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

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Abstract

Several investigators have suggested that certain hydroxylated metabolites of 17β-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), 16α-hydroxyestradiol (16α-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, 16α-OH E2, or 4-OH E1 did not induce any detectable mammary tumors. The serum levels of E2 in rats treated with 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, 16α-OH E2, and 4-OH E1 were inactive in inducing mammary tumors.


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 17β-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 16α-hydroxyestradiol (16α-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 16α-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 16α-OH E₁ was associated with a decreased risk of developing breast cancer after 5 years (Mutlu 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 diethylnitrosamine-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, 16α-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 E2 (n=8). In a second study, rats were implanted with a single 20 mg pellet containing cholesterol and 0 or 2 mg E2, or an equimolar dose of 2-OH E2, 4-OH E2, 16α-OH E2, or 4-OH E1 (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 E2 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 E2, and killed for E2 metabolism studies and serum E2 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 E2 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 µm) were prepared and stained with hematoxylin and eosin.

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**Hepatic microsomal metabolism of E2**

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.

NADP-dependent oxidation of E2 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 µM [3H]E2 (0.5 µCi) 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 methylene chloride. The reaction was extracted and 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 E2 and its metabolites in cholesterol pellets**

Pellets from rats treated with 2 mg E2, an equimolar amount of 2-OH E2, 4-OH E2, 16α-OH E2, or 4-OH E1, 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 E2 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 × 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 µl) of the pellet dilutions were injected into the HPLC. Fractions from the solutions of control and E2-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 E2, 4-OH E2, 16α-OH E2, or 4-OH E1 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
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 ± 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 ± 0·2 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, 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.
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 lesions 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 micro-glandular 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 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final body weight (g)</th>
<th>Organ wet weights (per 100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uterus* (mg)</td>
</tr>
<tr>
<td>6 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>161 ± 17</td>
<td>275 ± 15</td>
</tr>
<tr>
<td>1 mg E2</td>
<td>172 ± 6</td>
<td>313 ± 35</td>
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<tr>
<td>3 mg E2</td>
<td>175 ± 4</td>
<td>314 ± 12</td>
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<tr>
<td>12 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>187 ± 6</td>
<td>225 ± 11</td>
</tr>
<tr>
<td>1 mg E2</td>
<td>191 ± 3</td>
<td>224 ± 16</td>
</tr>
<tr>
<td>3 mg E2</td>
<td>185 ± 4</td>
<td>259 ± 16</td>
</tr>
<tr>
<td>24–36 weeks</td>
<td>206 ± 3</td>
<td>237 ± 20</td>
</tr>
<tr>
<td>Control</td>
<td>216 ± 6</td>
<td>266 ± 27</td>
</tr>
<tr>
<td>1 mg E2</td>
<td>206 ± 3</td>
<td>237 ± 20</td>
</tr>
<tr>
<td>3 mg E2</td>
<td>165 ± 5*</td>
<td>466 ± 47*</td>
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</tbody>
</table>

*Data from rats with unilateral uteri and kidney discarded.

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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final body weight (g)</th>
<th>Organ wet weights (per 100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver (g)</td>
</tr>
<tr>
<td>Control</td>
<td>219 ± 6*</td>
<td>3·0 ± 0·06</td>
</tr>
<tr>
<td>E2</td>
<td>189 ± 5</td>
<td>3·6 ± 0·13†</td>
</tr>
<tr>
<td>2-OH E2</td>
<td>230 ± 5*</td>
<td>2·8 ± 0·06</td>
</tr>
<tr>
<td>4-OH E2</td>
<td>220 ± 4*</td>
<td>2·8 ± 0·06</td>
</tr>
<tr>
<td>16α-OH E2</td>
<td>201 ± 4‡</td>
<td>3·0 ± 0·09</td>
</tr>
<tr>
<td>4-OH E1</td>
<td>221 ± 6*</td>
<td>2·8 ± 0·06</td>
</tr>
</tbody>
</table>

*Data from rats with unilateral uteri discarded.

†compared with cholesterol, 2-OH E2, 4-OH E2, 16α-OH E2, and 4-OH E1; ‡compared with 2-OH E2.
Hepatic microsomal metabolism of \(E_2\)

NADPH-dependent oxidation of \(E_2\) by hepatic microsomes from control rats were 2-OH \(E_2\) and \(E_1\) as previously reported (Mestia-Vela et al. 2002), and small amounts (~5%) of 4-OH \(E_2\) and 2-OH \(E_1\) were also detected. Treatment of rats with 1 or 3 mg pelleted \(E_2\) for 6 or 12 weeks resulted in a 50–70% increase in the hepatic microsomal formation of \(E_1\) (Table 3); however, the formation of 2- and 4-hydroxylated estrogens was not affected.

Table 3 Effect of \(E_2\) treatment on the NADPH-dependent oxidation of \(E_2\) by hepatic microsomes. Female ACI rats were implanted subcutaneously with 20 mg pellets containing cholesterol and 0 mg \(E_2\) (control; (▼)), 1 mg \(E_2\) (○), or 3 mg \(E_2\) (●). 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.

\[
\begin{array}{cccccc}
\text{Treatment} & \text{4-OH-E_2} & \text{2-OH-E_2} & \text{2-OH-E_1} & \text{E_1} \\
6 \text{ weeks} & & & & & \\
\text{Control} & 13 \pm 2 & 154 \pm 5 & 20 \pm 1 & 83 \pm 11 \\
1 \text{ mg } E_2 & 18 \pm 4 & 195 \pm 22 & 18 \pm 2 & 121 \pm 8^* \\
3 \text{ mg } E_2 & 14 \pm 4 & 191 \pm 18 & 21 \pm 4 & 125 \pm 12^* \\
12 \text{ weeks} & & & & & \\
\text{Control} & 13 \pm 2 & 154 \pm 12 & 17 \pm 3 & 93 \pm 7 \\
1 \text{ mg } E_2 & 9 \pm 1 & 150 \pm 29 & 18 \pm 3 & 150 \pm 18^* \\
3 \text{ mg } E_2 & 12 \pm 2 & 182 \pm 78 & 27 \pm 6 & 157 \pm 25^* \\
\end{array}
\]

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

Inactivity of 2-OH \(E_2\), 4-OH \(E_2\), 16\(\alpha\)-OH \(E_2\), and 4-OH \(E_1\) as mammary carcinogens

In the second study, we compared the oncogenic effects of \(E_2\), 2-OH \(E_2\), 4-OH \(E_2\), 16\(\alpha\)-OH \(E_2\), and 4-OH \(E_1\) in the mammary gland of female ACI rats. Since the data from our first study indicated that a 3 mg dose of \(E_2\) resulted in a 100% mammary tumor incidence while a 1 mg dose of \(E_2\) induced a 50% incidence of mammary tumors, we selected an intermediate dose, 2 mg \(E_2\), for this comparative study. ACI rats treated with 2 mg \(E_2\) 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 \(E_2\), 4-OH \(E_2\), 16\(\alpha\)-OH \(E_2\), or 4-OH \(E_1\) after the same time-period (Fig. 2).

Quantification of \(E_2\) 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 \(E_2\), the percentage of estrogen remaining in the pellets containing 2 mg \(E_2\) or equimolar amounts of 2-OH \(E_2\), 4-OH \(E_2\), 16\(\alpha\)-OH \(E_2\), or 4-OH \(E_1\) 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 \(E_1\) remained in the pellet at necropsy than was left in pellets from \(E_2\),
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 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 of E₂ 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.

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