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Physiology

Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses

(androgen receptors/benign prostatic hyperplasia/dose-response)

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ABSTRACT **On the basis of results of studies using high doses of estrogens, exposure to estrogen 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 mouse fetuses (released by a maternal Silastic 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 per g of body weight per day increased adult prostate weight, whereas a 200-ng-per-g dose decreased adult prostate weight in male offspring. Our findings suggest that a small increase in estrogen may modulate the action of androgen in regulating prostate differentiation, resulting in a permanent increase in prostatic 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 adult 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 refer to 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 *in utero* 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 2F 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 prostatic 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 2F 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 correlative study of intrauterine 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 dorsocranial, dorsocaudal, 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 prostatic lobe (4).

In the following series of experiments we examined the relationship between the levels of natural or man-made estrogen during fetal life and prostate size as well as number of prostatic 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 sexual differentiation is initiated. Our objective was to test the hypothesis that the enlarged prostate and elevated numbers of androgen receptors in 2F males were mediated by exposure to a small supplement in estradiol during fetal life.

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Abbreviations: DES, diethylstilbestrol; 1MF, positioned between one male and one female; ANCOVA, analysis of covariance.

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We thus determined the concentrations of total and free (not bound to plasma proteins) serum estradiol in untreated male mouse fetuses 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 androgen receptors. We then examined the effect of increasing free serum estradiol up to 8-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 1979 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 chow. Rooms 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 examined 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 1MF males per litter of 12 fetuses (6, 15).

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 CO_2 asphyxiation and cervical dislocation between 0900 and 1100 on gestation day 18 (one day before parturition). Blood from all 1MF male fetuses in each litter was collected in heparinized micropipettes, and one 1MF male fetus was randomly selected from each litter for prostate reconstruction (6 per group). The urogenital 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 (7 μm) through the fetal urogenital sinus as described previously (13). The outlines of the prostatic buds in the dorsal, lateral, and ventral budding lines were used to calculate the number of prostatic buds, the length of the line of buds along the urogenital sinus, the sum of the cross-sectional areas (total area) for buds in a budding line, and mean cross-sectional area for the buds in a budding line. The same measurements were made for the utriculus (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 tocopherol-stripped corn oil (ICN, catalog no. 901415), and 30 μl containing six different concentrations of DES [0 (vehicle controls), 0.002, 0.02, 0.2, 2, 20, and 200 ng/g body weight] were fed to pregnant mice (6–8 per group) once daily from gestation day 11 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 remained unhandled throughout pregnancy also was 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 micropipetter (Rainin Instruments) enabled delivery of an accurate volume of corn oil into the mouth. Mice readily consume corn oil, and this procedure was used instead of gavage 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 nurse their own offspring.

Postnatal Examination of the Prostate in 8-Month-Old Males. *Prenatal 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 unhandled throughout pregnancy was included (these females also were killed 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 within a normal range (19). 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 −70°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).

Prenatal DES Treatment. Males were weaned on postnatal day 23, and 2–4 males from the same litter were housed together. At 7 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 Statistical Analysis System, GLM procedure. Planned comparisons were made using the LSmeans 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 the variance in prostate weight on the basis of ANCOVA, prostate weight was reanalyzed by ANOVA. Results are presented as mean ± SEM.

Assays. *Radioimmunoassays.* Concentrations of total serum estradiol and testosterone were determined for control male fetuses and male fetuses exposed to different doses of estradiol

by maternal treatment using radioimmunoassay procedures described in detail elsewhere (18, 21). ^{125}I-labeled Estradiol and ^{125}I-labeled testosterone, as well as antisera, were obtained from ICN. Unlabeled estradiol and testosterone were obtained from Steraloids (Wilton, NH). Sensitivity for the assays was 0.5 pg for estradiol assay and 4.0 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.

Centrifugal Ultrafiltration Dialysis. Sera from 1MF males in each litter ($n = 6–8$ 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 1MF males then were pooled for determination of the percent free estradiol by centrifugal ultrafiltration 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 presented.

Prostatic Androgen Receptors. Cytosolic androgen receptors were measured as previously described (9), but with the following modifications. Cytosols were prepared from prostates in 10 mM Tris·HCl, pH 7.2/1.5 mM EDTA/1 mM $NaMoO_4$/1 mM DTT/1 mM phenylmethylsulfonyl fluoride/10% glycerol at 1–2 mg of protein per ml. Endogenous androgens were removed with dextran-coated charcoal, 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 nM with [^{3}H]dihydrotestosterone (DHT) (DuPont/NEN), 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 and normalized to cytosol protein (22) or to total DNA (23). Receptors occupied by endogenous ligand ("nuclear" receptors) also were determined in high-salt extracts as previously described (9), but these values were less than 10% of cytosolic receptors.

RESULTS

Total Serum and Free Serum Estradiol in Male Fetuses. The concentration of total serum estradiol in control 1MF male fetuses was 94 ± 7 pg/ml, corresponding to a free serum estradiol concentration of 0.21 pg/ml (0.21 parts per trillion or 0.77 pM). 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 pM) increase in the free serum estradiol concentration, resulting in a free serum estradiol concentration of 0.32 pg/ml (1.17 pM) in these male fetuses. The remaining capsule doses of estradiol (100, 200, and 300 μg) led to total serum estradiol concentrations of 232 ± 37, 355 ± 42, and 530 ± 26 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 0-, 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 fetuses exposed to the highest dose (300 μg) of estradiol, there was a significant increase ($P < 0.05$) in total serum testosterone to 3.5 ± 0.3 ng/ml (12.1 nM) relative to control males (0 dose), which had 2.4 ± 0.3 ng/ml (8.3 nM). The total serum testosterone concentration was not significantly different in control male fetuses and male fetuses from the other estradiol treatment groups.

A Low Dose of Estradiol Increases the Number of Prostatic Glands in Male Fetuses. 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 males carried by females implanted with the 25-μg dose of estradiol relative to control males (Table 1). Estrogen-treated males showed a significant increase ($P < 0.05$) in the total cross-sectional 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 estrogen-treated males. The increase in prostate size in response to the 25-μg dose of estradiol was most pronounced in the dorsal budding lines (Table 1; Fig. 1). Overall, the mean cross-sectional area of the individual epithelial buds was not significantly different between control and estrogen-treated males. These findings show that estrogen treatment increased the number of prostatic buds (gland genesis) and that the buds developed along a greater length of the urogenital sinus relative to control males (the overall volume 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 utriculus was significantly greater ($P < 0.05$) in estrogen-treated males (158 ± 8 μm) than in control males (124 ± 6 μm), although the total area of the utriculus 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 estrogen-treated males (61,636 ± 6078 μm^2) relative to control

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

Region	Treatment	No. of buds	Total area, μm^2	Length, μm	Mean area, μm^2
All	C	33.3 ± 6.6	29,637 ± 4,403	202 ± 26	896 ± 55
		*	*	*	NS
	E	46.8 ± 3.4	39,101 ± 4,403	279 ± 26	941 ± 55
Dorsal	C	14.4 ± 3.9	16,080 ± 4,732	194 ± 55	565 ± 40
		NS	*	*	*
	E	20.0 ± 3.1	27,260 ± 4,597	277 ± 50	739 ± 108
Lateral	C	9.5 ± 2.3	14,710 ± 3,766	127 ± 29	817 ± 71
		NS	NS	NS	NS
	E	11.5 ± 1.9	20,283 ± 3,827	180 ± 31	798 ± 77
Ventral	C	9.4 ± 1.3	58,120 ± 15,102	285 ± 62	1305 ± 169
		*	NS	NS	NS
	E	15.3 ± 3.8	69,761 ± 6,972	379 ± 33	1287 ± 39

Data are presented for all prostatic regions combined and separately for each region of budding. NS, not significantly different. *, statistically significant.

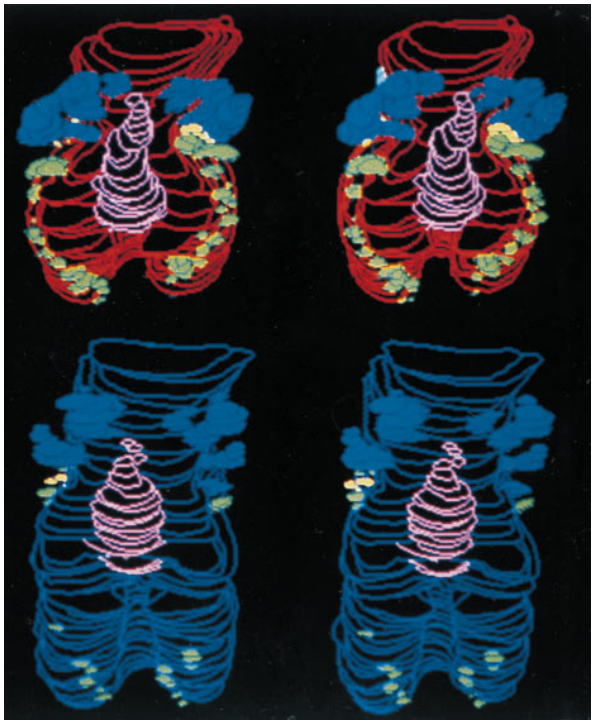

FIG. 1. Two stereo pair images (convergent, or cross-eyed viewing) of computer-assisted, serial-section reconstructions showing the dorsal portion of the prostate from two mouse fetuses. The prostate from a control male with 0.21 pg/ml free serum estradiol (blue urethra) is shown below. The top prostate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

males (77,375 ± 6078 μm^2), 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-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 capsule 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 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)-, 25-, 100-, 200-, and 300-μg estradiol groups were 36.2 ± 0.7, 35.6 ± 0.8, 33.8 ± 0.9, 33.2 ± 1.1, and 35.5 ± 1.1 g, respectively. Body weight and prostate weight were not significantly correlated ($r = 0.1$; $P = 0.5$; $n = 38$). ANCOVA showed that body weight did not account for a significant portion of the variance in prostate weight [$F(1, 32) = 0.5$, $P > 0.1$], and the data for prostate weight shown in Fig. 2 thus were not corrected for body weight.

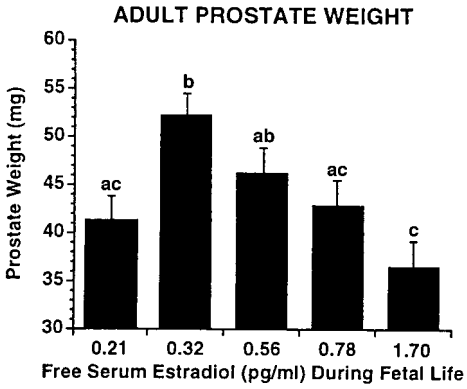

FIG. 2. Mean (+ SEM) prostate weight (mg) in 8-month-old male mice produced by mothers implanted s.c. with Silastic capsules containing 0, 25, 100, 200, or 300 μg of estradiol from day 13 to day 19 of pregnancy. The free serum estradiol concentration (in pg/ml) in male fetuses on gestation day 18 in response to these doses of estradiol (controls = 0.21 pg/ml) 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.

The number of prostatic androgen receptors was examined in control males and experimental males exposed *in utero* to 0.32 pg/ml free serum estradiol (Fig. 3). Protein and DNA were measured as the reference for expressing receptor numbers and to allow discrimination between hypertrophy and hyperplasia due to elevated estradiol during fetal life. Relative to adult controls exposed to 0.21 pg/ml free serum estradiol during fetal life, in 1MF males with 0.32 pg/ml free serum estradiol during fetal life, there was a significant increase (6-fold) in the total number of androgen receptors per prostate (per mg protein), a significant increase (2-fold) 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, $P < 0.05$).

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 influenced adult prostate weight (Fig. 4; $P < 0.001$). After correction for body weight by ANCOVA, relative to controls, males in the 0.02-, 0.2-, and 2-ng/g DES dose groups had significantly increased prostatic weights. In marked contrast, prostates in males in the 200-ng/g 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 [$F(1, 55) = 8.6$, $P < 0.01$], 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 [$F(6, 56) = 4.6$, $P < 0.001$]. Mean body weights for males exposed to the 0 (control), 0.002-, 0.02-, 0.2-, 2.0-, 20-, and 200-ng/g DES doses were 37.4 ± 0.7, 35.1 ± 0.8, 38.2 ± 0.7, 39.3 ± 1.9, 36.6 ± 1.1, 36.6 ± 0.6, 39.9 ± 0.8, and 34.0 ± 1.0 g, respectively. Body weight of males exposed to the 200-ng/g 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-ng/g dose ($P = 0.05$) tended to be lighter, while males exposed to the 20-ng/g 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

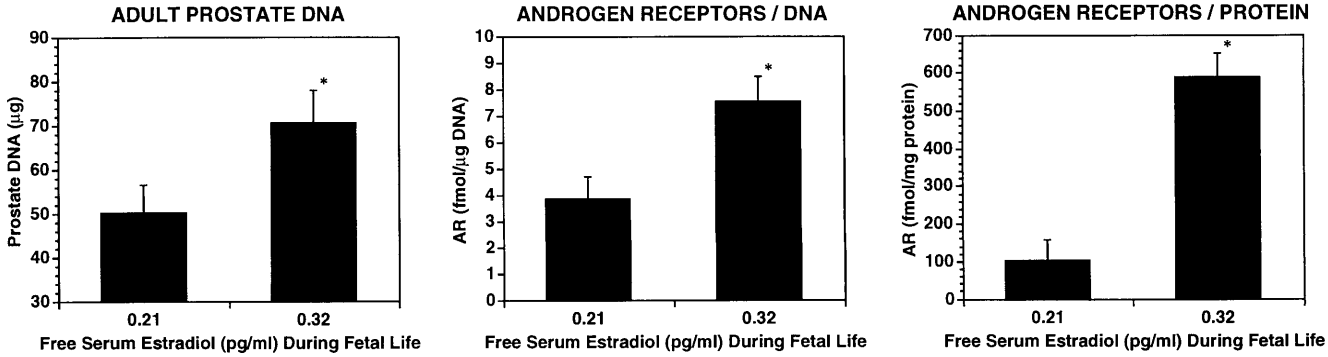

FIG. 3. For males exposed during fetal life to 0.21 pg/ml free estradiol (controls) and 0.32 pg/ml free estradiol (25 μg estradiol dose group), mean (+ SEM) total DNA (in μg), androgen receptors per mg of DNA (per cell), and androgen receptors per mg of protein are shown (these were determined after the prostate was weighed). * Statistically significant.

increased the number of developing prostatic glands by 40%. This increase in fetal estradiol subsequently led in adult 1MF 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 1MF 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 the total serum estradiol concentration in individual 2M male mouse fetuses (that are not exposed to a supplement of estradiol from an adjacent female fetus) as well as in individual 1MF and 2F male fetuses on gestation day 18; the highest estradiol value was 159 pg/ml, which was observed in a 2F male fetus (6). Our lowest-dose (25 μg) estradiol treatment (that produced prostate enlargement) increased total serum estradiol in 1MF male fetuses from a mean of 94 pg/ml to 146 pg/ml, and thus significantly shifted the population distribution for serum estradiol concentrations during fetal life in male mice.

When very low doses of DES (0.02, 0.2, and 2.0 ng/g of body weight per day) were fed to pregnant female mice, we again observed prostate enlargement in male offspring. We also have fed (using the same procedures described for DES) pregnant female mice 2.0 or 20 ng/g of body weight per day of bisphenol A (an estrogenic chemical that can leech 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).

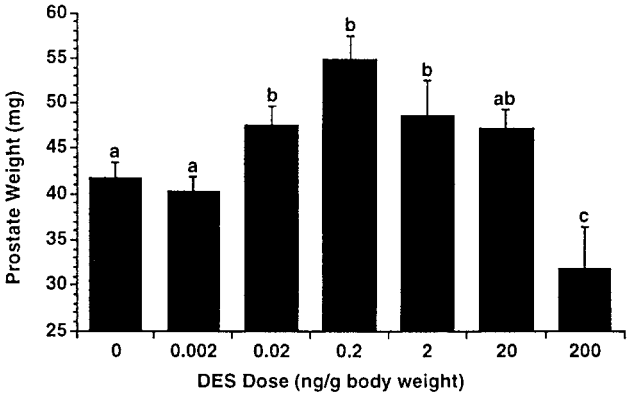

FIG. 4. Mean (+ SEM) prostate weight (mg) in 8-month-old CF-1 male mice produced by females fed different doses of DES from day 11 to day 17 of pregnancy. Group means that differed significantly are indicated by different letters.

Taken together, our findings suggest that the 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 diffusion from adjacent female fetuses in species in which there are multiple fetuses within the uterus (as in 1MF and 2F male mice) or transplacentally by maternal exposure to environmental estrogenic chemicals (such as pesticides 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 androgen 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 androgen, throughout adulthood induces prostate enlargement in mice (1), rats (27), and dogs (28). 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 prostate 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 urogenital sinus (Fig. 1), including the dorsocranial region. This finding suggests that effects of estrogen occur in glands within the mouse prostate that may be homologous to glands in the human prostate that bud from the dorsocranial region of the urogenital 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 CF-1 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 vitro* studies conducted over the last few decades with estrogen-responsive cells predicted that an increase in free estradiol as low as 0.1 pg/ml of serum would result in a biological response in organs with estrogen receptors, such as the fetal 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 (2 pM); a response to the growth-promoting action of estradiol can be seen at 0.1 pg/ml (0.4 pM) (31).

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 200-ng/g of body weight dose of DES. Effects on prostate 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 (tonic release of estradiol from a Silastic capsule 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 chemicals) to bind to receptors for other steroids, such as androgen receptors, resulting in antagonistic effects mediated via other receptor systems (35).

Even though inverted-U responses are not uncommon in physiology (36), the possibility of such nonmonotonic responses as dose increases has not been incorporated into methods of testing environmental chemicals that can mimic the action of estrogen or other hormones. Current testing methods for systemic toxicants, which includes estrogenic endocrine disruptors, are based on the assumption of a monotonic dose-response relationship, where the response to an environmental 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 in male offspring (25). The permanent increase 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 permanent 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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