The Prostate 30:256–262 (1997)

Effect of Combined Testosterone and Estradiol-17β Treatment on the Metabolism of E_2 in the Prostate and Liver of Noble Rats

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BACKGROUND. Long-term treatment of Noble (NBL) rats with testosterone (T) and estradiol-17β (E_2) induces dysplasia in the dorsolateral lobe (DLP) but not in the ventral lobe (VP) of the rat prostate. The aim of this study was to determine whether metabolic conversion of E_2 to catechol estrogens (CEs), which are potentially genotoxic, is a mechanism of estrogen carcinogenicity in this tissue.
METHODS. Male NBL rats were treated simultaneously with T and E_2, or left untreated, for 16 weeks after which time the liver, VP, and DLP were excised for microsomal preparations. ^{3}H-E_2 metabolites generated in microsomal incubates were separated by high-performance liquid chromatography (HPLC) and identified by coelution with known E_2 metabolites.
RESULTS. 2- and 4-hydroxyestrogens were detected at high levels in hepatic microsomal incubates, and at extremely low levels in prostatic microsomal incubates. T + E_2 treatment of rats did not increase the formation of these prostatic and hepatic metabolites.
CONCLUSIONS. These results do not support CE formation as a mediating step in estrogen-induced tumorigenesis in the rat prostate. *Prostate 30:256–262, 1997.*

KEY WORDS: **catechol estrogens; estrogen metabolism; estrogen genotoxicity**

INTRODUCTION

Despite a wealth of epidemiological evidence implicating prolonged disturbance of hormonal status as a risk factor for prostate cancer [1–4], the mechanisms of sex hormone-induced carcinogenesis in the prostate remain poorly understood. We have reported that the simultaneous treatment of intact Noble (NBL) rats with testosterone (T) and estradiol-17β (E_2) for 16 weeks consistently induces intraductal dysplasia, a preneoplastic lesion, in the dorsolateral prostate (DLP) but not in the ventral prostate (VP) [5–7]. Prolonged exposure (greater than 52 weeks) of the NBL rats to the combined T and E_2 treatment regimen resulted in a high incidence of prostatic carcinoma exclusively in the rat DLP [8–11]. Long-term treatment with T alone resulted in a low incidence of prostate tumors [8]. Similarly, Pollard and Luckert [12,13] described the induction of tumors in Lobund-Wistar rats following treatment with T but not with dihydrotestosterone (DHT). Recently, Ho and Roy [14] reported a significant increase in DNA strand breakage and accumulation of lipid peroxidation fluorescent products in the DLPs harboring the dysplasia. These findings suggest that the dual hormone treatment may elicit free radical-based DNA damage in the rat DLP, which may constitute the genetic basis of hormone-induced cellular transformation.

Although sex steroids are established mitogenic agents [15,16], they have not been shown to have direct genotoxic actions. Two schools of thought have emerged regarding possible mechanisms for hormone-induced cancers. One group believes that the carcinogenic properties of sex steroids stem from

Contract grant sponsor: National Cancer Institute; Contract grant numbers: CA 15776 and CA 60923.

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Received 20 September 1995; Accepted 5 December 1995

their ability to bind steroid receptors, turning on a series of cell proliferation events which increase the incidence of spontaneous genetic damage [17,18]. An alternative viewpoint suggests the metabolic conversion of sex steroids to metabolites which are known to be directly or indirectly damaging to DNA [19]. Metabolic conversion of E_2 into genotoxic metabolites has been proposed to be an underlying mechanism of E_2 carcinogenicity in the Syrian hamster kidney [20–24]. Formation of catechol estrogens (CEs), either by the NADPH-dependent estrogen-2/4 hydroxylase or by a peroxidatic, organic hydroperoxide-dependent estrogen-2/4-hydroxylase, has been documented in this tissue [25]. The CEs thus formed are believed to undergo redox cycling, thereby releasing reactive oxygen species, causing DNA damage and possibly tumor initiation. Additionally, oxidation of CEs by Cu (II) associated with guanines may produce site-specific oxidative DNA damage [26]. It is via these proposed mechanisms that sex hormones may be deemed potentially genotoxic.

In this study we wished to answer two questions regarding the potential role of CEs as causative factors of prostatic dysplasia: 1) Does the prostate gland have the ability to metabolize estrogen into CEs? 2) Does dual hormone treatment with T + E_2 elevate CE formation in the prostate and liver (since CEs formed in the liver may reach the prostate via the systemic circulation)? The profiles of ^{3}H-E_2 metabolites produced in DLP and VP microsomes of untreated and T + E_2-treated NBL rats were analyzed by high-performance liquid chromatography (HPLC) using coelution with known E_2 metabolites as reference standards. Estradiol metabolism in liver microsomes was studied in parallel as a positive control for method validation.

MATERIALS AND METHODS

Chemicals and Reagents

6,7-^{3}H-estradiol (47.2 Ci/mmol, > 96% pure by HPLC analysis) was obtained from Dupont-New England Nuclear (Boston, MA). Nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), sucrose, sodium phosphate, glycerol, cumene hydroperoxide (CuOOH), E_2, estrone (E_1), estriol (E_3), and T were purchased from Sigma Chemicals (St. Louis, MO). Ascorbic acid was obtained from the J.T. Baker Chem. Co. (Phillipsburg, NJ). HPLC grade methanol and ethyl acetate were supplied by Fisher Scientific (Fair Lawn, NJ), and reference compounds 2-hydroxyestradiol-17β (2-OHE_2), 2-hydroxyestrone (2-OHE_1), and 4-hydroxyestradiol-17β (4-OHE_2) were from Steraloids (Wilton, NH).

Animals and Treatment

Male NBL rats (5-6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and housed in departmental facilities. Hormonal treatment commenced at 11–12 weeks of age (280–300 g body weight). Hormone-filled Silastic™ implants were prepared as previously described [6]. T implants were 2 cm long and the E_2 implants were 1 cm long. The two experimental groups comprised simultaneously T and E_2 implanted (n = 6) and untreated (n = 6) animals. We have previously observed that the dual hormone treatment maintained plasma T at physiological levels (1.6 ± 0.2 ng/ml) and elevated plasma E_2 to approximately threefold (46.3 pg/ml) of that found in untreated rats. Sixteen weeks post-implantation, hormone-treated and age-matched untreated animals were euthanized by an overdose of Isoflurane (Ærrane, Anaquest, WI) anesthesia followed by decapitation. The VP, DLP, and liver pieces were rapidly removed for subcellular fractionation.

Preparation of Microsomes

Tissues were washed in three volumes of homogenization buffer (sucrose 0.32 M, sodium phosphate 40 mM, EDTA 1 mM, PMSF 1 mM, ascorbic acid 1 mM, glycerol 10%; pH 7.4) to remove extraneous blood. They were minced with scissors before homogenization with a Tissumizer (Tekmar, Cincinnati, OH) for five 10-sec bursts at increasing speeds (to minimize foaming). All tissue processing was performed at 4°C. Tissue homogenates were centrifuged at 3,000*g* for 15 min, the supernatant removed and recentrifuged at 12,000*g* for 20 min, followed by ultracentrifugation at 100,000*g* for 60 min. The resulting microsomal pellet was washed in buffer and centrifuged once more at 100,000*g* for an additional 60 min. Microsomes were aliquoted and stored at −80°C until enzyme analysis, within 3 months of preparation. Microsomal protein was determined by means of a Pierce BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Enzyme Incubation Conditions

Metabolism studies were conducted in a sucrose-based buffer (sucrose 0.32 M, sodium phosphate 40 mM, EDTA 1 mM, PMSF 1 mM, ascorbic acid 1 mM; pH 7.4). Routine assay conditions used were 50 μM radioinert E_2, 10^6 dpm ^{3}H-E_2, NADPH 1 mM, or cumene hydroperoxide 2.5 mM as indicated. In initial experiments, optimal protein concentrations, incuba-

tion times, and substrate concentrations were established at 1 mg ml^{-1}, 30 min and 50 μM E_2, respectively. Reactions were initiated by the addition of microsomes and immediate warming to 37°C, and stopped by rapid cooling to 4°C and the addition of 0.25 M perchloric acid and then stored in −80°C until HPLC analysis.

Separation of Metabolites by HPLC

Water-insoluble metabolites were extracted four times with ethyl acetate. Aliquots of a mixture of reference standards (1 mg ml^{-1}) were added to portions of organic extracts, which were dried under nitrogen and the residues resuspended in methanol. Analyses were carried out on a reverse-phase μBondapak C-18 column in a Waters ALC-201 HPLC with a UV detector set at 280 nm. E_2 metabolites were eluted using a gradient system, 50–60% methanol in double distilled water for 45 min at a flow rate of 1 ml/min. Radioactive metabolites were collected under the chromatogram peaks of reference standards (E_3, 2-OHE_1, 4-OHE_2, 2-OHE_2, E_1, and unmetabolized E_2), added to 10 ml Bio-Safe scintillant (Research Products International, IL), and counted on a Packard Tri-Carb liquid scintillation counter (1600TR, Meriden, CT). Relative amounts of each metabolite formed were expressed as a percentage of the total radioactivity recovered from the column. Microsome samples were prepared from individual animals, and data are shown as mean ± SEM. Statistical analyses were performed using a one-way analysis of variance (ANOVA) with a post-hoc Tukey test.

RESULTS

Optimization of Study Conditions

In the early experiments, several subcellular fractions were analyzed for their ability to metabolize E_2. Pellets were retained from 3,000*g* (crude nuclei), 12,000*g* (mitochondria-rich), and 100,000*g* (microsome fraction) centrifugation spins, resuspended in three volumes of buffer, and analyzed (1 mg ml^{-1} protein) alongside the soluble cytosolic fraction. For liver, VP, and DLP, the microsomal fraction was found to possess the largest metabolizing ability. In all subsequent experiments, the microsomal fraction was used (data not shown).

Using liver microsomes, we demonstrated that the rate of E_2 metabolism was dependent on substrate concentration (Fig. 1). From these data, 50 μM estradiol was chosen for subsequent metabolic studies, since it was within a nonlimiting substrate concentration. In addition to substrate dependency, the metabolism of estradiol in liver microsomes was time and protein concentration dependent. Initial studies revealed an incubation time of 30 min and a protein concentration of 1 mg ml^{-1} was optimal (data not shown).

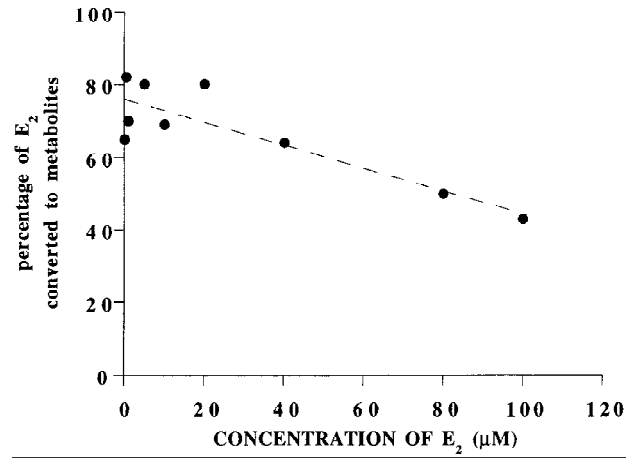

Fig. 1. Liver microsomes were incubated with increasing concentrations of E_2 to determine the substrate dependency of the enzymic reactions. Crude microsomal fractions (1 mg ml^{-1} protein) from livers of untreated rats were incubated for 30 min in the presence of 1 mM NADPH, 10^6 dpm ^{3}H-E_2, and concentrations of E_2 in the range 0.1–80 μM. Data are expressed as the percentage of total recovered radioactivity converted from E_2 into metabolites. The extent of metabolism was reduced as the concentration of E_2 added to the reaction increased. A nonlimiting concentration of E_2, 50 μM, was chosen for subsequent experiments.

To determine the identity of the major metabolites, microsomal incubates were mixed with known unlabeled E_2 metabolites. Column eluates were collected at minute intervals and the radioactivity of each fraction plotted against time. Unlabeled estrogenic reference standards were detected by UV spectrophotometry. When NADPH was used as a cofactor in the enzymic reactions, five major peaks of radioactivity were noted in liver microsome incubates (Fig. 2). Identities of these peaks were determined based on coelution with known E_2 metabolites. In order of increasing retention times, the peaks were recognized as E_3, 2-OHE_1, 2-OHE_2, E_1, and unmetabolized E_2. A minor peak was sometimes noted shortly before 2-OHE_2 and this was identified as 4-OHE_2 (not shown in Fig. 2). No other metabolites were identified with a retention time greater than E_2, and thus the above six reference compounds were used routinely as standard markers on the chromatograph for the identification of ^{3}H-E_2 metabolites. In contrast to E_2 metabolism in liver microsomes, the metabolites formed in the presence of VP and DLP microsomes were barely detectable. One peak was observed for VP and this was identified as E_1.

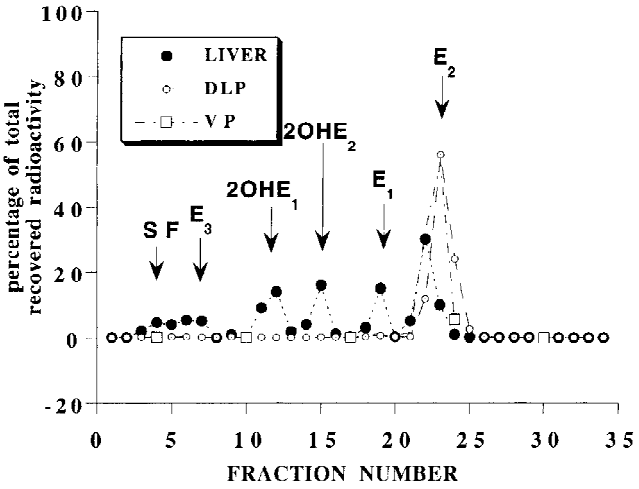

Fig. 2. Profile of metabolites of E_2 from crude microsomal preparations from liver, DLP, and VP of untreated NBL rats. Microsomes (1 mg ml^{-1}) were incubated with a nonlimiting concentration of E_2 (10^6 dpm ^3H-E_2 plus 50 μM unlabeled E_2) in the presence of NADPH 1 mM. Following a 30-min incubation, water-insoluble metabolites were extracted with ethyl acetate, dried under N_2, and reconstituted in methanol. The methanol extracts were injected onto a reverse-phase μbondapak C-18 HPLC column, utilizing a methanol-water solvent system. Eluate fractions were collected at minute intervals and radioactivity in each fraction was counted. Data are shown as a percentage of the total radioactivity eluted from the column. SF, solvent front.

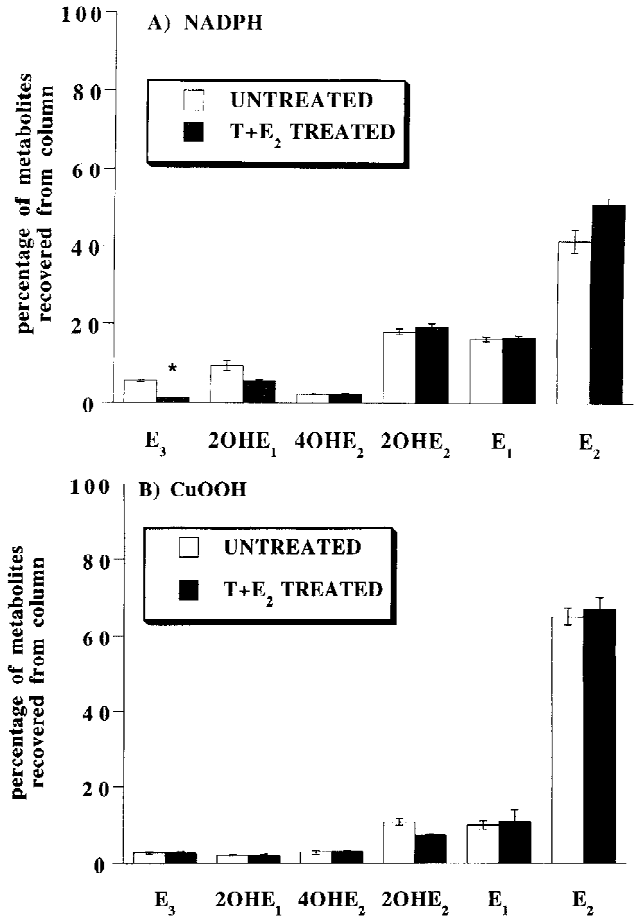

Fig. 3. Crude liver microsomal preparations (1 mg ml^{-1} microsomal protein) from untreated rats (n = 6), and rats treated with T and E_2 simultaneously (T + E_2 treated, n = 6), were incubated for 30 min in the presence of 1 mM NADPH (**A**) or 2.5 mM CuOOH (**B**), 10^6 dpm ^3H-E_2, and 50 μM unlabeled E_2. Organic extracts of the microsomal reaction were reconstituted in methanol and reference standards added. The methanol extracts were analyzed on a reverse-phase μbondapak C-18 HPLC column and the eluates collected. The radioactivity in each fraction was measured by scintillation counting. Data are shown as the percentage of total radioactivity recovered from the column. The asterisk indicates a significant difference in values obtained from microsomes from untreated and T + E_2-treated rats ($P < 0.05$).

Hepatic Estradiol Metabolism

Crude microsomal fractions prepared from the livers of untreated rats exhibited a marked ability to metabolize E_2. Utilizing 1.5 mM NADPH as an enzymic cofactor, the metabolites identified with this system following a 30-min incubation were 2-OHE_2 (18.2%), E_1 (16.4%), 2-OHE_1 (9.5%), E_3 (5.7%), and 4-OHE_2 (2.3%). Approximately 42% of radioactivity recovered from the column remained as unmetabolized E_2 (Fig. 3A). Following 16 weeks of treatment of rats with T + E_2, E_3 production was significantly reduced (1.2% in T + E_2-treated rats compared to 5.7% in untreated rats), whereas the extent of 2-OHE_2, 4-OHE_2, and E_1 formation was not significantly altered by T + E_2 treatment. 2-OHE_1 levels fell from 9.5% in untreated animals to 5.7% in T + E_2-treated animals (nonsignificant) (Fig. 3A).

When CuOOH was used as a cofactor in the hepatic metabolism of E_2, overall E_2 metabolism in liver microsomes was reduced compared to corresponding incubations using NADPH as a cofactor. The major metabolite under these conditions was 2-OHE_1, and the production of the metabolites was not altered by the hormonal treatments (Fig. 3B).

Prostate Microsomal Fraction Metabolism

VP microsomes, in the presence of NADPH as a cofactor, converted 6% of recovered radioactivity into E_1; in a 30-min incubation the remainder was recovered as unchanged E_2. In T + E_2-treated rats, VP metabolism of E_2 was reduced and the E_1 fraction decreased nonsignificantly to 1.9% (Fig. 4A). When CuOOH was utilized as a cofactor, E_2 metabolism was lower than with NADPH, and dual hormonal treatment did not affect E_2 conversion (Fig. 4B). Metabolism of E_2 in DLP was negligible, and was unaffected

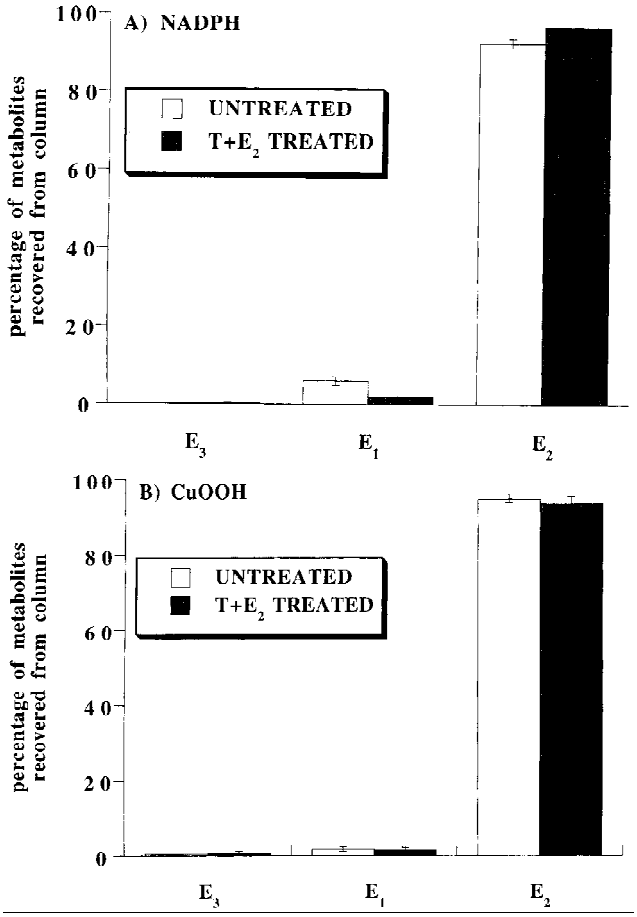

Fig. 4. Crude microsomal fractions (1 mg ml^{-1} microsomal protein) from VPs from untreated rats (n = 6) and rats treated with T and E_2 simultaneously (T + E_2 treated, n = 6) were incubated for 30 min in the presence of 1 mM NADPH (**A**) or 2.5 mM CuOOH (**B**), 10^6 dpm ^{3}H-E_2, and 50 μM unlabeled E_2. Organic extracts of the microsomal reaction were reconstituted in methanol and reference standards added. The methanol extracts were analyzed on a reverse-phase μbondapak C-18 HPLC column and the eluates collected. The radioactivity in each fraction was measured by scintillation counting. Data are shown as the percentage of total radioactivity recovered from the column.

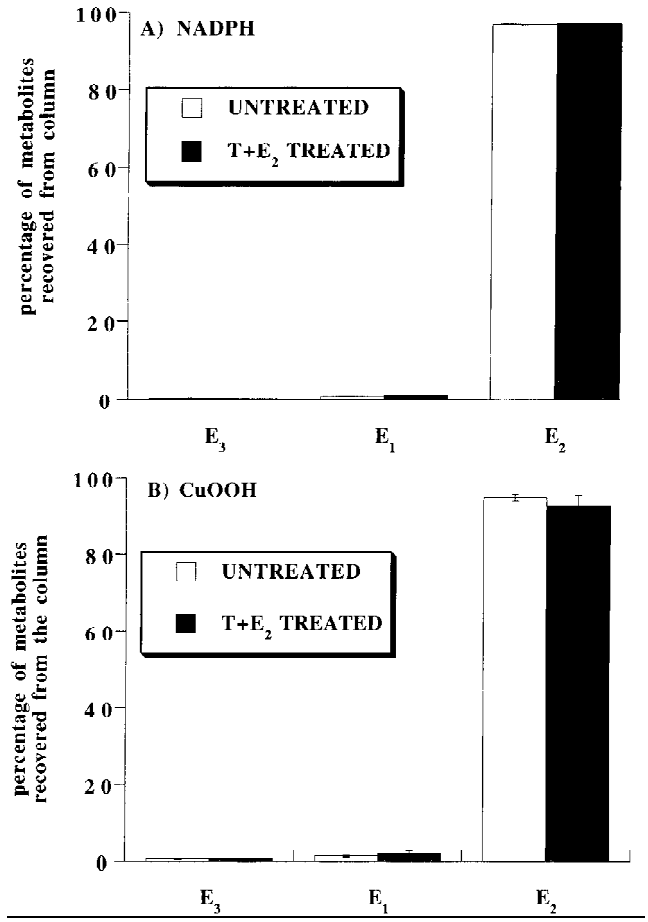

Fig. 5. Crude microsomal fractions (1 mg ml^{-1} microsomal protein) from DLPs from untreated rats (n = 6) and rats treated with T and E_2 simultaneously (T + E_2 treated, n = 6) were incubated for 30 min in the presence of 1 mM NADPH (**A**) or 2.5 mM CuOOH (**B**), 10^6 dpm ^{3}H-E_2, and 50 μM unlabeled E_2. Organic extracts of the microsomal reaction were reconstituted in methanol and reference standards added. The methanol extracts were analyzed on a reverse-phase μbondapak C-18 HPLC column and the eluates collected. The radioactivity in each fraction was measured by scintillation counting. Data are shown as the percentage of total radioactivity recovered from the column.

by chronic T and E_2 implantation in the presence of either NADPH or CuOOH (Fig. 5). CEs were not detected in any sample using this method of analysis.

In order to further analyze the very low to nondetectable level of E_2 metabolism in prostatic microsomes, an additional assay using a different buffer system and separation method was employed. Data from this study showed very similar findings to those detailed above. Samples were prepared in a Tris/HEPES/ascorbic acid buffer, and CEs were isolated using neutral alumina and separated by thin layer chromatography (TLC), following the methodology of Liehr and coworkers [23]. The amount of CEs formed in DLP microsomes was extremely low (0.31 pmol/mg protein/min for 4-OHE_2; 0.38 pmol/mg protein/min 2-OHE_2) or close to nondetectable. Moreover, T + E_2 treatment of rats did not induce a higher level of CE formation in DLP microsomes. The levels of CE formation in VP microsomes were similarly low and unaffected by hormonal treatments (0.22 pmol/mg protein/min 4-OHE_2, 0.32 pmol/mg protein/min 2-OHE_2). These values were close to nondetectable levels when compared to data reported for hepatic microsomes from male rats which formed 456 pmol/mg protein/min of 2-OHE_2 and 7 pmol/mg protein/min of 4-OHE_2 [25].

DISCUSSION

We have previously demonstrated that treatment of NBL rats with T + E_2 for 16 weeks consistently induces dysplasia in the DLP of all treated animals [5,6]. In contrast, liver dysplasia was not observed in any of the tissues from T + E_2-treated rats (unpublished observation). In this study we investigated the metabolism of E_2 in the DLP and VP of NBL rats to explore the possibility that CEs are formed via 2/4-hydroxylase activities. Using crude microsomes, a nonlimiting concentration of E_2, and two methods of analysis we found no significant CE formation under both NADPH- and CuOOH-dependent hydroxylation conditions in the two major lobes of the rat prostate. Furthermore, exposure of rats to a dysplasia-causing regimen of combined T and E_2 treatment for 16 weeks failed to induce CE formation in both prostatic lobes. Estradiol metabolism in VP microsomes was found to be minimal, with E_1 as the major metabolite, while the steroid remained largely unchanged in DLP microsomal incubates. In contrast, hepatic microsomes exhibited significant 2-hydroxylase activity and a lesser amount of 4-hydroxylase activity under similar experimental conditions. T + E_2 treatment also failed to alter the profile and extent of E_2 metabolism in liver microsomes.

In NBL rats, administration of estrogens to rats implanted with T caused dysplasia [5,6] and cancer development [11] in the DLPs of all treated animals [6]. Additional observations identified estrogens as obligatory factors in this dysplasia induction process [6]. It was originally presumed that the estrogens were incomplete carcinogens [18,27,28], and that they may simply exert promotional effects on prostatic cells initiated spontaneously by the action of intracellular oxidants [18,27–29]. Recently, however, increases in single-strand DNA breakage and lipid peroxidation fluorescent product accumulation were demonstrated in the DLPs of T + E_2-treated rats [14]. These findings raised the possibility that estrogens can exert direct or indirect genotoxic actions in rat DLP. In light of the data obtained from the hamster kidney studies [20–25], it was important to determine whether CEs were normally formed in the DLP of untreated rats, and further, whether T + E_2 treatment lead to an enhancement of CE formation in this prostatic tissue. Results from the present study demonstrated an absence of significant levels of 2/4-hydroxylase activities in the prostates of untreated and hormonally treated rats. Thus, even though metabolic conversion of E_2 to CEs may be implicated in estrogen carcinogenicity in the hamster kidney model [20–25], a parallel mechanism is apparently absent in the rat DLP. Further studies are needed to unveil the molecular basis of E_2 genotoxicity and/or carcinogenicity in this tissue.

Estradiol metabolism in the various lobes of the prostate has been previously studied [30,31]. The present investigation extended these findings to demonstrate for the first time that rat VP and DLP microsomes have low but different levels of E_2 metabolizing capacities. After a 30-min incubation, approximately 6% of the added E_2 was converted to E_1 by VP microsomes, but almost all the E_2 remained unmetabolized in incubates of DLP microsomes. Furthermore, T + E_2 treatment of NBL rats did not affect the E_2/E_1 oxidation-reduction equilibria in both prostatic preparations. Thus, it is reasonable to expect a slow but steady metabolic inactivation of E_2 in rat VP. In contrast, E_2 will remain unmetabolized in its hormonally active form for a much longer time in the DLP. Thus, differences in tissue E_2 availability may, in part, explain why the DLP is uniquely susceptible to the T + E_2 treatment in terms of dysplasia/cancer induction.

Lastly, it is feasible that CEs formed in the livers may enter the systemic circulation and elicit genotoxic or hormonal effects at a distant site such as the prostate. Although CEs are known to be extremely labile, it has been demonstrated that CEs, administered systemically, retained an ability to elicit effects at least 24 hr after injection [32], thus suggesting that the CEs could persist in the bloodstream. However, this proposed mechanism of long-range action of CEs is unlikely to be a probable cause of dysplasia induction in the DLP as treatment of rats with T + E_2 did not elevate the hepatic CE formation.

In summary, we have demonstrated close to nondetectable levels of CE formation in rat VP and DLP microsomal preparations. Thus, these potentially genotoxic metabolites of E_2 are unlikely to be involved in the hormonally induced dysplasia observed in this lobe.

ACKNOWLEDGMENTS

We thank Dr. Peter Ofner and Mr. Richard Vena for their advise and technical assistance with the HPLC system. We are grateful to Dr. J. Liehr for providing the expertise and facilities for TLC analysis of CEs. This study was supported in part by grants CA 15776 and CA 60923 awarded by the National Cancer Institute.

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