Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses
(androgen receptors, androgen-mediated hypertrophy, androgen receptors)

FREDERICK, S. VON SAAL,1, 2, BARRY G. TIMMS,1, 2, MOYER, M. MONTANO,1, 2, PAOLA PALANZA2, KRISTINA A. THAYER,1, 2, SUZAN N. NAGEL1, 2, MARISA D. DECK,1, 2, V. K. GANAM,1, 2, STEFANO PARMIGIANI,1, 2, AND WAVE V. WESBROOK1

1Division of Biobehavioral Sciences and 2Department of Veterinary Biobehavioral Sciences, University of Missouri-Columbia, Columbia, Missouri, USA 65211; Department of Anatomical and Structural Biology, University of South Dakota, Vermillion, SD 57069, and the Department of Evolutionary and Functional Biology, University of Pennsylvania, Philadelphia, USA 19104

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ABSTRACT On the basis of results of studies using high doses of estradiol, exposure to estradiol during fetal life is known to inhibit prostate development. However, it is recognized in endocrinology that low concentrations of a hormone can stimulate a tissue, while high concentrations can have the opposite effect. We report here that a 50% increase in free serum estradiol in male mice fetuses (reared by a maternal bilateral estradiol implant) induced a 40% increase in the number of developing prostatic glands during fetal life; subsequently, in adulthood, the number of prostatic androgen receptors per cell was permanently increased by 2-fold, and the prostate was enlarged by 30% (due to hyperplasia) relative to untreated males. However, as the free serum estradiol concentration in male fetuses was increased from 2 to 8-fold, adult prostate weight decreased relative to males exposed to the 50% increase in estradiol. As a model for fetal exposure to man-made estrogens, pregnant mice were fed diethylstilbestrol (DES) from gestation days 11 to 17. Relative to controls, DES doses of 0.02, 0.2, and 2.0 ng/g of body weight per day increased adult prostate weight, whereas a 200-ng-per-g-dose decrease adult prostate weight in male offspring. Our findings suggest that a small increase in estrogen may modulate the action of androgens in regulating prostate differentiation, resulting in a permanent increase in prostate androgen receptors and prostate size. For both estradiol and DES, prostate weight first increased then decreased with dose, resulting in an inverted-U dose-response relationship.

The possibility that estrogen is involved in both the normal process of prostate development and subsequent androgen prostate disease was raised 60 years ago (1). Recently, an increase in reproductive organ disorders and other disorders has been proposed to be linked to in utero exposure to endocrine-disrupting estrogenic chemicals in the environment (2, 3). The hypothesis is that elevated levels of estrogen (natural or man-made) during fetal life may alter development of reproductive organs, including the prostate, which thus may be predisposed for abnormal function and disease in later life, an outcome we seek to establish in the rat as a latent birth defect. During critical periods in cell differentiation, hormones are involved in "imprinting" (permanently turning on or off specific genes in cells with receptors for the hormones) (4). In addition, for genes that are turned on during critical periods in the differentiation of cells, the rate of gene transcription can be permanently set at different levels based on the concentrations of hormones to which the differentiating cells are exposed (5).

Our interest in the role of estrogen in prostate development resulted from an unexpected observation. We have reported that male mice positioned in utero between two female fetuses (2F males) are exposed to a supplement of serum estradiol (a 30% increase) in comparison to males that are positioned as mono twins between two male fetuses (2M males), due to transport of estradiol from adjacent female fetuses (6, 7). Fetal exposure to this very small supplement of estradiol in 2M male fetuses was associated with significant enlargement of the prostate (and changes in behavior) in adulthood. There was also a 3-fold permanent increase in prostate androgen receptors (although there was no effect on prostatic estrogen receptors), as well as differences in enzyme activity in a number of reproductive organs, in adult 2M male mice relative to adult 2M male mice (8, 9).

The developing prostate is responsive to androgen, which is the primary mediator of prostate differentiation (10). We proposed a modulating role for estrogen in prostate development based on our cumulative study of intraterine position. Mesenchyme (but not epithelium) in the embryonic tissues that develop into the mouse prostate expresses estrogen receptors (11). The development of prostatic glands begins with the formation of epithelial buds from the urogenital sinus just below the developing bladder. Epithelial buds begin forming in the dorsocentral, dorsosaudinal, and ventral regions of the urogenital sinus at approximately day 17 of gestation, followed by bud formation in the lateral and dorsomedial regions, under inductive influence of regional mesenchyme (12, 13). After extensive growth and branching, which continues through adolescence (4–8 weeks old), these epithelial buds form the glands of the different lobes (dorsal, lateral, and ventral) of the adult prostate (14). In the human fetus, the ventral buds regress, and thus there is no human homolog of the rodent ventral prostate lobes (15).

In the following series of experiments we examined the relationship between the levels of natural or man-made estrogen during fetal life and prostate development. Our major goal was to determine the number of prostate androgen receptors during fetal life and in adulthood. We first increased serum estradiol in all male fetuses to levels observed in 2F male fetuses during the last third of gestation, when actual differentiation is initiated. Our objective was to test the hypothesis that the enlarged prostate, and elevated numbers of androgen receptors in 2M male mice were mediated by exposure to a small supplement in estradiol during fetal life.

Abbreviations: DES, diethylstilbestrol; IMF, positioned between one male and one female; ANOVA, analysis of variance. To whom reprint requests should be addressed at: 114 Leveque Hall, Department of Biological Sciences, University of Missouri, Columbia, Missouri, USA 65211. Email: vonsaall@missouri.edu

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We thus determined the concentrations of total and free (not bound to plasma proteins) serum estradiol in untreated male mice and in male fetuses exposed to supplemental estradiol by maternal treatment. Specifically, we examined the effect of a 50% increase in serum estradiol on the structure of the prostate at the end of the first day of prostate development in male mouse fetuses on gestation day 18, as well as on subsequent adult prostate size, number of cells, and number of anogenital reproducers. We then examined the effect of increasing free serum estradiol up to 5-fold above control values (to supraphysiological levels) on the size of the adult prostate. Finally, a five-log range of doses of diethylstilbestrol (DES) was fed to pregnant mice, and the size of the prostate in adult male offspring was examined to assess the long-term effects of low vs. high doses of DES on the prostate as a model for fetal exposure to a man-made estrogenic chemical.

MATERIALS AND METHODS

Animals. CF-1 mice were purchased from Charles River Breeding Laboratories in 1978 and have been maintained as an outbred stock in a closed colony. Mice were housed in standard polypropylene mouse cages on corn cob bedding and fed Purina lab. chows. Room were kept at 23°C, with 12 h light and 12 h dark, and lights on at 1200 h.

Treatment of Pregnant Females with Estradiol. Females were time-mated by being placed with a stud male for 4 h between 0900 and 1300, and mating was confirmed by the presence of a copulatory plug (gestation day 0). On gestation day 13, pregnant females under methoxyflurane anesthesia were implanted s.c. with a 10-mm-long Silastic capsule (Dow 602-285) containing one of five doses of estradiol: 0 (surgical controls), 25, 100, 200, or 300 μg dissolved in 20 μl of sesame oil. We used only males positioned in utero between a male and a female fetus (1MF males), which are intermediate in fetal estradiol levels and subsequent phenotype between 2M and 2F males; on average, there are 3 MF males per litter of 12 fetuses (6). Collection of Fetal Blood and Computer-Assisted Reconstruction of the Fetal Prostate. For examination of serum estradiol and prostate morphology by computer-assisted reconstruction (in gestation day 18 fetuses, pregnant females treated with different doses of estradiol (6 per group) were killed by CO2 asphyxiation and cervical dislocation between 0900 and 1300 on gestation day 18 (one day before parturition). Blood from all 1MF male fetuses in each litter was collected in heparinized microcapillaries, and one 1MF male fetus was randomly selected from each litter for prostate reconstruction (6 per group). The ureteral sinus (along with the developing prostate) was removed, fixed in Bouin's solution, sectioned, and examined by computer-assisted, three-dimensional reconstruction. The computer-assisted, three-dimensional reconstruction technique involved tracing of serial histological sections through the developing prostate as described previously (13). The outlines of the prostate buds in the dorsal, lateral, and ventral budding lines were used to calculate the number of prostate buds, the length of the line of buds along the ureteral sinus, the sum of the cross-sectional area (total area) for each budding line, and mean cross-sectional area for the buds in a budding line. The same measurements were made for the urethra (the remnant of the Müllerian ducts, which is enclosed within the adult prostate). We also calculated the cross-sectional area of the urethra.

Treatment of Pregnant Females with DES. DES (Sigma) was dissolved in methocel-stripped corn oil (ICN, catalog no. 904141), and 30 μl containing six different concentrations of DES (0 [vehicle controls], 0.002, 0.02, 0.2, 2.0, and 200 ng body weight) were fed to pregnant mice (6–8 per group) once daily from gestation day 10 to day 17. Due to its long half-life relative to estradiol, DES still would be in the fetal circulation through gestation day 18 (16). An additional control group of females (n = 7) that received undiluted throughout pregnancy only was also included. A discussion of procedures to calculate doses of man-made estrogens to use in animal studies based on a new in vitro assay is presented elsewhere (17). An electronic micro-pipetter (Rainin Instruments) enabled delivery of an aqueous volume of corn oil into the mouth. Mice were fasted 1 h before feeding and each procedure was conducted instead of gauge to reduce stress, which can interfere with sexual differentiation (18). This experiment was conducted without knowledge of the prenatal intrauterine position of the male offspring, and the pregnant females were allowed to deliver and mate their own litters.

Postnatal Examination of the Prostate in 8-Month-Old Males. Prematral Estradiol Treatment. Pregnant females implanted with different doses of estradiol (6–8 per group) were killed between 0800 and 1000 on gestation day 19, just before parturition. 1MF male offspring were identified and reared along with littermates by foster dams that had delivered normally during the previous 24 h (males that were not from the 1MF intrauterine position were identified by a toe-clipping pattern). In this study, a second control group of 1MF male offspring carried by pregnant females that had remained untreated throughout pregnancy was included (these females also were weighed on gestation day 19), in addition to the zero-dose control group. Animals were weaned when 23 days old and housed 2–4 1MF males from the same litter per cage. When 7 months old, to control for litter effects, only one randomly selected 1MF male from each litter was individually housed. To control for any possible effects of prenatal treatment on the functioning of the brain-pituitary-testicular axis, 1 week after being individually housed, the 1MF males were castrated under nembutal anesthesia and implanted s.c. with a 10-mm-long Silastic capsule (Dow 602-285) containing 500 μg of testosterone dissolved in 20 μl of sesame oil. This procedure provides blood levels of testosterone sufficient to maintain accessory reproductive organ weight and testis volume. Males were killed 3 weeks later, and body weights were recorded. The entire prostate was removed, weighed, immediately placed in liquid nitrogen, and then stored at −80°C. We waited to collect the prostates from these males until they were 8 months old, because previous studies have shown that some adverse outcomes of developmental exposure to estrogenic chemicals are not expressed until middle age (20).

Prematral DES Treatment. Males were weaned on postnatal day 23, and 2–4 male mice from the same litter were housed together. At 47 months of age one randomly selected male from each litter (6–8 per group) was individually housed. One month later at 8 months of age, the males were killed, body weights were recorded, and the entire prostate was removed and weighed.

Statistical Procedures. For statistical comparisons we used the Student's t-test or Analysis of Variance (ANOVA) where appropriate. Statistical comparisons were made using the LMSmeas test, with the null hypothesis rejected at P < 0.05. The correlation between body weight and prostate weight in adulthood was determined using Pearson's correlation coefficient. Prostate weight also was analyzed by analysis of covariance (ANCOVA), where prostate weight was adjusted on the basis of body weight, to determine whether body weight accounted for a significant component of the variance in prostate weight. If prostate weight and body weight were not correlated, and body weight did not account for a significant component of variance in prostate weight on the basis of ANCOVA, prostate weight was recalculated by ANOVA. Results are presented as mean ± SEM.

Assays. Radioimmunoassay. Concentrations of total serum estradiol and testosterone were determined for control fetal males and male fetuses exposed to different doses of estradiol.
Physiological and behavioral responses to stress and physical activity in male and female rats

by maternal treatment using radioimmunoassay procedures described in detail elsewhere (18, 21). 125-I-labeled Estradiol and 125-I-labeled testosterone, as well as actinase, were obtained from ICN. Unlabeled estradiol and testosterone were obtained from Steraloids (Wilton, NH). Sensitivity for the assay was 0.5 pg for estradiol assay and 4.8 pg for testosterone. For estradiol, the intra- and inter-assay coefficients of variation were 3% and 11%, respectively, while for testosterone, the intra- and inter-assay coefficients of variation were 3% and 12%, respectively.

Counterstaining Ultrastructural Techniques. Sera from IMF males in each litter (n = 6 to 8 per group) were pooled, and aliquots were used to determine total serum estradiol (and testosterone) concentrations by radioimmunoassay. Within each treatment group, the remaining sera from all IMF males then were pooled for determination of the percent free estradiol by counterstaining ultrastructural dialysis as previously described (21). We calculated the free serum estradiol concentration by the formula: percent free estradiol \* total serum estradiol concentration (measured by radioimmunoassay) \* free serum estradiol concentration. Because the free serum concentration was calculated from one pool of serum for each group, no measure of variance is present.

Prostatic Androgen Receptors. Cytosolic androgen receptors were measured as previously described (9), but with the following modifications. Cytosols were prepared from pel- laces in 10 mM Tris-HCl, pH 7.2/1.5 mM EDTA/1 mM Na3MoO4/1 mM DTT/1 mM phenylmethanesulfonyl fluoride/10% glycerol at 1-2 mg of protein per ml. Endogenous androgens were removed with A-5000 Sepharose, and then 100 µl of cytosol was added immediately to 250 µl of 60% hydroxyapatite in 50 mM Tris-HCl and the mix was made 20 mM with [1H]dihydrotestosterone (DHT) (DuPont/NEC), with or without a 100-fold excess of nonradioactive DHT in a separate tube to measure nonspecific binding. After incubation overnight at 4°C to complete receptor occupancy and exchange, specific binding of DHT to the androgen receptors was determined by extraction and added to cytosol protein (22) to obtain total DNA (23). Receptors occupied by endogenous ligand ("bound" receptors) also were determined in high-salt extracts as previously described (9), but these values were less than 10% of cytosolic receptors.

RESULTS

The concentration of total serum estradiol in control IMF male and female rats was 94.7 pg/ml, corresponding to a free serum estradiol concentration of 0.21 pg/ml (0.2 parts per trillion or 0.77 pg/ml). The 25- µg capsule dose led to a 52 pg/ml increase in total serum estradiol to 146.7 pg/ml. This 52 pg/ml increase in total serum estradiol corresponded with a 0.11 pg/ml (0.4 pg/ml) increase in the free serum estradiol concentra- tion, resulting in a free serum estradiol concentration of 0.52 pg/ml (1.78 pg/ml) in these male flees. The remaining capsule dose increased serum estradiol (100, 200, and 300 µg) led to total serum estradiol concentrations of 228.3, 37.5, and 42.5 pg/ml, respectively (all groups differed significantly from each other, corresponding to free serum estradiol concentrations of 0.56, 0.78, and 1.70 pg/ml, respectively. The percent free estradiol in serum did not differ for the 25, 100, and 200- µg dose groups (mean = 0.23%), while the percent free estradiol for the 300- µg dose group was 0.32%. For male flees exposed to the highest dose (300 µg) of oestradiol, there was a significant increase (P < 0.05) in total serum testosterone to 3.5 ± 0.3 mg/ml (12.1 µM) relative to control males (0.00), which had 2.4 ± 0.3 mg/ml (8.3 µM). The total serum testosterone concentration was not signifi- cantly different in control male flees and male flees from the other estradiol treatment groups.

A Low Dose of Estradiol Increases the Number of Prostatic Glands in Male Flees. For all regions of the fetal prostate combined, there was a significant 40% increase (ANOVA, P < 0.05) in the number of prostatic glandular epithelial buds in male carried by females implanted with the 25- µg dose of estradiol relative to control males (Table 1). Estradiol-treated males showed a significant increase (P < 0.05) in the total cross-section area of buds (the sum of cross-sectional areas for buds), the length of the line of prostatic glandular buds along the urogenital sinus also was significantly increased in estradiol-treated males. The increase in prostate size in re- sponse to the 25- µg dose of estradiol was most pronounced in the dorsal budding lines (Table 2 and Fig. 3). Overall, the mean cross-sectional area of the individual epithelial buds was not significantly different between control and estradiol-treated males. These findings show that estradiol treatment increased prostate size in response to the capsule dose of estradiol and that the increase in prostate size developed along a greater length of the urogenital sinus relative to control males (the overall area of bud tissue was thus greater), although the size of the individual buds was not increased. The length of the urogenital sinus occupied by the urothelium was significantly greater (P = 0.005) in estradiol-treated males (158 ± 8 µm) than in control males (124 ± 6 µm), although the total area of the urothelium did not differ significantly. There was also a significant decrease (P < 0.05) in the mean cross-sectional area of the lumen of the prostatic region of the urethra (the canal running through the urogenital sinus) in estradiol-treated males (61.63 ± 0.78 µm²) relative to control males.

Table 1. Data from the three-dimensional computer-assisted reconstruction of the prostate in control (C) and estradiol-treated (E) male flees on gestation day 18

<table>
<thead>
<tr>
<th>Region</th>
<th>Treatment</th>
<th>No. of buds</th>
<th>Total area, µm²</th>
<th>Length, µm</th>
<th>Mean area, µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>C</td>
<td>33.9 ± 6.6</td>
<td>29.87 ± 4.407</td>
<td>202 ± 26</td>
<td>896 ± 55</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>48.8 ± 2.4</td>
<td>39.10 ± 4.403</td>
<td>279 ± 26</td>
<td>941 ± 35</td>
</tr>
<tr>
<td>Dorsal</td>
<td>C</td>
<td>14.4 ± 3.6</td>
<td>16.08 ± 4.732</td>
<td>194 ± 35</td>
<td>565 ± 40</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>20.8 ± 3.1</td>
<td>27.26 ± 5.587</td>
<td>275 ± 50</td>
<td>739 ± 108</td>
</tr>
<tr>
<td>Lateral</td>
<td>C</td>
<td>0.5 ± 0.2</td>
<td>14.71 ± 3.766</td>
<td>127 ± 29</td>
<td>817 ± 71</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>11.5 ± 2.9</td>
<td>20.28 ± 3.837</td>
<td>181 ± 31</td>
<td>1075 ± 137</td>
</tr>
<tr>
<td>Ventral</td>
<td>C</td>
<td>9.2 ± 3.3</td>
<td>58.12 ± 10.152</td>
<td>245 ± 62</td>
<td>1305 ± 169</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>15.2 ± 5.8</td>
<td>60.76 ± 9.972</td>
<td>379 ± 33</td>
<td>1327 ± 39</td>
</tr>
</tbody>
</table>

Data are presented for all prostatic regions combined and are given for each region of budding. NS, not significantly different. * , statistically significant.
The number of prostatic androgen receptors was examined in control males and experimental males exposed in utero to 0.32 μg/ml free serum estradiol (Fig. 3). Protein and DNA were measured as the reference for expressing receptor numbers and to allow discrimination between hyperplasia and hyperplasia due to elevated estradiol during fetal life. Relative to adult controls exposed to 0.21 μg/ml free serum estradiol during fetal life, in 1MF males with 0.32 μg/ml free serum estradiol during fetal life, there was a significant increase (3×) in the total number of androgen receptors per prostate (per mg protein), a significant increase (2.4×) in the number of androgen receptors per cell (relative to DNA), and a significant increase (40%) in DNA (and thus the number of cells) per prostate, demonstrating prostatic hyperplasia (for all comparisons, P < 0.01).

**Opposite Effects on Prostate Size of Low and High Doses of DES.** Unhandled and oil control groups were not significantly different on any measure and were combined into one control group. Prenatal exposure to DES significantly increased adult prostate weight (Fig. 4; P < 0.001). After correction for body weight by ANCOVA, relative to controls, males in the 0.002-, 0.02-, and 2-μg/d DES dose groups had significantly increased prostatic weights. In marked contrast, prostates in males in the 200-μg/d dose group were significantly smaller than prostates in control males and prostates in males in all other treatment groups. Prostate weight was corrected for body weight by ANCOVA, because body weight accounted for a significant portion of the variance in prostate weight (F(1, 53) = 4.9, P < 0.05), and prostate weight and body weight were significantly correlated (r = 0.32, P < 0.05; n = 63). Body weight differed significantly as a function of prenatal DES dose [F(6, 50) = 4.8, P < 0.001]. Mean body weights for males exposed to the 0 (control), 0.002-, 0.02-, 0.2-, 2-, and 200-μg/d DES doses were 37.4 ± 0.35, 38.2 ± 0.7, 39.3 ± 1.9, 36.6 ± 1.1, 36.6 ± 0.9, and 34.0 ± 1.0 g, respectively. Body weight of males exposed to the 200-μg/d body weight dose of DES was significantly decreased relative to control males (P < 0.005). Relative to control males, males exposed to the 0.002-μg/d dose (P = 0.05) tended to be heavier, while males exposed to the 20-μg/d dose tended to be heavier (P = 0.05).

**DISCUSSION.** The results of these experiments demonstrate that a 50% increase in free serum estradiol in 1MF male mouse fetuses

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**Fig. 2.** Mean (± SEM) prostate weight (mg) in 8-month-old male mice produced by mothers implanted s.c. with 0.03 μg estradiol benzoate in oil on day 18 of pregnancy. The free serum estradiol concentration (ng/ml) in female litters on gestation day 18 in response to these doses of estradiol is shown in relation to adult prostate weight. Group means that differed significantly are indicated by different letters, while groups with the same letter did not differ significantly.

**Fig. 1.** Two stereo-pair images (convergent, or cross-eyed viewing) of computer-assisted, serial-section reconstructions showing the dorsal portion of the prostate from two mouse fetuses. The prostate from a control male with 0.21 pg/ml free serum estradiol (blue areas) is shown below. The top prostate is reconstructed from a male fetus exposed to 0.32 pg/ml free serum estradiol (red areas). Glandular buds that form into the dorsal (green), lateral (yellow), and dorsocentral (blue) glands in the adult prostate can be seen as outgrowths of the fetal regressive sites (cervical buds are not visible). The urothelium (pink) is the remnant of the regressing embryonic female reproductive tract (Müllerian duct). Compared with controls, estradiol significantly increased the number of prostatic glandular buds and caused a reduction in the size of the lumen of the urothelium, which appears through the prostate.

**males (77,375 ± 6078 μm²), which could contribute to a restriction of urine flow (24).**

**A Low Dose of Estradiol Increases Adult Prostate Size and Number of Androgen Receptors: An Inverted-U Dose-Response for Prostate Size.** Unhandled and oil-control exposed controls did not differ significantly on any measure and were combined as one group. There was a significant effect of prenatal treatment on prostate weight (Fig. 2). ANOVA, P < 0.01. Specifically, relative to control 1MF males, prostate weight in 1MF males exposed to a 50% elevation in free serum estradiol (0.32 pg/ml) was significantly increased by (27%). In contrast, the highest dose of estradiol increased free serum estradiol during fetal life to 1.70 pg/ml and resulted in a significant decrease in adult prostate weight relative to control 1MF males exposed as fetuses to 0.32 or 0.56 pg/ml free serum estradiol, although males exposed to 1.70 pg/ml free serum estradiol did not differ significantly from control males. There was no significant effect of prenatal estradiol dose on adult body weight (P > 0.1). Mean (± SEM) body weights for the 0 (control), 0.25-, 1.00-, 2.00-, and 5.00-μg estradiol groups were 36.2 ± 0.7, 35.6 ± 0.8, 33.8 ± 0.8, 33.2 ± 1.1, and 35.5 ± 1.1 g, respectively. Body weight and prostate weight were not significantly correlated (r = 0.1; P = 0.5; n = 31). ANCOVA showed that body weight did not account for a significant portion of the variance in prostate weight (F(1, 32) = 0.3, P > 0.1), and the data for prostate weight shown in Fig. 2 thus were not corrected for body weight.
increased the number of developing prostatic glands by 40%. This increase in fetal estradiol subsequently led in adult I1MF males to a 30% increase in the size of the prostate, which showed a 40% increase in the number of cells (DNA). There was also a 6-fold increase in prostatic androgen receptors (per mg of protein), associated with a doubling of androgen receptors per cell (per DNA), leading to the prediction that the prostate in these estrogen-exposed I1MF adult males should show a permanent increase in sensitivity to testosterone and thus marked changes in function throughout the remainder of life.

For most previous work there has been no information provided concerning the increase (relative to controls) in estrogenic activity brought about by treatment with a natural or a man-made estrogen. In contrast, we previously determined that rat serum estradiol concentrations in individual 2M male rat femoral fat bodies that are not exposed to a supplement of estradiol from an adjacent female rat as well as in individual 1M1 and 2F1 male fat bodies at gestation day 18; the highest estradiol value was 159 pg/ml which was observed in a 2F1 male rat (6). Our lowest dose (25 μg) estradiol treatment (that produced prostatic enlargement) increased serum estradiol in 1M1 male fat bodies from a mean of 94 pg/ml to 146 pg/ml, and thus significantly shifted the population distribution on serum estradiol concentrations during fetal life in male mice. When very low doses of DES (0.02, 0.2, and 2.0 ng/g body weight per day) were fed to pregnant female mice, we again observed prostatic enlargement in male offspring. We also have been using the same procedures described for DEX for pregnant female mice 2.0 and 20 ng/g body weight per day of biphenyl A (an estrogenic estrogen that can leach out of polycarbonate plastic, the epoxy lining of metal food cans, and plastic dental sealants), and we observed a significant increase in adult prostate weight in male offspring relative to control males (17).

Taken together, our findings suggest that complex mechanisms mediating prostate differentiation and growth can be altered by exposure during fetal life to a very small increase in circulating estrogen, resulting in a permanent, irreversible (imprinted) enlargement of the prostate that can be detected by the end of the first day of fetal prostate development. Whether a fetus receives a small supplement of estrogen by dilution from adjacent female fetuses in species in which there are multiple fetuses within the uterus (as in 1M1 and 2F1 male mice) or by placental exposure to environmental estrogenic chemicals (such as bisphenols or bisphenol A) or estrogenic drugs (such as DES or ethinyl estradiol), our findings suggest that a significant shift in the population mean for numerous traits (6, 25), in addition to prostate size, will occur.

There has been speculation that during aging in men, estrogen interacts with androgens in the etiology of benign prostatic hyperplasia, the most common disease of aging in men (20, 26). Animal studies have shown that continuous, long-term treatment with a small supplement of estrogen, in combination with androgens, throughout adulthood induces prostatic enlargement in mice (1), rats (27), and dogs (29). Taken together with these studies using adult animals, our current findings suggest that at any time in life, a small supplement of estrogen can produce prostatic enlargement. Our findings also demonstrate that when this exposure occurs during fetal life, the effect is permanent. In male mouse fetuses exposed to a small increase in estradiol there was a significant increase in the number of prostatic glands throughout the dorsal urethral sinus (Fig. 1), including the dorsocentral region of the urethral sinus and are prone to the occurrence of benign prostatic hyperplasia during aging, while more caudal glands in men are prone to malignancy (20). In this regard, it has been proposed (29) that benign prostatic hyperplasia in aging men results from a reawakening of the same processes involved in embryonic growth of the prostate, and between 4 and 18 months of age in male CF1 mice, the prostate doubles in size (after correction for body weight) (20).

Only estradiol that is not bound to a plasma protein can diffuse through the lipid membrane of cells and thus be biologically active. In tissues, such as the prostate, in which there is essentially no metabolism of estradiol, only the free fraction of estradiol in serum contributes to the bioactive concentration of total circulating estradiol, because the protein-bound and free fractions of estradiol remain in equilibrium as blood rapidly flows through the tissue (30). We found
that a very low percent of total serum estradiol (0.2%) is free in male mouse fetuses, similar to previous findings in rats (21), and that a significant increase in prostate size occurred in response to an increase in free estradiol from 0.21 pg/ml to 0.32 pg/ml. Given the absence of any information from in vivo studies on developmental effects of low doses of estrogenic chemicals, it is interesting that in vivo studies conducted over the last few decades with estrogen-responsive cells predicted that an increase in free estradiol as low as 31 pg/ml of serum would result in a biological response in organ with estrogen receptors, such as the female prostate (11). Specifically, in studies with MCF-7 human breast cancer cells, the concentration of estradiol in culture medium sufficient to produce a half-maximal growth response is approximately 0.5 pg/ml of medium (22). A response to the growth-promoting actions of estradiol can be seen at 0.1 pg/ml (0.4 pM) (13).

Numerous studies have shown that exposure to a high dose of DES during development results in an abnormally small prostate and decreases the number of prostatic androgen receptors in adulthood (32, 33). We confirmed these previous findings in that there was a significant decrease in adult prostate weight in response to our 300-ng/kg body dose of DES. Effects on prostatic differentiation of high doses of natural and man-made estrogens are thus opposite to effects of low doses. As the dose of both estradiol and DES increased, we observed an inverted-U relationship between dose and response, although there was a much greater range of doses of DES compared with estradiol required to show the inverted-U dose-response curve in prostate weight. This is likely related to differences associated with route of maternal administration (aqueous release of estradiol from a sludicapsule vs. feeding of DES once per day). Potential mechanisms mediating a decrease in prostate weight in response to supraphysiological doses of estrogen include receptor down-regulation (34) and the capacity for estradiol (and possibly other estrogenic chemi- cals) to bind to receptors for other steroids, such as androgen receptors, resulting in antagonistic effects mediated via other receptor systems.

Even though inverted-U responses are not uncommon in physiology (36), the possibility of such nonmonotone re- sponses as dose increases has not been incorporated into methods of testing environmental chemicals that can mimic the antioxidative systems. Estrogenic Carcinogenic Testing Methods for systemic toxics, which includes estrogenic endocrine disrupters, are based on the assumption of a monotonic dose-response relationship, where the addition of an environ- mental chemical is assumed to increase or stay the same (but not increase and then decrease) as dose increases (37, 38). We show here that this assumption is invalid with respect to the effect of exposure to estrogenic chemicals during fetal life on adult prostate size. We also have previously demonstrated a similar inverted-U relationship between maternal dose of DES and territorial behavior is male offspring (25). The permanent change in the size of the prostate that we observed with very low (parts per trillion) doses of estradiol and DES was not predicted from previous studies in which much higher doses were used, and estrogen was reported to inhibit growth of the fetal prostate (33). The possibility now must be considered that prolonged enlargement of the prostate, as well as permanent alteration in the functioning of other estrogen-responsive organs in animals and humans, could occur due to exposure during fetal life to low doses of estrogenic chemicals present in drugs (birth control pills) or to environmentally relevant concentrations of estrogenic chemicals present in food, water, and air (from pesticides, components of plastics, detergents, hand creams, and other products).

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