



Role of Estrogen in Androgen-Induced Prostate Carcinogenesis in NBL Rats

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Abstract

Androgens are thought to cause prostate cancer, but the underlying mechanisms are unclear. Data from animal studies suggest that for androgens to cause prostate cancer, they must be aromatized to estrogen and act in concert with estrogen metabolites. We tested the hypothesis that androgen-receptor and estrogen receptor-mediated effects of androgen and estrogen are necessary, as well as genotoxicity of estrogen metabolites. NBL rats were treated with androgenic and estrogenic compounds for 16–75 weeks through slow-release silastic implants or pellets. Testosterone alone induced cancer in the prostate of 37% of rats. 5 α -Dihydrotestosterone, which cannot be converted to estradiol or testosterone, did not cause a significant prostate cancer incidence (4%). Addition of estradiol to 5 α -dihydrotestosterone treatment did not markedly enhance prostate cancer incidence (14%), unlike adding estradiol to testosterone treatment which induced a 100% tumor incidence. Testosterone plus estradiol treatment induced a DNA adduct detectable by ³²P-postlabeling, oxidative DNA damage (8-hydroxyguanosine), and lipid peroxidation at the site within the prostate where this treatment causes cancers, preceding later cancer formation. The non-estrogenic 4-hydroxy metabolite of estradiol, when combined with testosterone, induced prostatic dysplasia within 16 weeks and, after long-term treatment, a very low incidence of prostate cancer (21%). When an estrogen that cannot be hydroxylated (2-fluoroestradiol) was added to this combined treatment with testosterone and 4-hydroxyestradiol, dysplasia frequency after 16 weeks was doubled. These results strongly support the hypothesis, but additional definitive studies are needed which may identify new targets to interfere with these mechanisms that are clinically feasible in humans.

Keywords Prostate cancer · Estrogen · Androgen · Hormonal carcinogenesis

Nur Ozten and Katherine Vega contributed equally to this work.

Joachim Liehr is deceased. This paper is dedicated to his memory.

This paper is dedicated to the memory of Joachim G. Liehr who died in 2003. He developed the notion that estrogen genotoxicity is an essential component of estrogen carcinogenesis and created a substantive body of evidence in support of this mechanism.

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Introduction

Prostate cancer is the most frequently diagnosed cancer and the second most frequent cause of death due to cancer in US males. Although the causes of prostate cancer are not clear, hormones, particularly androgens and estrogens, are thought to be critically involved in human prostatic carcinogenesis [1]. The basis for this assumption is that the prostate gland is an androgen-dependent tissue and that prostate cancer is an androgen-dependent malignancy [1]. The underlying mechanism has been postulated to be androgenic stimulation of cell proliferation resulting in an increased risk of oncogenic genetic alterations [2]. However, there are no human data of the effects of androgen treatment on prostatic cell proliferation, but treatment of men with 5 α -dihydrotestosterone (DHT) did not alter androgen-regulated gene expression in one study [3]. Chronic testosterone (T) treatment of rats via subcutaneous silastic tubing implants or cholesterol pellets results in development of prostate adenocarcinomas in at least five different strains at incidences ranging from 7 to 19% [4, 5].

While prostate cancer risk increases steeply with age [6], circulating androgen levels decrease with aging. Estrogen levels remain unchanged or increase slightly resulting in an increase in the ratio of 17 β -estradiol (E2) to T with age, which has been suggested to point to a role of estrogen in prostate carcinogenesis [7]. Intraprostatic levels of DHT decrease with aging in epithelial cells, but not in stromal cells, while levels of E2 and estrone increase with age in the stromal compartment but not in the epithelium [8]. These observations suggest that prostate cancer risk may be associated with elevated concentrations of circulating and/or intraprostatic estrogens, but there is no direct evidence for such an association [9–11].

T can be converted to E2 by the enzyme aromatase, which is expressed in fat tissue and in the human and rodent prostate [12]. However, there is no evidence of an association between prostate cancer risk and single nucleotide polymorphisms in the aromatase (CYP19A1) gene that are associated with altered serum levels of total and free E2 [13]. Nonetheless, animal experiments suggest that estrogen may be involved in the aforementioned induction of prostate cancer by T in rats. We have shown that T treatment combined with E2 induces a prostate cancer incidence of 100% in NBL rats [14, 15]. These results have led us to hypothesize that estrogen and aromatization of T to E2 play a critical role in prostate carcinogenesis, at least in the rat. Of note, estrogen treatment alone results in shutdown of LH production and endogenous androgen production, resulting in prostatic atrophy. Therefore, experiments to determine whether E2 can cause prostate cancer require joint administration of androgen to restore physiological levels of testosterone.

E2 and estrone (E1) can be enzymatically metabolized to 2- and 4-hydroxyestradiol and 2- and 4-hydroxyestrone by CYP1A1 and CYP1B1. These so-called catecholestrogens

can be converted to highly reactive estrogen semiquinones and estrogen quinones by the process of redox cycling [16, 17]. These reactive intermediates can adduct to DNA and redox cycling additionally causes generation of reactive oxygen species (ROS). ROS can induce lipid peroxidation, resulting in the formation of lipid hydroperoxides, and both can damage DNA and potentially lead to mutations [16, 17]. The 4-hydroxyestradiol (4OH-E2)-quinone-DNA adducts rapidly result in apurinic sites in the DNA which, when repaired by error-prone DNA repair mechanisms, can lead to mutations [18]. Detection of quinone-DNA adducts has been difficult, because they depurinate with a very short half-life, leading to apurinic sites that are difficult to detect. However, with highly sensitive analytical methods (LC-MS/MS), such adducts have been observed after estrogen treatment of DNA, cells, and tissues [19–21]. In the rodent [22] and human prostate [*E. cavaleri* & E. Rogan, personal communication], and in studies of urinary levels of estrogen metabolites and adducts in men with or without prostate cancer [23, 24], evidence has been found of enzymatic conversion of E2 and E1 by CYP1A1 and CYP1B1 to the catecholestrogens and subsequently the quinones.

We hypothesized that for T to be carcinogenic to the rat prostate, it must be aromatized to E2 which acts as a chemical carcinogen through the above described metabolism to catecholesterogen and subsequent redox cycling leading to DNA damage [25]. We have demonstrated that these reactions can take place in the rat prostate [22] and we identified a major DNA adduct by ³²P-postlabeling selectively in the periurethral area of their prostates, the site of later cancer development [26], following treatment for 16 weeks with T plus E2 of NBL rats. A low level of this adduct was also found at this location in control rats, perhaps indicating sensitivity of this tissue to DNA damage. In the present paper, studies are described that provide further evidence that T is carcinogenic for the prostate and that aromatization of T to E2 and estrogen genotoxicity are involved in hormonal prostate carcinogenesis in the NBL rat model.

Methods

Animals and Animal Care

Male NBL/Cr rats 4–5-week-old were obtained from Charles River (Kingston, NY). The animals were housed in solid bottom cages, 2–3 to a cage under conventional conditions (22 \pm 2 $^{\circ}$ C, 40–70% relative humidity, 12 h light/12 h dark); they had free access to tap water and an open formula, natural ingredient diet, NIH 07 (Zeigler Bros., Gardners, PA). Body weights were recorded weekly. The experiments were reviewed and approved by the NYU School of Medicine Institutional Animal Care and Use Committee.

Hormone Treatments

At 8–9 weeks of age, the animals received the following hormone treatment: two silastic implants (Dow Corning (Midland, MI), type 602 305, I.D. 1.6 mm; O.D. 3.2 mm, sealed with General Electric (Waterford, NY), RTV-108 silicone rubber adhesive sealant), each containing a tightly packed filling of T or DHT (Steraloids, Wilton, NH) over a length of 2 cm. E2 was administered via one such implant containing a tightly packed 1-cm-long filling of the steroid (Steraloids). The implants were placed subcutaneously on the back of the rats. T and E2 implants were replaced at 6-month intervals; at the end of each interval, the implants still contained a substantial amount of the steroids. This treatment was previously shown to stably increase circulating E2 levels 148% from 3.3 ± 0.7 to 49 ± 4.0 pg/ml similar to peak estradiol levels in female rats, while keeping T concentrations at normal levels [27]. Control rats received empty implants or control pellets. Ninety-day (~13 week) slow release pellets containing 4OH-E2 or 2F-E2 (obtained from Steraloids) were made for this project by Innovative Research of America (Sarasota, FL) and replaced every 13 weeks.

Autopsy and Tissue Sampling Procedures

Animals were euthanized by a pentobarbital overdose and exsanguination after 13 or 16 weeks or, in long-term experiments when they became moribund or at the end of the experiments at 75 weeks after initiation of treatment. Immediately following death, the entire accessory sex gland complexes together with the urinary bladder were removed in to and either fixed in 4% neutral buffered aqueous formaldehyde solution or rapidly snap frozen in liquid nitrogen following micro-dissection to separate the dorsolateral, ventral, and anterior prostate, periurethral area, and seminal vesicles the secretion of which was emptied before freezing. These tissues were stored at -80 °C until they were analyzed following pulverization using a Microdismembrator-II (Braun, Melsungen, Germany) in liquid nitrogen cooled stainless steel ball containing capsules.

Histology and Immunohistochemistry Methods

The accessory sex glands were dissected after fixation as described previously [28] to separate the ventral lobes, anterior prostate (= coagulating gland) plus seminal vesicle complex, and dorsolateral prostate complex, including the periurethral area plus prostatic urethra. These tissues were processed to and embedded in a paraffin wax, the dorsolateral prostate complex cut in two halves. Five-micrometer-thick sections were prepared and stained with hematoxylin and eosin (H&E). Accessory sex glands were step sectioned at approximately 250- μ m intervals, producing six sections per tissue for

the dorsolateral prostate complex and coagulating gland plus seminal vesicles and one section for the ventral prostate.

For staining of 8-OHdG, a mouse monoclonal antibody was obtained from the Japan Institute for the Control of Aging (Fukuroi City, Shizuoka, Japan). Sections were autoclaved with 0.62 M ZnSO₄ at 100 °C for 15 min and then treated with 2 M HCl at 37 °C for 30 min. Sections were pre-incubated with 10% normal horse serum for 30 min at 37 °C and then treated with an avidin/biotin block (Vector Laboratories, Burlingame, CA) before overnight incubation at 4 °C with the primary antibody against 8-OHdG (diluted 1/40). After incubation with a secondary peroxidase conjugated anti-mouse antibody (DakoCytomation, Glostrup, Denmark), immunoreactivity was visualized with DAB (DakoCytomation). Human seminal vesicle was used as a positive control tissue [29].

Measurement of 4-OHE2-Induced DNA Adducts

4-OHE2-induced DNA adducts were measured essentially as described by Li et al. [20] with some modifications. Briefly, tissues were ground to a powder in liquid nitrogen and suspended in 50 mM ammonium acetate, pH 4.4, containing 1 mg/ml ascorbic acid, with a final concentration of 30% methanol. They were passed through a Certify II solid phase extraction column and eluted with 70% methanol in the buffer. The eluant was evaporated to dryness and resuspended in 0.2 ml methanol. LC/MS analyses were carried out with a Waters Aquity Ultra Pressure Liquid Chromatography (UPLC) system connected with a high-performance Quattro Micro triple quadrupole mass spectrometer designed for LC-MS/MS operation. Analytical separations on the UPLC system were conducted using Aquity UPLC BEH C18 1.7 μ column (1 \times 100 mm) at a flow rate of 0.15 ml/min. The analytical gradient started with 80% A (0.1% formic acid in H₂O) and 20% B (0.1% formic acid in CH₃CN). Eighty percent A changed to 79% over 4 min, followed by a 6 min linear gradient to 45% A, resulting in total separation time of 10 min. The elutions from the UPLC column were introduced to the mass spectrometer.

The electrospray ionization (ESI) method was used in both positive ion (PI) and negative ion (NI) mode with a capillary voltage of 3.0 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 600 l/h. Cone gas flow was set at 60 l/h. Desolvation temperature and source temperature were set to 200 and 100 °C, respectively. Tandem mass spectrometry (MS/MS) was performed in a multiple-reaction monitoring (MRM) mode to produce structural information about a compound by fragmenting specific parent ions inside the mass spectrometer and identifying the resulting fragment ions. Pure standards were used to optimize the LC/MS conditions prior to analysis. Due to small amounts of tissue, prostate tissues

within each group of this study were pooled together. Analyses were performed in duplicate and data are presented as the mean of two replicates. The limits of detection for DNA adducts varied between 1.82×10^5 and 4.94×10^5 pmol/g tissue.

Measurement of 8-Hydroxyguanosine (8-OHdG)

8-OHdG was measured by HPLC according to Hofer and Moller [30]. DNA was extracted and digested using a modified alkaline phosphatase-nuclease P1 method, adding DNase I, phosphodiesterases I and II, and an iron chelator, as described in detail elsewhere [31]. Quantitation of nucleosides and 8-OHdG was done using a 4.6×250 mm Microsorb C18 column (Varian, Palo Alto, CA) and reverse-phase ESA HPLC with electrochemical detection of 8-OHdG by a Coulochem II electrochemical detector (ESA Biosciences, Chelmsford, MA) and of nucleosides by spectrophotometry according to Hofer and Moller [30]. Data presented are expressed as 8-OHdG/ 10^5 dG,

Measurement of Lipid Hydroperoxides

Lipid hydroperoxides were measured after isopropanol extraction of pulverized tissue (1:3, *w/v*) with a colorimetric assay [32] using a kit (LPO-CC) from Kamiya Biomedical (Seattle, WA) with cumene hydroperoxide as standard, as described by Wang and Liehr [33].

Measurement of DNA Adducts by ^{32}P -Postlabeling

DNA adducts were detected and quantified by ^{32}P -postlabeling as described previously in detail [26]. Briefly, DNA was isolated by phenol/chloroform extraction and enzymatic digestion according to Gupta [34]. DNA was assayed for adducts using the nuclease Pi-enhanced ^{32}P -postlabeling procedure of Reddy and Randerath [35]. To quantify adduct levels, adduct spots were excised from chromatograms and subjected to Cerenkov counting and values were expressed as relative adduct labeling (RAL) according to Reddy and Randerath [36].

Statistical Analysis

Mortality data were analyzed using the Kruskal-Wallis test and Dunn's multiple comparison test. For body weight data, analysis of variance followed by Dunnett's multiple comparison test or a 2 sample *t* test was used. Tumor and lesion incidences were analyzed using the Fisher exact probability test. Data on tissue adduct and lipid peroxide levels were analyzed using a 2 sample *t* test or ANOVA followed by Student-Newman-Keuls or Tukey multiple comparison test.

P values of ≤ 0.05 (one- or two-sided, as applicable) were considered significant.

Results

Testosterone Induced Prostate Cancer and Estradiol Enhanced the Incidence, but DHT with or Without Estradiol Did Not (Tables 1 and 2)

First, we tested the idea that T needs to be aromatized to E2 to be carcinogenic by treating NBL rats with the 5α -reduced T metabolite DHT, which cannot be aromatized or converted back to T. We used a DHT dose that increased ventral prostate weight following 16 weeks of treatment to a similar degree as treatment with the dose of T we used previously and in this study (Table 1). The incidence of prostate carcinomas induced by chronic T treatment was 37% (11 of 30 rats), but DHT induced prostate cancer in only one of 27 treated rats (4%) (Table 2). Based on our previous observation that T plus E2 induced prostate cancers in 100% of treated NBL rats [14], we expected that addition of E2 to the chronic DHT treatment would produce a high prostate cancer incidence but only 14% of animals developed these tumors (Table 2). However, DHT plus E2 did induce a 100% incidence of dysplastic lesions in the dorsal and particularly lateral prostate after 16 weeks (Table 1) and after long-term treatment (data not shown), as we have previously observed for T plus E2 treatment [14, 27].

E2 is known to induce pituitary adenomas in rats and we have previously found a 100% incidence of such tumors after T plus E2 treatment, markedly reducing survival [14]. E2 when combined with DHT, however, produced pituitary tumors in only 30% of rats (Table 2), even though pituitary weights were increased after 16 weeks of treatment (Table 1). Furthermore, compared to controls, the effects of DHT and the DHT plus E2 combination on weights of seminal vesicle/anterior prostate complex were fundamentally different from those of T and T plus E2 treatment for 16 weeks, slightly reducing rather than increasing seminal vesicle/anterior prostate weights and reducing testicular weights far more (Table 1).

The dysplastic lesions found after 16 weeks of E2 plus T or E2 plus DHT treatment were morphologically identical to those described in detail previously [27], as were the mostly microscopic-size adenocarcinomas originating in the periurethral ducts found following these treatments [14]. The adenocarcinomas found in the dorsolateral prostate (eleven) and anterior prostate (one) of rats treated with only T or DHT were grossly visible and histologically well to moderately differentiated, similar to those we described following treatment with a chemical carcinogen and T [28]. The precise origin of most of these large carcinomas was not clear as they involved both glands in the periphery of the prostate and prostatic ducts leading towards the prostatic urethra; in several cases, these

Table 1 Effect of 16-week treatment of NBL rat with T or DHT with and without E2 on the weights of accessory sex glands, pituitary, and testes (mean ± SEM), and dysplasia and inflammation of the dorsolateral prostate (number (%) of animals with lesions)

Treatment	N	Body weight (g)	Relative weights (mg/kg body weight)				Testes (absolute weight, mg)		
			Ventral prostate	Dorsolateral prostate	Seminal vesicle/Anterior prostate	Pituitary	Dysplasia	Inflammation	
<i>Study a</i>									
Control ^a	10	394 ± 5	96 ± 4	136 ± 5	266 ± 13	2.5 ± 0.2	3623 ± 44	0	0
E2 plus T ^a	10	294 ± 8**	175 ± 11***	318 ± 11***	573 ± 36** ^{b,c}	9.9 ± 0.6 ^c	1435 ± 53*** ^c	10 (100%) ^d	10 (100%) ^d
<i>Study b</i>									
Control	10	402 ± 8	94 ± 2	134 ± 8	232 ± 52	2.8 ± 0.3	3643 ± 59	0	0
T	10	361 ± 7**	139 ± 7***	230 ± 9***	387 ± 27** ^{b,c}	3.1 ± 0.2 ^c	1988 ± 30*** ^c	0	0
DHT	11	342 ± 8*** ^b	139 ± 10***	203 ± 9***	219 ± 14 ^b	2.5 ± 0.2 ^c	1445 ± 20*** ^c	0	0
E2 plus DHT	11	299 ± 5*** ^b	139 ± 6***	201 ± 6***	185 ± 12 ^c	8.9 ± 1.0*** ^c	912 ± 17*** ^c	11 (100%) ^d	11 (100%) ^d

p* < 0.05, *p* < 0.01, ****p* < 0.001, *****p* < 0.0001 for difference with control values in each study (ANOVA with Tukey post hoc test)

^a Data taken from reference [14]

^b *p* < 0.01 for difference between groups with same suffix (ANOVA with Tukey post hoc test)

^c *p* < 0.001 for difference between groups with same suffix (ANOVA with Tukey post hoc test)

^d *p* < 0.0001 for difference with all other groups (Fisher exact test; one-sided)

Table 2 Induction of lesions in accessory sex glands by chronic treatment of NBL rats with T or DHT with and without E2 of lesions in accessory sex glands

Treatment	N	Median survival (weeks)	Periurethral ducts of Dorsolateral or Anterior Prostate	Peripheral glands of:			All Accessory Sex Glands tissues	Pituitary adenoma
				Dorsolateral Prostate	Anterior Prostate	Seminal vesicles		
<i>Study a^a</i>								
Control ^a	8	72	0	0	0	0	0	0
E2 + T ^a	12	44*	12 (100%)*	0	0	0	12 (100%)*	12 (100%)*
<i>Study b</i>								
Control	30	61	0	0	0	0	0	3 (10%)
T	30	67	0	10 (33%)*	1 (3%)	0	11 (37%)*	1 (3%)
DHT	27	69	0	1 (4%)	0	0	1 (4%)	0
E2 + DHT	28	57	4 (14%)*	0	0	0	4 (14%)*	8 (30%) ^b
<i>Study c</i>								
E2 + T-16 weeks	24	71	1 (4%)	0	0	0	1 (4%)	0
E2 + T – 16 weeks + chronic T	24	66	17 (71%)*	0	2 (4%) ^d	7 (29%)* ^c	18 (75%)*	0
E2 + T-8 weeks + chronic T	24	75	7 (28%)* ^c	1 (4%)	9 (38%)* ^f	2 (8%)* ^g	15 (63%)*	0

* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ for difference with control values (study a and study b) or E2 + T-16 weeks (study c) (Fisher exact test; one-sided)

^aData taken from reference [14]

^b $p \leq 0.002$ for difference with DHT only group and for difference with T group (Fisher exact test; two-sided)

^c $p < 0.01$ for difference with E2 + T – 16 weeks + chronic T group

^dBoth animals also had periurethral prostate tumors

^eSix of these seven rats also had periurethral prostate tumors

^f $p < 0.05$ for difference with E2 + T – 16 weeks + chronic T group; four of these nine rats also had periurethral prostate tumors

^gThese two rats only had seminal vesicle tumors but no prostate tumors

tumors were so large that they caused urethral obstruction. One of the carcinomas concentrically involved the periurethral ducts spreading out to the periphery of the prostate, suggesting that larger tumors after hormone treatment may also have originated from these ducts. However, one of the carcinomas in T-treated rats was smaller and clearly confined to the periphery of the dorsal and lateral prostate and no small cancers were found in the periurethral ducts of rats treated with only T or DHT. Metastases were found in the spleen, stomach, pancreas, liver, and/or lungs of three of the ten rats (30%) with prostate carcinomas after treatment with only T.

Short-Term Treatment with Estradiol Combined with Long-Term Testosterone Administration Caused a High Incidence of Prostate Cancer (Table 2)

To determine whether a short course of E2 treatment would induce prostate cancer but not induce pituitary tumors and impact survival in rats chronically treated with T, we gave NBL rats treatment with E2 plus T for 16 or 8 weeks at which time the E2 implant was removed, but the T implants were left in place for the duration of the study, until the animals became

moribund or died (Table 2). Animals lived 22–31 weeks longer than when the E2 implant was not removed, did not develop pituitary tumors, and they had carcinomas that originated in the periurethral ducts of the dorsolateral prostate (Table 2). In both treatment groups, some animals also developed carcinomas of the seminal vesicles and anterior prostate (Table 2) which we have never observed after continuous E2 treatment together with T. In the 16-week E2 group, the incidence of carcinomas of prostatic origin (75%) was driven by the presence of periurethral or dorsolateral prostate tumors. One animal from the 8-week E2 group developed a small adenocarcinoma in the periphery of the dorsolateral prostate and one rat from the 16-week E2 group had a periurethral adenocarcinoma that was grossly visible and extended into the dorsolateral prostate periphery. This tumor had metastasized to other abdominal organs. All of the seminal vesicle and six of the eleven anterior prostate tumors were grossly visible and many of the large tumors had metastasized, mostly to other abdominal organs. There was one undifferentiated sarcoma of the seminal vesicle in the 16-week E2 group (not listed in Table 2).

An additional group of rats received T plus E2 treatment for 16 weeks at which time both the T and the E2 implants

were removed and the animals followed until moribund. The animals also lived longer than those in which T plus E2 treatment was continued, but only one animal developed a periurethral prostate carcinoma (Table 2).

Long-Term Treatment with the Catechol Estrogen 4-Hydroxyestradiol in Combination with Testosterone Induced Prostate Cancer and Dysplasia at Low Incidence, but Required Additional Estrogenic Stimuli to Produce a High Dysplasia Incidence (Table 3)

To circumvent aromatization of T and the antiandrogenic effects of E2, we exposed NBL rats to 4OH-E2, the catechol metabolite of E2, alone or together with T using silastic tubing implants containing T and custom-made slow release pellets for 4OH-E2 administration. Because we used slow-release pellets with 13-week release duration, we first compared 13 weeks of E2 plus T with 16 weeks of this hormone treatment and found an identical 100% dysplasia incidence and comparable degrees of inflammation in the lateral prostate (data not shown). The dose of 4OH-E2 used in these studies, 5 µg/day given by slow release pellets, was based on the result of a preliminary 21-day experiment in which we found that this dose resulted in the formation of depurinating DNA adducts detectable by mass spectrometry (Fig. 1). We identified the following depurinating adducts in lateral, dorsal, and anterior prostate tissue: 4OH-

E2–1-N7-guanine, 4OH-E1-1-N7-guanine, 4OH-E2-1-N3-adenine, and 4OH-E1-1-N3-adenine. In the pooled prostate tissues of 4OH-E2-treated rats, we detected a total amount of these adducts of 2.10 pmol/g tissue, whereas in control prostates, we found a total of 0.45 pmol/g tissue, indicating that the 4OH-E2 dose we used did induce depurinating estrogen-DNA adducts.

T plus 4OH-E2 induced inflammation but no dysplasia after 13 weeks, while this treatment for 52 weeks induced carcinomas in 4 (28%) NBL rats, three in the prostate (21%) and one in the seminal vesicle. The latter treatment also induced a 43% incidence of dorsolateral prostate dysplasia, in addition to inflammation. One or two lesions classified as carcinoma in situ [28] were found in all 52-week treatment groups (Table 3). By contrast, we previously showed [14] that treatment with T plus E2 induced a 100% incidence of adenocarcinomas in the periurethral prostate and inflammation and dysplasia in the dorsolateral prostate of NBL rats (see Table 2). Treatment with only 4OH-E2 also induced dorsolateral prostate inflammation and dysplasia and a couple of carcinomas in situ and one prostate adenocarcinoma.

We then examined whether adding an estrogenic stimulus to the T plus 4OH-E2 treatment would potentiate their effects on the prostate. We used 2-fluoro-estradiol (2F-E2) which has been used successfully in the past to separate genotoxicity from estrogenicity in the male hamster kidney model to

Table 3 Incidence of carcinomas, dysplasia, and inflammation in the NBL rat prostate (no pituitary tumors developed in any rat) following treatment with T, 4OH-E2, and/or 2F-E2 for 13 or 52 weeks

Treatment	N	Periurethral prostatic ducts		Dorsolateral prostate			Adenocarcinoma in:		
		Adenocarcinoma	Carcinoma in situ	Inflammation	Dysplasia	Carcinoma in situ	Anterior prostate	Seminal vesicles	All glands ^a
<i>13 weeks study 1</i>									
Control	10	0	0	3 (30%)	0	0	0	0	0
4OH-E2	10	0	0	2 (20%)	0	0	0	0	0
4OH-E2 + T	9	0	0	7 (77%)* ^b	0	0	0	0	0
<i>13 weeks study 2</i>									
Control	6	0	0	2 (33%)	0	0	0	0	0
2F-E2	5	0	0	3 (60%)	0	0	0	0	0
2F-E2 + T	10	0	0	10 (100%)* ^c	3 (30%)	0	0	0	0
2FE2 + 4OH-E2 + T	10	0	0	9 (90%)*	6 (60%)* ^c	0	0	0	0
<i>52 weeks study</i>									
Control	14	0	1 (7%)	2 (14%)	3 (21%)	1 (7%)	0	0	0
4OH-E2	13	1 (8%)	2 (15%)	8 (62%)* ^c	3 (23%)	0	0	0	1 (8%)
4OH-E2 + T	14	1 (7%)	1 (7%)	7 (54%)*	6 (43%)	1 (7%)	2 (14%)	1 (7%)	4 (28%)*

* $p \leq 0.05$, ** $p < 0.02$, *** $p < 0.001$, for difference with control values in each group (Fisher exact test; one-sided)

^a All accessory sex glands combined: periurethral, dorsolateral, and anterior prostate and seminal vesicles

^b $p < 0.05$ for difference with 4OH-E2 group (Fisher exact test; one-sided)

^c $p < 0.05$ for difference with 4OH-E2 group (Fisher exact test; one-sided)

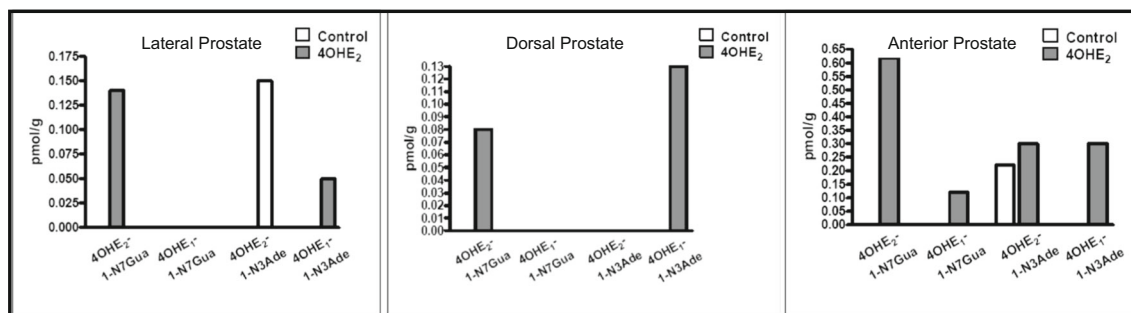


Fig. 1 4-OHE₂-induced DNA adducts. Levels of DNA adducts in dorsal (DP), lateral (LP), and anterior prostate (AP) of NBL rats following treatment with 5 μg/day 4-OHE₂ or placebo for 21 days. LC-MS/MS analysis

of pooled prostate tissue. Data are expressed as mean of two pooled tissue samples from five animals each

demonstrate that estrogen genotoxicity is required for estrogens to induce tumors in that classical model [37]. First, we identified a 2F-E₂ dose (5 μg/day) that produced estrogenic activity in NBL rats when delivered by slow release pellets; this dose increased pituitary weights from 3.30 ± 0.24 to 4.45 ± 0.21 mg/100 g body weight ($p = 0.005$; 2 sided t test). Following 13 weeks of treatment, 2F-E₂ alone increased the percentage of rats with dorsolateral prostate inflammation; and combined 2F-E₂ plus T caused inflammation in 100% of rats and dorsolateral prostate dysplasia in 30%. The combination of 2F-E₂ plus T plus 4OH-E₂ produced inflammation in 90% of rats and dysplasia in 60%, short of the 100% incidence of both lesions observed previously after T plus E₂ treatment [14]. However, although pituitary weights were increased by 2F-E₂ alone (see above), addition to T or T plus 4OH-E₂ reduced the weight to control levels (3.20 ± 0.93 and 3.67 ± 0.51 mg/100 g body weight, respectively) suggesting that this reduced estrogenicity of 2F-E₂.

Induction by Testosterone, Estradiol, and Testosterone Plus Estradiol of DNA Adducts Detectable by 32P-Postlabeling, 8-Hydroxyguanosine, and Lipid Hydroperoxides (Table 4)

We previously identified by 32P-postlabeling the formation of a major DNA adduct selectively in the periurethral area of the NBL rat after treatment with E₂ plus T for 16 weeks [14]. This finding demonstrated the induction of DNA damage by this hormone treatment at the precise site of later cancer development and preceding tumor development in time. Because we found that T alone caused a 37% incidence of prostate carcinomas, we measured the formation of this adduct in prostate tissues following treatment with just T and compared this with exposure to only E₂ and E₂ plus T. By 16-week treatment with E₂ plus T, we confirmed the induction of a major DNA adduct spot (P1) that was chromatographically identical to the adduct that we had previously identified selectively in the periurethral prostate [26] (Supplemental Fig. 1A).

Interestingly, T alone also induced this adduct at about half the amount and low levels of the adduct spot were found after E₂ treatment and in control rats (Table 4). The adduct spot was not detectable in the dorsolateral prostate (Supplemental Fig. 1A) or the ventral prostate (data not shown).

As a measure of oxidative DNA damage, we determined the formation of 8-OHdG in the NBL rat prostate by HPLC and immunostaining. Following 16-week treatment with E₂ plus T, we detected elevated levels of 8-OHdG by HPLC in the periurethral region of the prostate, which was the only prostatic area that had detectable levels in control animals (Table 4). The treatment also induced low levels of this oxidized DNA base in the dorsolateral prostate where the combined hormone treatment induced inflammation and dysplasia, but no cancer. 8-OHdG was undetectable by HPLC in the ventral prostate. Immunostaining essentially confirmed these findings (Supplemental Fig. 1B). Interestingly, the seminal vesicles stained strongly for 8-OHdG in control animals, as they do in humans [29], but E₂ plus T treatment reduced staining intensity (data not shown); we have never observed lesions in the seminal vesicles of E₂ plus T-treated rats.

The formation of lipid hydroperoxides, indicators of lipid peroxidation that can be induced by ROS, followed the same general pattern as the generation of 8-OHdG (Table 4). The highest levels were found in the periurethral and dorsolateral prostate of E₂ plus T-treated rats, and lowest levels in the ventral lobe of control rats, while intermediate levels were found in the target regions of control animals, suggesting that these tissues are particularly sensitive to or poorly protected from oxidative DNA damage. The difference from control values was almost significant for the periurethral region and became significant when the data for the periurethral and dorsolateral prostate were combined.

Discussion

We obtained novel evidence that estrogen is involved in prostate cancer induction by T and strongly suggestive evidence of

Table 4 Sixteen week treatment of NBL rats with T, E2, and T plus E2 induced: (A) a DNA adduct^a detectable by ³²P-postlabeling (P1 in Supplementary Figure 1A), (B) 8-OHdG^b, and (C) lipid hydroperoxides^c in the dorsolateral prostate^d

Treatment	N	Periurethral area	Peripheral dorsolateral prostate tissue	Periurethral area and peripheral tissue combined	Ventral prostate
A - DNA adduct detectable by ³²P-postlabeling^a					
Control	4	2.7 ± 0.53	n.d.	n/a	n.d.
E2	4	2.9 ± 0.84	n.d.	n/a	n.d.
T	4	5.6 ± 0.50 ^e	n.d.	n/a	n.d.
T plus E2	4	10.2 ± 2.47 ^f	n.d.	n/a	n.d.
B - 8-OHdG^b					
Control	3	0.32 ± 0.28	n.d.	0.32	n.d.
T plus E2	4	2.11 ± 1.37	0.14 ± 0.14	2.25	n.d.
C - Lipid hydroperoxides^c					
Control	3	1.41 ± 0.52	1.97 ± 1.58	1.69 ± 0.75 (n = 6)	0.20, 0.59 (n=2)
T plus E2	4	4.52 ± 1.28 ^g	3.82 ± 1.34	4.17 ± 0.87 ^h (n = 8)	1.55 ± 0.44

^a Data expressed as Relative Adduct Levels (RAL) x 10⁹

^b Data are expressed as 8-OHdG/10⁵ dG

^c Data are expressed as nmol hydroperoxides per gram wet tissue

^d Data are presented as mean ± SEM

^e p < 0.01 for difference with control and E2 groups (ANOVA followed by Student-Newman-Keuls post-hoc test)

^f p < 0.01 for difference with control and E2 groups and p < 0.05 for difference with the T only group (ANOVA followed by Student-Newman-Keuls post-hoc test)

^g p = 0.053 for difference with control value (1-sided t-test)

^h p = 0.031 for difference with control value (1-sided t-test)

n/a = not applicable; n.d. = not detectable

a critical contribution of a combination of androgen- and estrogen-receptor mediation as well as DNA damage, probably caused by estrogen metabolites; the latter notion is supported by our previous findings that generation of catecholesterogen and redox cycling can occur in the prostate of rats treated with estrogen [22]. The results indicate (a) that T plus E2 treatment causes DNA damage and lipid peroxidation in the rat prostate at the site of origin of carcinomas that develop later with continued hormone treatment; and (b) that E2 is essential for the high prostate cancer response to treatment with T plus E2, suggesting that aromatization of T to E2 may be critical for the tumor response to T alone. Unfortunately, we were not able to conduct a study with T treatment plus an aromatase inhibitor. The results of combination treatments with 4OH-E2 and T, with or without addition of the estrogen 2F-E2 which cannot be converted to reactive species, provide evidence supporting the notion that catecholesterogen formation and redox cycling of 4-hydroxylated E2 are important factors and that estrogen receptor activation (by 2F-E2) is also necessary to generate prostatic dysplasia and cancer. However, the 2F-E2 dose we used was probably too low (inducing only a modest pituitary enlargement) to have a sufficiently strong estrogenic effect and result in a high tumor response; additional experiments with higher doses of this estrogenic compound are needed to definitively test the hypothesis that a combination of androgen and estrogen

receptor mediation and estrogen metabolite-induced DNA damage are the mechanism by which T, with or without additional E2, causes prostate cancer in the NBL rat.

Aromatase knockout mice [38] and aromatase overexpressing mice [39, 40] suffer from androgen metabolism abnormalities that limit their potentially interesting use to address this hypothesis [41]. Aromatase knockout mice lack estrogen production, have elevated circulating T levels, and their prostates are enlarged, but they do not develop cancer [38]. In aromatase-overexpressing mice estrogen production is elevated, while T levels are considerably reduced and no neoplastic or preneoplastic prostate lesions develop [39, 40]. These observations are consistent with the idea that both hormones are necessary for prostate carcinogenesis.

Both estrogen receptors (ER)-α and -β are expressed in the rat and human prostate and they may mediate some or all of the prostatic effects of estrogens [42, 43]. When Thompson et al. [44] treated NBL rats with T plus E2 in combination with the pure antiestrogen ICI182,780, development of prostatic dysplasia (a putative preneoplastic lesion comparable to human prostatic intraepithelial neoplasia or PIN) was inhibited. It has long been known that E2 induces pituitary tumors that produce prolactin in rats. Lane et al. [45] demonstrated that bromocriptin treatment, which reduces prolactin secretion, reduces the induction of prostatic dysplasia by T plus E2 in

NBL rats, suggesting that prolactin plays a role in the development of this lesion. Importantly, the dysplasia in NBL rats treated with E2 plus T occurs in a different region of the prostate (dorsolateral prostate) than the carcinomas we find following this treatment, which originate from the periurethral prostatic ducts [14], and this dysplasia rarely progresses to cancer [unpublished observations]. Thus, the findings of Thompson et al. [44] and Lane et al. [45] may not have a bearing on cancer development in the prostate of T plus E2-treated rats. This notion is supported by our observation that chronic treatment with T alone or T combined with E2 for the first 8–16 weeks induces prostate cancer in 37, 63, and 75% of rats, respectively, without inducing pituitary tumors. Overall, these data suggest that estrogen receptors and prolactin do not play a role in T plus E2 induction of prostate cancer in NBL rats, but conclusive experiments are still lacking.

We also obtained evidence to indicate that T alone induces cancer in the prostate of the NBL rat at a 35–40% incidence, which is considerably higher than reported previously by us and others for this and other rat strains [4, 5]. The mechanistic basis for the high susceptibility of NBL rats to prostate cancer induction by T is not known. These tumors were locally invasive adenocarcinomas that were capable of metastasizing. The T dose we used elevated circulating T non-significantly by 17%, well within the physiological range of T levels in NBL rats [5]. These findings indicate that T is a carcinogen for the rat prostate and suggest that this androgen could be the cause of human prostate cancer at relevant circulating concentrations [5]. The evidence for this from human epidemiological studies is weak; there are only two reports of a positive association between free serum T levels and risk of prostate cancer [46, 47], which would be consistent with our findings in rats. Interestingly, a recent report indicated that men with very low circulating free T levels have a low risk of prostate cancer [48].

Indirect evidence that androgens are involved in human prostate carcinogenesis is derived from clinical studies with the 5 α -reductase inhibitors finasteride and dutasteride which both reduced risk of developing prostate cancer by 23–24% over a 4–7-year intervention period [49, 50]. However, the duration of the intervention was short in view of the known slow growth of prostate cancer, and the study subjects were middle-aged men who have a high frequency of small cancers in their prostates [51]. Thus, these studies are unlikely to provide much insight into whether androgens are involved in the process of prostate carcinogenesis as such or only influence growth and progression of pre-existing cancer.

The observation that DHT, which cannot be aromatized to E2 or converted back to T, is a very weak prostate carcinogen at best in the NBL rat suggests that E2 generated from T by aromatase (CYP19) is critically involved in the carcinogenic activity of T for the rat prostate. However, adding E2 to DHT treatment did not substantially increase its prostate cancer-inducing efficacy, unlike our previous findings with the

combination of E2 with T [14, 15]. DHT also reduced the pituitary tumor-inducing effect of E2 considerably, from a 100 to a 30% incidence, and had effects on accessory sex gland weights that were quite different from those of T, regardless of whether the androgens were combined with E2. Conceivably, DHT treatment alters androgen and estrogen metabolism or action in ways that reduces their prostate cancer-inducing activity. Alternatively, 3 β -androstanediol, the downstream metabolite of DHT, is known to bind and activate ER- β and inhibits human prostate cancer cell proliferation [52]. Of note, we observed by immunohistochemistry that the regions of the NBL rat prostate that are most susceptible to the carcinogenic effects of T + E2 have low ER- α expression and very high ER- β expression [unpublished observations].

In conclusion, our findings provide strong support for the hypothesis that for T to be carcinogenic to the rat prostate, it must be aromatized to E2 which acts as a chemical carcinogen through the above described metabolism to catecholestrogen and subsequent redox cycling leading to DNA damage. We also obtained evidence to suggest that estrogen receptor mediation is important for this carcinogenic effect, in addition to androgenic mechanisms. More research is warranted to provide further proof of the latter notion. These findings may identify druggable targets that are clinically feasible for prostate cancer chemoprevention.

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Compliance with Ethical Standards

The animal experiments described in this paper were reviewed and approved by the NYU School of Medicine Institutional Animal Care and Use Committee and all applicable national and institutional guidelines for the care and use of animals were followed.

Conflict of Interest The authors declare that they have no conflicts of interest.

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