The effects of steroidal estrogens in ACI rat mammary carcinogenesis: 17 β -estradiol, 2-hydroxyestradiol, 4-hydroxyestradiol, 16 α -hydroxyestradiol, and 4-hydroxyestrone

V K Turan^{1,6}, R I Sanchez^{1,2}, J J Li⁴, S A Li⁴, K R Reuhl^{1,3},
P E Thomas^{1,6}, A H Conney^{1,6}, M A Gallo^{1,5}, F C Kauffman^{1,2}
and S Mesia-Vela²

¹Joint Graduate Program in Toxicology, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854 and University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School, USA

²Laboratory of Cellular and Biochemical Toxicology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA

³Laboratory for Neurotoxicology, Department of Pharmacology and Toxicology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA

⁴Hormonal Carcinogenesis Laboratory, Department of Pharmacology and Toxicology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA

⁵Department of Environmental and Occupational Medicine, Environmental and Occupational Health Sciences Institute, and The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA

⁶Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA

(Requests for offprints should be addressed to S Mesia-Vela, Laboratory for Cellular and Biochemical Toxicology, 41B Gordon Road, Piscataway, New Jersey 08854, USA; Email: smvela@yahoo.com)

Abstract

Several investigators have suggested that certain hydroxylated metabolites of 17 β -estradiol (E₂) are the proximate carcinogens that induce mammary carcinomas in estrogen-sensitive rodent models. The studies reported here were designed to examine the carcinogenic potential of different levels of E₂ and the effects of genotoxic metabolites of E₂ in an *in vivo* model sensitive to E₂-induced mammary cancer. The potential induction of mammary tumors was determined in female ACI rats subcutaneously implanted with cholesterol pellets containing E₂ (1, 2, or 3 mg), or 2-hydroxyestradiol (2-OH E₂), 4-hydroxyestradiol (4-OH E₂), 16 α -hydroxyestradiol (16 α -OH E₂), or 4-hydroxyestrone (4-OH E₁) (equimolar to 2 mg E₂). Treatment with 1, 2, or 3 mg E₂ resulted in the first appearance of a mammary tumor between 12 and 17 weeks, and a 50% incidence of mammary tumors was observed at 36, 19, and

18 weeks respectively. The final cumulative mammary tumor incidence in rats treated with 1, 2, or 3 mg E₂ for 36 weeks was 50%, 73%, and 100% respectively. Treatment of rats with pellets containing 2-OH E₂, 4-OH E₂, 16 α -OH E₂, or 4-OH E₁ did not induce any detectable mammary tumors. The serum levels of E₂ in rats treated with a 1 or 3 mg E₂ pellet for 12 weeks was increased 2- to 6-fold above control values (~30 pg/ml). Treatment of rats with E₂ enhanced the hepatic microsomal metabolism of E₂ to E₁, but did not influence the 2- or 4-hydroxylation of E₂. In summary, we observed a dose-dependent induction of mammary tumors in female ACI rats treated continuously with E₂; however, under these conditions 2-OH E₂, 4-OH E₂, 16 α -OH E₂, and 4-OH E₁ were inactive in inducing mammary tumors.

Journal of Endocrinology (2004) **183**, 91–99

Introduction

Breast cancer is a major cause of morbidity and mortality in women in both developed and developing countries (Sasco 2001). Epidemiological and clinical evidence indicate that estrogens are critical factors in the etiology of sporadic breast cancer. Hormonal risk factors relating to reproductive history may reflect longer life-time exposures to endogenous ovarian estrogens. Early age at menarche, late age at menopause, late first full-term pregnancy, and

nulliparity increase the risk of developing breast cancer (Kelsey & Bernstein 1996). Other factors that increase risk include postmenopausal obesity, never having been married, and higher socioeconomic status (Kelsey & Bernstein 1996). Removal of the ovaries at an early age (Kelsey & Bernstein 1996, Kreiger *et al.* 1999), regular exercise (Bernstein *et al.* 1994), pregnancy, and longer lactation periods (Yuan *et al.* 1988) all decrease the risk of developing breast cancer. These observations suggest that increasing the number of ovulatory cycles and the

subsequent increase in body burden of estrogens may increase the risk of developing breast cancer, while reducing the number of cycles may be protective.

Numerous rodent studies have shown that administration of 17 β -estradiol (E₂) is carcinogenic. E₂ administration decreased the latency for the development of mammary adenocarcinoma, and increased the incidence of cervical, uterine, vaginal, and bone tumors in mice (Rudali *et al.* 1975, Highman *et al.* 1980, 1981), and increased the incidence of mammary and pituitary tumors in rats (Shull *et al.* 1997). Naturally occurring and synthetic estrogens are kidney carcinogens in castrated male hamsters (Li *et al.* 1983). Despite extensive studies, the carcinogenic mechanism(s) of estrogens remain unresolved.

Two major hypotheses have been put forth to explain the role(s) of estrogens in mammary cancer. The longest standing is the estrogen receptor agonist hypothesis (Preston-Martin *et al.* 1990, Henderson & Feigelson 2000, Santen 2002) which concludes that E₂ and other ovarian estrogens cause proliferation of selective proliferating genes, secondary to the binding of E₂ to estrogen receptor- α and estrogen response elements in up-stream promoter regions. The second hypothesis is based on some hydroxylated metabolites (2-hydroxyestradiol (2-OH E₂), 4-hydroxyestradiol (4-OH E₂), or 4-hydroxyestrone (4-OH E₁) primarily) being able to form catechol estrogen quinones that are genotoxic, inducing mutations that may lead to mammary cancer (Cavalieri *et al.* 2000, Liehr 2000, Yue *et al.* 2003).

E₂ is metabolized to multiple hydroxylated metabolites, including the catechol estrogens 2-OH E₂, 4-OH E₂, and 4-OH E₁, by rat liver microsomes (Suchar *et al.* 1996, Mesia-Vela *et al.* 2002), human liver microsomes (Lee *et al.* 2001), and by isolated human cytochrome P450 enzymes (Lee *et al.* 2003). 4-OH E₂ and 16 α -hydroxyestradiol (16 α -OH E₂) possess estrogenic activity and have been proposed as carcinogenic metabolites of E₂. 4-OH E₂, but not 2-OH E₂, has strong carcinogenic activity in the Syrian hamster kidney (Liehr *et al.* 1986, Li & Li 1987) and in the uterus of CD-1 mice (Newbold & Liehr 2000). In addition, 4-hydroxylation of E₂ was reported to be elevated in human breast tumor biopsies over that of normal breast tissue samples (Liehr & Ricci 1996, Rogan *et al.* 2003). Treatment with 16 α -OH E₂ caused mammary tumors in mice (Rudali *et al.* 1975) and adrenal, mammary, and pituitary tumors in rats (Noble *et al.* 1975). Urine analyses of premenopausal women showed that a higher ratio of urinary 2-OH E₁ to 16 α -OH E₁ was associated with a decreased risk of developing breast cancer after 5 years (Muti *et al.* 2000). Catechol estrogens (or the ortho quinones derived from these compounds) are believed to react with DNA and result in depurinating DNA adducts (Stack *et al.* 1996, Cavalieri *et al.* 1997, 2000, 2002) and oncogenic mutations (Chakravarti *et al.* 1995, 2001).

There are marked strain differences in susceptibility to estrogen-induced mammary oncogenesis in both mice and rats (Young & Hallowes 1973), and several rodent models are dependent upon treatment with 7,12-dimethylbenz(a)anthracene, *N*-nitroso-*N*-methylurea, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine, and/or radiation to induce tumor formation (Broerse *et al.* 1989, Ip 1996, Snyderwine 1999). The ACI rat, a cross between the August and Copenhagen-Irish strains (Altman & Katz 1979), appears to be uniquely sensitive to estrogen-induced formation of mammary tumors (Cutts & Noble 1964, Holtzman *et al.* 1981, Shull *et al.* 1997, Spady *et al.* 1999, Harvell *et al.* 2000). Treatment of intact female ACI rats with E₂ causes mammary tumors in 80–100% of the animals within a few months. In addition, the ACI strain has a very low incidence (11%) of spontaneous mammary tumors after 2 years (Segaloff & Maxfield 1971, Maekawa & Odashima 1975). After Dunning *et al.* (1947) first reported on the susceptibility of the ACI rat to diethylstilbestrol-induced mammary carcinogenesis, many other naturally occurring and synthetic estrogens such as E₂, estrone (E₁), and ethinylestradiol have also been shown to induce mammary tumors in this strain (Dunning & Curtis 1952, Stone *et al.* 1979, Holtzman *et al.* 1981). We chose to study the ACI rat model because the estrogen-induced mammary tumors closely resemble human breast tumors histologically and in many salient molecular aspects more closely than do the tumors induced in other rat strains by chemical or environmental carcinogens (Li *et al.* 2002a). The purpose of this study was to compare the *in vivo* carcinogenic potential of E₂ versus the hypothesized carcinogenic metabolites.

In the present study, we have demonstrated that treatment of female ACI rats with E₂ caused a dose-dependent induction of mammary tumors. We also found that administration of several metabolites of E₂, including 2-OH E₂, 4-OH E₂, 16 α -OH E₂, and 4-OH E₁, which have been implicated as possibly playing a role in estrogen-induced mammary cancer, did not exhibit detectable oncogenic effects in the female ACI rat mammary gland.

Materials and Methods

Animals and treatments

Female ACI rats were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN) at 7–8 weeks of age. The rats were housed individually in an American Association for Accreditation of Laboratory Animal Care-accredited barrier facility under controlled temperature, humidity, and lighting conditions, and provided with AIN-76A diet, a phytoestrogen-free, semi-synthetic diet (Dyets Inc., Pennsylvania, PA, USA and Research Diets, New Brunswick, NJ, USA) and water *ad libitum*. Treatment began 4–7 days after arrival. In one study, rats were

implanted with a single 20 mg pellet containing cholesterol and 0, 1, or 3 mg E_2 ($n=8$). In a second study, rats were implanted with a single 20 mg pellet containing cholesterol and 0 or 2 mg E_2 , or an equimolar dose of 2-OH E_2 , 4-OH E_2 , 16 α -OH E_2 , or 4-OH E_1 ($n=11-12$). All pellets in the second study contained 5% ascorbic acid to stabilize the catechol estrogens. Since all of the pellets contained either 20 mg cholesterol alone (control) or with a corresponding amount of cholesterol replaced by an estrogen, we will refer to the E_2 pellets by the amount of estrogen present.

The pellet implantation procedure, performed in a sterile environment, was adapted from a previously described method (Li *et al.* 1968). An incision was made in a shaved area in the middle of the back in rats anesthetized with an i.p. injection of 80 mg/kg ketamine and 12 mg/kg xylazine. Pellets were implanted subcutaneously between the scapulas through the incision site, which was closed with a single wound clip. Rats were monitored daily for a week after which the wound clip was removed. Pellets with or without estrogens (Sigma Chemical Co., St Louis, MO, USA) were purchased from Hormone Pellet Press (Shawnee Mission, KS, USA).

The rats were palpated for tumors twice weekly and weighed every 2 weeks for the duration of the study. The dates of appearance and location of tumors were noted. Rats were terminated when a mammary tumor reached approximately 3 cm² in size or at the end of experimental periods (36 weeks). Additional rats were implanted with 0, 1, or 3 mg E_2 , and killed for E_2 metabolism studies and serum E_2 measurements after 6 or 12 weeks of treatment, which was prior to the development of mammary tumors ($n=6$).

Blood and tissue collection

Blood was collected by cardiac puncture during anesthesia immediately prior to necropsy. The blood was allowed to clot at 4 °C for 6 h, and centrifuged. The serum was collected and stored at -80 °C. Serum levels of E_2 were determined by RIA, using Coat-A-Count estradiol RIA kits, according to the manufacturer's instructions (Diagnostic Products Corporation, Los Angeles, CA, USA).

At necropsy, all the rats were subjected to gross pathologic examination, and the presence of mammary tumors was recorded. The mammary glands and the mammary tumors were quickly removed, portions were fixed in Carnoy's solution for 4 h and processed for paraffin embedding, while unfixed portions of tissues were immediately frozen in liquid nitrogen and stored at -80 °C. Pituitary gland, thymus, liver, uterus, kidneys, and adrenals were removed and weighed. Portions of pituitary gland and liver were fixed in Carnoy's solution. For morphological examination, tissue sections (6 μ m) were prepared and stained with hematoxylin and eosin.

Hepatic microsomal metabolism of E_2

Hepatic microsomes were prepared by differential centrifugation as described previously (Thomas *et al.* 1983) and stored at -80 °C until analyzed. Protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the supplier's instructions, using bovine serum albumin as a standard.

NADPH-dependent oxidation of E_2 was carried out as previously described (Suchar *et al.* 1995). Hepatic microsomes were incubated with 5 mM ascorbic acid, 3 mM magnesium chloride, 50 mM sodium-phosphate buffer (pH 7.4), and 50 μ M [³H] E_2 (0.5 μ Ci) for 20 min at 37 °C. The enzyme reaction was initiated by the addition of 2 mM NADPH and terminated by the addition of 5 ml spectral grade ethyl acetate and shaken to extract E_2 and its metabolites. The ethyl acetate extracts were evaporated to dryness under nitrogen. The residues were resolubilized in methanol and analyzed for estrogen metabolite formation by HPLC as previously described (Suchar *et al.* 1995).

Quantification of E_2 and its metabolites in cholesterol pellets

Pellets from rats treated with 2 mg E_2 , an equimolar amount of 2-OH E_2 , 4-OH E_2 , 16 α -OH E_2 , or 4-OH E_1 , and corresponding controls were removed at necropsy and kept frozen at -20 °C until HPLC analysis. The pellets to be analyzed were those removed from rats treated with 2 mg E_2 for 35-36 weeks ($n=6$), and those removed from the other treatment groups after 36 weeks ($n=11-12$). After removal of adherent tissue from each pellet, they were individually dissolved in 1 ml of a mixture consisting of methanol:chloroform (1:2). Twenty microliters of this solution were diluted in 1 ml methanol for HPLC analysis.

The HPLC system consisted of a Shimadzu SCL-6B system controller, Shimadzu CR4-A integrator (Shimadzu Scientific Instruments, Columbia, MD, USA), Luna Phenomenex, Torrance, CA, USA 250 $\times$ 2.0 mm C-18 column maintained at 25 °C, and a u.v. detector set at 280 nm. Mobile phase A consisted of 0.1% acetic acid, and mobile phase B (B) consisted of methanol:acetonitrile (20:80) containing 0.1% acetic acid. Aliquots (10 μ l) of the pellet dilutions were injected into the HPLC. Fractions from the solutions of control and E_2 -containing pellets were eluted at a constant flow rate of 0.25 ml/min with a 20 min linear curve from 35 to 90% mobile phase B, 2 min 90% B, 2 min linear curve from 90 to 35% B, and re-equilibrated for 12 min at 35% B. Similarly, fractions from the solutions of pellets containing 2-OH E_2 , 4-OH E_2 , 16 α -OH E_2 , or 4-OH E_1 were eluted at a constant flow rate of 0.25 ml/min with a 2 min linear curve from 35 to 70% B, 18 min convex gradient (SCL-6B time/program menu - 10) to 90% B, 2 min 90% B, 2 min linear curve from 90 to 35% B, and re-equilibrated for

Figure 1 Cumulative incidence of palpable mammary tumors in ACI rats treated with different doses of E₂. Female ACI rats ($n=8$) were implanted subcutaneously with 20 mg pellets containing cholesterol and 0 mg E₂ (control; (▼)), 1 mg E₂ (○), or 3 mg E₂ (●).

12 min at 35% B. The amount of estrogen present in each pellet was calculated by fitting the data to standard curves that were created from serial dilutions and HPLC analysis of authentic steroids (Steraloids, Wilton, NH, USA).

Statistical analysis

The data presented represent the means $\pm$ S.E.M. Differences between means were assessed using two-way ANOVA followed by Bonferroni post hoc test using GraphPad Prism software (San Diego, CA, USA). P values <0.05 were considered significant.

Results

Dose-response and time-course for E₂-induced formation of mammary gland tumors

In the first study, we compared the formation of mammary tumors in female ACI rats treated with 1 or 3 mg E₂ (Fig. 1) while, in the second study, we examined the effect of a 2 mg E₂ pellet (Fig. 2). A 50% incidence of palpable mammary tumors was observed after 18 weeks for 3 mg E₂, 19 weeks for 2 mg E₂, and 36 weeks for 1 mg E₂. At termination after 36 weeks, the incidence of palpable mammary tumors was 100% for 3 mg E₂, 73% for 2 mg E₂, and 50% for 1 mg E₂ (Figs 1 and 2). A 100% incidence of mammary tumors was observed after 24 weeks of treatment with 3 mg E₂.

After 27 weeks of treatment of the rats with 3 mg E₂ there were 3.2 ± 2.6 mammary tumors/tumor-bearing rat. After treatment for 36 weeks with 2 mg E₂ there were 2.1 ± 0.4 mammary tumors/tumor-bearing rat, while 1 mg E₂ resulted in 1.0 ± 1.7 mammary tumors/tumor-bearing rat. Our data regarding latency, final incidence,

Figure 2 Lack of effect of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ on the formation of palpable mammary tumors in female ACI rats. Female ACI rats ($n=11-12$) were implanted subcutaneously with 20 mg pellets containing cholesterol and 2 mg E₂ (■) or an equimolar dose of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁, or 0 mg E₂ (control) (▼).

and number of palpable tumors/rat at necropsy clearly represents a dose-response for the formation of E₂-induced mammary tumors.

Body and organ weight responses

Treatment of ACI rats with 1 or 3 mg E₂ for 6 or 12 weeks had no effect on body weight, uterine weight, or adrenal weights, while the weights of the liver and pituitary gland were significantly increased and those of the thymus were significantly decreased (Table 1). Kidney weights were significantly increased in rats at all time-periods of 3 mg E₂ treatment. Rats treated with 1 or 3 mg E₂ for 24–36 weeks exhibited significant decreases in body weights, and significant increases in liver and pituitary weights.

Hepatic morphological changes observed in rats treated with 3 mg E₂ included very mild bile duct proliferation, scattered hepatocellular necrosis, and focal cholestasis. Treatment with 1 or 3 mg E₂ for 6 weeks resulted in increased hepatic mitotic activity and scattered single cell degeneration. These effects became less evident after prolonged treatment.

Treatment of ACI rats with 2 mg E₂ had similar effects as were observed after treatment with 1 or 3 mg E₂ for 24–36 weeks. Liver and pituitary weights were significantly increased with 2 mg E₂ treatment, while body weight was significantly reduced compared with control rats (Table 2). The hepatic morphologic changes observed after treatment with 2 mg E₂ were similar to those described above for rats treated with 1 and 3 mg E₂. Treatment with 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ did not affect liver histology or body, uterus/ovary, liver, or pituitary weights.

Table 1 Body and organ weights of ACI rats treated with E₂. Female ACI rats were implanted subcutaneously with 20 mg pellets containing cholesterol and 0 (control), 1, or 3 mg E₂ for the indicated times. Values are the mean ± S.E.M.

	Final body weight (g)	Organ wet weights (per 100 g body weight)					
		Uterus ^a (mg)	Liver (g)	Thymus (mg)	Pituitary (mg)	Adrenals (mg)	Kidneys ^a (mg)
Treatment							
6 weeks (<i>n</i> =6)							
Control	161 ± 17	275 ± 15	3.0 ± 0.1	150 ± 7	5.6 ± 0.2	35 ± 2	741 ± 29
1 mg E ₂	172 ± 6	313 ± 35	4.5 ± 0.1*	106 ± 4*	15 ± 1*	35 ± 2	860 ± 39
3 mg E ₂	175 ± 4	314 ± 12	4.4 ± 0.1*	87 ± 4*	21 ± 2*	30 ± 2	897 ± 21*
12 weeks (<i>n</i> =6)							
Control	187 ± 6	225 ± 11	3.0 ± 0.1	107 ± 4	5.2 ± 0.4	28 ± 0.6	664 ± 6
1 mg E ₂	191 ± 3	224 ± 16	4.2 ± 0.1*	63 ± 8*	17 ± 1*	26 ± 3	812 ± 22
3 mg E ₂	185 ± 4	259 ± 16	4.0 ± 0.1*	51 ± 3*	30 ± 2*	29 ± 1	870 ± 41*
24–36 weeks (<i>n</i> =8) ^b							
Control	221 ± 6	266 ± 27	3.2 ± 0.05	55 ± 4	5 ± 0.4	28 ± 2	695 ± 25
1 mg E ₂	206 ± 3	237 ± 20	3.8 ± 0.1	63 ± 7	11 ± 2	28 ± 1	774 ± 15
3 mg E ₂	165 ± 5*	466 ± 47*	4.4 ± 0.2*	89 ± 14	33 ± 1*	38 ± 3*	858 ± 33*

^aData from rats with unilateral uteri and kidney discarded.^bData were obtained from rats killed at different time-periods, 24–27 weeks for 3 mg E₂, 24–36 weeks for 1 mg E₂, and 36 weeks for control.*Statistically different from control group (ANOVA followed by Bonferroni post hoc test, *P* < 0.05).

Histopathology of E₂-induced mammary and pituitary lesions

Histopathological examination of mammary glands from rats treated with cholesterol for 6, 12, or 36 weeks showed complete absence of mammary lesions. Rats treated for 36 weeks with 1 mg E₂ exhibited an increased number of mammary gland ducts and proliferation of the terminal lobular units; however, only 50% of the rats presented palpable mammary tumors within that time-period. The terminal duct units were dilated and filled with epithelial and myoepithelial cells and displayed intraductal epithelial hyperplasia. The mammary glands of ACI rats treated with E₂ for as little as 6 weeks presented atypical epithelial hyperplasia. The rats treated with 1 and 3 mg E₂ that developed palpable mammary tumors showed classical

morphological features of mammary ductal adenocarcinomas with a variety of histological patterns. Most of the mammary tumors displayed solid phase histology, with masses of closely packed neoplastic cells with large zones of necrosis. Other mammary tumors showed a classical cribriform phenotype or a 'ribbon' pattern consisting of long rows of cells, often three to five deep, aligned in undulating strips. Generally, most mammary tumors appeared encapsulated by bands of connective tissue, while others showed compression of adjacent stroma and microglandular invasion. Considerable nuclear pleomorphism was present and mitotic figures were abundant within the tumors. There was no evidence of distant metastasis.

At necropsy, the pituitaries of the E₂-treated rats appeared tan-brown to frankly hemorrhagic and displayed

Table 2 Body and organ weights of ACI rats treated with E₂, 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁. Female ACI rats were subcutaneously implanted with 20 mg pellets containing cholesterol and 0 mg E₂ (control), 2 mg E₂ or an equimolar amount of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ for 24–36 weeks for 2 mg E₂ and 36 weeks for all other treatment groups (n=11–12). Values are the mean ± S.E.M.

Treatment	Final body weight (g)	Organ wet weights (per 100 g body weight)		
		Liver (g)	Pituitary (mg)	Uterus/ovary ^a (mg)
Control	219 ± 6*	3.0 ± 0.06	5.5 ± 0.7	362 ± 19
E ₂	189 ± 5	3.6 ± 0.13†	37 ± 14†	413 ± 34
2-OH E ₂	230 ± 5*	2.8 ± 0.06	5.4 ± 0.3	325 ± 27
4-OH E ₂	220 ± 4*	2.8 ± 0.06	5.0 ± 0.3	273 ± 23*
16α-OH E ₂	201 ± 4‡	3.0 ± 0.09	5.1 ± 0.5	297 ± 21*
4-OH E ₁	221 ± 6*	2.8 ± 0.06	5.2 ± 0.4	312 ± 24

^aData from rats with unilateral uteri discarded.Significantly different (ANOVA, followed by Bonferroni post hoc test, *P* < 0.05): *compared with E₂;†compared with cholesterol, 2-OH E₂, 4-OH E₂, 16α-OH E₂, and 4-OH E₁; ‡compared with 2-OH E₂.

Figure 3 Serum E₂ levels in ACI rats treated with different doses of E₂. Female ACI rats (n=6) were implanted subcutaneously with 20 mg pellets containing cholesterol and 0 mg E₂ (control; (▼)), 1 mg E₂ (○), or 3 mg E₂ (●). Each value represents the mean ± S.E.M., as determined by RIA. Error bars are not visible for some data points because the standard error range was very small.

modest to massive enlargement. By 12 weeks, the pituitaries of rats treated with 3 mg E₂ were increased by as much as 6-fold in weight, by which time the masses had begun to erode the sella turcica and compress the overlying cerebrum. Similar features were observed in 2 mg E₂-treated rats after 24–36 weeks. Morphologically, the pituitaries consisted predominantly of hyperplastic lactotrophic pituicytes, often interspersed with large zones of hemorrhage and necrosis. Mitotic figures were frequent in many visual fields.

Serum E₂ levels in rats treated with E₂

Circulating serum E₂ levels were determined at 6, 12, 27, and 36 weeks in rats treated with 1 and 3 mg E₂ (Fig. 3). In control rats, serum E₂ levels remained relatively constant at 30 ± 5 pg/ml. After 12 weeks of treatment with 1 or 3 mg E₂, the serum E₂ level increased 2.2- or 6.4-fold above control levels respectively. No further changes in serum E₂ levels were observed at later time-intervals.

Hepatic microsomal metabolism of E₂

NADPH-dependent oxidation of E₂ by hepatic microsomes produced several metabolites (Table 3). The major products of E₂ oxidation in hepatic microsomes from control rats were 2-OH E₂ and E₁ as previously reported (Mesia-Vela *et al.* 2002), and small amounts (~5%) of 4-OH E₂ and 2-OH E₁ were also detected. Treatment of rats with 1 or 3 mg pelleted E₂ for 6 or 12 weeks resulted in a 50–70% increase in the hepatic microsomal formation of E₁ (Table 3); however, the formation of 2- and 4-hydroxylated estrogens was not affected.

Table 3 Effect of E₂ treatment on the NADPH-dependent oxidation of E₂ by hepatic microsomes. Female ACI rats were implanted subcutaneously with 20 mg pellets containing cholesterol and 0 (control), 1, or 3 mg E₂ for 6 or 12 weeks. Liver microsomes (0.5 mg protein) were incubated at 37 °C for 20 min with 50 µM sodium-phosphate buffer, 5 mM ascorbic acid, 3 mM magnesium chloride, 50 µM [³H]E₂, and 2 mM NADPH. Each value is the mean ± S.E.M. obtained from two separate experiments (n=5)

Treatment	Estradiol metabolites formed (pmol/mg per min)			
	4-OH-E ₂	2-OH-E ₂	2-OH-E ₁	E ₁
6 weeks				
Control	13 ± 2	154 ± 5	20 ± 1	83 ± 11
1 mg E ₂	18 ± 4	195 ± 22	18 ± 2	121 ± 8*
3 mg E ₂	14 ± 4	191 ± 18	21 ± 4	125 ± 12*
12 weeks				
Control	13 ± 2	154 ± 12	17 ± 3	93 ± 7
1 mg E ₂	9 ± 1	150 ± 29	18 ± 3	150 ± 18*
3 mg E ₂	12 ± 2	182 ± 78	27 ± 6	157 ± 25*

*Statistically different from control group (ANOVA followed by Bonferroni post hoc test, P<0.05).

Inactivity of 2-OH E₂, 4-OH E₂, 16α-OH E₂, and 4-OH E₁ as mammary carcinogens

In the second study, we compared the oncogenic effects of E₂, 2-OH E₂, 4-OH E₂, 16α-OH E₂, and 4-OH E₁ in the mammary gland of female ACI rats. Since the data from our first study indicated that a 3 mg dose of E₂ resulted in a 100% mammary tumor incidence while a 1 mg dose of E₂ induced a 50% incidence of mammary tumors, we selected an intermediate dose, 2 mg E₂, for this comparative study. ACI rats treated with 2 mg E₂ exhibited a 73% incidence of mammary tumors after 36 weeks of treatment. No mammary tumors were detected in any of the rats treated with equimolar doses of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ after the same time-period (Fig. 2).

Quantification of E₂ and its metabolites in cholesterol pellets

To ensure that the catechol estrogens present in the pellets were released in an amount similar to that of E₂, the percentage of estrogen remaining in the pellets containing 2 mg E₂ or equimolar amounts of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ was determined at necropsy. The HPLC retention times observed for each of the individual estrogens were the same as those for their authentic respective standards, indicating that the remaining estrogen present in the pellets was not altered from its original form. Estrogens were not detected in any of the 20 mg cholesterol pellets (control). In the estrogen-containing pellets, significantly less 4-OH E₁ remained in the pellet at necropsy than was left in pellets from E₂,

Figure 4 Percentage of E₂ or metabolite remaining in pellets 35–36 weeks after subcutaneous implantation. Female ACI rats ($n=11$ – 12) were implanted subcutaneously with 20 mg pellets containing cholesterol and 2 mg E₂, or an equimolar dose of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁. Only pellets from rats treated with 2 mg E₂ that remained on treatment for 35–36 weeks were included in these analyses ($n=6$). Each value represents the means $\pm$ S.E.M., as determined by HPLC. Statistically different (ANOVA followed by Bonferroni post hoc test, $P<0.01$): * from E₂, ** from E₂, 2-OH E₂, 4-OH E₂, and 16α-OH E₂.

2-OH E₂, 4-OH E₂, or 16α-OH E₂, and significantly less 4-OH E₂ remained compared with E₂ (Fig. 4).

Discussion

E₂ alone induces mammary tumors, both ductal carcinoma *in situ* and adenocarcinoma, in female ACI rats (Shull *et al.* 1997, Spady *et al.* 1999, Harvell *et al.* 2000, Li *et al.* 2002a,b). These tumors have been shown to closely resemble human sporadic breast cancer in many salient molecular aspects (Li *et al.* 2002a). We have therefore studied the time-course and dose-dependency of induction of mammary tumors by E₂, as well as by 2-OH E₂, 4-OH E₂, 16α-OH E₂, and 4-OH E₁, administered in cholesterol pellets in the estrogen-sensitive female ACI rat.

The data presented herein indicated that only low amounts of exogenous E₂ are required to consistently induce mammary tumors in female ACI rats. The incidence and the latency periods for mammary tumors observed in these studies are similar to those induced by diethylstilbestrol, using a similar pellet delivery system in ACI rats (Stone *et al.* 1979). We observed highly mitotic solid, cribriform, and papillary patterns in some mammary tumor samples, but not the carcinomas with invasive and metastatic features that have been reported elsewhere (Meites 1972, Harvell *et al.* 2000).

Estrogens have been proposed to trigger breast cancer through oxidative stress induced by catechol estrogens formed by NADPH-dependent oxidation of E₂ (Liehr 2000). We studied alterations in catechol estrogen formation by isolated microsomes from livers of rats treated with

E₂. In our studies, no alterations in the amount of 2- or 4-hydroxylation of E₂ by hepatic microsomes from ACI rats treated with E₂ were found at any of the doses or time-periods studied. This suggests that E₂ treatment did not significantly alter the expression of hepatic cytochrome P450 1A, 2B, or 3A isoforms responsible for the NADPH-dependent oxidation of E₂ to catechol estrogens in rats. Strain differences in estrogen metabolite formation can be ruled out, as we have shown previously that the liver of the ACI rat generates similar levels of catechol estrogens as the Sprague–Dawley rat (Mesia-Vela *et al.* 2002), which is less sensitive to mammary tumors induced by E₂ treatment.

Additionally, the possible role of 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ in mammary carcinogenesis was studied by investigating their effects on tumor incidence when these metabolites were administered exogenously. No mammary tumors were induced by 2-OH E₂, 4-OH E₂, 16α-OH E₂, or 4-OH E₁ when administered in a manner similar to E₂ and at doses equimolar to 2 mg E₂. The absence of mammary tumors with these metabolites of E₂ was not due to poor release of these metabolites from the cholesterol pellets. The amount of metabolites recovered from the pellet at necropsy was similar to or less than the amount of E₂ recovered (Fig. 4). This indicates that the total exposure to each of these E₂ metabolites was either greater (4-OH E₂ and 4-OH E₁) than that of E₂ or was comparable with that of E₂. Since all of these metabolites are more water soluble than E₂, they could be cleared faster. It may be critical for these metabolites to be formed in or near the mammary tissues to be effective as initiators of carcinogenesis. Although unlikely, one or more of the metabolites could have initiated mammary carcinogenesis in a few cells but the circulating levels of endogenous estrogens was insufficient to promote those cells to a stage that was palpable or visible at the time of necropsy. It is of interest that El-Bayoumy *et al.* (1996) injected E₁-3,4-quinone under the nipples of 30 day old female CD rats but, detected no tumors 44 weeks post treatment. All of the metabolites in our study are markedly less effective estrogen receptor agonists compared with E₂ itself (Martucci & Fishman 1979, Kuiper *et al.* 1997). The low doses of E₂, but not any of the metabolites, were sufficient to affect various estrogen-responsive organs such as liver, uterus, and pituitary gland.

In summary, the results have indicated that female ACI rats treated with cholesterol pellets containing low doses of E₂ are a useful animal model for evaluating the mechanisms of estrogen-induced breast cancer. This is the first report to our knowledge that shows a dose dependency for E₂-induction of mammary tumors using an amount of E₂ that approximates physiological concentrations. Collectively, the lack of increased formation of reactive catechol estrogens by hepatic microsomes during continuous E₂ administration together with the failure of these hydroxylated estrogen metabolites administered via

cholesterol implants to exhibit any oncogenic potential suggests that the E₂ induction of mammary cancer in the ACI rat occurs as a consequence of molecular events arising primarily from E₂ or from E₂ metabolites generated within or in close proximity to oncogenic targets in the mammary gland.

Acknowledgements

Portions of this work were supported by ES 05022, ES 11256, GM 44982 and a Collaborative Research Award from The Cancer Institute of New Jersey. The work was presented in part at the 42nd Annual Meeting of the Society of Toxicology, Salt Lake City, UT, USA, March 2003.

References

- Altman PL & Katz DD 1979 Inbred strains: rat. In *Inbred and Genetically Defined Strains of Laboratory Animals*, part 1, pp 238–239. Eds PL Altman & DD Katz. Bethesda, Maryland: Federation of American Societies for Experimental Biology.
- Bernstein L, Henderson BE, Hanisch R, Sullivan-Halley J & Ross RK 1994 Physical exercise and reduced risk of breast cancer in young women. *Journal of the National Cancer Institute* **86** 1403–1408.
- Broerse JJ, van Bekkum DW & Zurcher C 1989 Radiation carcinogenesis in experimental animals. *Experientia* **45** 60–69.
- Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL & Rogan EG 1997 Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *PNAS* **94** 10937–10942.
- Cavalieri E, Frenkel K, Liehr JG, Rogan E & Roy D 2000 Estrogens as endogenous genotoxic agents – DNA adducts and mutations. *Journal of the National Cancer Institute Monographs* **27** 75–93.
- Cavalieri EL, Li KM, Balu N, Saeed M, Devanesan P, Higginbotham S, Zhao J, Gross ML & Rogan EG 2002 Catechol ortho-quinones: the electrophilic compounds that form depurinating DNA adducts and could initiate cancer and other diseases. *Carcinogenesis* **23** 1071–1077.
- Chakravarti D, Pelling JC, Cavalieri EL & Rogan EG 1995 Relating aromatic hydrocarbon-induced DNA adducts and c-H-ras mutations in mouse skin papillomas: the role of apurinic sites. *PNAS* **92** 10422–10426.
- Chakravarti D, Mailander PC, Li KM, Higginbotham S, Zhang HL, Gross ML, Meza JL, Cavalieri EL & Rogan EG 2001 Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene. *Oncogene* **20** 7945–7953.
- Cutts JH & Noble RL 1964 Estrone-induced mammary tumors in the rat: I. Induction and behavior of tumors. *Cancer Research* **24** 1116–1123.
- Dunning WF & Curtis MR 1952 The incidence of diethylstilbestrol-induced cancer in reciprocal F1 hybrids obtained from crosses between rats of inbred lines that are susceptible and resistant to the induction of mammary cancer by this agent. *Cancer Research* **12** 702–706.
- Dunning WF, Curtis MR & Segaloff A 1947 Strain differences in response to diethylstilbestrol and the induction of mammary gland and bladder cancer in the rat. *Cancer Research* **7** 511–521.
- El-Bayoumy K, Ji BY, Upadhyaya P, Chae YH, Kurtzke C, Rivenson A, Reddy BS, Amin S & Hecht SS 1996 Lack of tumorigenicity of cholesterol epoxides and estrone-3,4-quinone in the rat mammary gland. *Cancer Research* **56** 1970–1973.
- Harvell DM, Strecker TE, Tochacek M, Xie B, Pennington KL, McComb RD, Roy SK & Shull JD 2000 Rat strain-specific actions of 17 beta-estradiol in the mammary gland: correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers. *PNAS* **97** 2779–2784.
- Henderson BE & Feigelson HS 2000 Hormonal carcinogenesis. *Carcinogenesis* **21** 427–433.
- Highman B, Greenman DL, Norvell MJ, Farmer J & Shellenberger TE 1980 Neoplastic and preneoplastic lesions induced in female C3H mice by diets containing diethylstilbestrol or 17 beta-estradiol. *Journal of Environmental Pathology and Toxicology* **4** 81–95.
- Highman B, Roth SI & Greenman DL 1981 Osseous changes and osteosarcomas in mice continuously fed diets containing diethylstilbestrol or 17 beta-estradiol. *Journal of the National Cancer Institute* **67** 653–662.
- Holtzman S, Stone JP & Shellabarger CJ 1981 Synergism of estrogens and X-rays in mammary carcinogenesis in female ACI rats. *Journal of the National Cancer Institute* **67** 455–459.
- Ip C 1996 Mammary tumorigenesis and chemoprevention studies in carcinogen-treated rats. *Journal of Mammary Gland Biology and Neoplasia* **1** 37–47.
- Kelsey JL & Bernstein L 1996 Epidemiology and prevention of breast cancer. *Annual Reviews in Public Health* **17** 47–67.
- Kreiger N, Sloan M, Cotterchio M & Kirsh V 1999 The risk of breast cancer following reproductive surgery. *European Journal of Cancer* **35** 97–101.
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138** 863–870.
- Lee AJ, Kosh JW, Conney AH & Zhu BT 2001 Characterization of the NADPH-dependent metabolism of 17 beta-estradiol to multiple metabolites by human liver microsomes and selectively expressed human cytochrome P450 3A4 and 3A5. *Journal of Pharmacology and Experimental Therapeutics* **298** 420–432.
- Lee AJ, Cai MX, Thomas PE, Conney AH & Zhu BT 2003 Characterization of the oxidative metabolites of 17 beta-estradiol and estrone formed by 15 selectively expressed human cytochrome P450 isoforms. *Endocrinology* **144** 3382–3398.
- Li JJ & Li SA 1987 Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism. *FEBS Letters* **46** 1858–1863.
- Li JJ, Kirkman H & Hunter RL 1968 Sex difference and gonadal hormone influence on Syrian hamster kidney esterase isozymes. *Journal of Histochemistry and Cytochemistry* **17** 386–393.
- Li JJ, Li SA, Klicka JK, Parsons JA & Lam LK 1983 Relative carcinogenic activity of various synthetic and natural estrogens in the Syrian hamster kidney. *Cancer Research* **43** 5200–5204.
- Li JJ, Papa D, Davis MF, Weroha SJ, Aldaz CM, El-Bayoumy K, Ballenger J, Tawfik O & Li SA 2002a Ploidy differences between hormone- and chemical carcinogen-induced rat mammary neoplasms: comparison to invasive human ductal breast cancer. *Molecular Carcinogenesis* **33** 56–65.
- Li SA, Weroha SJ, Tawfik O & Li JJ 2002b Prevention of solely estrogen-induced mammary tumors in female ACI rats by tamoxifen: evidence for estrogen receptor mediation. *Journal of Endocrinology* **175** 297–305.
- Liehr JG 2000 Is estradiol a genotoxic mutagenic carcinogen? *Endocrine Reviews* **21** 40–54.
- Liehr JG & Ricci MJ 1996 4-Hydroxylation of estrogens as marker of human mammary tumors. *PNAS* **93** 3294–3296.
- Liehr JG, Fang WF, Sirbasku DA & Ari-Ulubelen A 1986 Carcinogenicity of catechol estrogens in Syrian hamsters. *Journal of Steroid Biochemistry* **24** 353–356.
- Maekawa A & Odashima S 1975 Spontaneous tumors in ACI/N rats. *Journal of the National Cancer Institute* **55** 1437–1445.

- Martucci CP & Fishman J 1979 Impact of continuously administered catechol estrogens on uterine growth and luteinizing hormone secretion. *Endocrinology* **105** 1288–1292.
- Meites J 1972 Relation of prolactin and estrogen to mammary tumorigenesis in the rat. *Journal of the National Cancer Institute* **48** 1217–1224.
- Mesia-Vela S, Sanchez RI, Li JJ, Li SA, Conney AH & Kauffman FC 2002 Catechol estrogen formation in liver microsomes from female ACI and Sprague–Dawley rats: comparison of 2- and 4-hydroxylation revisited. *Carcinogenesis* **23** 1369–1372.
- Muti P, Bradlow HL, Micheli A, Krogh V, Freudenheim JL, Schunemann HJ, Stanulla M, Yang J, Sepkovic DW, Trevisan M & Berrino F 2000 Estrogen metabolism and risk of breast cancer: a prospective study of the 2:16 alpha-hydroxyestrone ratio in premenopausal and postmenopausal women. *Epidemiology* **11** 635–640.
- Newbold RR & Liehr JG 2000 Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Research* **60** 235–237.
- Noble RL, Hochachka BC & King D 1975 Spontaneous and estrogen-produced tumors in Nb rats and their behavior after transplantation. *Cancer Research* **35** 766–780.
- Preston-Martin S, Pike MC, Ross RK, Jones PA & Henderson BE 1990 Increased cell division as a cause of human cancer. *Cancer Research* **50** 7415–7421.
- Rogan EG, Badawi AF, Devanesan PD, Meza JL, Edney JA, West WW, Higginbotham SM & Cavalieri EL 2003 Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: potential biomarkers of susceptibility to cancer. *Carcinogenesis* **24** 697–702.
- Rudali G, Apiou F & Muel B 1975 Mammary cancer produced in mice with estriol. *European Journal of Cancer* **11** 39–41.
- Santen RJ 2002 To block estrogen's synthesis or action: that is the question. *Journal of Clinical Endocrinology and Metabolism* **87** 3007–3012.
- Sasco AJ 2001 Epidemiology of breast cancer: an environmental disease? *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **109** 321–332.
- Segaloff A & Maxfield WS 1971 The synergism between radiation and estrogen in the production of mammary cancer in the rat. *Cancer Research* **31** 166–168.
- Shull JD, Spady TJ, Snyder MC, Johansson SL & Pennington KL 1997 Ovary-intact, but not ovariectomized female ACI rats treated with 17 beta-estradiol rapidly develop mammary carcinoma. *Carcinogenesis* **18** 1595–1601.
- Snyderwine EG 1999 Mammary gland carcinogenesis by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in rats: possible mechanisms. *Cancer Letters* **143** 211–215.
- Spady TJ, Harvell DM, Lemus-Wilson A, Strecker TE, Pennington KL, Vander Woude EA, Birt DF, McComb RD & Shull JD 1999 Modulation of estrogen action in the rat pituitary and mammary glands by dietary energy consumption. *Journal of Nutrition* **129** S587–S590.
- Stack DE, Byun J, Gross ML, Rogan EG & Cavalieri EL 1996 Molecular characteristics of catechol estrogen quinones in reactions with deoxyribonucleosides. *Chemical Research in Toxicology* **9** 851–859.
- Stone JP, Holtzman S & Shellabarger CJ 1979 Neoplastic responses and correlated plasma prolactin levels in diethylstilbestrol-treated ACI and Sprague–Dawley rats. *Cancer Research* **39** 773–778.
- Suchar LA, Chang RL, Rosen RT, Lech J & Conney AH 1995 High-performance liquid chromatography separation of hydroxylated estradiol metabolites: formation of estradiol metabolites by liver microsomes from male and female rats. *Journal of Pharmacology and Experimental Therapeutics* **272** 197–206.
- Suchar LA, Chang RL, Thomas PE, Rosen RT, Lech J & Conney AH 1996 Effects of phenobarbital, dexamethasone, and 3-methylcholanthrene administration on the metabolism of 17 beta-estradiol by liver microsomes from female rats. *Endocrinology* **137** 663–676.
- Thomas PE, Reik LM, Ryan DE & Levin W 1983 Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochromes P-450c and P-450d, by structurally diverse xenobiotics. *Journal of Biological Chemistry* **258** 4590–4598.
- Young S & Hallows RC 1973 Tumours of the mammary gland. *International Agency for Research in Cancer Science Publications* **5** 31–73.
- Yuan JM, Yu MC, Ross RK, Gao YT & Henderson BE 1988 Risk factors for breast cancer in Chinese women in Shanghai. *Cancer Research* **48** 1949–1953.
- Yue W, Santen RJ, Wang JP, Li Y, Verderame MF, Bocchinfuso WP, Korach KS, Devanesan P, Todorovic R, Rogan EG & Cavalieri EL 2003 Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. *Journal of Steroid Biochemistry and Molecular Biology* **86** 477–486.

Received 24 June 2004

Accepted 13 July 2004

Made available online as an

Accepted Preprint 23 July 2004