulted in almost complete loss of AR immunoexpression from the testis, with only some weak peritubular myoid cell staining remaining (Figure 3). Similarly, AR immunoexpression in the epithelium of the rete testis (not shown), the caput and cauda epididymis and vas deferens appeared greatly reduced in intensity, whereas immunoexpression in the periductal stroma was virtually abolished (Figure 3). Immunostaining for AR was, however, detected in the cytoplasm of epithelial cells in the cauda epididymis and proximal vas deferens of animals in the 10 μg DES treatment group (Figure 3). Although this finding is surprising, it was consistent, and cytoplasmic staining was never seen in controls or in any other treatment group. In contrast to the effects of 10 μg DES treatment on AR immunoexpression, treatment with GnRHa or flutamide was without effect in the tissues examined, except that the intensity of AR immunostaining in the

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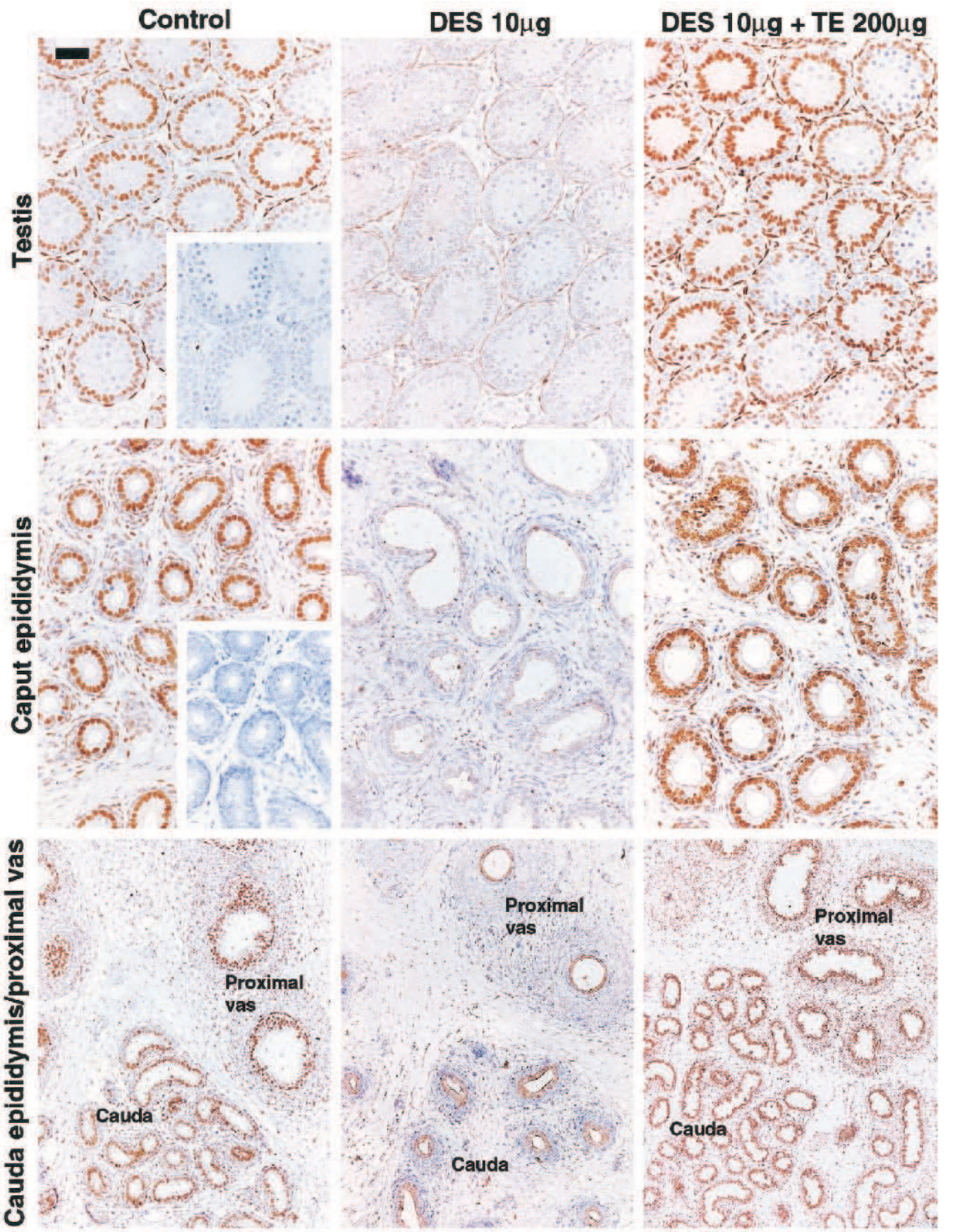
of DES + TE partially prevented lumenal distention of the efferent ducts (asterisks) but restored normal epithelial cell height (indicated by black lines in 2nd row) compared with DES treatment alone. Efferent ducts were immunostained for ERα and the cauda epididymis/proximal vas for ERβ to highlight cell nuclei and illustrate the absence of any major change in immunoexpression of the two ERs in the 3 treatment groups. Scale bar = 200μm (top and 3rd rows) or 100 μm (2nd and bottom rows).


Control
DES 10μg
DES 10μg + TE 200μg
Testis
Caput epididymis
Cauda epididymis/proximal vas
Proximal vas
Cauda
Proximal vas
Cauda
Proximal vas
Cauda

testes of flutamide-treated animals was consistently weaker than in controls (Figure 3). Treatment with the lower dose (1 μg) of DES was without effect on AR immunoexpression either in the testis (Figure 4) or any other tissue (results not shown, but summarized in Table).

To demonstrate the specificity of the observed changes in AR immunoexpression, the effect of neonatal DES treatment on immunoexpression of AR and ERs was compared. Neonatal treatment with either 10 μg or 1 μg DES had no detectable effect on immunoexpression of ERβ in the testis (Figure 4), and ERβ immunoexpression in all other tissues examined was also unaffected by DES or any other treatment (results not shown). Similarly, immunoexpression of ERα in the efferent ducts was unaffected by DES (Figure 4) or by any other treatment (not shown).

Leydig (3β-HSD-Positive) Cell Volume per Testis

The studies described above showed fundamental differences in the effect of DES (10 μg) and GnRHa treatment on AR immunoexpression in reproductive tract tissues. To establish whether these differences could be explained by differential effects of the 2 treatments on Leydig cell development, the volume of Leydig (3β-HSD-positive) cells per testis was measured. This showed that treatment with either 10 μg DES or GnRHa resulted in substantial (>80%) and significant ($P < .001$) reduction in Leydig cell volume per testis, whereas treatment with 1 μg DES had no effect on this parameter (Figure 5). It is presumed that this reduction will have led to similar reductions in the blood levels of testosterone in rats treated neonatally with DES (10 μg) or with GnRHa, however, our attempts to measure plasma testosterone levels accurately in these groups was prevented by nonspecific interference in the assay (perhaps from plasma binding proteins).

Effect of Coadministration of DES and Testosterone on Reproductive Tract Abnormalities, AR Immunoexpression, and Testis Weight

As induction of gross morphological abnormalities of the reproductive tract was only observed in animals in which loss of AR immunoexpression was also induced by the high dose of DES, it was reasoned that maintenance of AR expression by testosterone (ie, TE) administration (Bremner et al, 1994) might prevent the DES-induced abnormalities. Animals were therefore treated simultaneously with 10 μg DES + 200 μg TE or with either treatment alone. With one exception, all of the morphological changes resulting from DES (10 μg) treatment were completely prevented by coadministration of DES + TE (Figure 6), and this coincided with restoration of normal immunoexpression of AR in all of the tissues examined (Figure 7). The one DES-induced abnormality that was not entirely prevented by coadministration of TE was lumenal distention of the efferent ducts; this change was only partially prevented (Figure 6). In addition, testis weight in DES + TE-treated rats was not restored to control levels, though weights were significantly higher than in the group treated with DES (10 μg) alone and were comparable to animals treated with TE alone (Figure 2). The TE treatment alone induced no gross abnormalities of the reproductive tract, though it was noted that epididymal and vas deferens development in this group were advanced compared with controls, having a morphological appearance similar to that of an animal aged 22–25 days (not shown). This advance was equally evident in the DES + TE-treated group (Figures 6 and 7).

AR Protein Expression in the Testis—Western Analysis

The changes in testicular AR immunoexpression in control, DES-treated (10 μg), DES + TE–treated, and flutamide-treated animals were confirmed by Western blotting of testis protein extracts. The AR antibody recognized a protein band of ~113 kilodaltons (kd), corresponding to the molecular weight of the full-length AR protein, which was prominently expressed in extracts from control and DES + TE–treated animals (Figure 8). This band was virtually absent in extracts from DES-treated animals, and was only weakly expressed in extracts from flutamide-treated animals (Figure 8). Prominent bands at ~45 and ~60 kd, presumed to be AR breakdown products, were also absent in extracts from DES-treated animals but were present in the other treatment groups. Preabsorption of the antibody with the immunizing peptide resulted in the complete loss of detection of the 113-kd band, as well as the lower molecular weight bands (Figure 8).

Discussion

The present study has shown that neonatal treatment of male rats with a high dose (equivalent to >350μg/kg/day), but not a lower dose (~35μg/kg/day), of DES induces major morphological changes throughout the repro-

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Figure 7. Effect of neonatal treatment with vehicle (control), 10 μg DES or cotreatment with 10 μg DES and testosterone (DES + TE) on immunoexpression of AR in the testis (top row), caput epididymis (middle row), and cauda epididymis and proximal vas deferens (bottom row) at 18 days of age. Note that coadministration of DES + TE was able to completely prevent loss of AR immunoexpression in all of the tissues when compared with animals treated with DES alone. Note also the cytoplasmic AR staining in epithelial cells of the cauda/proximal vas in the group treated with DES alone (bottom row, middle panel). Insets show negative controls in which the antiserum was substituted by (normal) blocking serum. Scale bar = 100 μm.

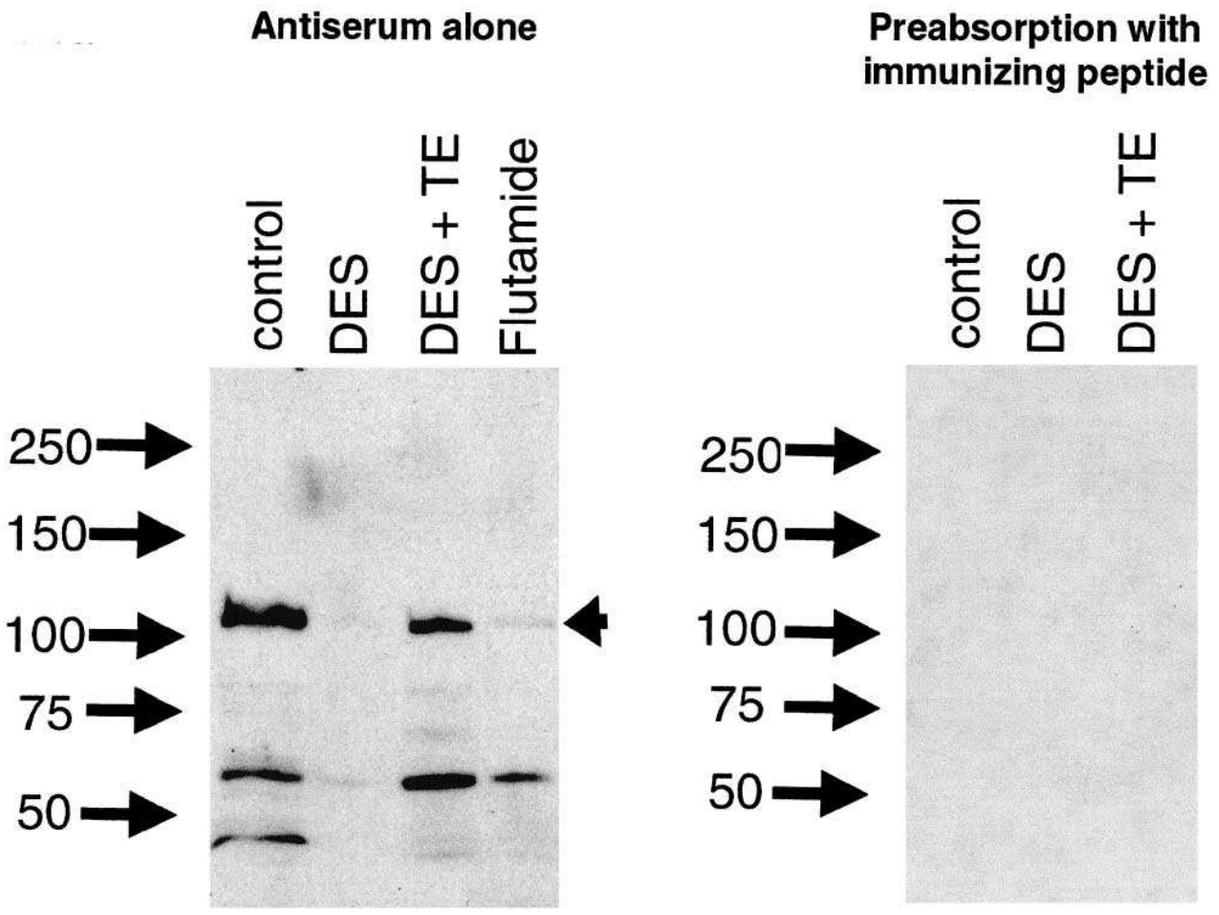

Figure 8. Western analysis of AR expression in the testis of 18-day-old rats treated neonatally with vehicle (control), DES (10 μg), DES (10 μg) + TE (200 μg), or with flutamide. Numbers show the position of molecular weight markers in kd. The panel to the left shows a blot probed with the AR antiserum while the panel to the right shows a blot probed after preabsorption of the antiserum with the immunizing peptide. Arrowhead points to the expected position of the full length AR protein; lower molecular weight bands in the left-hand panel are presumed to be breakdown products of the full-length AR as they are reduced markedly in the DES-treated group and are absent after preabsorption with the immunizing peptide (right-hand panel).

ductive tract at 18 days of age. These changes were associated with almost complete loss of AR immunoexpression in the affected tissues as well as in the testis. All of the affected tissues also expressed ERβ (Saunders et al, 1997, 1998; Williams et al, 2000; unpublished data) and, in a few instances, ERα (Fisher et al, 1997; Hess et al, 1997). However, no change was found in ERβ immunoexpression in any of the tissues, nor of ERα immunoexpression in the efferent ducts, from DES-treated animals. Together with the loss of AR immunoexpression there was major retardation of Leydig cell development in rats treated with the higher dose of DES, and it is reasonable to presume that this led to a drop in blood levels of testosterone. At face value, these findings are therefore consistent with the DES-induced reproductive tract abnormalities resulting from interference with androgen action. However, parallel studies in which Leydig cell development and androgen production (GnRHa) or androgen action (flutamide) were inhibited, without administering exogenous estrogen, failed to reproduce the reproductive tract abnormalities or the widespread loss of AR expression seen in animals treated with the higher dose of DES. This suggests that although suppression of androgen action appears to be closely associated with the induction of DES-induced abnormalities of the reproductive tract in males, the effects cannot be explained by *anti-androgenic* activity alone.

An alternative explanation for our findings is that induction of reproductive tract abnormalities in the male by neonatal high-dose DES treatment results not just from increased estrogen exposure or reduced androgen exposure, but from *gross* disturbance of the normal androgen: estrogen balance (ie, *grossly* elevated estrogen action plus concomitant *grossly* lowered androgen action). This interpretation is supported by our findings that 1) simply suppressing androgen action by treatment with GnRHa or

*Summary of the relationship between gross morphological changes to the reproductive tract induced by various neonatal treatments and the intensity of androgen receptor (AR) immunoexpression in the same tissue relative to control (not shown, but scored as +++ for all tissues examined)**

Gross Morphological Changes Induced by DES Treatment	DES 10 μg		DES 1 μg		GnRH Antagonist		Flutamide	
	Morphology	AR	Morphology	AR	Morphology	AR	Morphology	AR
Testis								
Retardation of development	Major	−/+†	Minor	+++	Major	+++	Minor	++/+
Size reduction	72%		38%		68%		40%	
Rete testis								
Distension and overgrowth	Major	−/+	Minor	+++	Normal‡	+++	Normal‡	++
Efferent ducts								
Distension/cell height reduction	Major	−/+	Minor	+++/++	Normal‡	+++	Normal‡	+++
Epididymis/vas deferens								
Relative stromal overgrowth + relative epithelial undergrowth	Major	−/+	Normal	+++	Normal‡	+++	Normal‡	+++
Convoluted proximal vas	Present§		Absent		Absent		Absent	

* Summary data are based on a minimum of 6–12 animals per group from at least 2 separate experiments. DES indicates diethylstilbestrol.
† Most animals were immunonegative for AR, but occasional animals showed sporadic positive immunostaining of some cells.
‡ Morphological development retarded so that appearance is comparable to that of a (normal) younger animal.
§ Present in most, but not all, animals.

flutamide merely retarded normal development of the reproductive tract without inducing major abnormalities, with the exception of cellular changes in the seminiferous tubules of the testis (see below); 2) simply raising estrogen levels by injection of a lower (but still substantial) dose of DES (1 μg) that did not suppress Leydig cell development was not sufficient to induce reproductive tract abnormalities and also had no effect on AR immunoexpression; 3) major reproductive tract abnormalities were induced only after injection of a very high dose (10 μg) of DES, which was also capable of suppressing Leydig cell development and AR immunoexpression; and 4) simultaneous treatment with high doses of both DES and TE prevented nearly all of the effects induced by DES alone, and also restored normal AR immunoexpression. This interpretation also fits with other pieces of published data that demonstrate that disturbance of the androgen: estrogen balance in males results in various abnormalities, such as gynecomastia in adult men (reviewed in Sharpe, 1998).

The male reproductive tract abnormalities induced by neonatal DES treatment in the present studies are consistent with earlier studies in which DES or other potent estrogens have been administered neonatally (Arai et al, 1983; Bellido et al, 1990; Fisher et al, 1998; Khan et al, 1998; Sharpe et al, 1998) or in utero (McLachlan et al, 1975; Thomson et al, 1981; Newbold et al, 1985) to mice, rats, hamsters, or monkeys. The underdevelopment of epithelial cells/tissue observed in the efferent ducts, caput and cauda epididymis, and vas deferens of DES-treated (10 μg) rats in the present studies is also matched by similar changes reported in the prostate (Rajfer and Coffey, 1979; Chang et al, 1999) and seminal vesicles (Newbold and McLachlan, 1985; Williams et al, 2000) of neonatally estrogen-treated rats. Furthermore, our finding that DES-induced abnormalities are associated with the suppression of AR immunoexpression in the affected tissues is consistent with studies showing a similar association between morphological effects and AR immunoexpression in the prostate of rats treated neonatally with estrogens (Prins, 1992; Prins and Birch, 1995). Examination of the doses administered in these various experiments indicates that induction of major reproductive tract abnormalities *in the male* requires administration of extremely high doses (>100 μg/kg) of potent estrogens, such as DES or ethinyl estradiol. Data from human males exposed in utero to DES, in whom similar reproductive tract abnormalities have been reported as in rodents, also involved administration of similarly high doses (5–150 mg/day or ~100–3000 μg/kg/day) to the pregnant mothers (Stillman, 1982; Toppari et al, 1996). Our own findings in male rats exposed neonatally to DES show that only these very high doses are able to induce major reproductive tract abnormalities (see also Sharpe et al, 1998; Atanassova et al, 1999; Fisher et al, 1999). Such evidence seems at odds with the high affinity of both ERα and ERβ for DES (Kuiper et al, 1997). Our present findings offer a possible mechanistic explanation for this puzzling dose-response relationship; namely, that only very high doses of DES are able to suppress AR expression, and it is the latter change in concert with the elevated

estrogen action that results in the reproductive tract abnormalities.

It is unclear from our study why only a very high dose of DES was able to suppress AR expression in reproductive tract tissues; the mechanism of this induction is also unclear. The most obvious mechanism is that the suppression of Leydig cell development induced by 10 μg DES led to reduced testosterone levels, which in turn led to a reduction in AR expression, based on findings in adult males in which testosterone levels are experimentally lowered (Rajfer and Coffey, 1979; Blok et al, 1992; Bremner et al, 1994). Although we were unable to measure plasma testosterone in the present study, it is well established that exposure of males to estrogens, including to DES, can suppress both Leydig cell development and steroidogenesis (Abney, 1999). However, this explanation would fail to explain why AR immunoexpression was unaffected in males treated neonatally with GnRHa in which we found a reduction in Leydig cell development of similar magnitude to that induced by DES (10 μg). We also have no ready explanation as to why 10 μg, but not 1 μg, DES can affect Leydig cell development and AR immunoexpression elsewhere, when either of these doses will elevate endogenous estrogen levels to supranormal levels.

A notable exception to the above discussion concerning DES-induced reproductive abnormalities are the changes induced in testis weight. Neonatal treatment with GnRHa was able to induce a similar reduction in testis weight to that induced by the high dose (10 μg) of DES, and coadministration of DES + TE only partially ameliorated the DES-induced decrease. Indeed, administration of TE alone also significantly reduced testis weight to the same extent as did DES + TE, and this reduction was similar in magnitude to that induced by flutamide or by the lower dose (1 μg) of DES. Detailed comparison of testis cell composition and numbers has not yet been undertaken for all of these treatment groups, though we have previously established that numbers of Sertoli cells, germ cells, and apoptotic germ cells are very similar at day 18 in rats treated with DES (10 μg) or with GnRHa (Sharpe et al, 1998). In the latter 2 groups there is comparable retardation of pubertal development that may be secondary to lowered FSH as well as, or instead of, lowered testosterone levels (Sharpe et al, 1998). It is therefore reasoned that some of the effects of DES (10 μg) treatment on the testis are not attributable to suppression of testosterone action and that, correspondingly, they are not preventable by coadministration of DES + TE.

There are alternative interpretations of the present findings. For example, although our results point to involvement of suppressed androgen action in the adverse reproductive effects induced by DES in males, this does not exclude the possibility of direct effects of DES on the target tissues, all of which express ERs (Hess et al, 1997; Saunders et al, 1997, 1998; Williams et al, 2000). In addition, it is probable that the TE treatment elevated endogenous testosterone levels above normal, at least in some of the reproductive tract tissues. Evidence for this included advancement of epididymal development and increase in size of the seminal vesicles (unpublished) in TE-treated and DES + TE–treated animals. This ''accelerated'' development, although not pronounced, might somehow protect against the effects of DES treatment. We are unable to discount this possibility. Based on the doses of TE and DES that were coadministered we restored an androgen:estrogen ratio of 20:1, though factors such as different half-lives and so on mean that this is very much an approximation. Nevertheless, this value is markedly lower than the ratio of 1000:1 or greater reported in the literature for adult rats (there are no data for neonates; de Jong et al, 1973, 1974). It therefore seems highly unlikely that we have grossly elevated the androgen:estrogen ratio compared with normal. Future studies in which different doses of TE are coadministered with DES or in which TE administration is delayed with respect to DES treatment may provide further insight on these issues. Other options are to combine treatment with an antiandrogen with a dose of DES that is ineffective on its own.

Irrespective of the mechanisms involved, our findings suggest strongly that for neonatal exposure to estrogens to result in major disruption of *male* reproductive development, there must also be a concomitant suppression of androgen action. However, suppression of androgen action alone, without exogenous estrogen, is insufficient to induce reproductive tract abnormalities comparable to those induced by DES. The suppression of androgen action by DES occurs only when very high doses are administered, although it is not clear why this is so. This dose-response suggests that weaker estrogens, for example, ''environmental'' estrogens, will be unable to induce major adverse changes to the male reproductive system as a result of their estrogenicity. Indeed, in similar studies with extremely high doses of 3 ''weaker'' environmental estrogens (octylphenol, bisphenol A, genistein) we have found no evidence for effects on any of the parameters reported in the present studies (Atanassova et al, 2000; unpublished data). Our working hypothesis to explain the present findings is that gross disturbance of the androgen:estrogen balance may underlie the DES-induced reproductive tract abnormalities in males. If this hypothesis is correct, it would add to health concerns about identified environmental anti-androgens (some phthalates, vinclozolin, p′p′-DDE, etc), and in particular about chemicals that

possess both estrogenic and anti-androgenic activity in the same molecule.

Acknowledgments

We are grateful to Professor Ian Mason (Edinburgh) for antiserum to 3β-HSD and to Dr R Deghenghi and Europeptides for their generous gift of GnRH antagonist. We are indebted to Jim MacDonald for expert assistance.

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