Advances in Brief

Lack of Tumorigenicity of Cholesterol Epoxides and Estrone-3,4-quinone in the Rat Mammary Gland


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Abstract

The purpose of this study is to test the long-standing hypothesis that endogenous agents found in human breast fluid and plasma are potential initiators of breast cancer. Therefore, we evaluated the tumorigenicity in the mammary glands of female CD rats of cholestan-5α,6α-epoxy-3β-ol (cholesterol-α-epoxide), cholestan-5β,6β-epoxy-3β-ol (cholesterol-β-epoxide), and 1,5(10)estradiene-3,14,17-trione (estrone-3,4-quinone). As a positive control, trans-3,4-dihydroxy-α,α′-l,2-epoxy-l,2,3,4-tetrahydrobenzo[chlphenanthrene (BcPDE) was used. Rats were fed a high-fat AIN-76A diet (23.5% corn oil) to mimic the Western dietary composition. Because literature data suggest that the endogenous agents tested in this study are weak electrophiles, the total doses of cholesterol epoxides (12.3 μmol/rat) and of estrone-3,4-quinone (30 μmol/rat) were 10- and 25-fold higher, respectively, than that of BcPDE (1.2 μmol/rat). Each agent was dissolved in DMSO, and one-sixth of the total dose was injected under each of six nipples on the left side of the rat, whereas DMSO only was injected under the nipples on the right side. The thoracic glands of the rats were treated at 30 days of age, and those located in the inguinal area were treated on the following day. The experiment was terminated 44 weeks after treatment. Consistent with our previous study, BcPDE was a strong mammary carcinogen. However, there were no differences between rats treated with DMSO alone and those receiving DMSO containing cholesterol-α-epoxide, cholesterol-β-epoxide, or estrone-3,4-quinone. The results of this study clearly indicate, for the first time, that metabolites derived from cholesterol and estrone lack tumorigenic activity in the rat mammary gland, at least under the conditions of the present protocol.

Introduction

Breast cancer is second only to lung cancer as the leading cause of death from cancer in American women (1). Although modifiers of breast cancer have been described in the literature, there is a relative paucity of knowledge about the initiators of carcinogenesis in breast tissue. An array of different stimuli may qualify as initiators of breast cancer. There appears to be a link between ionizing radiation and induction of breast cancer in humans (2, 4). Moreover, exposure to xenobiotics is an accepted cause of various types of cancer in humans, with diet being a major source of exposure to carcinogens. Dietary chlorinated hydrocarbons have also been suggested to contribute to the development of breast cancer (13).

There is considerable interest in estrogens acting as initiators of tumorigenesis because of their covalent binding to and/or damage to cellular macromolecules (14). Literature data also suggest a role for epoxides derived from cholesterol and lipids in breast cancer development (15). Thus, examining whether endogenous and exogenous agents can induce mammary tumors in rodents may provide important leads toward understanding the role of such compounds in human breast cancer. In the present study, with BcPDE as a positive control (10, 11) and a high-fat diet to mimic the composition of the Western diet, we tested by direct injection into the rat mammary glands whether selected endogenous agents, namely cholesterol-α-epoxide, cholesterol-β-epoxide, and estrone-3,4-quinone (Fig. 1), were initiators of carcinogenesis in the breast tissue. The protocol used in this study has been employed successfully in the evaluation of several bioassays with xenogenous environmental agents, as well as with their ultimate genotoxic derivatives (10-12, 16).

Materials and Methods

Chemicals. Cholesterol-α-epoxide and cholesterol-β-epoxide are commercially available (Steraloids Inc., Wilton, NH). However, we synthesized these compounds to obtain sufficient amounts at a reasonable cost for animal bioassays. The synthetic procedure was adopted from the literature (17, 18). With m-chloroperbenzoic acid as the oxidizing agent in CH₂CN, the major product was cholesterol-α-epoxide (92%). However, perphthalic acid as the oxidizing agent in CH₂OH yielded a mixture containing substantial amounts of cholesterol-β-epoxide (35%). The compounds were separated by normal-phase HPLC with slight modifications from the conditions described in the literature (19). A system using 4.2% isopropanol in hexane at a flow of 3 ml/min was used to separate the isomers on a Partisil 10 ODS-3 Magnum 9 column (10 μm; 0.94 × 50 cm; Whatman, Inc., Clifton, NJ). Differential refractometry (Differential Refractometer R 401, Waters Associates, Milford, MA) was used for detection. Under these conditions, the α-epoxide and β-epoxide eluted after 40 and 44 min, respectively; cholesterol itself eluted after 17 min. The purity of each isomer was >99%, and both were highly stable when kept at room temperature for several days.

Estrone-3,4-quinone was prepared according to a procedure developed by Abu-Hajj (20). Activated MnO₂ (219 mg, 0.42 mmol) was added to a stirred solution of 4-hydroxyestrone (120 mg, 0.52 mmol) in CDC₁₇ (60 ml) at ~30°C under N₂ atmosphere. The reaction was complete in ~30 min. The reaction mixture was filtered very rapidly to remove MnO₂ and to give a dark yellow solution. Normal-phase HPLC analysis, using isocratic conditions, indicated a single product that eluted after 51 min [column: 0.46 × 25 cm, Si 60 (EM Industries Inc., Gibbstown, NJ); program: 4.2% isopropanol in hexane at a flow rate of 1 ml/min]. However, in a reverse-phase HPLC system using isocratic conditions [column: 0.41 × 30 cm Versapack C₁₈-10 μm (Alltech/Applied Science, Deerfield, IL); program: 60% CH₂OH in H₂O at a flow rate of 1 ml/min] the product eluted after 7.5 min. It was stable

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¹ The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; nitro-PAH, nitropolycyclic aromatic hydrocarbon; HAA, heterocyclic aromatic amine; BcPDE, trans-3,4-dihydroxy-α,α′-l,2-epoxy-l,2,3,4-tetrahydrobenzo[chlphenanthrene; cholesterol-α-epoxide, cholestan-5α,6α-epoxy-3β-ol; cholesterol-β-epoxide, cholestan-5β,6β-epoxy-3β-ol; estrone-3,4-quinone, 1,5(10)-estradiene-3,14,17-trione; HPLC, high-performance liquid chromatography.
for at least 1 month at −30°C in CDCl₃. To avoid decomposition when preparing a solution in DMSO for bioassay, the solvent was added before complete evaporation of CDCl₃. Traces of CDCl₃ were then evaporated at room temperature. The ¹H nuclear magnetic resonance spectrum of this compound is identical to that reported previously (20, 21); (360 MHz, CDCl₃) δ 0.93 (s, 3H, 18-CR₂), 1.2–2.8 (m, 14H), 6.22 (d, 1H, J = 10.4 Hz), 7.18 (d, 1H, 1-H, J = 10.4 Hz).

BcPDE was synthesized for use as a positive control in the bioassay (22). Its structure was confirmed by ¹H nuclear magnetic resonance, mass spectrometry, and UV spectral analysis. Its purity was assessed by HPLC as being greater than 99% (11); this compound was stable in DMSO for several days, as determined by normal-phase HPLC analysis. DMSO was obtained from Aldrich Chemical Co. (Milwaukee, WI). Solutions of the test compounds in DMSO were prepared immediately before injections.

Bioassay. Female CD rats 21 days old [Crl:CD(SD)BR] were obtained from Charles River Breeding Laboratories (Kingston, NY) and housed under standard conditions (16). They were maintained on water ad libitum and were allowed free access to a high-fat AIN-76A diet throughout the course of the experiment (23, 24). All diet ingredients were obtained from Dyets, Inc. (Bethlehem, PA) and were then mixed in our laboratory and fed as a powder. The composition of this semipurified diet was as follows: casein, 23.5%; DL-methionine, 0.3%; corn starch, 32.0%; dextrose, 8.3%; corn oil, 23.5%; Alphacel, 5.9%; mineral mix (AIN-76), 4.2%; vitamin mix (AIN-76A), 1.8%; and choline bitartrate, 0.24% (24). Rats were randomly assigned to treatment groups as summarized in Table 1. The dosages of test compounds were determined on the basis of previous studies with PAHs, nitro-PAHs, and PAH diol epoxides (10–12, 16).

Because literature data point to the fact that the endogenous agents tested here are weak electrophiles, by comparison to certain PAH diol epoxides (e.g., BcPDE), the total doses of cholesterol epoxides (12.3 µmol/rat) and of estrone-3,4-quinone (30 µmol/rat) were 10-fold and 25-fold higher, respectively, than that of BcPDE (1.2 µmol/rat). The first injections of DMSO or test compound in DMSO were given at 30 days of age. The second injections were administered on the following day. The protocol was identical to that described previously (16). The mammary tissue underneath each of the three left thoracic nipples was injected with 100 µl DMSO containing 0.2 µmol (group 1), 2.04 µmol (groups 2 and 3), or 5 µmol (group 4) of test compound. The corresponding areas of the three nipples on the right side were treated with 100 µl of DMSO only (group 5). On the second day, the inguinal nipple areas received the same treatments. All injections were carried out under light ether anesthesia. Body weights were measured weekly during the first month, then monthly until termination. Rats were inspected and palpated weekly for the presence of mammary tumors beginning 5 weeks after treatment and then every 2 weeks for the remainder of the experiment. Animals were sacrificed when large or ulcerated tumors developed or when moribund. The experiment was terminated 44 weeks after the injections. At necropsy, all organs, especially the mammary glands, were examined macroscopically for any gross lesions or abnormalities. The tissues were fixed in 10% buffered formalin, processed for paraffin sections, and stained with H&E. Mammary tumors were classified according to Russo et al. (25). Tumor incidence data were analyzed by the χ² test; body weight gains over time were statistically treated by repeated measures ANOVA and tumor multiplicity data by Student’s t test.

Table 1 Mammary tumors in rats treated with BcPDE, cholesterol-α-epoxide, cholesterol-β-epoxide and estrone 3,4-quinone

<table>
<thead>
<tr>
<th>Group</th>
<th>Total dose, µmol</th>
<th>No. of rats</th>
<th>No. of rats with tumors (%)</th>
<th>No. of mammary tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>1. BcPDE</td>
<td>1.2</td>
<td>20</td>
<td>20 (100)b</td>
<td>46c</td>
</tr>
<tr>
<td>2. Cholesterol-α-epoxide</td>
<td>12.3</td>
<td>20</td>
<td>3 (15)</td>
<td>2</td>
</tr>
<tr>
<td>3. Cholesterol-β-epoxide</td>
<td>12.3</td>
<td>20</td>
<td>3 (15)</td>
<td>2</td>
</tr>
<tr>
<td>4. Estrone-3,4-quinone</td>
<td>30.0</td>
<td>20</td>
<td>4 (20)</td>
<td>1</td>
</tr>
<tr>
<td>5. DMSO</td>
<td>20</td>
<td>20</td>
<td>3 (15)</td>
<td>1</td>
</tr>
</tbody>
</table>

* Each rat received 6 injections of test compound (% of total dose), one under each nipple on its left side; see "Materials and Methods" for details.
* Significantly different from groups 2–5; P < 0.01, χ² test.
* Significantly different from groups 2–5; P < 0.01, Student’s t test.
* Dysplastic fibroadenoma, four dysplastic adenoma, three fibroma, four carcinomas, and three sarcoma.
* Dysplastic adenoma.
* In situ carcinoma.
Results

Mean body weights of rats treated with the test agents are shown in Fig. 2. ANOVA was used to examine the overall group differences in weight gain over time. Rats treated with BcPDE (group 1) or estrone-3,4-quinone (group 4) had body weights similar to those treated with DMSO alone (group 5) during the first 16 weeks after injections. Thereafter, the rats in groups 1 and 4 had significantly lower body weights than those in group 5 (P < 0.002). Body weights of rats treated with cholesterol-α-epoxide (group 2) and cholesterol-β-epoxide (group 3) were not different from those treated with DMSO (group 5).

All animals treated with cholesterol-α-epoxide, cholesterol-β-epoxide, or DMSO survived the full 44 weeks of the bioassay. With the exception of one rat that was sacrificed 1 month before termination, the animals that received estrone-3,4-quinone survived for the duration of the bioassay. Mean survival time in the group treated with BcPDE was 32.3 ± 11.0 weeks.

As shown in Fig. 3, the incidence of palpable mammary tumors rose rapidly in the BcPDE group, reaching 50% after 8 weeks and 100% after 25 weeks. BcPDE was significantly more active in eliciting tumors than was the vehicle control or the other compounds (P < 0.01). The incidence of palpable mammary tumors in rats treated with cholesterol-α-epoxide, cholesterol-β-epoxide, or estrone-3,4-quinone was similar to that found in rats treated with DMSO alone.

Histological findings are summarized in Table 1. Virtually all tumors were found on the left side (the treatment site). Adenocarcinomas and fibroadenomas were the most common tumors. Both incidence and multiplicity of adenocarcinomas were significantly higher in rats treated with BcPDE than in any of the other groups (P < 0.01). There were no differences between tumor incidences or multiplicities among rats receiving DMSO (group 5) and those treated with cholesterol-α-epoxide (group 2), cholesterol-β-epoxide (group 3), or estrone-3,4-quinone (group 4).

Discussion

Nipple aspirates of human breast fluid contain various chemical substances, including those of exogenous origin (such as the cigarette smoke constituents nicotine and cotinine), and mutagenic agents of undetermined origin (15). Identification of such agents is of particular importance in the etiology of breast cancer. Likely exogenous compounds to be found in breast fluid include chlorinated hydrocarbons, PAHs, NO2-PAHs, and HAAs (5). Nipple aspirates also contain constituents of endogenous origin, including cholesterol and estrogens and their metabolites that may have potential significance in the etiology of breast cancer. Therefore, this study was designed to provide insights into the possible contribution of cholesterol-α-epoxide, cholesterol-β-epoxides, and estrone-3,4-quinone to initiation of mammary tumors in CD rats. The results of this study clearly indicate the lack of activity of these endogenous agents in the rat mammary gland.

Our earlier studies had established the sensitivity of rat mammary tissue to the carcinogenic effects of exogenous carcinogens such as PAHs, nitro-PAHs, HAAs, and selected diol-epoxide metabolites of PAHs (10–12, 16, 26). BcPDE represents the latter class of agents, and it is administered by intramammary injection in this study as a positive control to compare its carcinogenic activity with that of endogenous agents known to be present in human serum and breast fluid (15). Consistent with our previous study, BcPDE was a strong mammary carcinogen (11).

Although several substances of endogenous origin have been identified in human breast fluid, we selected for the present study cholesterol epoxides and estrone-3,4-quinone because literature data pointed to their role as genotoxic agents under certain conditions (14, 27, 28). Moreover, their concentrations in nipple-aspirate breast fluid are significantly higher than those in serum (15). Therefore, prolonged contact of breast epithelium with these genotoxic agents could, conceivably, contribute to the initiation of carcinogenesis in this tissue. The results of our assays clearly indicate that these agents do not contribute to tumor-initiating activity in the rat mammary gland under the conditions of the present protocol.

To verify whether these substances have a promoting and/or cocarcinogenic effect will require further studies. Although cholesterol and its oxidation products had no enhancing effect on N-methyl-N-nitrosourea-induced mammary carcinogenesis (29, 30), cholesterol itself showed a promoting effect on dimethylbenz[a]anthracene-induced mammary carcinogenesis (31). However, the promoting effect of cholesterol epoxides on dimethylbenz[a]anthracene-initiated mammary carcinogenesis or in conjunction with other initiators also requires further investigation.

There are two potential mechanisms that may account for the previously observed tumorigenicity and cytotoxicity of estrogens in rats, hamsters, mice, and guinea pigs (32). These are modification of DNA and/or generation of reactive oxygen species. Covalent binding of DNA with quinones derived from estrogen metabolism has been
demonstrated in vitro (21, 28). Results from studies conducted in the hamster model support a role of free radicals in the development of kidney carcinogenesis (33). Reactive oxygen species (H₂O₂, -OH, and semiquinone) as a result of redox cycling of estrogen quinone have the potential to be genotoxic and cytotoxic. The role of reactive oxygen species in the initiation and, even more, in the promotion phase of carcinogenesis has frequently been suggested. On the basis of our observations and those published in the literature, the combined carcinogenesis has frequently been suggested. On the basis of our species in the initiation and, even more, in the promotion phase of potential to be genotoxic and cytotoxic. The role of reactive oxygen gland in animal models.

References

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