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HORMONAL EFFECTS ON THE ESTROGEN RECEPTOR SYSTEM IN THE EPIDIDYMIS AND ACCESSORY SEX ORGANS OF SEXUALLY IMMATURE RABBITS

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Summary—The epididymis and male accessory sex organs (vesicular gland, prostate, and bulbourethral gland) of sexually immature rabbits contain a functional estrogen receptor system which is regulated in an organ-specific manner by various hormones. In both intact and castrated animals, acute estrogen challenge causes depletion of estrogen receptor from the cytosolic fraction and its appearance in the nuclear fraction of these tissues. A considerable amount of unoccupied nuclear receptor was detected both before and after estrogen challenge. An estrogen-activated, receptor-processing mechanism is operable in these organs since chronic treatment (daily for 14 days) with estradiol benzoate modified the levels of total estrogen receptor, and altered the relative amounts of occupied to unoccupied nuclear receptor present following estrogen challenge. Chronic treatment with estradiol benzoate, Tamoxifen, and testosterone propionate (alone and in combination) had differential, organ-specific effects on the ability of subsequent estrogen challenge to cause accumulation of nuclear receptor. The vesicular gland was the most responsive to estrogen treatment and the bulbourethral gland the least responsive.

INTRODUCTION

We have previously demonstrated that the rabbit epididymis [1–2] and accessory sex organs [3] (vesicular gland, proprostate, prostate, and bulbourethral glands as defined by Holtz and Foote [4]), contain estrogen receptors and we have shown that there is an age-dependent decrease in the concentration of available cytosolic estrogen receptor in the epididymis and in the accessory sex organs [3]. We have further noted that there is a shift in the sedimentation coefficient of the cytosolic estrogen receptor of the epididymis on low ionic strength (0.01 M KCl), 5–20% sucrose gradients from an $\simeq$8S form in immature rabbits to an $\simeq$4S form in adult animals [5]. The presence of estrogen receptors in the male reproductive tract and the changes in their concentration and physicochemical properties with age suggested to us that estrogens might be involved in the physiological regulation of the tract.

To approach an understanding of the effects of estrogens on the male reproductive tract and to determine if there might be interactions between estrogens and androgens in regulating the development and maintenance of the tract, we conducted a series of experiments involving the administration of hormones to intact sexually immature rabbits or involving testis ablation and hormone replacement. When the epididymides and accessory sex organs from these animals were weighed and evaluated histologically [6], the data indicated that the administration of exogenous estrogens resulted in specific differential alterations in the histology of the organs. The studies further showed that estradiol alone was able to increase the weight of the epididymis, the vesicular gland, and the prostatic complex above intact control values. To examine the biochemical mechanisms that might underlie the effects of estrogen on the male reproductive tract and to gain information concerning the possible hormonal regulation of the estrogen receptor, we studied the effects of hormone treatment on the level and distribution of estrogen receptor in the classically defined cytosolic and nuclear compartments of the epididymis and accessory sex organs of intact and castrated sexually immature rabbits. The result of these studies are presented in this communication.

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EXPERIMENTAL

Chemicals

[2,4,6,7-^{3}H]Estradiol (115 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). Periodic evaluation of the compound by thin-layer chromatography indicated that its radiochemical purity was greater than 95% during the period of use. Estradiol benzoate (EB, 40 mg/ml in sesame oil) was obtained from Consolidated Midland Corp. (Brewster, NY). Testosterone propionate (TP, 50 mg/ml in sesame oil) was obtained from Eli Lilly and Co. (Indianapolis, IN), Tamoxifen citrate (Tamoxifen) was a gift from Stuart Pharmaceuticals (Wilmington,

DE). The following suspensions for injection were prepared in sesame oil: EB, 250 μg/ml, TP 20 mg/ml, Tamoxifen, 2.5 mg/ml. Diethylstilbestrol (DES), neutralized charcoal (Norit I), Dextran 60C, and sucrose (Grade I) were obtained from Sigma (St Louis, MO). Unlabeled estradiol was obtained from Steraloids (Wilton, NH). Nembutal was obtained from Abbott Laboratories (North Chicago, IL). Spectrafluor was obtained from Amersham–Searle (Arlington Heights, IL). All other chemicals were reagent or analytical grade.

Animals

Sexually immature (approx 6 weeks of age at the start of treatment) New Zealand White rabbits were used for these studies. The animals were either left intact or were castrated via an abdominal incision under Nembutal anesthesia (approx 30 mg/kg). Animals were either untreated or received daily injections (s.c.) of the test compound (sesame oil, 0.1 ml/kg; EB in sesame oil, 25 μg/kg; Tamoxifen in sesame oil, 250 μg/kg; TP in sesame oil, 2 mg/kg; or combinations of these treatments for 2 weeks. The doses of EB and Tamoxifen were based on those used by Koseki *et al.*[7]. The dose of TP was based on that of Danzo and Eller[8]. Either 24 or 72 h after the last administration, animals were challenged or not with 200 μg of unlabeled estradiol (0.2 ml of 1 mg/ml estradiol in ethanol plus 0.8 ml of 0.85% saline, i.v.). This estradiol dosage is comparable to that shown to induce maximal accumulation of estrogen receptor in nuclei of the immature rat uterus [9]. One hour after this injection, animals were killed with a lethal dose of Nembutal. Epididymides and accessory sex organs were removed and trimmed of fat and connective tissue. The accessory sex organs were divided into vesicular gland, prostatic complex (which includes the proprostate and prostate), and bulbourethral glands. All tissues were immersed in ice-cold TE buffer (10 mM Tris–HCl, pH 7.5, 1.0 mM EDTA). Epididymides were processed immediately, while accessory sex organs were sometimes frozen on solid carbon dioxide, stored at −70°C, and processed at a later date.

Tissue fractionation

All operations were performed at 0–4°C. The tissues were weighed, minced, and homogenized 1:4 (w/v) in TE using motor driven, glass–glass homogenizers. The homogenates were centrifuged for 10 min at 1500 ***g*** to obtain a nuclear-myofibrillar pellet, and an initial supernatant, which was centrifuged at 249,000 ***g*** for 30 min to yield the cytosol fraction. The pellets were washed twice with 8 ml TE and resuspended in 4.5 ml TE.

Cytosol binding assay

Unoccupied estrogen binding sites in the cytosol fraction were quantified by incubating 300 μl of cytosol (in triplicate) with 6.7 nM [^{3}H]estradiol alone, or in combination with 1.3 μM DES, in a total vol of 0.5 ml for 18 h at 0–4°C. At the conclusion of the incubation, 0.5 ml of a 0.5% charcoal–0.05% dextran suspension in TE was added to each sample. Following agitation on a Vortex mixer, samples were kept on ice for 10 min. The charcoal was then sedimented by centrifugation for 5 min at 1500 ***g***, and the supernatants containing macromolecular bound steroid were decanted into scintillation vials and counted in 5 ml of scintillation fluid containing Triton X-100, toluene, and spectrafluor (1230:2365:100, by vol.). The counting efficiency was 32%. Specifically bound ([^{3}H]estradiol was calculated as the difference between that bound in the absence and presence of DES.

Nuclear binding assay

An exchange assay based on that of Andersen *et al.*[9] was used to measure total estrogen-binding activity in the nuclear fraction. The exchange assay consisted of incubating 500 μl of the resuspended nuclei with approx 6.7 nM [^{3}H]estradiol alone, or in combination with 1.3 μM DES, in a final volume of 0.7 ml for 1 h in a shaker water bath at 36°C. To quantify unoccupied estrogen binding sites in the nuclear fraction, a parallel set of samples was incubated for 18 h at 0–4°C. At the conclusion of the incubation, 8 ml of ice-cold TE were added to each sample. The nuclei were sedimented by centrifugation at 1500 ***g*** for 10 min. The wash step was repeated once. The pellets from the second wash step were resuspended in 3 ml of room temperature 100% ethanol by agitation on a Vortex mixer. The ethanol was decanted into scintillation vials and the samples were counted in 10 ml of scintillation fluid containing Spectrafluor and toluene (100:2365, v/v). The counting efficiency was 55%. Occupied receptor was calculated as the difference between total and unoccupied receptor.

Sucrose gradient analysis

Aliquots (200 μl) of cytosol were incubated with 6.7 nM [^{3}H]estradiol alone, or in combination with 1.3 μM DES in a total volume of 250 μl for 2 h at 0–4°C. Samples were then extracted for 10 min with charcoal pellets that had been obtained by sedimenting 0.5 ml of the above charcoal–dextran suspension, and aliquots (200 μl) of the extracted samples were layered onto sucrose gradients. Linear 5–20% sucrose gradients containing 10% glycerol and 0.01 M KCl were prepared, run, and analyzed as previously described [10].

Circulating hormone levels

In some cases, blood samples (approx 15 ml collected *via* ear vein into heparinized tubes) were obtained from animals 24 h after final hormonal administration, and just prior to estrogen challenge. Plasma fractions were stored at −20°C and subsequently assayed for testosterone, 5α-dihydrotestosterone and estradiol as described by Abraham *et al.*[11].

Statistical evaluation

The significance of differences in receptor levels among treatment groups was determined by analysis of variance followed by Duncan's Multiple Range Test [12]. The significance of differences in organ weights and plasma hormone levels was determined using Student's t-test [12]. Differences in all cases were only considered to be significant when $P < 0.05$.

RESULTS

Estrogen effects on tissue weight and cytosolic estrogen receptor levels in the epididymis and vesicular gland of sexually immature rabbits

While previous studies have indicated that reproductive tract organs of the sexually immature male rabbit contain physicochemically normal estrogen receptors [1, 3, 5], the data presented in [2] and Table 1 provide evidence that these receptors are also functionally normal. In intact, oil-treated, non estrogen-challenged control animals (group 1), the epididymis and vesicular gland yielded similar concentrations (fmol/100 mg tissue) of cytosolic estrogen receptor. Following removal of the testes (group 2), both organs underwent a less than 50% reduction in weight but the receptor concentration increased 3.4-fold (epididymis) and 2.0-fold (vesicular gland). As previously observed [6], chronic estrogen exposure (group 3) caused a precocious increase in the weight of the epididymis and vesicular gland. Compared with intact controls (group 1), the 14 days of estrogen treatment enhanced epididymal weight by 2.6-fold ($P < 0.001$) and vesicular gland weight by 9.5-fold ($P < 0.001$).

Chronic estrogen exposure (group 3) caused the organ content (fmol/organ) of cytosolic estrogen receptor to decrease by 73% in the epididymis (vs group 2) and to increase slightly in the vesicular gland (vs group 2). However, owing to the increase in organ weights, a decrease in receptor concentration occurred (>90% group 3 compared to group 2). Estrogen challenge 1 h prior to sacrifice (group 4) brought the concentration and content of cytosolic receptor in both tissues to nearly undetectable levels. These results demonstrate estrogen-dependent depletion of cytosolic receptor. Therefore, we studied the influence of various hormone treatments on estrogen-induced accumulation of receptor in the nuclear compartment of various reproductive tract organs.

Circulating hormone levels

To determine the level of endogenous and exogenous hormones to which the reproductive tract tissues of the various experimental groups were exposed, we measured the plasma levels of androgens and estrogens. In intact animals (Table 2), estradiol administration, alone or in combination with Tamoxifen, resulted in a statistically significant ($P < 0.01$) depression of androgen levels. In the absence of exogenous estradiol, there was no detectable estradiol in the plasma. Tamoxifen alone did not alter plasma androgen levels, suggesting that it lacks an antigonadotrophic action at the dose level used. Androgen treatment alone and in combination with estrogen had no statistically significant effect on testosterone levels in the intact animal, but did cause a significant ($P < 0.01$) drop in DHT levels. As anticipated, the plasma androgen levels in the cas-

Table 1. Estrogen effects on tissue weight and cytoplasmic estrogen receptor levels in the epididymis and vesicular gland of sexually immature rabbits

					Cytosolic estrogen receptor	
Group	Treatment	E_2 challenge	Organ	wt (g)[a]	fmol/ organ	fmol/ 100 mg tissue
1	Intact oil	—	Epididymis	0.21 ± 0.03 (8)	256	116
			Vesicular	0.12 + 0.02 (4)	185	154
2	Castrate oil	—	Epididymis	0.16 ± 0.01 (7)	610	394
			Vesicular gland	0.07 ± 0.01 (4)	280	314
3	Castrate EB	—	Epididymis	0.54 ± 0.05 (8)*	165	31
			Vesicular gland	1.14 ± 0.12 (4)*	358	31
4	Castrate EB	+	Epididymis	0.49 ± 0.05 (7)*	30	6
			Vesicular gland	1.02 ± 0.24 (4)*	48	5

Groups of 4 rabbits were either left intact or castrated and then injected (s.c.) daily for 14 days with vehicle (sesame oil) or 25 μg/kg estradiol benzoate (EB). All animals were killed 24 h after the last injection. One of the castrated plus EB-treated groups received an injection (i.v.) of 200 μg estradiol 1 h prior to sacrifice (E_2-challenge). The epididymides and vesicular glands from each animal in a group were weighed and then pooled for cytosol preparation. Both the concentration (fmol/100 mg tissue) and content (fmol/organ) of estrogen receptor in a cytosol sample were determined as described in the Experimental section.

[a]The weights are the mean ± SEM of (n) organs. Treatments were compared to control (group 1) using Student's t-test. Values marked with an asterisk are significantly different ($P < 0.05$) from control. Values for cytoplasmic estrogen receptor levels are the means for each pool of tissues.

Table 2. The effects of various treatments on plasma androgen and estrogen levels in intact rabbits

Treatment	T (ng/ml)	DHT (ng/ml)	E_2 (pg/ml)
Control	2.54 ± 0.67 (14)	1.18 ± 0.31 (14)	ND (4)
EB	0.09 ± 0.03 (4)*	0.14 ± 0.11 (4)*	7.5 ± 4.8 (4)*
Tamoxifen	1.83 ± 1.50 (3)	0.62 ± 0.37 (3)	ND
EB + Tamoxifen	0.10 ± 0.03 (4)*	0.06 ± 0.01 (4)*	10 ± 0 (4)*
TP	1.02 ± 0.51 (4)	0.30 ± 0.04 (4)*	ND
EB + TP	2.18 ± 1.04 (4)*	0.60 ± 0.25 (4)*	12.5 ± 4.8 (4)*

Abbreviations: EB, estradiol benzoate; TP, testosterone propionate; T, testosterone; DHT, dihydrotestosterone; E_2, estradiol; ND, not detectable. Groups of intact animals were injected (s.c.) daily for 14 days with either vehicle (sesame oil, control), EB (25 μg/kg), Tamoxifen (250 μg/kg), TP (2 mg/kg), or combinations of these agents. Blood was collected 24 h following the last injection, and plasma levels of T, DHT, and E_2 were measured by radioimmunoassay. The values shown are the mean ± SEM of (n) observations. Treatments were compared to control using Student's t-test. Values marked with an asterisk are significantly different ($P < 0.05$) from intact controls.

Table 3. The effect of various treatments on plasma androgen and estrogen levels in castrated rabbits

Treatment	T (ng/ml)	DHT (ng/ml)	E_2 (pg/ml)
Control (castrated)	0.08 ± 0.02 (4)*	0.02 ± 0.01 (4)*	ND (4)
EB	0.07 ± 0.01 (4)*	0.02 ± 0.02 (4)*	20 ± 7.0 (4)*
Tamoxifen	0.15 ± 0.08 (4)*	0.09 ± 0.02 (4)*	ND (4)
EB + Tamoxifen	0.08 ± 0.01 (4)*	0.06 ± 0.0.01 (4)*	8.6 ± 3.5 (4)*
TP	1.04 ± 0.05 (4)*	0.34 ± 0.14 (4)*	ND (4)
EB + TP	2.58 ± 0.80 (4)	1.46 ± 0.67 (4)	8.25 ± 4.44 (4)*

Abbreviations: See legend for Table 2. Groups of castrated animals were treated and assayed for plasma hormone levels as described in legend for Table 2. The values shown are the mean ± SEM of (n) observations. Treatments were compared to control using Student's t-test. Values marked with an asterisk are significantly different ($P < 0.05$) from intact control.

trated rabbits were low (Table 3). Neither estradiol nor Tamoxifen, alone or in combination, influenced these levels. Testosterone administered alone failed to maintain plasma androgen levels at intact values. However, the combination of testosterone and estradiol did maintain normal testosterone and DHT levels. Plasma estradiol was detected in only those animals that had received exogenous estrogen (Tables 2 and 3).

Total nuclear estrogen receptor levels following estrogen challenge

See Orgebin-Crist *et al.* [6, group A, Tables 1–4] for weights of the epididymides and accessory sex organs used for this study.

Epididymis. Following estrogen challenge, appreciable levels of total estrogen receptor were detected in epididymal nuclei of intact (Table 4) and castrated (Table 5) animals, regardless of the hormone pretreatment regimen. Because of wide variability among the animals in their response, no statistically significant differences among the treatment groups were seen. However, some general trends can be noted.

In intact animals (Table 4), the age-dependent (14-day) increase in epididymal weight [6] was accompanied by an increase in receptor content (Table 4, zero-time control vs intact oil treated control), and, therefore, the receptor concentration remained the same. Although 14 days of estrogen treatment caused greater than a 2-fold increase in epididymal weight, Table 1 and [6], both the estrogen receptor content and concentration (fmol/100 mg tissue) were reduced as compared to oil-treated controls. This is a reflection of the fact that there was not an increase in receptor levels proportional to the increase in organ weight. Tamoxifen alone, which failed to influence the epididymal growth response [6], reduced the accumulation of nuclear receptor (vs zero-time and oil controls). As the dosage used, Tamoxifen failed to block the estrogen-induced reduction in nuclear receptor concentration (Table 4). Androgen supplementation had no effect on epididymal weight [6] or on the accumulation of nuclear estrogen receptor when given alone or in combination with estrogen (Table 4).

Table 4. Total nuclear estrogen receptor content and concentration in epididymides of intact immature rabbits

Treatment	Total nuclear estrogen receptor	
	fmol/ epididymis	fmol/ 100 mg tissue
Zero-time (6)[a,b] control	726 ± 290	677 ± 260
Intact oil (6) control	1089 ± 360	546 ± 142
Intact (5) plus EB	480 ± 90	105 ± 20
Intact (6) plus Tamoxifen	393 ± 120	238 ± 82
Intact (6) EB plus Tamoxifen	569 ± 150	143 ± 39
Intact (6) plus TP	1038 ± 570	477 ± 234
Intact (6) EB plus TP	1135 ± 470	409 ± 170

Abbreviations: See Table 2. Animals were treated or not as described in the legend to Table 2 and 24 hr after the last treatment were challenged with estradiol (200 μg, i.v.). All animals were killed 1 h later and epididymal levels of total nuclear estrogen receptor were determined as described in Experimental Sections. [a]Zero time control indicates animals assayed upon their arrival at the laboratory. They received no treatment. [b]The numbers in parentheses indicate the number of surviving animals in each group. The values represent the mean ± SEM of triplicate determinations on epididymides from (n) animals. When the values are compared using Duncan's Multiple Range Test [12], no statistically significant differences among the groups were obtained ($P < 0.05$).

Table 5. Total nuclear estrogen receptor content and concentration in epididymides of 2-week castrated immature rabbits

Treatment	Total nuclear estrogen receptor	
	fmol/ epididymis	fmol/ 100 mg tissue
Intact oil (6)[a] control	1089 ± 360	546 ± 142
2 wk castrated (6) control	265 ± 30	280 ± 33
2 wk castrated (5) plus EB	1038 ± 270	245 ± 71
2 wk castrated (6) plus Tamoxifen	776 ± 360	738 ± 313
2 wk castrated (4) EB plus Tamoxifen	929 ± 560	192 ± 95
2 wk castrated (5) plus TP	732 ± 270	374 ± 150
2 wk castrated (4) EB plus TP	692 ± 460	280 ± 220

Abbreviations and experimental procedures were the same as described in legend to Table 4, except that castrated animals were used. [a]The numbers in parentheses indicate the number of surviving animals in each group. The values represent the mean ± SEM of triplicate deterninations on epididymides from (n) animals. When the values were compared using Duncan's multiple range test [12], no statistically significant differences among the groups were obtained ($P < 0.05$).

Castration eliminated the 14-day growth response [6] and resulted in a reduction in nuclear estrogen receptor content and concentration (Table 5) as compared to both intact zero-time controls (Table 4) and intact oil-treated controls. The reduced accumulation of nuclear receptor in castrated animals following estrogen treatment and estrogen challenge was unexpected since castration increased the level of available cytoplasmic receptor in the epididymis (Table 1, group 1 vs group 2) of animals that were not treated with estradiol. In intact and castrated animals, estrogen induced similar increases in the weight of the epididymis Table 1 and [6], but both the content and concentration of nuclear receptor in castrated, estrogen-treated animals were twice that present in similarly treated, intact animals (Table 5 vs Table 4). Thus, estrogen treatment caused the content of nuclear estrogen receptor in castrated animals to equal that measured in intact oil-treated controls. Tamoxifen treatment of castrated animals had no effect on epididymal weight [6] but it maintained the receptor content and concentration of the epididymis near intact control levels (Table 5). Tamoxifen did not antagonize the estrogen-induced increase in receptor content over castrate levels (Table 5). Androgen administration maintained the epididymal weight [6] and the level of nuclear estrogen receptor in castrated animals at that of intact animals (Table 5). Neither of these androgen-induced effects in the castrated animals was affected by estrogen. These results indicate that various hormone treatments influence the ability of the estrogen challenge to induce nuclear accumulation of estrogen receptor in the epididymis of immature rabbits. Differences in results obtained using castrated and intact animals suggest that the testes can modulate this process.

Accessory sex organs of intact rabbits. In intact zero-time controls, the weight [6] and post estrogen-challenge content of total nuclear estrogen receptor (Fig. 1A) was greatest in the prostate. Compared to oil treatment (Fig. 1B), estrogen treatment resulted in increased weight [6] and receptor content in all organs (Fig. 1C), but only in the vesicular gland was the increase in receptor content statistically significant. Nuclear receptor content in the organs of animals treated with estrogen plus Tamoxifen (Fig. 1E) or androgen (Fig. 1G) was not significantly different from that in organs of oil-treated animals (Fig. 1B). Treatment with Tamoxifen alone or with androgen alone (which induced approx a 2-fold weight gain in all organs [6]) had no significant effect on receptor content in any organ (Fig. 1D and Fig. 1F respectively vs Fig. 1B).

Examination of the post estrogen-challenge concentration of total nuclear estrogen receptor showed an age-dependent (but not statistically significant) increase in all organs (Fig. 2A vs 2B). Compared to oil treatment (Fig. 2B), estrogen treatment, either alone (Fig. 2C) or in combination with Tamoxifen (Fig. 2E) or androgen (Fig. 2G), significantly ($P < 0.05$) reduced the receptor concentration in all organs except the bulbourethral glands. Androgen treatment alone also reduced the estrogen receptor concentrations (Fig. 2F vs Fig. 2B), but this effect was statistically significant only in the prostate. Tamoxifen treatment alone had no effect on receptor concentration in any organ (Fig. 2D vs Fig. 2B).

Accessory sex organs of castrated rabbits. The hormone response patterns of estrogen-induced accumulation of total nuclear estrogen receptor, in terms of both content (Fig. 3) and concentration (Fig. 4) was the same in castrated animals as that observed in intact animals. The results with both the intact and castrated animals indicate that different pretreatments can modulate subsequent nuclear accumulation of estrogen receptor.

Estrogen receptor dynamics in the epididymis and vesicular gland of sexually immature rabbits

While the preceding results demonstrate receptor depletion from the cytosol (Table 1) and its appearance in the nuclear compartment of male reproductive tract organs following estrogen exposure (Table 4 and 5, Figs 1–4), they do not provide direct evidence for estrogen-dependent translocation of the receptors. Therefore, we performed simultaneous receptor measurements in the cytosol and nuclear compartments of selected tissues (the epididymis and vesicular glands) of animals that had or had not been given an estrogen challenge. Nuclear receptor levels were sub-divided into total and occupied components to provide a clearer indication of the amount of translocated receptor present in a particular tissue.

In a castrated animal treated for 14 days with estrogen and then killed 24 h after the last estrogen injection (Fig. 5A), the concentrations of cytosolic estrogen receptor in the epididymis and vesicular

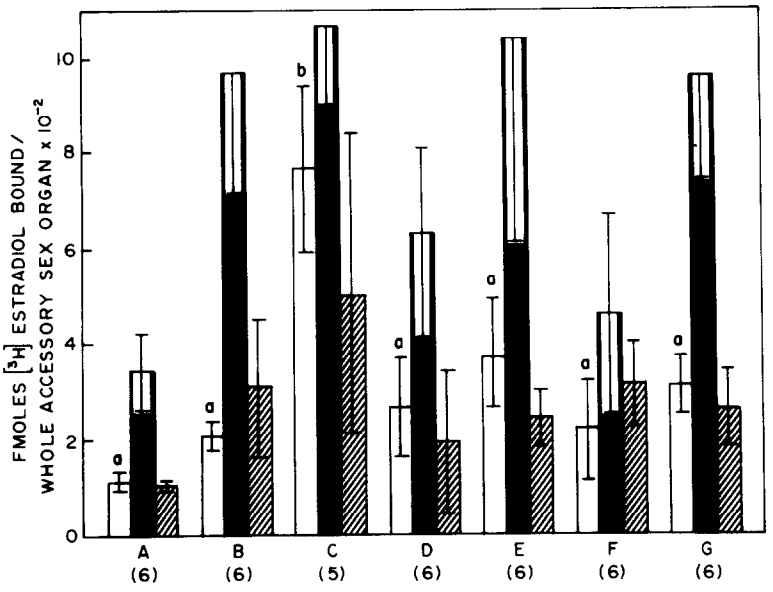

Fig. 1. Total nuclear estrogen receptor content of the vesicular gland (□), Prostatic complex (■), and bulbourethral gland (▨) of intact immature rabbits. Receptor levels were determined in accessory sex organs removed from rabbits upon their arrival at the laboratory, zero-time controls (A), or after having received daily subcutaneous injections of sesame oil (B), EB (C), Tamoxifen (D), EB plus Tamoxifen (E), TP (F), or EB plus TP (G) for 2 weeks. All animals were challenged with estradiol 24 h after the last injection and 1 h prior to sacrifice. Further experimental details are provided in the Experimental section. The values plotted represent the mean ± SEM of triplicate determinations on organs of (n) individual animals. Statistical evaluation of the data was performed using Duncan's Multiple Range test [12]. Bars referring to a given organ bearing the same letter are statistically the same. The absence of a letter above the bar indicates that the analysis of variance indicated no significant differences ($P < 0.05$).

gland were similar and comparable to those measured previously (Table 1, group 3). Although nuclear receptor was detected in both organs, it existed primarily in the unoccupied form. In a similarly treated, but estrogen-challenged animal (Fig. 5B), both organs contained less cytosol receptor and more nuclear receptor. Furthermore, the increase in total nuclear receptor levels reflected the appearance of

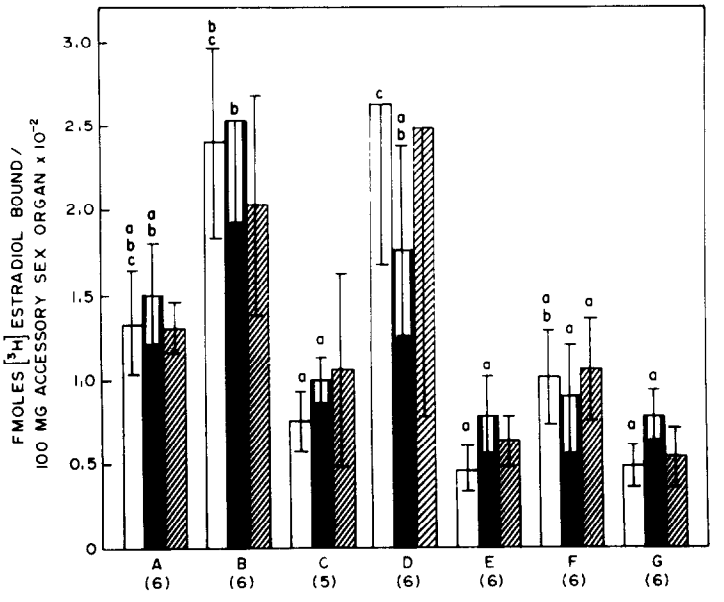

Fig. 2. Nuclear estrogen receptor concentration (fmol/100 mg tissue) in the accessory sex organs of sexually immature intact rabbits. Vesicular gland (□), prostate (■), bulbourethral gland (▨). Treatment groups A, zero-time control; B, intact oil control; C, EB; D, Tamoxifen; E, EB + Tamoxifen; F, TP; G, EB + TP. See legend to Fig. 1 and the Experimental section for experimental details and statistical evaluation.

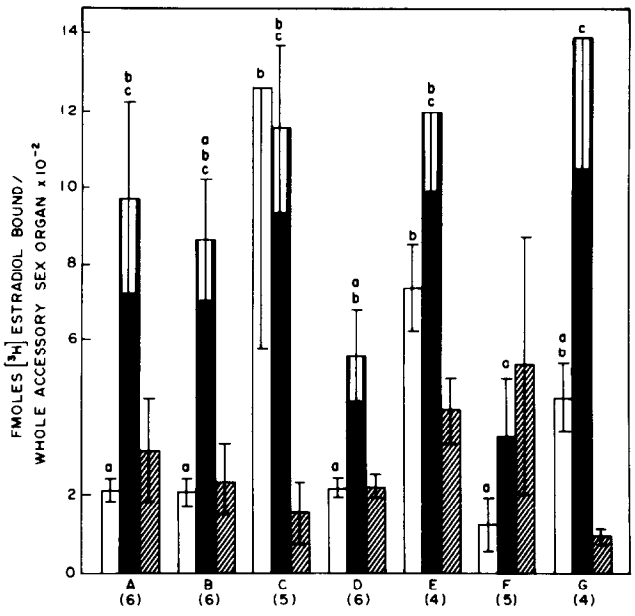

Fig. 3. Nuclear estrogen receptor content of the accessory sex organs of sexually immature castrated rabbits. Vesicular gland (□), prostate (■), bulbourethral gland, (▨). Treatment groups: A, intact oil control; B, 2 week castrate; C, castrate + EB; D, castrate + Tamoxifen; E, castrate + EB + Tamoxifen; F, castrate + TP; G, castrate + EB + TP. See legend to Fig. 1 and the Experimental section for experimental details and statistical evaluation.

occupied receptors. These results provide direct evidence for translocation of estrogen receptor in both organs.

We also observed time-dependent replenishment of estrogen-depleted cytosolic estrogen receptors in both the epididymis and vesicular gland. Extending the estrogen withdrawal period to 72 h had no effect on tissue weights (data not shown), but allowed cytosolic receptor concentrations in the epididymis and vesicular gland (Fig. 5C) to approach levels measured in castrated, untreated animals (Table 1, group 2). Furthermore, the cytosolic receptor content of the

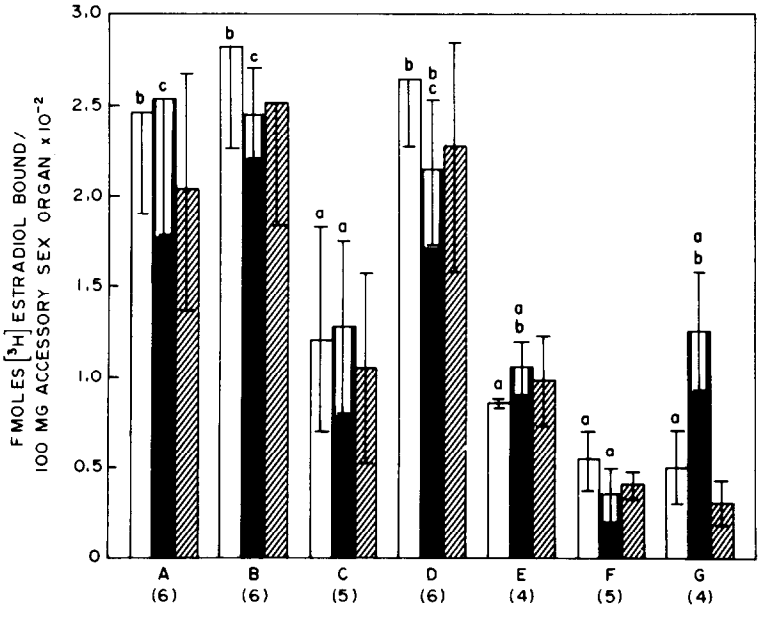

Fig. 4. Nuclear estrogen receptor concentration (fmol/100 mg tissue) in the accessory sex organs of sexually immature castrated rabbits. Vesicular gland (□), prostate (■), bulbourethral gland, (▨). Treatment groups: A, intact oil controls; B, 2 week castrate; C, castrate + EB; D, castrate + Tamoxifen; E, castrate + EB + Tamoxifen; F, castrate + TP; G, castrate + EB + TP. See legend to Fig. 1 and the Experimental section for experimental details and statistical evaluation.

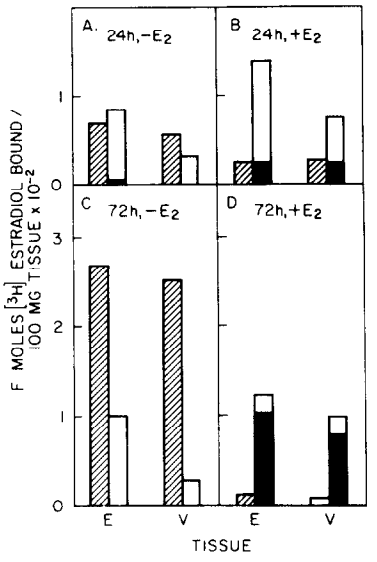

Fig. 5. Estrogen receptor dynamics in the epididymis and vesicular gland of sexually immature castrated rabbits. After castration, animals were treated for 14 days with EB (25 μg/kg/day). Either 24 h (A,B) or 72 h (C,D) after the last injection, animals were challenged (B,D) or not (A,C) with an intravenous injection of estradiol (200 μg) and killed 1 h later. The concentration of unoccupied cytoplasmic estrogen receptor (▨), total nuclear estrogen receptor (□) and occupied nuclear estrogen receptor (■) in the epididymis (E) and vesicular gland (V) of each animal was determined as described in the Experimental section.

epididymis and vesicular gland in an animal withdrawn from estrogen for 72 h (1.23 and 1.57 pmol, respectively) were considerably higher than in animals withdrawn for 24 h (Table 1, group 3) or in untreated animals (Table 1, group 2). It is also clear that the total receptor content of these organs increases between 24 and 72 h after estrogen withdrawal since the concentration of total nuclear receptor remained the same during this period (Fig. 5A,C). In addition, note that the nuclear receptors present 72 h after estrogen withdrawal are entirely in the unoccupied state (Fig. 5C). when an estrogen challenge was given to the 72 h withdrawn animals (Fig. 5D), a depletion of cytosolic receptor occurred. This was accompanied by the appearance of primarily occupied receptor in the nuclei (Fig. 5D).

When cytosolic receptor obtained from the epididymis and vesicular gland 72 h after estrogen withdrawal was analyzed on hypotonic sucrose gradients, it sedimented primarily in the oligomeric form (≥8S) along with a minor ~4S component (Fig. 6A,B). Identical behavior has been observed with cytosol receptor from untreated immature rabbits [3]. Both gradient (Fig. 6C,D) and charcoal analysis (Fig. 5D) reveal that estrogen challenge induces depletion of essentially all cytosolic receptor that is present in the epididymis and vesicular gland 72 h after estrogen withdrawal. Although this depletion phenomenon was accompanied by striking increases in the level of occupied nuclear receptors (Fig. 5D), not all the binding activity lost from the cytosol compartment of the organs was recovered in the nuclear compartment. It is also important to note, especially for the epididymis, that neither the duration of estrogen withdrawal nor estrogen challenge had an appreciable effect on the level of total nuclear receptor. These results further demonstrate that reproductive tract organs of immature rabbits possess a functional estrogen receptor system.

DISCUSSION

Our studies indicate that the estrogen receptor system present in reproductive tract organs of sexually immature male rabbits is subject to dynamic regulation by hormones. The interactions of estradiol, Tamoxifen, and androgens in this process occur in a tissue-specific and differential manner. The vesicular gland is the most responsive to estrogens and the bulbourethral gland is the least responsive. Castration results in an increase in the content and concentration (fmol/100 mg tissue) of unoccupied cytosolic estrogen receptor in those organs examined (epididymis and vesicular gland). It is unlikely that the precastration levels of circulating testosterone or dihydrotestosterone would have been sufficient to cause translocation of cytosolic estrogen receptor to the nuclei, because high levels are required for this to

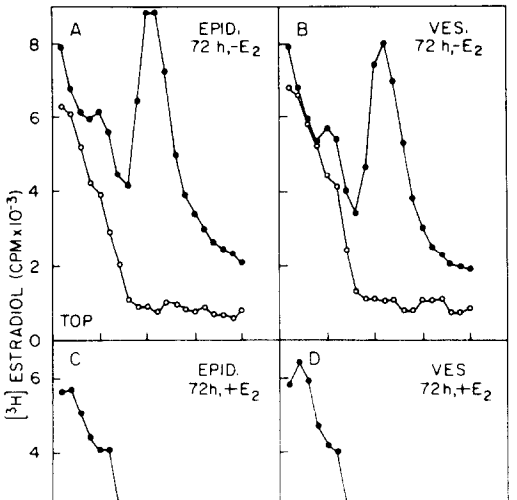

Fig. 6. Sucrose gradient analysis (0.01 M KCl) of estrogen binding activity in epididymal and vesicular gland cytosol of sexually immature castrated rabbits. Cytosol prepared from the epididymides (A,C) and vesicular glands (B,D) of those animals described for Fig. 5C (A,B) and Fig. 5D (C,D) were labeled with [^{3}H]estradiol alone (●) or in combination with DES (○) and analyzed on hypotonic (0.01 M KCl) sucrose gradients as described in the Experimental section.

occur [13–14]. The possibility exists that other androgen metabolites with a greater affinity for the estrogen receptor [15] may have caused depletion of cytosolic estrogen receptor in intact animals. However, the most likely explanation for the increase in available cytosolic estrogen receptor following castration is that endogenous estrogens, though undetectable in blood using our assay, caused nuclear translocation of the receptor in the intact animals.

Chronic estrogen treatment of castrated and intact rabbits results in further depletion of the cytosolic estrogen receptor. Twenty-four and 72 h after estrogen withdrawal, estrogen challenge resulted in loss of available estrogen receptor from the cytosolic compartment and accumulation in the nuclear compartment. The levels of receptor were strikingly different at the two time points. In animals that were not given an estrogen challenge, total estrogen receptor (cytosolic plus nuclear) increased with duration of estrogen withdrawal. In the 72 h withdrawn animal, estrogen challenge caused a decrease in the level of total receptor. These results suggest that estrogen treatment activates a receptor processing mechanism similar to that observed in cultured breast tumor cells [16] and in the mouse uterus [17]. Since occupied nuclear receptors were absent at the 24 h time point, synthesis [18] and/or reactivation [17], rather than simple recycling of receptors [19], must be responsible for the replenishment seen under these conditions.

The level of total nuclear estrogen receptor remained constant regardless of whether the animals were withdrawn for 24 or 72 h prior to estrogen challenge. Thus, considerable unoccupied nuclear estrogen receptor was detected at the 24 h point. Although some investigators report that unoccupied nuclear receptors are an artifact resulting from cytoplasmic contamination of crude nuclear preparations [20], others have presented evidence ruling out the cytoplasm as the source of this activity [21, 22]. Several studies have also shown that exposing cells and whole tissues to estrogen resulted in depletion of unoccupied cytosolic receptor, but did not alter the level of unoccupied nuclear receptor [21–23]. We observed similar results 24 h after estrogen withdrawal, but 48 h later, estrogen challenge reduced unoccupied cytosolic and nuclear receptor. Our results indicate that chronic estrogen treatment can modify the relative amounts of occupied and unoccupied nuclear receptor in the epididymis and vesicular gland. In the studies we conducted on the effects of hormones on nuclear estrogen receptor levels, only total receptor was examined. Therefore, if changes in the ratio of occupied to unoccupied receptor occurred, they would not have been detected.

The testis appears to regulate the ability of chronic exogenous estradiol and Tamoxifen treatment to modify subsequent estrogen-induced accumulation of nuclear receptor in the epididymis. Although estrogen treatment enhanced epididymal weight in intact and castrated rabbits to the same extent [6], nuclear receptor levels were higher in castrated animals. Tamoxifen treatment, which had no effect on epididymal weights in either group [6], maintained nuclear estrogen receptor in castrated animals at levels comparable to intact controls. Others [7] have observed that chronic treatment of ovariectomized rats with a dose of Tamoxifen comparable to that used in this study failed to support uterine growth. Such doses did, however, cause estrogen receptors to be retained in uterine nuclei for several days, while receptor reappeared in the cytosol [7]. Thus, in both the ovariectomized rat uterus and the immature castrated rabbit epididymis, it appears that Tamoxifen is defective in inducing the processing step which is thought to remove estrogen receptor complexes from the nucleus [16]. Nevertheless, Tamoxifen failed to block the estrogen-induced growth response [6] and the differential estrogen-dependent modification of nuclear receptor levels in epididymides and accessory sex organs of intact or castrated rabbits.

In intact animals, androgen treatment had no effect on the weight of the epididymis [6] or on nuclear estrogen receptor levels, but it did antagonize the ability of estradiol to enhance epididymal weight [6] and to reduce the concentration of nuclear receptors. Androgen treatment of castrated animals maintained epididymal weights at intact levels and blocked further stimulation by estradiol [6], but it failed to maintain the intact level of nuclear estrogen receptor either in the presence or absence of estrogen.

No differences in response patterns of the accessory sex organs to hormones between castrated and intact animals were noted. Consistent with organ weight responses [6], estrogen treatment induced the greatest increase in nuclear estrogen receptor content in the vesicular gland. Estrogen treatment had no effect on the histology of the bulbourethral gland [6] nor did it affect nuclear estrogen receptor levels in that organ. The response of the prostate to estrogen treatment was intermediate between the above extremes. Estrogen has been shown to increase the weight and nuclear estrogen receptor content and concentration in the dog prostate [24]. In the rabbit accessory sex organs, the increase in receptor content did not keep pace with the increase in organ weight; therefore, a decrease in estrogen receptor concentration occurred.

In terms of weight [6] and nuclear receptor accumulation, Tamoxifen had no antiestrogenic effect on the rabbit accessory sex organs. These results differ from those of Funke *et al.*[25] who showed that Tamoxifen was able to reverse the estrogen-induced morphological and biochemical changes in the dog prostate. Although the treatment period used in the dog study [25] was 6 months as compared to the 2 weeks used in our study, the doses of estrogen and Tamoxifen were comparable. The dose of Tamoxifen that we used was equivalent to about 80% of the

estradiol dose as determined by *in vitro* studies on its relative affinity for the estrogen receptor [3]. If Tamoxifen were antiestrogenic in our system, we would have anticipated that such a dose would have at least partially antagonized the effects of estradiol. The lack of antagonism may be due to failure of *in vivo* metabolic processes to convert Tamoxifen to an active form [26]. Our previous histological studies [6] clearly indicate that Tamoxifen affects only the epithelium of the rabbit epididymis and accessory sex organs, while estradiol affects both the epithelium and the stroma. The ability of Tamoxifen to stimulate the epithelium [6] implies that it can exert estrogenic activity in these tissues. The primary effects of estradiol are in the stroma where it causes a dramatic hypertrophy and hyperplasia [6]. Therefore, the apparent inability of Tamoxifen to inhibit estrogen action in the rabbit male reproductive tract may be due to its failure to antagonize estrogen in the stroma, which constitute a greater portion of the organs than does the epithelium. Thus, anti-estrogenic action on the epithelium would be masked when the whole organ is examined. Species and organ differences in the action of Tamoxifen, therefore, may be due to differences in its metabolic conversion and differences in the proportion of epithelium and stroma that constitute the organs being examined.

Although androgen treatment induced a moderate increase in the weight of the rabbit accessory sex organs [6], the content of total nuclear receptor stayed the same or decreased. Thus, the nuclear receptor concentration was reduced to the same level or lower levels than those observed following estrogen treatment. The effects of androgen plus estrogen treatment on the weights [6] and nuclear estrogen receptor levels were not different from the effects of estrogen alone. However, histological examination of the tissues indicated that androgen administration reduced somewhat the estrogen-induced hyperplastic effect on the stroma of the glands; prevented the epithelial metaplasia, which in its absence would have been caused by estrogen; and caused typical androgenic stimulation of the epithelium [6]. Despite these clear antagonistic effects of androgens on estrogen-induced histological changes, antagonistic effects on estrogen-induced changes in nuclear receptor levels were not clearly demonstrable. However, the data did show a statistically significant reduction in the estrogen receptor content of the prostate of castrated animals following testosterone administration. The responses in the rabbit differ from those observed in the guinea pig accessory sex organs where the androgen-induced increase in seminal vesicle weight was inhibited by estrogen treatment [27]. The responses in the rabbit also differ from those in the castrated dog prostate, where androgens and estrogens synergize to enhance organ weight, but where androgens antagonize the estrogen-induced increase in cytosolic and nuclear estrogen receptor [24]. These data further emphasize the species and organ-specific effects of hormones in regulating reproductive tract organs and highlight the possibility that many of the effects may be due to differences in the composition of the tissues comprising the organs.

The data discussed here have important implications for studies on normal development and pathological processes in male reproductive tract organs. Of particular interest is whether endogenous estrogens are involved in the physiological regulation of the development and function of the male reproductive tract. The data provide additional evidence for species-specific differences in the responses of these organs to hormonal stimuli and demonstrate that a given hormone can induce differential effects in individual organs of the same animal. Further analysis of such considerations will be of extreme importance in the development and evaluation of animal model systems for studying the etiology and treatment of benign and malignant growth of the human prostate and other accessory sex organs.

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