Relative Carcinogenic Activity of Various Synthetic and Natural Estrogens in the Syrian Hamster Kidney ¹

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ABSTRACT

Both synthetic and natural estrogens have been studied for their ability to induce renal carcinomas in castrated male hamsters after 9.0 months of treatment. Tumor foci were detected in frozen serial sections stained histochemically for esterase activity. Both diethylstilbestrol (DES) and 17β-estradiol had equal ability (100%) to induce renal tumors (~20.5 ± 3 [S.E.] tumor foci) in these animals. Hexestrol induced the same incidence and number of renal carcinoma foci as DES or 17β-estradiol. However, α-dienestrol and DES 3,4-oxide showed an 86 to 88% incidence of renal tumors in hamsters (~10.8 ± 3). When equilenin and δ-equilenin, components of therapeutic conjugated estrogens, were tested, only equilenin had a 76% incidence of renal tumor foci (5.5 ± 0.9). The ability of these stilbene and steroidal estrogens to compete for renal tumor estrogen receptor generally correlated well with their ability to cause renal tumorigenesis in the hamster with one notable exception. Although ethinyl estradiol competed as well as did DES or 17β-estradiol for estrogen receptor, had similar ability to induce renal progesterone receptor, and led to similar high serum prolactin levels as either DES or 17β-estradiol, it had only weak carcinogenic activity (21%) in the hamster kidney (0.6 ± 0.5 foci). These data represent the first detailed analysis of the relative carcinogenic activity of different estrogens within a given tumor-inducing system, and based on the carcinogenicity data of hexestrol and α-dienestrol presented herein, they suggest that epoxidation of the olefinic double bond and the p-quinone metabolite of DES probably are not involved significantly in its carcinogenic activity. Moreover, the poor carcinogenic activity of ethinyl estradiol in this system, despite strong estrogenicity, suggests that estronic activity alone may not be sufficient to effect renal tumorigenesis in the hamster.

INTRODUCTION

For over a decade, evidence has continued to accumulate indicating a causal link between estrogens and a variety of human cancers involving vaginal, hepatic, breast, endometrial, and cervical organ sites (1–3, 17, 23). Although some of the tumorigenic effects of estrogen exposure are due undoubtedly to their pro-motive effects on cellular differentiation, there is a growing awareness of the carcinogenic potential of both natural and synthetic estrogens (4, 14, 18, 21). At present, there are no detailed studies demonstrating the relative carcinogenic activity of various estrogens within a given animal tumor system.

The hamster renal adenocarcinoma has provided a unique model to investigate both hormonal and carcinogenic aspects of estrogen-induced tumorigenesis (5). Early studies in our laboratory strongly support a hormonal role for the induction of these tumors by estrogen. The demonstration of a specific high-affinity estrogen receptor in renal cytosols of castrated hamsters which is enhanced nearly 3.5-fold after prolonged estrogen administration supports this contention (8). Pertinent also is the finding that estrogen-induced renal tumorigenesis in the hamster can be completely blocked by those antiestrogens which inhibit markedly estrogen receptor-binding activity (8). Moreover, estrogen treatment resulted in at least a 10-fold rise in cytosolic progesterone receptor concentration in the untransformed kidney, and this elevated receptor can be modulated by antiestrogens, androgens, and some synthetic progestins (11, 15). However, our recent result concerning the inhibition of estrogen-induced kidney transformation by α-naphthoflavone cannot be readily explained by hormonal mechanisms alone (12). In addition, the marked suppression of kidney aryl hydrocarbon hydroxylase activity, not evident in the liver, supports a pertinent role for P-450 multisubstrate monooxygenases in effecting the tumorigenic response of the hamster to estrogens (14).

In an effort to discern the structural and hormonal requirements for estrogen carcinogenicity in the hamster kidney and to elucidate the significance of particular intermediates in a pathway, we have undertaken to determine the carcinogenic potential of various synthetic and natural estrogens to transform the hamster kidney. These data are correlated with the ability of these estrogens to compete for estrogen receptor binding, their ability to induce renal progesterone receptor in the untransformed kidney, and in some instances their ability to elevate serum PRL levels as determined by the Nb2 rat lymphoma cell bioassay.

MATERIALS AND METHODS

Chemicals and Reagents. 17β-[2,4,6,7-3H]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, Mass. Radioinert estradiol and progesterone, chromatographic grade, were obtained from Calbiochem-Behring, San Diego, Calif., and all other nonlabeled steroids were purchased from Sigma Chemical Co., St. Louis, Mo. DES 3,4-oxide was synthesized by a procedure modified from that

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of Metzler and McLachlan (22) using m-chloroperbenzoic acid. Trizma supplied by Sigma. Ultrapure sucrose (RNase free) was obtained from Schwarz/Mann, Orangeburg, N. Y. Ovine PRL (NIAMDD-o-PRL-14, 31 IU/mg) was a gift of the National Hormone and Pituitary Program, Baltimore Md. FM for leukemic cells of mice, fetal bovine serum, L-glutamine, and antibiotic-antimycotic (100x) were purchased from Grand Island Biological Co., Grand Island, N. Y. Horse serum was supplied by Sterile Systems, Logan, Utah.

TED (0.01 M Tris-HCl, 0.0015 M EDTA, and 0.001 M dithiothreitol) and TEDG (TED plus 5% glycerol) buffers, pH 7.4, were prepared at room temperature as indicated previously (8, 11, 13).

Animals and Renal Tumor Induction. Adult castrated-male Syrian golden hamsters (LAK-LVG, outbred strain) were obtained from Charles River Lakeview Hamster Colony, Wilmington, Mass. Similar hypophysectomized male hamsters were also supplied from the same source. All animals weighed between 85 and 95 g (50 to 55 days old) and were acclimated at least 1 to 2 weeks prior to treatment or use. Pure estrogen pellets, prepared without binder, were implanted in the shoulder region as described earlier (9). All animals were exposed to different estrogens for 9.0 to 9.2 months for induction of renal carcinomas. To maintain constant estrogen levels in these hamsters, hormone pellets were reimplanted at 3-month intervals. Ethinyl estradiol pellets, however, were absorbed more rapidly and were therefore reimplanted every 2 months. For progesterone receptor induction, hamsters were treated for 3.0 months with various estrogens as reported in detail elsewhere (11, 15).

The estrogen pellets used weighed 20 ± 1.4 (S.E.) mg. The mean daily absorption expressed in µg for the estrogens used was: DES, 170 ± 20 (S.E.); α-dienestrol, 160 ± 10; hexestrol, 185 ± 10; DES, 3,4-oxide, 101 ± 5; 17β-estradiol, 112 ± 15; estrone, 100 ± 10; ethinyl estradiol, 210 ± 10; equilin, 147 ± 22; and d-equilin, 145 ± 15 (8).

High-Performance Liquid Chromatography Analyses of the Estrogens. High-performance liquid chromatography analyses were performed on the estrogens investigated following pellet preparation and after implantation for 3 to 6 months in the hamster to determine whether chemical integrity of the hormones was maintained. Estrogen samples (10 µg) were dissolved in tetrahydrofuran (10 µl) and injected into a Hewlett-Packard Model 1084B liquid chromatograph equipped with a variable UV detector set at 284 nm. Separation was accomplished using a reverse-phase column (0.46 x 25 cm; IBM Instruments, Inc., Danbury, Conn.) eluted with a linear gradient of 45 to 58% methanol in water over 60 min at a flow rate of 2.0 ml/min. The column temperature was maintained at 35°. The retention times of the estrogen samples were compared to authentic standards. No apparent changes in chemical purity were observed following either pellet preparation or after prolonged pellet implantation.

Detection of Renal Tumor Foci. Analyses of the frequency of different synthetic and natural estrogens to induce renal adenocarcinomas in hamsters is summarized in Table 1. Both DES and 17β-estradiol induced a 100% incidence of bilateral and multiple renal tumors in castrated male hamsters treated for 9.0 months. Of the stilbene estrogens, hexestrol was equally effective and produced the lowest number of combined tumor foci in each animal. On the other hand, the synthetic estrogens, α-dienestrol and DES, 3,4-oxide, produced a slightly lower renal tumor incidence and correspondingly decreased number of tumor foci compared to either DES or hexestrol for the same time period. Although estrone treatment resulted in a reduced incidence (80%) of renal tumors as well as a lower number of renal tumor foci compared to either DES- or 17β-estradiol-treated animals, this latter difference was not statistically significant. Estrone tumor-inducing ability, however, was notably slower when examined at an earlier treatment period (8.0 months) compared to the above-cited estrogens (data not shown). Of the 2 nonprime natural estrogens, equilin treatment resulted in a 75% incidence of renal tumors in male hamsters with a significantly lower number of combined renal tumor foci, whereas no detectable tumor foci were found after prolonged d-equilin treatment. Remarkably, ethinyl estradiol resulted in only a 20% incidence of renal tumors in hamsters and a markedly diminished number of tumor foci in these animals. Moreover, the renal carcinoma foci were considerably smaller than all other effective estrogens when administered for the same time period.

RESULTS

Induction of Renal Tumors by Various Estrogens. The ability of different synthetic and natural estrogens to induce renal adenocarcinomas in hamsters is summarized in Table 1. Both DES and 17β-estradiol induced a 100% incidence of bilateral and multiple renal tumors in castrated male hamsters treated for 9.0 months. Of the stilbene estrogens, hexestrol was equally effective and as either DES or 17β-estradiol in producing renal carcinomas following the same treatment period with a similar number of combined tumor foci in each animal. On the other hand, the synthetic estrogens, α-dienestrol and DES, 3,4-oxide, produced a slightly lower renal tumor incidence and correspondingly decreased number of tumor foci compared to either DES or hexestrol for the same time period. Although estrone treatment resulted in a reduced incidence (80%) of renal tumors as well as a lower number of renal tumor foci compared to either DES- or 17β-estradiol-treated animals, this latter difference was not statistically significant. Estrone tumor-inducing ability, however, was notably slower when examined at an earlier treatment period (8.0 months) compared to the above-cited estrogens (data not shown). Of the 2 nonprime natural estrogens, equilin treatment resulted in a 75% incidence of renal tumors in male hamsters with a significantly lower number of combined renal tumor foci, whereas no detectable tumor foci were found after prolonged d-equilin treatment. Remarkably, ethinyl estradiol resulted in only a 20% incidence of renal tumors in hamsters and a markedly diminished number of tumor foci in these animals. Moreover, the renal carcinoma foci were considerably smaller than all other effective estrogens when administered for the same time period.
Table 1
Carcinogenicity and estrogenicity of various synthetic and natural estrogens in the Syrian hamster kidney

<table>
<thead>
<tr>
<th>Estrogens*</th>
<th>Structure</th>
<th>No. of animals</th>
<th>Animals with tumors</th>
<th>% with tumors</th>
<th>Combined no. of tumor nodules in both kidneys</th>
<th>Competitive binding (% of inhibition)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DES</td>
<td><img src="image1" alt="Structure" /></td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>22.0 ± 3e</td>
<td>90.1 ± 0.9 (7)e</td>
</tr>
<tr>
<td>α-Dienestrol</td>
<td><img src="image2" alt="Structure" /></td>
<td>8</td>
<td>7</td>
<td>88</td>
<td>10.4 ± 3a</td>
<td>88.6 ± 1.4 (6)</td>
</tr>
<tr>
<td>Hexestrol</td>
<td><img src="image3" alt="Structure" /></td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>25.8 ± 6</td>
<td>90.4 ± 0.8 (6)</td>
</tr>
<tr>
<td>DES 3,4-oxide</td>
<td><img src="image4" alt="Structure" /></td>
<td>7</td>
<td>6</td>
<td>86</td>
<td>11.4 ± 3</td>
<td>90.0 ± 1.8 (6)</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td><img src="image5" alt="Structure" /></td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>18.0 ± 4</td>
<td>89.8 ± 0.7 (8)</td>
</tr>
<tr>
<td>Estrone</td>
<td><img src="image6" alt="Structure" /></td>
<td>10</td>
<td>8</td>
<td>80</td>
<td>10.4 ± 5</td>
<td>85.2 ± 1.3 (4)</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td><img src="image7" alt="Structure" /></td>
<td>15</td>
<td>3</td>
<td>20</td>
<td>0.6 ± 0.5f</td>
<td>90.5 ± 1.1 (6)</td>
</tr>
<tr>
<td>Equilin</td>
<td><img src="image8" alt="Structure" /></td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>5.5 ± 0.9f</td>
<td>78.4 ± 1.7 (5)</td>
</tr>
<tr>
<td>d-Equilenin</td>
<td><img src="image9" alt="Structure" /></td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>49.2 ± 5.5 (6)</td>
</tr>
</tbody>
</table>

* Duration of estrogen treatment was 8.7 months. After the initial pellet implantation, additional hormone pellets were implanted every 3 months except for ethinyl estradiol. Because of the rapid absorption of the ethinyl estradiol pellets, this synthetic estrogen was implanted every 2 months.

* Competitive binding of radioinert estrogens for estrogen receptor was carried out on cytosols obtained from hamster estrogen-induced renal carcinomas. [³H]-Estradiol concentration in these tumor cytosols (4 to 5 mg/ml protein concentration), without competitor, corresponded to 0% inhibition.

* Mean ± S.E. of the number of tumor foci per animal in each group.

* Numbers in parentheses, number of individual determinations.

* p < 0.05 versus DES treated.

* p < 0.001 versus DES treated.

It should also be noted that neither the incidence nor the combined number of renal tumor foci was enhanced following continuous treatment with ethinyl estradiol for 10.3 months (data not shown).

**Competitive Binding for Estrogen Receptor.** As an indicator of their relative estrogenicity in the hamster, natural and synthetic estrogens were compared for their ability to compete with [³H]-estradiol for renal tumor cytosolic estrogen receptor at 1- to 100-fold excess concentrations. These data are shown in Chart 1. At low competitor concentrations (1- and 5-fold excess), both d-equilenin and equilin were poor competitors and could easily be distinguished from the more potent estrogens. Estrone and α-dienestrol also did not exhibit appreciable competition for estrogen receptor binding at similar low concentrations. In contrast, DES 3,4-oxide, hexestrol, and ethinyl estradiol all had nearly identical abilities to displace labeled hormone from renal tumor estrogen receptor as either DES or 17β-estradiol at all concentrations used. At 100-fold excess concentrations, only d-equilenin was markedly less effective in competing for estrogen receptor in the hamster renal tumor, while all the other estrogens examined showed similar binding capabilities (Table 1; Chart 1).

**Induction of Progesterone Receptor in Untransformed Kidneys.** Specific progesterone binding was determined in kidneys following in vitro incubation of cytosol fractions derived from...
castrated male hamsters treated for 3.2 months with different estrogens. Table 2 summarizes the results of these experiments. Compared to untreated castrate levels, progesterone receptor was elevated about 10-fold after either DES or 17β-estradiol treatment as reported previously (11). Except for d-equilenin, all estrogens studied elicited similar increases in specific progesterone receptor using either radiolabeled progesterone or R5020. These results are consistent with the ability of these synthetic and natural estrogens to compete with [3H]estradiol for renal carcinoma cytosolic estrogen receptor as shown earlier.

**Assay of Serum PRL.** Using the Nb2 node rat lymphoma replication bioassay, hamster serum PRL concentration was assessed in hypophysectomized and castrated hamsters as well as in estrogenized animals treated for 1 to 4 months. Replication of Nb2 node rat lymphoma cells has been shown to be a sensitive and specific bioassay for hormonal lactogens derived from both human (25) and animal sources (7) and appears to be effective in assessing serum PRL activities in the hamster as well. Serial dilutions of hamster pituitary extract and pooled hamster sera yielded Nb2 node replication curves that were parallel to PRL standards. Hypophysectomized hamsters exhibited serum PRL levels of <10 ng/ml (n = 6), which is at the detectable threshold of the bioassay and indistinguishable from zero PRL controls. On the other hand, serum PRL levels in castrated male hamsters were 46 ± 6.2 ng/ml (S.E., n = 11). In the 3 estrogen-primed groups, serum PRL levels were elevated about 7- to 12-fold after 3.0 months of treatment with either ethinyl estradiol, 17β-estradiol, or DES (Chart 2). Although the serum levels of this pituitary hormone were slightly lower in ethinyl estradiol-treated hamsters at all treatment periods tested, in each instance, the levels were not significantly different from PRL levels found after similar treatment with 17β-estradiol and were of only borderline significance in relation to DES-treated animals.

**DISCUSSION**

At present, it is not known whether the oncogenic effects of estrogens are exerted through their hormonal properties or whether they behave as chemical carcinogens. In this initial series, we have attempted to elucidate the structural requirements for estrogen carcinogenicity and to determine whether selected estrogens, both stilbene and steroidal types, are capable of transforming the hamster kidney. Such data should aid in identifying the importance of a given metabolic pathway germane for the carcinogenic effect of these hormones. The present results confirm that both 17β-estradiol and DES are equally carcinogenic in the hamster kidney (5). The finding that hexestrol is a potent tumorigenic agent in this organ demonstrates clearly that epoxidation of the olefinic double bond is not necessary for estrogen carcinogenesis in the hamster kidney, since hexestrol, lacking the stilbene double bond, is unable to convert to DES 3,4-oxide, a metabolite which has been suggested previously as a possible reactive intermediate (19). Consistent with this observation is that DES 3,4-oxide possesses a lower ability to effect renal transformation in this species. Moreover, since both α-dienestrol and hexestrol are active tumorigenic agents in this

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Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone receptor concentration (fmol/mg cytosol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>3.1 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DES</td>
<td>29.5 ± 3.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Dienestrol</td>
<td>20.3 ± 2.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>27.7 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>31.2 ± 2.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Estrone</td>
<td>27.5 ± 5.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Equinol</td>
<td>28.0 ± 6.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>d-Equilenin</td>
<td>9.2 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethinyl estradiol</td>
<td>35.0 ± 5.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Castrated male hamsters were treated with estrogens for 3.2 months; hormone pellets were withdrawn 24 hr before killing.
<sup>b</sup> Mean ± S.E. of at least 3 separate determinations.
<sup>c</sup> Statistically significantly different from control (p < 0.001).
system, the \( p \)-quinone metabolites (4,4\(^{-}\)-quinones) are unlikely to be pertinent, as these stilbene-type estrogens have not been shown to form these oxidative intermediates (20, 22). In contrast, it should be noted that both hexestrol and \( \alpha \)-dieneestrol have been shown to be poor inducers of \textit{in vitro} neoplastic transformation of Syrian hamster embryo fibroblasts (18), indicating that \textit{in vitro} models in some respects do not adequately mimic \textit{in vivo} tumor-inducing systems. Of the natural estrogens, estrone appeared slightly less carcinogenic in its ability to transform the hamster kidney, and this could be partially due to the lower rate of absorption of this hormone. Of the nonprimate natural estrogens studied, equilin was found to be carcinogenic in the hamster kidney, whereas \( \delta \)-equilenin was completely ineffective. It is apparent that, to be consistent with the results presented herein, the most probable reactive electrophilic intermediates which may be critical in transformation of the hamster kidney would involve metabolism in the A ring to either arene oxide or \( \alpha \)-semiquinone:quinone (or both) of stilbene and steroid estrogens. It is also conceivable that other hydroxylated intermediates in the catechol pathway which possess these above-mentioned electrophilic groups may also be involved in transforming the hamster kidney.

Although the nonprimate natural estrogens as well as estrone and \( \alpha \)-dieneestrol can be distinguished easily at low concentrations with respect to their competitive binding for estrogen receptor, at higher excess concentrations (100x), except for \( \delta \)-equilenin, all estrogens examined exhibited similar ability to compete for radiolabeled hormone (6, 13) and to induce progesterone receptor. Most interesting, however, is the finding that ethinyl estradiol is a relatively poor inducer of renal carcinomas while retaining its strong estrogenicity in the hamster kidney in terms of its competitive binding for estrogen receptor, ability to induce progesterone receptor, and ability to elevate serum PRL concentrations in the hamster to essentially the same level as either 17\( \beta \)-estradiol or DES. While our previous studies have suggested an intimate relationship between hormonal and carcinogenic effects of estrogens on the tumorigenic process in this organ site, this observation indicates that these 2 effects are partially dissociable. It also further suggests that, while hormonal activity may be necessary for renal tumorigenesis, it may not itself be sufficient to cause transformation of the hamster kidney.

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REFERENCES

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