Increase in Testis Luteinizing Hormone Receptor by Estrogen in Mice Susceptible to Leydig Cell Tumors

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ABSTRACT

In an investigation comparing two strains of mice (BALB/c, susceptible to estrogen-induced Leydig cell tumors and C3H, resistant to such tumors), we found that the Leydig cell-luteinizing hormone (LH) receptors increase in BALB/c mice and decrease in C3H mice during estrogen treatment. In the BALB/c strain, LH receptor content in the testes of mice treated 1, 2, 4, 6, or 8 weeks with diethylstilbestrol (DES) was 2.4- to 5.4-fold greater than that in the testes of untreated littersmates. By 24 weeks of treatment, the receptor number had increased 10-fold. Likewise, two weeks of estradiol benzoate treatment in BALB/c mice resulted in a dose-dependent increase in LH receptor content. In contrast, in C3H mice, DES treatment resulted in a transient initial increase (60%), followed by a time-dependent decrease in testicular LH receptor number: 38 and 17% that of normal by six and eight weeks of treatment, respectively. In both strains of mice, DES-induced changes in 125I-labeled human chorionic gonadotropin binding reflected changes in LH receptor number rather than in receptor affinity. The tests of BALB/c mice remained normal during DES treatment, whereas those of the C3H decreased with time. Sprague-Dawley rats, resistant to estrogen-induced Leydig cell tumors, like C3H mice, also underwent testicular atrophy and lost LH receptors during DES treatment. The present study demonstrates that estrogen treatment induces diagnostically opposed change in testicular LH receptor number in the two strains of mice with different susceptibilities to Leydig cell tumorigenesis.

INTRODUCTION

Chronic administration of estrogenic compounds, be they steroidal or nonsteroidal in nature, results in the genesis of Leydig cell tumors in some strains of mice but not in others (1, 7, 11, 22). Tumor-susceptible strains, such as BALB/c, maintain testis weight and, to a considerable degree, spermatogenesis during the 8 to 11 months of tumor induction, whereas the testes of tumor-resistant strains, such as C3H, atrophy upon chronic estrogen treatment (23).

Estrogen can act directly on the testis. Not only have estrogen receptors been identified in the testis (19, 28), but studies in hypophysectomized mice and rats have shown that estrogens can directly inhibit testicular steroidogenic enzymes and steroid production (6, 9, 17, 18). Moreover, susceptibility to Leydig cell tumor induction in mice apparently resides in the testis itself, as evidenced by experiments in which parental-type testes were transplanted into F1 hybrids of tumor-susceptible and tumor-resistant strains (26). Upon chronic estrogen treatment, Leydig cell tumors developed only in the transplanted testes of the tumor-susceptible strain. However, estrogen can also act on the pituitary (24); in addition to testicular genetic predisposition and estrogen, an as yet unidentified pituitary factor(s) also plays a role in tumor induction and growth (11).

We have undertaken a study to identify the differences, as well as similarities, in the testes of estrogen-treated BALB/c and C3H mice during the preneoplastic stage of tumor formation. This comparison may allow us to identify changes that are either key in the transformation of normal Leydig cells into tumors or indicative that such a transformation will occur. In the testis, LH receptors are present only in the Leydig cells (6, 21) and, therefore, are a specific marker of Leydig cell function. In this report, we demonstrate that the tumor-susceptible BALB/c mice respond to DES, a synthetic estrogenic compound, with a large, sustained increase in testicular LH receptors and that the tumor-resistant C3H mice respond to DES with a loss of these receptors.

MATERIALS AND METHODS

Animals and Treatment Regimen. Mice of the BALB/c and C3H strains were purchased from the Leonell C. Strong Foundation (San Diego, Calif.). At 8 weeks of age, one-half of the mice of each strain were given implants s.c. with a 5- to 6-mg pellet composed of 20% DES and 80% cholesterol, as described by Wieder and Shimkin (29). The remaining mice were untreated and served as controls. At 1, 2, 4, 6, 8, and 24 weeks after the DES pellets were implanted, animals were withdrawn randomly from the control and treatment groups. They were decapitated, and their testes were excised, decapsulated, weighed, and prepared for the receptor assay. Only those treated animals in which the implanted hormone pellet was localizable at the time of death were used.

The tissue and hormone specificities of the assay were determined using testes from untreated 6-month-old C3H mice. Testes of BALB/c mice exhibited similar specificities. For testing the effect of estradiol benzoate on LH receptors, 12-week-old BALB/c mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). Animals were given s.c. injections once each day for 2 weeks, with their assigned dose of estradiol benzoate dissolved in corn oil (2.5 mg/ml); controls received a daily injection of the vehicle.

Sprague-Dawley rats were purchased from Hilltop Lab Animals, Inc. (Chatsworth, Calif.). When they were between 65

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and 70 days of age, one-half of the animals were given implants s.c. with a 1-cm Silastic implant (inside diameter, 0.062 inch; outside diameter, 0.125 inch) containing DES (Sigma Chemical Co., St. Louis, Mo.). The remaining half served as untreated controls. At 3, 7, 14, and 28 days after estradiol treatment had begun, animals were decapitated, and their testes were excised, decapsulated, weighed, and prepared for the receptor assay.

**Hormones.** DES and cholesterol were purchased from Steraloids, Inc. (Wilton, N. H.) and Calbiochem (San Diego, Calif.), respectively. Estradiol benzoate was purchased from Sigma. Highly purified HCG (HCG-CR121; 13,450 IU Second International Standard HCG per mg by bioassay) was obtained from the Center for Population Research, National Institute of Child Health and Human Development. Ovine prolactin (NIH-PRL-S1; 26 IU/mg) was obtained from the National Pituitary Agency, NIAMDD, and contains less than 0.2 unit of NIH-FSH-S1 per mg and less than 0.004 unit of NIH-LH-S1 per mg. Ovine FSH (Papkoft Lot G4-150C; FSH potency equal to 50 NIH-FSH-S1 units per mg, LH potency less than 0.01 NIH-LH-S1 unit/mg) was kindly provided by Dr. H. Papkoff, University of California, San Francisco. Rat LH (NIAMDD-14; LH potency, 1.0 NIH-LH-S1; FSH potency, less than 0.04 NIH-FSH-S1 unit/mg), human FSH ß subunit (LER-1793-B), and ovine TSH (NIH-TSH-S8; 1% U.S.P. unit/mg; LH potency, less than 0.01 NIH-LH-S1 unit/mg) were the generous gifts of the National Pituitary Agency, NIAMDD. Pregnyl, used to determine nonspecific binding in the HCG-binding studies, was obtained from Organon Diagnostics (West Orange, N. J.).

**Testicular LH Receptor Assay.** The concentration and affinity of LH receptors in the testis were determined using an in vitro receptor-binding assay with 125I-labeled HCG as radioligand. HCG was iodinated using a modification of the lactoperoxidase method (25): 20 µg (20 µl) of HCG were mixed with 1 mCl (35 µl) of 125I (Amersham/Searle Corp., Arlington Heights, Ill.); 1 µg (10 µl) of lactoperoxidase (Calbiochem); and a 1/80,000 dilution (10 µl) of a 30% solution of H2O2 (Mallinckrodt, Inc., St. Louis, Mo.). Reaction time was 50 sec. Radioiodinated HCG was separated from free iodine on a Sephadex G-100 column. The specific activity of the labeled hormone was determined by self-displacement analysis in the radioligand receptor assay (14) and ranged from 20,000 to 50,000 cpm/ng. The fraction of the 125I molecules capable of reacting with an excess of testicular receptors (i.e., the maximum binding activity) was determined for each tracer preparation; it ranged from 50 to 60%. Specific counts bound were converted into ng of hormone bound, using the specific activity and maximum binding values (14).

Decapsulated testes were homogenized in ice-cold PBS containing 0.1% BSA (pH 7.2) with a tissue grinder with a Teflon pestle. Homogenates were centrifuged at 27,000 × g for 30 min, and the resulting pellets were washed, recentrifuged, and diluted with ice-cold PBS containing BSA such that one testis corresponded to 1.5 ml of homogenate, except in the case of atrophied C3H testes, in which one testis = 0.25 to 0.50 ml of homogenate. Serial dilutions of homogenates were incubated with a saturating concentration of labeled hormone (5 to 10 ng) in a total reaction volume of 400 µl. Specific binding (total binding minus nonspecific binding) was determined for 3 dilutions of each homogenate. Total binding tubes contained PBS with BSA, tissue homogenate, and radioligand. Non specific binding tubes contained, in addition to these constituents, unlabeled HCG (100 IU) in excess of the radioligand concentration. After incubation at room temperature (22 to 24°) for 16 to 18 hr, the mixtures were diluted with 3 ml ice-cold PBS and centrifuged at 1500 × g for 30 min. After one washing, the pellets were counted in a γ spectrometer (Nuclear-Chicago) with a 125I-counting efficiency of about 60%. The amount of specifically bound radioactivity at saturation was expressed in terms of ng bound hormone per testis or per mg testis (wet weight).

To determine the dissociation constant (Kd), the HCG receptor assay was performed as described above by incubating testicular homogenate with 1 ng of 125I-labeled HCG alone or with graded doses of nonradioactive HCG. The resulting competition curves were converted into saturation curves, from which Kd's were calculated, using Scatchard analysis (20).

**Hormone and Tissue Specificity of 125I-Labeled HCG Binding.** LH and HCG bind to the same receptor in the rat testis (6). That the mouse is similar to the rat in this regard was tested and verified (Chart 1); HCG and rat LH competed significantly with 125I-labeled HCG for binding sites. It is not possible to compare on a weight basis the abilities of unlabeled HCG and rat LH to compete with labeled HCG for receptor sites, because the preparation of LH is considerably less pure than the HCG preparation; nevertheless, both hormones competed at high concentrations, reaching the same degree of displacement. Ovine prolactin and the ß subunit of human FSH did not compete at all, even though they were added in µg amounts. Although ovine FSH and ovine TSH could compete, the extent was slight and occurred only at high levels of hormone. At such levels, the known LH contamination of these preparations could account for any competition seen. Therefore, we conclude that, as in the rat, HCG is binding to the LH receptor exclusively and, therefore, is a valid measure of the number of LH receptor sites in the mouse testis. Moreover, HCG was found to bind specifically only to the mouse testicular homogenate and not to homogenates of mouse spleen, lung, seminal vesicles, cerebral cortex, or kidney, thereby fulfilling the requirement of bona fide receptors that specific hormone binding occurs only in known target tissues (Table 1).

**Statistical Analysis.** Statistical analysis was performed using the analysis of variance and Student’s t test.
RESULTS

Effect of Treatment with DES upon Testis Weight and LH Receptor Content in BALB/c and C3H Mice. BALB/c mice were able to maintain a normal testis weight notwithstanding sustained DES treatment for 6 months, whereas the testes of C3H mice progressively atrophied with treatment time (Chart 2). Testis weight in C3H mice decreased to 63% of the control level by 6 weeks of treatment. By the end of 8 weeks of DES treatment, the testis weight was 16% of that found in untreated animals.

DES treatment also elicited markedly contrasting responses in the 2 strains in the pattern of testicular LH receptor content and concentration: in the BALB/c testes, a striking, sustained increase in the number of LH receptors; in the C3H testes, a relatively short-lived initial increase followed by a progressive decrease in the receptor number (Chart 3). In BALB/c mice, the HCG bound per testis (Chart 3A) or per testis weight (Chart 3B) increased approximately 2.5-fold above control by 1 week of treatment (p < 0.01). At 2, 4, 6, and 8 weeks, the total testicular LH receptor content per testis increased to 2.4-, 4.2-, 5.4-, and 3.1-fold above normal, respectively. At every time point studied, the testicular LH receptor content in DES-treated animals is significantly higher than in untreated animals (p < 0.01). However, the LH receptor levels in DES-treated animals 1, 2, 4, 6, and 8 weeks after treatment were not significantly different (p > 0.05). By 24 weeks of DES treatment, the number of LH receptors per testis had increased up to 10-fold, 9-fold expressed on a testis weight basis. Moreover, by 24 weeks, one testis of an animal not included in the graphed data was grossly enlarged (325 mg) and necrotic. This obvious Leydig cell tumor, combined with the animal's contralateral testis of normal weight range (64 mg), possessed a 34-fold increase in binding ability. In these experiments, the testes of each animal were combined, and the resulting testicular homogenate of each animal was assayed separately. In every DES-treated BALB/c mouse at every time tested, the testicular HCG-binding ability was increased.

In contradistinction to the findings in BALB/c mice, the pattern seen in C3H animals treated with DES was basically one of a decline in testicular HCG-binding ability. By 1 week of DES treatment, a transient increase (60%) in 125I-labeled HCG-

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Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>125I-labeled HCG bound (cpm/mg tissue)</th>
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</thead>
<tbody>
<tr>
<td>Testis</td>
<td>21,019</td>
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<tr>
<td>Spleen</td>
<td>933</td>
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<tr>
<td>Lung</td>
<td>901</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>1,813</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>1,125</td>
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<tr>
<td>Kidney</td>
<td>263</td>
</tr>
</tbody>
</table>

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Chart 2. Effect in BALB/c and C3H mice of DES treatment on testis weight as a function of treatment time. Each treated animal had a DES pellet implanted s.c.; controls were untreated. Points, means; bars, SE. Numbers on curves, number of animals examined at each time point. To calculate the mean testes weight at the zero time point, the weights of all control animals were averaged.
binding ability occurred. By 2 weeks, however, the increase had disappeared when computed on a per testis basis, dropping back to the control level (p > 0.05). Thereafter, the binding ability decreased progressively, falling to 85% that of normal by 4 weeks, to 38% at 6 weeks, and to approximately 20% at 8 and 24 weeks of DES treatment. If the data of the C3H testes are expressed on a testis weight basis, HCG-binding ability was increased 50% at 1 and 2 weeks of treatment, returned to normal by 4 weeks, and thereafter paralleled the drastic reduction in testis weight.

In order to test the possibility that changes in 125I-labeled HCG binding in the testes in mice treated with DES are due to changes in the binding affinity of the LH receptor, testis homogenates were incubated with 125I-labeled HCG alone or with increasing concentrations of nonradioactive HCG to determine the receptor dissociation constant (Kd). The affinity of the testicular LH receptors did not change in either BALB/c or C3H mice during DES treatment at any time tested (Table 2). Moreover, only one class of receptors was present in the various experimental groups. Therefore, the increases in LH-binding ability measured are a reflection of an increase in the number and not the affinity of the receptor.

Effect of DES Treatment upon Testis Weight and LH Receptor Content in Rats. The testes of rats, like those of C3H mice, atrophied when DES was chronically administered (Chart 4); a decrease in testicular 125I-labeled HCG-binding ability also occurred. The decrease in binding ability was not only more rapid and severe than in the testes of C3H mice, but no initial, transient increase in testicular HCG-binding ability could be detected after 1 week of DES treatment. Whether the data were expressed on a per testis (Chart 4) or on a testis weight basis (data not shown), the number of LH receptors was below normal (p < 0.01).

Effect of Treatment with Estradiol upon Testis Weight and on LH Receptor Content in BALB/c Mice. Since the stimulatory effect of DES in BALB/c mice on testicular LH receptors is presumably due to its estrogenic properties, we investigated whether estradiol benzoate, a derivative of a natural estrogen, could also increase receptor content. Daily treatment of BALB/c mice with increasing concentrations (5, 50, and 500 µg/day) of estradiol benzoate for 2 weeks increased the testicular 125I-

Table 2

<table>
<thead>
<tr>
<th>Treatment time (wk)</th>
<th>BALB/c</th>
<th>C3H</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.1 ± 0.6a</td>
<td>3.5 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>12</td>
<td>1.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a Mean ± S.E.
treated animal received s.c. a 1-cm Silastic implant containing DES; controls were untreated. Each group had 4 animals at each time point. Points, means; bars, S.E. In A, the standard error was less than 5% in all cases except at 4 weeks of treatment, at which time, it was 10%. To calculate the mean binding value at the zero time point, we averaged the HCG-binding values of all the control animals.

DISCUSSION

We have identified an early, differential testicular response to DES, which may contribute to the understanding of the early tumorigenic changes initiated by estrogen. Chronic administration of DES or estradiol benzoate to mice of the tumor-susceptible BALB/c strain resulted in a large, sustained increase in their testicular LH receptors (Charts 3 and 5). In contrast, testes of tumor-resistant C3H mice eventually lost 80% of their LH receptors during chronic DES treatment (Chart 3). Likewise, the number of testicular LH receptors in Sprague-Dawley rats, also resistant to induction of Leydig cell tumors by estrogens, declined drastically when DES was chronically administered (Chart 4).

The testis weights of the BALB/c mice remained normal when estrogen or DES was given. Therefore, the total testicular LH receptor increase reflected an increase in the number of receptors in each mg of testis. Since Leydig cell volume does not increase substantially for at least 2 weeks after DES treatment (23), the average number of receptors on each Leydig cell was presumably increasing during this period.

In C3H mice, the treated testes had, at first, an increase in the number of LH receptors, but this increase proved to be transient and had disappeared by 2 weeks of treatment. Then, the decrease in receptor number paralleled the decrease in testis weight, such that at later times of treatment, although the total testicular receptor number was drastically reduced, the receptor number in each mg of testis was normal (Chart 3). Since Shimkin et al. (23) observed none to minimal loss of interstitial tissue mass over 90 days of treatment with DES in C3H mice, the present result probably reflects an estrogen-induced decrease in LH receptor per Leydig cell. In rat testes, a striking reduction in the total number of LH receptors, as well as the number in each mg of testis, was evident within 3 days after the start of DES treatment and continued for at least 4 weeks (Chart 4). Since the method of DES administration was different in the C3H mice and the rats, the responses of the 2 species cannot be compared with respect to time course and extent of response. The end result, however, was the same in the tumor-resistant mice and rats, a drastic, sustained loss of the total number of LH receptors. This decrease contrasted sharply with the 5- to 10-fold increase in the testicular LH receptors of BALB/c mice.

It should be noted that, in contrast to the up to 10-fold difference in receptor number between the 2 strains during DES treatment, the levels of testicular LH receptor in normal, untreated adult C3H and BALB/c mice were similar, those of the former being consistently slightly higher (Chart 3).

LH stimulates Leydig cells to proliferate (5, 8), as well as being the regulator of the differentiated functions of the Leydig cells. A possible prolonged increase in LH responsiveness as mediated by LH receptor may be related to tumorigenesis. It has been reported that excessive stimulation with gonadotropins of neonatal rat ovaries and testes and neonatal mouse ovaries transplanted to the spleen of castrated animals results in tumor formation (3). Although such attempts to induce Leydig cell tumors in mice bearing intrasplenic transplants of neonatal testes have not been successful (15), optimal conditions for tumorigenesis in male mice may require concerted action of LH and estrogen on the testis. Huseby (10) reported that administration of LH to intact male mice bearing stilbestrol pellets produced an impressive interstitial cell hyperplasia.

Even if this DES-induced increase in Leydig cell LH receptors in BALB/c mouse testes is not essential to the transformation of a normal to a tumor cell, it may prove an invaluable tag for following the population of cells that will eventually become tumorous or serve as a clue as to which other factors are involved in the estrogen response.

Whether estrogen is directly responsible for this increase in testicular LH receptors is unknown. The identification of estrogen receptors in the testes of BALB/c mice (19) and the suppression of testicular steroidogenic enzymes directly by estrogen (17, 18) make this a possibility. This notion is further
supported by a recent report (12), demonstrating the ability of intrasplenic estrogen pellets to induce Leydig cell tumors in adjacent testis grafts but not in testis grafts placed in the mammary gland of the same animal. However, another possibility is that prolactin may be responsible for the increase. In BALB/c mice during DES treatment, plasma prolactin levels are elevated 6- to 8-fold. Prolactin has been found capable of increasing LH receptors in the prolactin-deficient dwarf mouse (4), in the seasonally regressed hamsters (2), and in the hypophysectomized immature (30) or adult (16) rats. A delineation of the actions of prolactin in BALB/c testis would be of obvious interest, since a pituitary factor is apparently needed for tumor growth (11).

In conclusion, only 2 potentially important differences in the response to estrogens at the cellular level of tumor-resistant and tumor-susceptible mice have been reported heretofore. (a) When estrogen is administered to the BALB/c mouse strain, a spurt of testicular DNA synthesis occurs during the first week; no such DNA spurt could be detected in A × C3H F1 mice, which, although susceptible to estrogen-induced Leydig cell tumors, were less so than BALB/c (13, 27) or tumor-resistant strains (11). (b) Testicular content of the nuclear estrogen receptor is greater, and its binding ability to chromatin is stronger in BALB/c mice than in C3H mice (19). Our discovery of the differential response to DES in the number of testicular LH receptors in the BALB/c and C3H strains represents a third difference. This finding of a differential response in an element that is key in the regulation of Leydig cell function points to new avenues of investigation that should bring us closer toward clarifying the role of estrogen in Leydig cell tumorigenesis.

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