Carcinogenic Activities of Various Steroidal and Nonsteroidal Estrogens in the Hamster Kidney: Relation to Hormonal Activity and Cell Proliferation

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ABSTRACT

The therapeutic use of estrogens has been associated with an increased risk of some of the most predominant, as well as less prevalent, cancers in women. The estrogen-induced renal tumor is one of the primary animal models to evaluate the carcinogenic properties of estrogens. Correlations were made with various estrogens by using parameters of estrogenicity end points such as competitive binding, progesterone receptor induction, and alterations in prolactin levels; in vitro renal proximal cell proliferation; and in vivo estrogen-induced carcinogenicity. The most potent estrogens were Moxestrol (MOX), diethylstilbestrol (DES), and 17β-estradiol, followed by indenestrol B, 16α-hydroxyestrone, and 11β-methoxyestradiol with moderate estrogenic activities, whereas 11β-methylestradiol, 17α-estradiol, indenestrol, and deoxoestrone were all relatively weaker. As expected, hydrolyzed Premarin (unconjugated estrogens) was strongly estrogenic. Of the estrogens tested, MOX was the most potent carcinogenic estrogen in the hamster kidney. Both 16α-hydroxyestrone and 11β-methoxyestradiol induced intermediate tumor incidences with distinctly lower frequencies of renal tumor foci compared to the most potent carcinogenic estrogens. However, hamsters treated for 9.0 months with 11β-methylestradiol, 17α-estradiol, deoxoestrone, and indenestrol exhibited no tumors. In contrast, treatment with estrone, equilin plus δ-equilenin, and hydrolyzed Premarin for the same time period resulted in 100% renal tumor incidences and numerous tumor foci. Cell proliferation studies of cultured hamster kidney proximal tubule cells were carried out at varying estrogen concentrations (0.01–100 nM). Exposure to MOX resulted in consistently high renal cell proliferative response over a concentration range of 0.1–10 nM. Strongly carcinogenic estrogens such as estrone had a maximal renal cell proliferation response (2.4-fold above untreated control levels) between 0.1 and 10 nM, DES and 17β-estradiol responded at 1.0 nM, and 4-hydroxyestradiol responded at 10 nM. Interestingly, exposure to ethinylestradiol, a potent estrogen, at similar or higher doses as those used for DES and 17β-estradiol, yielded only a 10% renal tumor incidence and induced only a 1.7-fold increase in proximal tubule cell proliferation. In contrast, 17α-estradiol, deoxoestrone, indenestrol, and 11β-methylestradiol, all weakly estrogenic and noncarcinogenic agents, had relatively little effect on tubule cell proliferation. The hydrolyzed Premarin exhibited a maximal 2.0-fold cell proliferative response at 10 nM.

The present results provide clear evidence that, in the hamster kidney, the degree of carcinogenicity of a given estrogen correlates with its ability to induce proximal tubule cell proliferation in vitro. Therefore, the ability of estrogen to enhance tubule cell proliferation is a more accurate indicator of its carcinogenicity in this system than either the estrogen-responsive end points used or the amount of catechol metabolites generated in this tissue as reported earlier.

INTRODUCTION

Numerous epidemiological studies have established causal relationships between estrogen use and increased risk for some of the predominant cancers in women, namely, breast and endometrium (1–3). Ingestion of estrogens has also been associated with some less prevalent cancers at hepatic, cervico-vaginal, and ovarian sites (4–6). It is estimated that at least 20 million women in the United States take estrogenic hormones, largely but not solely for contraception, for the relief of menopausal symptoms, and to reduce the risk of osteoporosis and cardiovascular disease that afflicts aging women. On a worldwide basis, this number is most certainly multiplied many times. Menopausal estrogens have become one of the most widely prescribed drugs, with a current usage rate of over 32% among women ages 50–70 years (7). Despite increasing study, there is still little understanding of the cellular and molecular mechanisms whereby these estrogen-related neoplastic events occur.

The estrogen-induced renal tumor has emerged as one of the primary experimental models to evaluate the carcinogenic properties of estrogens, whether they possess steroidal or nonsteroidal structures (8–10). Recent advances in estrogen carcinogenesis indicate that cell proliferation may play a critical early role in estrogen-induced tumorigenic processes (11, 12). Interestingly, this concept has received considerable support from earlier studies using numerous experimental models in hormonal carcinogenesis employing estrogens as well as other sex steroids (13–17).

The current report presents data correlating in vivo carcinogenicity data of estrogens and in vitro cell proliferation of primary renal epithelial hamster cells in culture, grown in serum-free chemically defined conditions, as well as other parameters of estrogenicity.

MATERIALS AND METHODS

Chemicals and Reagents. [2,4,6,7-3H]-17β-estradiol (115 Ci/mmol), [1,2,6,7-3H]-progesterone (103 Ci/mmol), and [17α-methyl-3H]-R5020 (86 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Radioinert chromatographic grade estradiol, estrone, and progesterone were purchased from Calbiochem (Behring, CA), and all other nonlabeled steroids were obtained either from Sigma Chemical Co. (St. Louis, MO) or Steraloids (Wilton, NH). Deoxoestrone was prepared by Dr. Luke K. T. Lam (LKT Laboratories, Minneapolis, MN). Indenestrol B and indenestrol were generously provided by Dr. Manfred Metzler, University of Kaiserslautern, Kaiserslautern, Germany; MOX5 (11β-methoxyestrone) by Dr. J. P. Raynaud (Roussel UCLAF, Paris, France); and 16α-hydroxyestrone by Dr. Jack Fishman (IVAX Corp., Miami, FL). All estrogens investigated exhibited purities of at least 95% as shown by HPLC analyses by using a Waters model 840 liquid chromatograph equipped with a Waters 490 programmable multiwavelength detector. Estrogen samples (10 µg), dissolved in tetrahydrofuran, were injected and eluted on two tandem octadecyl (C18) columns, 0.46 x 25 cm (IBM, Wallingford, CT), by using 29% acetonitrile in water isocratically at a flow rate of 3 ml/min. Conjugated estrogens were hydrolyzed from Premarin (Wyeth-Ayerst, Philadelphia, PA) under defined conditions as previously provided by Dr. Manfred Metzler (University of Kaiserslautern, Kaiserslautern, Germany). Amino acid analysis of the hydrolysate was performed as described by C. Raynaud and J. P. Raynaud (18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: MOX, Moxestrol; PRL, prolactin; ER, estrogen receptor; DES, diethylstilbestrol; PR, progesterone receptor; EE, ethinylestradiol.

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Animals and Treatments. Young adult castrated male Syrian golden hamsters, weighing 85-95 g, were purchased from Harian Sprague-Dawley (Indianapolis, IN). All animals were acclimated 1 week before treatment or use. Hamsters were housed in compliance with the guidelines of the United States Department of Health and Human Resources (NIH). Animals were housed in a facility certified by the American Association for the Accreditation of Laboratory Animal Care. Hamsters were maintained on a 12-h light/12-h dark cycle and fed rodent certified chow 5012 Purina diet (Ralston Purina Co., Richmond, IN) and tap water ad libitum. Castrated animals in the treatment groups (9-12/group) were implanted s.c. with the following pellets (20 mg): 17ß-estradiol, MOX, 11ß-methoxyestradiol, 11ß-methylestradiol, 16a-hydroxyestrone, 17α-estradiol, deoxoestrone, DES, indenestrol B, indanestrol, or hydrolyzed Premarin as described previously (18, 19). To maintain estrogenic levels of each compound constant, additional pellets were implanted every 2.5 months. The pellets were prepared by Hormone Pellet Press (Westwood, KS), and their release rates were adjusted so that mean daily absorptions were similar (111 ± 11 μg). However, 11β-methoxyestradiol, like EE2, exhibited a mean daily absorption rate of 259 ± 12 μg. For PR induction and serum PRL levels, hamsters were treated for 3.0 and 9.0 months, respectively, with various estrogens as reported elsewhere (19-21).

Competitive Binding Studies. For ER, pure renal tumors were excised from hamsters treated with DES for 9-11 months as described previously (22), homogenized in 9.0 volumes of TED buffer (10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4), and centrifuged at 100,000 × g. Competitive binding studies of the 8S ER were carried out on filtered cytosols (22-μm Millipore filter; Millipore) as described earlier, by using a-naphthyl butyrate as substrate (18, 19). To maintain estrogenic levels of each compound constant, additional pellets were implanted every 2.5 months. The pellets were prepared by Hormone Pellet Press (Westwood, KS), and their release rates were adjusted so that mean daily absorptions were similar (111 ± 11 μg). However, 11β-methoxyestradiol, like EE2, exhibited a mean daily absorption rate of 259 ± 12 μg. For PR induction and serum PRL levels, hamsters were treated for 3.0 and 9.0 months, respectively, with various estrogens as reported elsewhere (19-21).

Absorbance (280 nM) vs. Time (min)

![Fig. 1. HPLC separation of hydrolyzed Premarin (unconjugated estrogens) hydrolyzed by sulfatase for 5 h. Percentage composition is in descending order of quantity. Peak 10: estrone; Peak 9: 8-deoxyestrone; Peak 8: equilenin; Peak 7: 17α-dihydroequilenin; Peak 6: equilin; Peak 5: 17α-dihydroequilenin; Peak 4: 17α-estradiol; Peak 3: 17α-dihydroequilenin; Peak 2: 17β-dihydroequilenin.](image-url)
Carcinogenic Activity of Estrogens and Cell Proliferation

Table 1. Estrogenicity and carcinogenicity of various steroidal and stilbene estrogens in the Syrian hamster kidney

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>Renal tumor % of ER competitive binding*</th>
<th>Hamster kidney induction of PR' (fmol/mg protein)</th>
<th>Serum prolactin (ng/ml)</th>
<th>No. of animals with tumors (%)</th>
<th>Combined no. of tumor foci in both kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroidal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17ß-Estradiol</td>
<td>55 ± 2</td>
<td>48 ± 6</td>
<td>390 ± 76</td>
<td>100</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>11ß-Methoxy EE (MOX)</td>
<td>52 ± 4</td>
<td>60 ± 2</td>
<td>330 ± 65</td>
<td>100</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>16α-Hydroxyestrone</td>
<td>48 ± 3</td>
<td>45 ± 7</td>
<td>387 ± 35</td>
<td>38</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>11ß-Methoxyestradiol</td>
<td>30 ± 6</td>
<td>35 ± 6</td>
<td>389 ± 17</td>
<td>25</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>17ß-Methyltestosterone</td>
<td>14 ± 1</td>
<td>18 ± 2</td>
<td>149 ± 69</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>34 ± 5</td>
<td>6 ± 1</td>
<td>129 ± 17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Deoxoestrone</td>
<td>14 ± 1</td>
<td>8 ± 1</td>
<td>94 ± 20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stilbene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>46 ± 4</td>
<td>50 ± 4</td>
<td>449 ± 89</td>
<td>100</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>Indenestrol B</td>
<td>46 ± 2</td>
<td>49 ± 5</td>
<td>281 ± 90</td>
<td>100</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Indanestrol</td>
<td>10 ± 1</td>
<td>29 ± 5</td>
<td>103 ± 40</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Duration of estrogen treatment was 9.0 months. Each treatment group contained 9–12 animals. Data, mean ± SEM.

b Competitive binding of radioinert estrogens to ER was carried out in renal tumor cytosolic fractions obtained from DES-induced renal carcinomas. Estradiol concentration in these tumor cytosols (4 mg of protein/ml) without competitor corresponded to 0% inhibition. [3H]-estradiol at 5 nm ± cold competitor at 1.0-fold excess.

c Determined by affinity labeling of cytosolic fractions from 4.0-month DES-treated hamsters. [3H]-progesterone at 5 nm ± cold competitor at 100-fold excess.

RESULTS

Estrogenicity Studies. To assess the relative estrogenicity of the various steroidal and nonsteroidal estrogens studied in the hamster, competitive binding to kidney tumor cytosolic ER, induction of PR, and changes in serum PRL levels were studied. Untreated normal levels of PR and PRL in castrated male hamsters were 3.3 ± 0.2 fmol/mg protein and 34 ± 5 pg/ml, respectively. Generally, there was a good correlation among these parameters concerning the relative estrogenic potency of the steroidal and nonsteroidal compounds studied (Table 1).

Utilizing the hormonal end points described herein, it is evident that MOX, 17ß-estradiol, and DES were the most potent estrogens, followed closely by indenestrol B, 16α-hydroxyestrone, and 11ß-methoxyestradiol with moderate estrogenic potencies, whereas 11ß-methyltestosterone, 17α-estradiol, indanestrol, and deoxoestrone exhibited distinctly lower estrogenic activities (Table 1). The expected strong estrogenicity of hydrolyzed Premarin (unconjugated estrogens) was confirmed by its competitive binding to renal tumor ER (1.0-fold excess) at 42 ± 3%, its induction of hamster kidney PR to 53 ± 2 fmol/mg protein, and its elevation of serum PRL levels to 339 ± 93 ng/ml.

Carcinogenic Activity of Various Estrogens. As reported previously (19, 26), 17ß-estradiol and DES were equally carcinogenic in the hamster kidney. These potent carcinogenic estrogens served as positive references for the rest of the steroidal and nonsteroidal estrogens studied. MOX was found to be the most potent carcinogenic estrogen in the hamster kidney (Table 1), affecting a 100% tumor incidence and more numerous individual tumor foci even at shorter treatment periods (6.0–9.0 months) compared to either DES or 17ß-estradiol (data not shown). Both 16α-hydroxyestrone- and 11ß-methoxyestradiol-treated hamsters exhibited moderate tumor incidences and distinctly lower frequencies of renal tumor foci compared to MOX, DES, or 17ß-estradiol. In contrast, in hamsters treated for 9.0 months with either 17α-estradiol, deoxoestrone, 11ß-methylestradiol, or indanestrol exhibited no tumors. These evidently noncarcinogenic estrogens did not induce renal tumors even when the dose was increased to 40 mg (2 pellets) and implanted every 2.5 months for 9.0 months (data not shown).

Interestingly, hamsters treated for 9.0 months with estrone, equilin plus d-equilenin, or hydrolyzed Premarin exhibited 100% renal tumor incidences and abundant tumor foci for the same treatment period (Table 2).

Renal Tubule Cell Proliferation. Fig. 2 depicts the cell proliferation of cultured hamster renal proximal tubule cells with several steroidal estrogens at varying concentrations (0.01–100 nM) under serum-free chemically defined conditions. Estrone attained a maximal 2.4-fold increase in renal cell proliferation between 0.1 and 10 nM; 4-hydroxyestradiol exhibited a similar maximum proliferative response at 10 nM. Additionally, 4-hydroxyestrone exposure resulted in a maximal 2.1-fold rise in tubule cell proliferation at 1.0 nM. It is noted that 2-hydroxylated metabolites of 17β-estradiol and estrone exhibited lower renal cell proliferative activity compared to their corresponding 4-hydroxylated counterparts (data not shown). Interestingly, renal proximal tubule cell proliferation increased only a maximal of 1.7-fold after exposure to EE. At 100 nM, all of the above indicated estrogens exhibited a decline in tubular cell proliferation.

DISCUSSION

Previous studies from our laboratory (19, 26, 27) have concluded that a good correlation exists between hormonal activity and carcinogenicity.

Table 2. Carcinogenicity of unconjugated estrogens

<table>
<thead>
<tr>
<th>Estrogen</th>
<th>No. of animals with tumors (%)</th>
<th>Combined no. of tumor foci in both kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone</td>
<td>100</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Equilin + d-Equilenin</td>
<td>100</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Premarin</td>
<td>100</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

a Duration of estrogen treatment was 9.0 months. Each group contained 6–8 animals. Data, mean ± SEM.
CARCINOGENIC ACTIVITY OF ESTROGENS AND CELL PROLIFERATION

**Fig. 2. Effect of various steroidal estrogens (0.1-100 nM) on proliferative outgrowth of kidney tubule cells grown on PF-HR9 basement membrane in serum-free chemically defined medium.** Hamster cortical explants (~500/flask) were plated in separate 25 cm² flasks. Twelve days after initiation of the culture after ± estrogen exposure, all tubules were washed and treated with 0.1% EDTA. Both parent explants and monolayer cells were removed and disaggregated into single cells. The cells were then counted in a hemocytometer. Points, mean of six counts from three individual flasks; bars, mean ± SEM. The data are expressed as a stimulation index, the ratio of the number of cells grown in the presence of estrogen versus the number of cells grown in the absence of estrogen. The steroidal estrogens shown are estrone (•), 4-hydroxyestradiol (○), 4-hydroxyestrone (●), ethinylestradiol (□), and 17α-estradiol (▲).

Fig. 3. Effect of different steroidal estrogens (▲) and nonsteroidal stilbene estrogens (●) at varying concentrations (0.01-100 nM) on proliferative outgrowth of kidney tubule cells grown on PF-HR9 basement membrane in serum-free chemically defined medium. All culture conditions are the same as described in Fig. 2. A, stimulation by steroidal estrogens 17β-estradiol (○), Mox (▲), hydrolyzed Premarin (▼), 16α-hydroxyestrone (□), and 11β-methoxyestradiol (●). B, stimulation by nonsteroidal stilbene estrogens DES (●), indenestrol B (□), and indenestrol (▲).
that in human and animal studies, it is the unconjugated or "free" form of Premarin that is the hormonally active form because the conjugated form has been found to be hydrolyzed by target-tissue sulfatases (44, 45).

Therefore, it is possible that the disparity observed among some estrogens in the hamster kidney, as it concerns hormonal activity and the response to estrogen-dependent cell proliferation, may be due to the fact that elevation of serum PRL and renal PR (46) requires the expression of only a few genes, which may be more readily expressed by virtually all estrogens to varying degrees. However, cell proliferation elicited by most potent estrogens likely requires the expression of about 24 genes in a highly coordinated and concerted process (47, 48) which may not necessarily be elicited by all estrogens in a given tissue. The data presented in this study are consistent with recent evidence implicating estrogen-mediated cell proliferation as an early critical event in a multistage process of estrogen carcinogenesis in the hamster kidney (11, 12).

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REFERENCES


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