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Differential hormonal regulation of estrogen receptors ERα and ERβ and androgen receptor expression in rat efferent ductules

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Abstract

Estrogen receptors, in addition to the androgen receptor (AR), are expressed at high levels in efferent ductules of the male reproductive tract and it is now well recognized that estrogen receptor (ER) α is required for the maintenance of normal structure and function of the ductules. However, little is known regarding the hormonal regulation of the receptors themselves in the male. In the present study, efferent ductule ligation and castration, followed by replacement with testosterone, dihydrotestosterone (DHT) or estradiol was used to investigate the relative importance of circulating and luminal sources of steroid for the modulation of ERα, ERβ and AR in rat efferent ductules. Uni- or bilateral castration and ligation did not affect the expression of ERα and ERβ, but bilateral castration caused down-regulation of AR. Replacement with DHT and testosterone alone or in combination with estradiol caused the recovery of AR expression to control levels. A slight recovery of AR was also observed after estrogen replacement. ERα expression was decreased to nearly undetectable levels after estrogen replacement. On the other hand, ERβ did not show evident effects following any of the treatments, suggesting a constitutive expression of this receptor. This differential modulation of the steroid hormone receptors highlights the importance of maintaining a physiological androgen-estrogen balance to regulate the structure and function of efferent ductules in the male.

Introduction

Androgens have a well-established role in regulating the male reproductive tract. However, studies in several different experimental animal models have revealed that estrogens also play an essential role in the normal structure and function of the male reproductive tract (Lubahn *et al.* 1993, Smith *et al.* 1994, Morishima *et al.* 1995, Hess *et al.* 1997*a*, Couse & Korach 1998, Krege *et al.* 1998, Fisher *et al.* 1999, Robertson *et al.* 1999, Lee *et al.* 2000, Turner *et al.* 2000, Atanassova *et al.* 2001, Oliveira *et al.* 2001, 2002, Zhou *et al.* 2001*a*, Cho *et al.* 2003). The effects of androgens are mediated through the androgen receptor (AR) and the estrogen actions are mediated through two estrogen receptors, ERα and ERβ, which are co-localized in some regions of the male reproductive tract (Nie *et al.* 2002, Zhou *et al.* 2002). Despite the known expression of one or both ERs in the male tract (Hess *et al.* 1997*b*, Fisher

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et al. 1997, Nielsen *et al.* 2001, Saunders *et al.* 2001, Nie *et al.* 2002, Zhou *et al.* 2002), the full extent of estrogen's action is not completely understood and the interaction of the various steroid receptors in the same cells of the male has not been examined.

Disruption of ERα function caused major effects in the efferent ductules, which resulted in male infertility (Eddy *et al.* 1996, Hess *et al.* 1997*a*, 2001, Oliveira *et al.* 2001). It is consistent across species that the efferent ductule is the site having the highest concentration of ERα in the male tract (Fisher *et al.* 1997, Goyal *et al.* 1997, Hess *et al.* 1997*b*, Nielsen *et al.* 2001, Nie *et al.* 2002, Zhou *et al.* 2002). The functional significance of ERα in the efferent ductule is under current scrutiny (Hess *et al.* 1997*a*, Lee *et al.* 2001, Zhou *et al.* 2001*a*, Cho *et al.* 2003), but the regulation of ERα expression itself in the efferent ductules remains to be determined. Even less information is available regarding ERβ, the estrogen receptor subtype with the widest distribution in the male (Hess *et al.* 1997*b*, Atanassova *et al.* 2001, Saunders *et al.* 2001). Targeted disruption of ERβ (βERKO) did not promote significant abnormalities in the male reproductive organs (Krege *et al.* 1998, Dupont *et al.* 2000); therefore, the biological function of ERβ in these organs, as well as the factor(s) involved in ERβ regulation, are at present unknown. It should be emphasized that continued expression of ERβ in the efferent ductules of the αERKO was unable to compensate for loss of ERα (Rosenfeld *et al.* 1998) and the double knockout of ER receptors (αβERKO) resulted in a phenotype resembling that of αERKO.

The efferent ductules and epididymis have two sources of estrogens and androgens, either the rete testis luminal fluid or the circulating blood in the vasculature. The relative importance of each source of steroid for the efferent ductules is not known (Goyal *et al.* 1998). However, circulating androgens do not appear to be sufficient to maintain the structure of the initial segment epididymis which is dependent upon luminal dihydrotestosterone (DHT), but do maintain the structure in the corpus and cauda (Fawcett & Hoffer 1979, Robaire & Viger 1995). The efferent ductules have high concentrations of AR (Schleicher *et al.* 1984, Roselli *et al.* 1991) and bind DHT (Schleicher *et al.* 1984), but in contrast with the initial segment, there is little or no 5α-reductase activity (Roselli *et al.* 1991) and the testosterone concentration in the luminal fluid (29 ng/ml) is much higher than that of DHT (2 ng/ml) (Vreeburg 1975). Accordingly, very little androgen dependence has been described for the efferent ductules (Goyal & Hrudka 1980, Ilio & Hess 1994, Hess 2002). Considering that efferent ductules express greater amounts of ER than the initial segment of the epididymis (Hess *et al.* 1997*b*, Mansour *et al.* 2001), it is reasonable to suggest that estrogens entering the efferent ductules may play a major role in ductal epithelial function, similar to the role that DHT plays in the initial segment of the epididymis (Robaire & Viger 1995).

There is little known about the hormonal regulation of the AR and ER in efferent ductules (Ilio & Hess 1994, Hess 2002, Hess *et al.* 2002). The only study to have focused on ER regulation in efferent ductules was carried out in the goat (Goyal *et al.* 1998). That study was based on castration and testosterone replacement, but the authors did not investigate the action of testosterone metabolites, DHT and estradiol, or the regulation of ERβ expression. Therefore, an investigation of this issue in rodents is warranted. The regulation of AR and ER by androgens and estrogens shows tissue and organ specificity, with both increases and decreases in receptor mRNA and protein in reproductive and non-reproductive tissues, depending on several physiological factors (Barton & Shapiro 1988, Goyal & Williams 1988, Quarmby *et al.* 1990, Lauber *et al.* 1991, Prins 1992, Gonzalez-Cadavid *et al.* 1993, Prins & Birch 1997, Prins *et al.* 1998, Yeap *et al.* 1999, Agarwal *et al.* 2000, Lynch & Story 2000, Zhu *et al.* 2000, Turner *et al.* 2001, Zhou *et al.* 2001*b*). These studies reveal the complexity associated with steroid receptor regulation across species, as well as organ- and cell-specific responses (Yeap *et al.* 1999). Thus, the potential for diverse steroid receptor responses to estrogens and androgens in the efferent ductules would be expected.

The present experiments were designed to investigate whether the expressions of ERα, ERβ and AR in the rat efferent ductules are regulated by estrogens, androgens or other factors derived from testicular fluid and/or blood. For this purpose, ligation and castration, followed by replacement with testosterone, DHT or estradiol were used. Differential expression of ERα, ERβ and AR was found in the rat efferent ductules. Estrogen caused down-regulation of ERα, and androgens promoted up-regulation of AR, whereas ERβ was not affected by these hormones or by the ligation and castration procedures, suggesting a constitutive expression of this receptor in the efferent ductules.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Harlan Bioproducts, Indianapolis, IN, USA), 90 days old, were used for this study. Upon arrival, the rats were housed under constant conditions of light (12 h light: 12 h darkness) and temperature (22 °C) and allowed to adapt to the vivarium conditions for at least 7 days before the experiment. They were fed a commercial diet (Teklad Chow–Harlan Teklad, Madison, WI, USA) and tap water was available *ad libitum.* All animal experiments and surgical procedures were approved by the University of Illinois Division of Animal Resources and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996).

The rats were randomly divided into 13 groups of three animals each, which were subjected to one of the following treatments: unilateral or bilateral ligation, unilateral or bilateral orchidectomy, sham-operation, bilateral orchidectomy followed by daily replacement with different doses of testosterone, DHT or estradiol, or left intact to serve as controls.

Experimental procedures

The rats were anesthetized with an i.p. injection of sodium pentobarbital (0.08 ml/100 mg body weight (BW)) and surgery was carried out in aseptic conditions. The body weight was recorded before surgery for later comparison with the weight at death. The scrotal skin was shaved and an incision in the mid-line ventral scrotal skin was performed to expose the testis-epididymis, in order to proceed to the ligation and/or castration. With bilateral castration, deprival of both luminal and circulating hormones will be achieved, while ligation and unilateral castration will deprive the efferent ductules of the luminal but not the circulating source of sex hormones. Three animals were not submitted to any treatment and served as intact experimental controls. Sham-operations were performed in one group of males to expose, manipulate and then reinsert the testes intact into the scrotum.

Ligation—After testis-epididymis exposure, the fat surrounding the extratesticular rete testis and the initial part of the efferent ductules was carefully dissected. The ducts were carefully separated from the pampiniform plexus vessels, which were kept intact. Ligation was performed at the extratesticular rete testis level, using a nonabsorbable silk suture placed as close to the testis as possible. For unilateral ligation, the extratesticular rete testis was ligated on one side and the contralateral testis/epididymis was left intact and used as control. After ligation, the testis-epididymis was returned to the scrotum and the scrotal incision was closed by suture. The rats were killed 15 days after ligation.

Castration—The initial procedure for castration followed the same protocol as that for ligation. The extratesticular rete testis together with the testicular blood vessels were ligated, as close to the testis as possible and then the testes were removed. The ligated efferent ductules and epididymis were placed back into the scrotum and the scrotal incision was closed by suture.

When unilateral castration was performed, the contra-lateral testis/epididymis remained intact and served as control. The rats were killed 15 days after castration.

At the end of surgery, the scrotal incision was closed by suture and the wound was treated with Betadine. Postoperative conditions of the animals, including food and water ingestion, defecation, and surgical site were monitored daily.

Hormone replacement

Starting on the same day as the bilateral orchidectomy, the animals were treated once per day, for 15 days, with s.c. injections of 5 mg 5α-DHT/day (Sigma, St Louis, MO, USA), 1 mg or 5 mg testosterone propionate/day (JT Baker Chemicals, Phillipsburg, NJ, USA), 75 μg or 400 μg 17β-estradiol-3-benzoate/day (Sigma) or both testosterone propionate (5 mg/day) and 17β-estradiol-3-benzoate (400 μg/day), dissolved in a volume of 0.1 ml corn oil as vehicle. The rats were killed after 15 days of treatment. The castration control group received the same volume of vehicle only.

A high dosage of estradiol (400 μg) was used because this was found to be effective for inducing complete estrogenization in male rats without inducing significant regression of the efferent ductules (Hansen *et al.* 1997, Tena-Sempere *et al.* 2000). The dosage of 75 μg estradiol was equivalent to that shown to induce a response in reproductive organs, with minimal histological alterations (Turner *et al.* 2001). The dose regimen of 1 mg testosterone was used to mimic physiological serum testosterone levels and that of 5 mg testosterone and DHT was based on previous studies showing that this concentration reproduces that which is normally found in the epididymis (Hansen *et al.* 1997, Fan & Robaire 1998, Goyal *et al.* 1998). DHT, the non-aromatizable metabolite of testosterone, was used to get around the problem of whether the potential effects of testosterone were direct or were dependent upon aromatization to estrogen or 5α-reduction to DHT.

Tissue preparation and morphometry

Fifteen days after surgery (ligation or castration) and initiation of hormone replacement, the rats were anesthetized (i.p. sodium pentobarbital 0.1 ml/100 g BW), weighed and perfused intracardially with 10% neutral buffer formalin (NBF). After fixation, the testis, epididymis with the attached efferent ductules, ventral prostate and seminal vesicles with coagulating glands were removed and weighed. The efferent ductules were dissected out from the epididymis, embedded in paraffin, sectioned (5 μm) and mounted on electrostatically charged glass slides. The sections were stained with hematoxylin and eosin for histological study or were used for immunohistochemistry staining. The lumens of the efferent ductules were measured at the widest diameter of five sections of tubules from the proximal area nearest the rete testis. The height of the epithelium of the efferent ductules was measured from the basement membrane to the microvillus bases in areas of straight sections from 25 cells with an evident nucleus. The luminal and epithelial measurements were performed using a calibrated ocular micrometer coupled to a 10 × and 40 × objective, respectively.

Hormone measurements

Plasma concentrations of testosterone and estradiol were measured by RIA. Blood was collected by cardiac puncture, immediately before death. The plasma was separated by centrifugation and stored at −20 °C for subsequent hormone assays. All samples were measured in duplicate. The reported concentrations of the hormones were corrected for extraction losses.

The antibody used for assaying testosterone was developed by Dr O D Sherwood (University of Illinois, Urbana, IL, USA) and the procedures have been described previously (Jackson *et al.* 1991, Oliveira *et al.* 2002). For the assay, plasma was extracted with toluene:petroleum

ether (2:5 v/v). The efficiency of the extraction was 89.7%, the sensitivity of the assay was 0.2 ng/ml and the assay coefficient of variation was 8.3%.

Estradiol concentration was estimated using a double antibody ultra-sensitive estradiol RIA-DSL 4800 Kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). This kit uses an antibody with high affinity for estradiol and low cross-reactivity with other estrogens and with testosterone. For the assay, plasma was extracted with toluene; the efficiency of the extraction was 95.3%. The limit of detection of the assay was 0.8 pg/ml and the assay coefficient of variation was 10.8%.

Immunohistochemistry

Changes in the expression of ERα, ERβ and AR in the efferent ductules were investigated by immunohistochemistry in all experimental rats, following the protocol previously described (Oliveira *et al.* 2003). Tissue sections from animals of each experimental group were run in parallel, and the staining was performed in three different sets using one animal of each group per set to confirm the results. Fixed tissues were embedded in paraffin; sections were subjected to microwave antigen retrieval before incubation with primary antibody. The antibodies used were a monoclonal mouse anti-human ERα antibody (NCL-ER-6F11-Novocastra Laboratories, Newcastle, UK), a polyclonal sheep anti-human/rat ERβ antibody (S-40, raised against a peptide in the hinge domain of hERβ; Saunders *et al.* 2000) and a polyclonal rabbit anti-rat/human androgen receptor antibody (PG21–29; Prins *et al.* 1991). Sections were incubated overnight at 4 °C with diluted primary antibody (1:500 for ERα and AR; 1:1000 for ERβ). For negative controls, the sections received PBS in place of the primary antibody. After washing in PBS, the sections were exposed for 1 h to a biotinylated secondary antibody - goat anti-mouse (for ERα) (Dako, Carpinteria, CA, USA), rabbit anti-sheep (for ERβ) (Vector Laboratories, Burlingame, CA, USA) and goat anti-rabbit (for AR) (Dako), all used at 1:100 dilution. After this step, the sections were incubated with the avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories) for 30 min and the immunoreaction was visualized using diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. Sections stained for ERβ were slightly counterstained with Mayer's hematoxylin.

Statistical analysis

A two-way ANOVA was applied to analyze the effect of ligation, castration and hormone replacement on testosterone and estradiol blood concentrations, body weight, efferent ductules/epididymis and accessory sex gland weights, and luminal diameter and epithelial height of efferent ductules. The *post hoc* Tukey's test was used for multiple comparisons between the experimental groups.

Results

Hormone levels

After unilateral castration and efferent ductule ligation on one or both sides, as well as sham operation, testosterone levels did not differ significantly from those of the intact controls, but following bilateral orchidectomy the testosterone concentration fell to a level similar to or below the limit of detection of the assay (0.2 ng/ml) (Table 1). Similar results were observed when corn oil or estradiol was administered following castration. By contrast, supplementation with DHT or testosterone (1 or 5 mg), alone or combined with estradiol, resulted in plasma testosterone levels significantly higher than controls. This increase in testosterone level was dose dependent, reaching levels above the limit of detection (20 ng/ml) with the high dose of testosterone (5 mg). Plasma levels of estradiol were not significantly different from intact controls in any experimental group, except after estradiol replacement, alone or combined with

testosterone, which resulted in an increase in plasma estradiol to levels above the upper limit of the assay (>80 pg/ml) (Table 1).

Body and organ weights

No significant difference in body weight was observed when initial and final weights were compared in most experimental groups, but there was a significant ($P < 0.05$) loss of weight over the 15 days of estradiol replacement (12 to 24% decrease) (Table 1). Compared with the control group, the weights of the ventral prostate and paired seminal vesicle/coagulating glands, as well as the weight of the efferent ductules/epididymis were significantly diminished after bilateral castration (Table 2). Estradiol alone was not able to restore the weight of either the accessory sex glands or the efferent ductules/epididymis. Replacement with testosterone or DHT resulted in sex gland weights even higher than intact controls (Table 2). Although androgen treatment greatly increased the weight of the efferent ductules/epididymis, they were still below the intact control weight. On the other hand, when testosterone was combined with estradiol, the efferent ductules/epididymis weight was restored to control levels. Combined testosterone and estrogen also resulted in heavier sex glands than those treated with testosterone alone. Unilateral castration and uni- or bilateral ligation, as well as sham operation, did not affect significantly the weight of the accessory glands (Table 2). Similarly, the weight of the efferent ductules/epididymis was not greatly affected by sham operation, but a significant decrease in weight at the operated side was observed when unilateral ligation (32%) and unilateral castration (28%) were compared with the contra-lateral intact efferent ductules/epididymis, or when the bilateral ligated (18–22%) was compared with the intact control (Table 2).

Epithelial cell height

Regression of the efferent ductules, characterized by a reduction in luminal diameter and epithelial height, was observed as a result of either ligation or castration, but the effects on luminal diameter were more dramatic (Fig. 1). Efferent ductule luminal diameter was significantly increased by 400 μg estradiol, when compared with castrated control animals, but androgens had no effect. In contrast, testosterone and DHT replacement, as well as estradiol, caused a partial recovery in the height of the epithelium, but higher epithelium was seen after resupplementation with testosterone plus estradiol. However, in all cases the epithelial height was still below the control level.

Expression of androgen receptors

The AR expression in control efferent ductules was found in the nuclei of ciliated and nonciliated epithelial cells, in addition to the peritubular and some stromal cells (Fig. 2A). Similarly, the initial segment of the epididymis was strongly positive for AR in the epithelial, peritubular and stromal cell nuclei. AR expression was not affected by sham operation, unilateral or bilateral ligation, or unilateral castration. On the other hand, bilateral castration caused a remarkable down-regulation in AR protein expression (Fig. 2B). Testosterone, alone or associated with estradiol, as well as DHT restored the AR to the intact control levels (Fig. 2C–F). The AR immunostaining intensity in the efferent ductules was partially restored by estradiol supplementation (Fig. 2G, H). The effects on AR expression in the initial segment were similar to those of the efferent ductule, including the recovery of staining after estradiol replacement (Fig. 2I–L).

Expression of ERα

Immunohistochemical localization of ERα in the efferent ductules was restricted to the ciliated and nonciliated cell nuclei (Fig. 3A). ERα immunostaining in the efferent ductules after unilateral or bilateral ligation and castration was unchanged when compared with that of intact

controls (Fig. 3B). The administration of corn oil, testosterone and DHT following castration had no effect on ERα expression (Fig. 3C, D, E). However, ERα was greatly reduced in the epithelium of the efferent ductules after estradiol replacement, in a dose-dependent manner (Fig. 3G, H). The higher dose of estradiol (400 μg) promoted down-regulation of ERα to nearly undetectable levels, both when used alone and in combination with testosterone (Fig. 3F, H). The lower dose of estradiol (75 μg) caused less, but still a dramatic, reduction in immunodetectable ERα in the efferent ductules (Fig. 3G). The initial segment of the epididymis was negative for ERα (Fig. 3I); however resupplementation with estradiol induced slight ERα immunoexpression in the epithelium (Fig. 3J).

Expression of ERβ

The pattern of ERβ localization in efferent ductules and initial segment was similar to that of the AR, with positive staining detected in the nuclei of epithelial cells, as well as in peritubular and some stromal cells (Fig. 4A). Bilateral and unilateral ligation or castration, as well as hormonal replacement did not change ERβ expression or, at the very least, caused a very slight decrease in ERβ (Fig. 4B-H). A decrease in staining was observed after testosterone treatment.

Discussion

This study provided evidence that expression of ERα, ERβ and AR proteins in the rat efferent ductules is differentially regulated by androgens and estrogens. These results partially differed from those in the goat (Goyal *et al.* 1998), but agree with recent findings that AR and both ERs respond differently to the antiestrogen ICI 182,780, with ERα expression being dramatically down-regulated, but ERβ and AR levels remaining unchanged in the rat efferent ductule (Oliveira *et al.* 2003). These data suggest that there may be important interspecies differences in the regulation of ER expression in the male reproductive tract.

The effects of castration and hormone replacement on body and sex gland weight, and blood concentrations of testosterone and estradiol were similar to previously reported data for the rat (Podesta *et al.* 1975, Roselli & Resko 1984). The effects of androgens on sex gland weight, which was much higher than controls, was expected because a high supplemental dose of hormone was used to maintain the elevated concentrations needed to stimulate the testis-epididymal region. The rat efferent ductule epithelium regressed and the efferent ductule/epididymal weight decreased following castration, and both parameters were restored by androgens but not to control levels. Circulating estrogen alone does not appear to be sufficient to maintain the efferent ductule structure. However, concomitant administration of testosterone and estradiol was the only treatment able to restore efferent ductule/epididymal weight to the control level and induced a higher recovery of epithelial height, indicating that a cooperative action of these steroids may be necessary.

The effects of androgens on AR expression in the efferent ductule are in agreement with previous studies showing increased AR protein following testosterone or DHT treatment (Calandra *et al.* 1975, Sar *et al.* 1990, Goyal *et al.* 1998, Yeap *et al.* 1999, Zhu *et al.* 2000, Turner *et al.* 2001). Others have shown up-regulation of AR protein even in the presence of decreased mRNA (Quarmby *et al.* 1990, Yeap *et al.* 1999). These data indicate that the mechanism underlying AR regulation by androgens involves differential transcriptional and/or post-transcriptional events, which will likely be cell- and tissue-specific (Yeap *et al.* 1999). In this sense, recent findings in the prostate gland showed that AR is down-regulated by androgen withdrawal as well as estrogen treatment, through a post-transcriptional pathway mediated by proteasome (Woodham *et al.* 2003). In the present study, ligation of the efferent ductule did not affect levels of immunodetectable AR, nor did unilateral castration. Taken together, these data indicate that deprivation of circulating androgens, but not of luminal androgens or other testicular factors such as androgen binding protein (ABP), affected AR

expression, suggesting that AR expression in rat efferent ductules is mainly dependent on circulating androgens. This result is in agreement with that found for efferent ductules in the goat (Goyal *et al.* 1998). ABP is a Sertoli cell product important for the maintenance of high levels of intratubular androgen (Danzo *et al.* 1977, Dohle *et al.* 2003). Some chemical agents that alter AR expression in the testis also alter the expression of ABP (Tirado *et al.* 2004). Altered expression of ABP has been associated with testis and male tract alterations similar to those caused by alterations of AR (Danzo *et al.* 1977, Jeyaraj *et al.* 2002). Therefore, it would be possible that ABP could have some effect on AR expression. We cannot exclude this possibilitiy, but ABP transgenic mice showed no significant changes in androgen receptor, which may indicate that the deleterious effects of aberrant levels of ABP may be caused by some other factor than AR modulation (Munell *et al.* 2002).

It was interesting to note that estrogen replacement also caused a partial recovery of AR expression in the efferent ductule following castration. Modulation of AR by estrogen has been reported in the rat testis (Turner *et al.* 2001) and in some areas of the rat brain (Lynch & Story 2000). In the prostate, developmental estrogenization has been shown to induce a transient up-regulation of ERα and a down-regulation of AR and ERβ levels in adult prostate epithelium (Prins & Birch 1997, Prins *et al.* 2001). Estrogen also appears to interfere in some physiological events usually attributed to androgens, such as modulation of prostate androgen responsive genes (prostate specific androgen (PSA), Rosner *et al.* 1998; C3 and sulphated glycoprotein-2 (SGP-2), Turner *et al.* 2001) and stimulation of spermatogenesis in hypogonadal males (Ebling *et al.* 2000), suggesting that the effects of estradiol are mediated via cross-reactivity with AR. Physical interactions between AR and ERα, which result in estradiol-induced modulation of AR transcriptional activity by ERα (Panet-Raymond *et al.* 2000) and the ability of estradiol to activate AR genes when AR complexes with the coactivator ARA70 (Yeh *et al.* 1998) or with sex hormone binding globulin (SHBG)-SHBG receptor (Rosner *et al.* 1998), have been described and they indicate alternative pathways to explain receptor interplay. It is more likely that interaction between androgen and estrogen action may exist in the male reproductive tract, similar to other tissues (Migliaccio *et al.* 2000, Ochiai *et al.* 2004).

The distribution of ERα was found to be restricted to the epithelium of the efferent ductules, consistent with findings across species investigated to date (Hess 2003). The only dramatic decrease in ERα expression in the efferent ductules was observed after estrogen replacement. ERα down-regulation by estradiol was dose dependent, confirming previous findings that ERα proteolysis is modulated by ligand concentration (Preisler-Mashek *et al.* 2002). Both ligation and castration were unable to disturb ERα expression in the efferent ductules. Because plasma levels of estradiol were not altered significantly by these procedures, it would appear that circulating estradiol might play a role in regulating ERα in the efferent ductule epithelium. Considering that aromatase mRNA was recently detected in the epithelium of epididymal cells *in vitro* (Wiszniewska 2002), the contribution of locally produced estrogen cannot be ruled out. However, it is important to point out that studies in several species have detected aromatase protein and activity in the sperm that traverse the male tract, but not in the epithelium of the ductal system (Janulis *et al.* 1996, 1998).

The efferent ductule is the segment of the male tract more sensitive to altered levels of estrogen or disruption of estrogen action, as shown by experiments using high doses of estrogens, antiestrogens, as well as knockout of estrogen receptors, all of them resulting in efferent ductule abnormalities, especially in the luminal diameter (Hess *et al.* 2002, Oliveira *et al.* 2001, 2002, Rivas *et al.* 2002, 2003, Cho *et al.* 2003). Also in the present study, estrogen (400 μg) was the treatment that caused greater dilation in the efferent ductules after castration. These results are in agreement with recent data showing that the primary function of estrogens/ERα in the efferent ductules is to regulate the expression of proteins involved in fluid reabsorption (Zhou *et al.* 2001*a*, Lee *et al.* 2001, Oliveira *et al.* 2002), in addition to the

maintenance of the epithelial morphology (Hess 2003), although the last function appears to be more dependent on an androgen–estrogen balance (Rivas *et al.* 2003).

In contrast to the effects on efferent ductules, estrogen replacement induced a weak expression of ERα in the initial segment of the epididymis, a region that was negative for ERα in the control animals. Depending on the tissue and the antibodies used, estrogens have been shown to cause autologous ER up-regulation in some tissues (liver, Barton & Shapiro 1988; prostate epithelium, Prins & Birch 1997; uterus, Zou & Ing 1998; bone, Zhou *et al.* 2001*b*; thyroid, Banu *et al.* 2002). Considering that in most of these tissues ERα expression is usually low, it is possible that the difference between ERα expression in response to estrogen may be determined by the relative levels of expression of this receptor. In sites where ERα is abundant, such as the efferent ductule, it appears that down-regulation of ERα is the mechanism responsible for regulating the duration of physiological response to the activating ligand. On the other hand, in sites with low abundance or no ERα expression there is a likelihood that estrogens will up-regulate ERα.

Differing from AR and ERα, there was no dramatic effect on the ERβ expression in efferent ductules after any treatment presently investigated. Similar to the present result, ERβ immunoreaction was not affected by androgen/estrogen withdrawal or by steroid replacement in rat testis and prostate (Turner *et al.* 2001). Also, exposure of rats to the phytoestrogen genistein (Cotroneo *et al.* 2001) and diethylstilbestrol (Atanassova *et al.* 2001, McKinnell *et al.* 2001) caused an alteration in ERα expression, but not ERβ, in the female and male tract respectively. However, in contrast to the efferent ductules, ERβ mRNA may be regulated by androgens in the prostate. Castration down-regulates ERβ mRNA in the prostate, and testosterone restores it (Prins *et al.* 1998, 2001, Shughrue *et al.* 1998).

The antiestrogen ICI 182,780 also has no effect on ERβ, although it leads to a dramatic down-regulation of ERα in the rat efferent ductules (Oliveira *et al.* 2003). In agreement with these results, target disruption of ERβ has failed to cause major abnormalities in the male reproductive tract (Krege *et al.* 1998). Given the widespread expression of ERβ along the male reproductive tract, this lack of response to ligation, castration or hormonal modulation may be indicative that ERβ has a constitutive expression and a non-essential function in the male tract. Another possibility is that the factor that regulates ERβ is most likely of extra-testicular origin. A plausible candidate for regulation of ERβ in the efferent ductules would be luteinizing hormone (LH). It has already been shown that LH receptors (LHR) are present in the male tract (Derecka *et al.* 1999), and that there are correlations between ERβ and LH/LHR concentrations (Bao *et al.* 2000, Guo *et al.* 2001).

Using a similar experimental approach to the present one, Goyal *et al.* (1998) did not find any difference in the regulation of AR and ER in the efferent ductule of the goat. In the goat, both receptors appeared to be regulated by circulating androgens. However, it is necessary to highlight the fact that ER subtypes were not differentiated in that study, nor was the regulatory effect of estrogen and DHT investigated. In addition, other studies have shown differences in sex steroid receptor expression and steroid hormone action along the female reproductive tract between ruminants and other mammals (Miller *et al.* 1979, Stone *et al.* 1982), pointing to species differences in ER regulation. Differences in ER distribution in the male tract of rat and goat can be exemplified by the negative staining of goat efferent ductule ciliated cells and Leydig cells in the testis, both of them being positive for ERs in the rat. Confirming that diverging results may be attributed to species differences, to date the only species showing agonist uterotropic action for the antiestrogen ICI 182,780 is the ewe (al-Matubsi *et al.* 1998), in contrast to rat, mice, pig, monkey and human (Wakeling *et al.* 1991, Dukes *et al.* 1993, Thomas *et al.* 1994, Branham *et al.* 1996, Tarleton *et al.* 1999).

In conclusion, the present observations indicate that ERα, ERβ and AR are differentially regulated in the efferent ductule, where AR and ERα are selectively modulated by their own ligand, but ERβ appears to be constitutively expressed. The potential for multiple factors to be regulating the expression of several steroid hormone receptors in some cells, highlights the importance of maintaining a physiological androgen and estrogen balance to regulate the structure and function of efferent ductules in the male.

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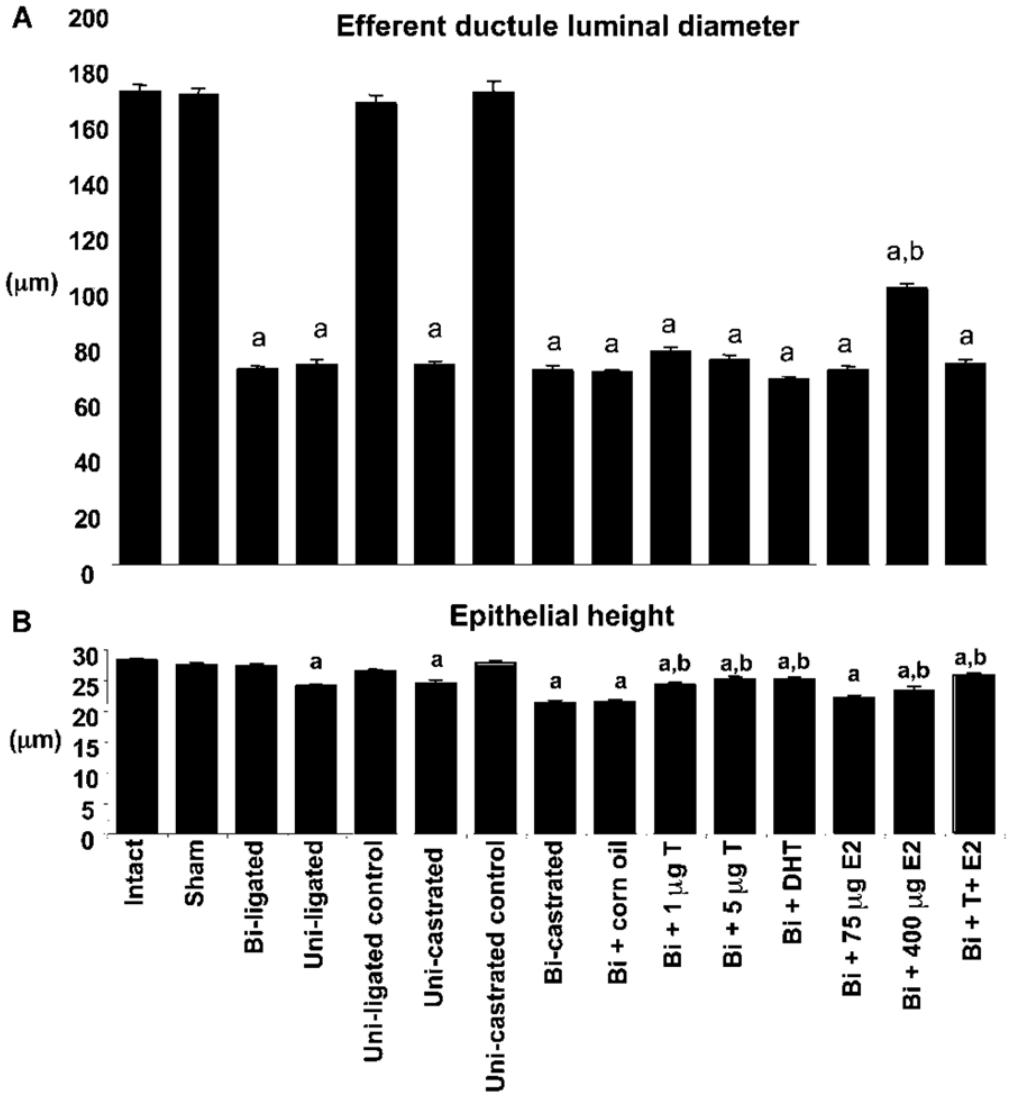

Figure 1.

Effects of ligation, castration and hormone replacement on the efferent ductule. (A) Compared with the intact rat, the luminal diameter of the efferent ductule was significantly (a) decreased in ligated and castrated rats, following or not hormone replacement. However, compared with the castrated control animal, the luminal diameter was significantly (b) higher after treatment with the high dose of estradiol (E2; 400 μg). (B) The height of the epithelium decreased significantly (a) after unilateral ligation or castration followed or not by hormone replacement. Testosterone (T; 1 mg and 5 mg), DHT (5 mg) and estradiol (75 μg and 400 μg) caused a partial recovery of the epithelial height, compared with the castrated rat. Higher epithelium was seen after replacement with testosterone (5 mg) + estradiol (400 μg). Values represent means ±S.E.M. ($n = 3$ in each group). a, $P \leq 0.05$ compared with intact rat; b, $P \leq 0.05$ compared with castrated rat. Uni, unilateral; Bi, bilateral.

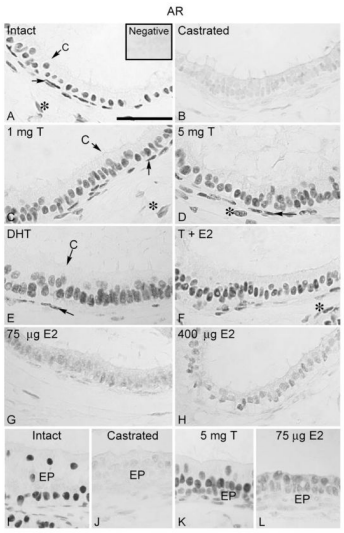

Figure 2.

Regulation of AR in the efferent ductule of rat. (A) AR was intensely expressed in the nuclei of the efferent ductule epithelial, peritubular (arrow) and some stromal cells (*) of intact control rats. C, ciliated cell. Insert represents the negative control. (B) After castration, there was a dramatic decrease in the AR expression in the efferent ductule. Replacement with (C) 1 mg testosterone (T), (D) 5 mg testosterone, (E) 5 mg DHT, as well as (F) 5 mg testosterone associated with 400 μg estradiol (E2) restored AR immunostaining to the control levels. A slight recovery of AR staining was observed after estradiol (75 μg and 400 μg) replacement in the efferent ductules (G, H). The initial segment of the epididymis (EP) was positive for AR (I). The AR staining decreased after castration (J), but testosterone replacement restored the positive reaction (K). Estradiol replacement also induced a slight recovery of AR staining in the initial segment (L). The results regarding the efferent ductule of sham-operated, unilateral or bilateral ligated and unilateral castrated rats were similar to those of intact controls (not shown). Bar = 100 μm.

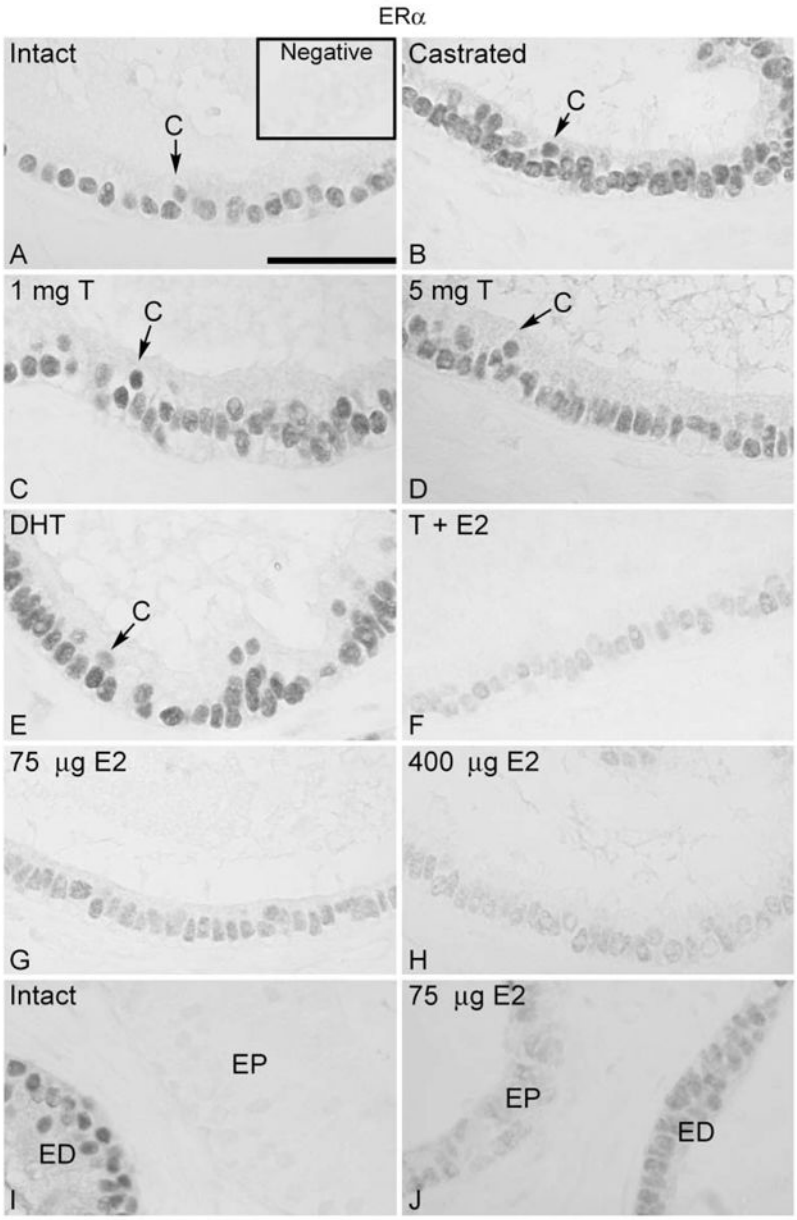

Figure 3.

Regulation of ERα in the efferent ductule of the rat. (A) ERα was expressed in the nuclei of the ciliated (C) and nonciliated cells of efferent ductule epithelium in intact control rats. Insert represents the negative control. Castration (B) or castration followed by androgen replacement (C, D, E) did not affect ERα expression. After replacement with 400 µg estradiol (E2) associated with 5 mg testosterone (T) (F), or with estradiol (75 µg and 400 µg) alone (G, H) there was a remarkable decrease in ERα expression. The higher dose of estradiol induced a greater decrease in ERα (F, H). (I) The initial segment of the epididymis (EP) was negative for ERα in control animals. ED, distal efferent ductules. (J) After replacement with estradiol (75 µg and 400 µg) a slight staining for ERα was found in the initial segment epithelium (EP). The results from efferent ductules of sham-operated, unilateral or bilateral ligated and unilateral castrated rats were similar to those of intact controls (not shown). Bar = 100 µm.

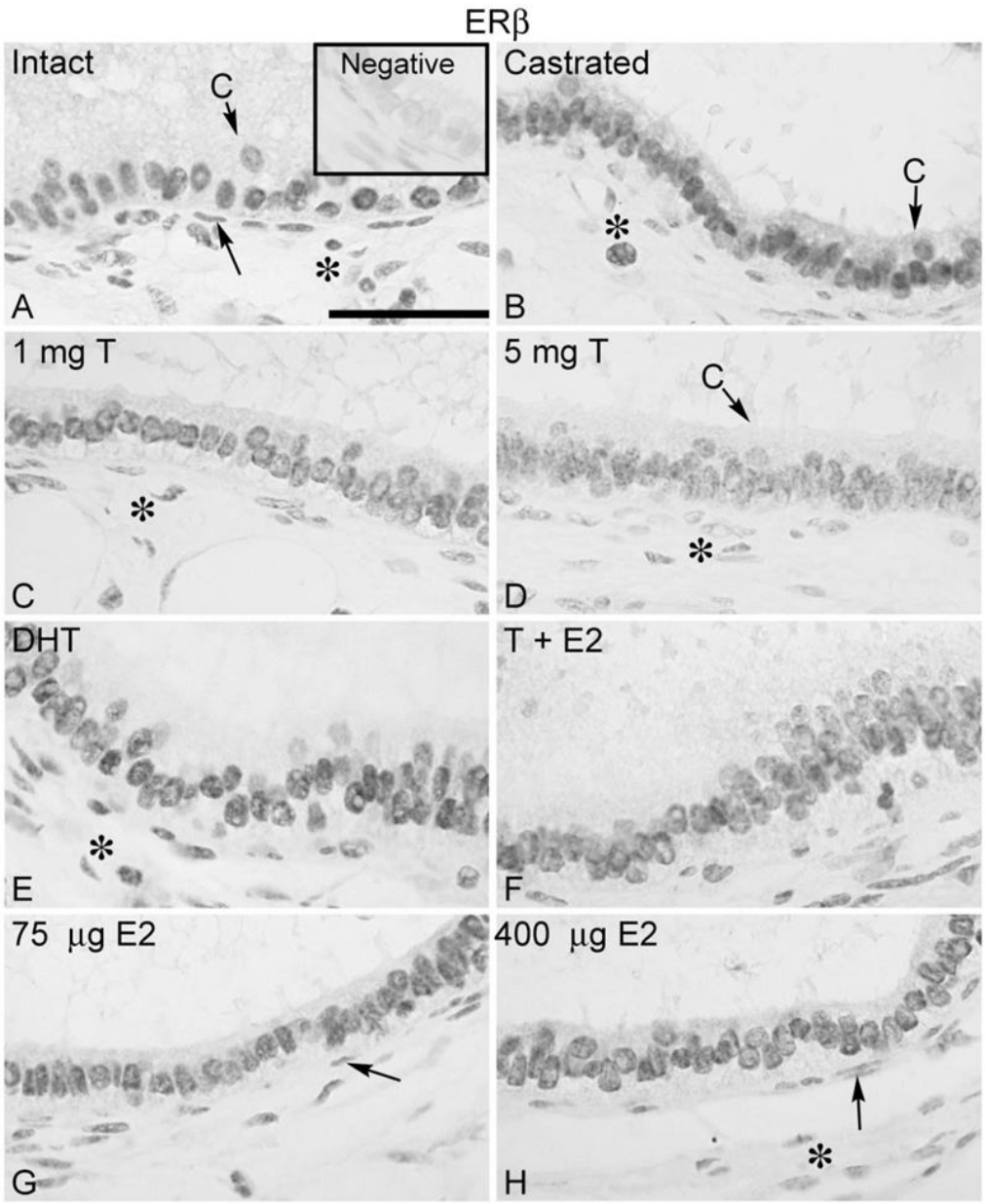

Figure 4.

Regulation of ERβ in the efferent ductule of the rat. (A) ERβ was expressed in the nuclei of the efferent ductule epithelium, peritubular (arrow) and some stromal cells (*) in intact control rats. C, ciliated cells. Insert represents the negative control. Castration (B) or castration followed by replacement with 5 mg testosterone (T) (C, D), 5 mg DHT (E), 400 µg estradiol (E2) associated with 5 mg testosterone (F) or with estradiol alone (75 µg and 400 µg) (G, H) did not cause evident effects in ERβ expression, except for a slight decrease in staining in some areas after testosterone replacement (D). The results from efferent ductules of unilateral or bilateral ligated and unilateral castrated rats were similar to those of bilateral castrated rats (not shown). Bar = 100 µm.

Table 1

Effects of rat efferent ductile ligation, castration and hormonal replacement on plasma testosterone (T) and estradiol (E2) concentrations and on body weight. Values are means±S.E.M.

			Body weight	
Treatment	**Testosterone (ng/ml)**	**Estradiol (pg/ml)**	**Initial (g)**	**Final (g)**
Intact control	3.6 ± 0.5	17.8 ± 5.8	ND	396.1 ± 2.7
Bilateral ligation	3.4 ± 0.8	7.9 ± 1.3	360.4 ± 3.2	375.6 ± 2.4
Unilateral ligation	4.2 ± 0.8	8.1 ± 2.2	365.9 ± 8.0	406.3 ± 9.0
Unilateral castration	3.3 ± 0.2	4.9 ± 1.1	366.7 ± 4.4	389.9 ± 11.0
Bilateral castration	0.1 ± 0.0*	5.7 ± 1.5	355.1 ± 16.0	389.0 ± 9.2
Sham operation	3.3 ± 0.7	3.9 ± 0.4	367.5 ± 3.2	400.2 ± 12.9
Bicastration + corn oil (control)	0.2 ± 0.0*	4.9 ± 1.0	366.0 ± 5.7	381.9 ± 1.9
Bicastration + T (1 mg)	10.8 ± 1.3*	7.2 ± 0.4	375.6 ± 4.0	384.8 ± 2.4
Bicastration + T (5 mg)	>20.0 ± 0.0*	6.7 ± 1.1	381.7 ± 2.1	393.2 ± 5.2
Bicastration + DHT (5 mg)	10.4 ± 0.7*	3.9 ± 0.2	375.6 ± 10.4	400.6 ± 10.7
Bicastration + E2 (75 μg)	0.2 ± 0.0*	>80.0 ± 0.0*	378.2 ± 3.2	331.5 ± 8.8*
Bicastration + E2 (400 μg)	0.1 ± 0.0*	>80.0 ± 0.0*	372.6 ± 5.7	284.5 ± 4.6*
Bicastration + T (5 mg) + E2 (400 μg)	14.1 ± 3.4*	69.0 ± 8.3*	372.7 ± 2.1	319.9 ± 3.7*

* $P \leq 0.05$ compared with intact control; ND, not determined.

Table 2

Effects of efferent ductile ligation, castration and hormonal replacement on the weights of the efferent ductile/epididymis, ventral prostate, and combined seminal vesicles (SV)/coagulating glands (CG) in the rat. Values are means±S.E.M.

	Efferent ductules + epididymis			
Treatment	**Left (mg)**	**Right (mg)**	**Ventral prostate (mg/100 g BW)**	**SV + CG (mg/100 g BW)**
Intact control	170.6 ± 4.7	170.0 ± 2.8	257.1 ± 8.0	481.1 ± 24.0
Bilateral ligation	133.3 ± 2.7*	136.7 ± 4.8*	172.6 ± 16.3	428.3 ± 35.7
Unilateral ligation	161.6 ± 5.8	110.6 ± 5.9**	203.1 ± 11.1	398.8 ± 20.3
Unilateral castration	169.5 ± 9.4	122.9 ± 5.5**	252.9 ± 35.8	464.2 ± 18.1
Bilateral castration	55.4 ± 0.9*	60.3 ± 3.2*	26.3 ± 1.2*	67.7 ± 4.3*
Sham operation	187.8 ± 10.9	161.5 ± 17.8	196.9 ± 27.2	440.5 ± 14.4
Bicastration + corn oil (control)	56.8 ± 0.2*	56.6 ± 5.7*	25.0 ± 0.7*	74.8 ± 2.9*
Bicastration + T (1 mg)	148.4 ± 2.9*	148.8 ± 4.9*	348.7 ± 38.6*	950.2 ± 33.0*
Bicastration + T (5 mg)	147.5 ± 10.8*	147.5 ± 13.2*	379.6 ± 11.3*	929.2 ± 3.2*
Bicastration + DHT (5 mg)	147.1 ± 11.9*	142.8 ± 8.2*	362.4 ± 19.4*	866.8 ± 10.8*
Bicastration + E2 (75 μg)	63.8 ± 4.2*	81.7 ± 13.7*	42.5 ± 3.6*	103.2 ± 15.5*
Bicastration + E2 (400 μg)	69.1 ± 7.6*	90.7 ± 18.5*	51.0 ± 9.4*	104.6 ± 9.0*
Bicastration + T (5 mg) + E2 (400 μg)	157.6 ± 4.5	169.8 ± 5.1	466.8 ± 18.5*	1206.9 ± 56.7*

T, testosterone; E2, 17β-estradiol.

* $P \leq 0.05$ compared with intact control;

** $P \leq 0.05$ compared with castrated control.