ABSTRACT

The possible contribution of endocrine disruptors to human disease, particularly those compounds that modulate the estrogen receptor (ER), has recently drawn considerable attention. The tissue specificity of effects mediated by the ER is well recognized, although the mechanism of this specificity is not understood sufficiently to predict the effects of a particular ligand in different target tissues. Although the divergence of ER-mediated effects in the breast, bone, and uterine endometrium has been described, a frequently overlooked site of estrogen action is the smooth muscle of the uterus. The uterine myometrium is the tissue of origin of an extremely common hormone-responsive tumor, uterine leiomyoma, a tumor with a significant impact on women’s health and a possible environmental influence. This report describes an in vitro/in vivo system for identifying the effects of ER ligands in the myometrium and elucidating their mechanism of action. Several natural and synthetic xenoestrogens were evaluated at the cellular and molecular level for their ability to mimic estrogen action in uterine myometrial tissues. Diethylstilbestrol, coumestrol, genistein, naringenin, and endosulfan were able to activate the AF2 function of the ER in vitro and demonstrated agonist activity in estrogen-responsive myometrial cells, as determined by induction of proliferation and increased message levels of progesterone receptor. Compounds that could not activate AF2 function (4-hydroxy-tamoxifen, LY117018, and LY317783) did not act as estrogen agonists. For agonists, rank order of potency was predicted by receptor affinity; however, endosulfan displayed a surprising degree of activity, with negligible receptor binding. Additionally, diethylstilbestrol and tamoxifen demonstrated prototypical agonist and antagonist effects, respectively, in the intact myometrium of sexually mature rats. The results presented here suggest that some exogenous ER ligands may mimic the effects of endogenous estrogens on uterine leiomyoma and may contribute to a complex hormonal milieu that impacts both normal and neoplastic myometrium.

INTRODUCTION

Compounds with the biochemical capacity to disrupt normal endocrine function are numerous and abundant in the environment and in dietary sources and are used as pharmacological agents. The adverse sequelae of exposure to compounds that mimic or oppose steroid hormone function include developmental abnormalities, reproductive dysfunction, and proliferative disorders, including malignancies (1, 2). The mechanism by which endocrine disruptors produce these disorders is poorly understood, despite considerable study. Exogenous ER3 ligands are among the most thoroughly investigated of xenobiotic compounds from a biochemical standpoint, and yet their physiological effects are not predictable solely on the basis of structure or receptor affinities. It is known that a given ligand can mimic estrogen function (agonist), oppose it (antagonist), or have a mixed function; the agonist/antagonist dichotomy is determined, to a large extent, by the target tissue, but the molecular determinants have yet to be fully defined.

Endogenous estrogens have critical functions not only in the female reproductive tract and mammary gland but also in bone, the cardiovascular system, and the central nervous system during development and throughout life. There is controversy about whether estrogen replacement therapy after the onset of menopause puts women at increased risk for hormonally responsive cancer, even as it protects against bone loss and cardiovascular disease (3). For breast cancer patients and for women at high risk for breast cancer development, antiestrogen therapy has proven beneficial, although opposing estrogen action is detrimental in some tissues. As a result, considerable energy has been focused on developing agents with the capacity to modulate hormone activity in a tissue-specific manner (4, 5). A well-known example of differential effects of a synthetic ER ligand is the compound tamoxifen, which is used clinically to inhibit estrogen-responsive breast cancer and is currently undergoing clinical trials for use as a prophylactic agent. Although tamoxifen is an effective estrogen antagonist in mammary tissue, it can act as an estrogen agonist in the uterine endometrium, an effect that is observable both at the biochemical level and biologically in an increased risk of endometrial carcinoma in women undergoing tamoxifen therapy (6). Thus, a beneficial modulation of ER-mediated effects in one tissue may have profound and sometimes adverse effects in another tissue.

Investigations of the mechanism of the tissue-specific effects of tamoxifen have had a tremendous impact on our understanding of ligand-dependent activation of the ER as well as the effects of estrogen agonism and antagonism in mammary tissue. This knowledge, however, has not translated into an equivalent understanding of ER ligand effects in other tissues. Relatively little is known about the effect of ER modulation in the myometrial compartment of the uterus (7, 8). The uterine myometrium is the site of an extremely common neoplasm, uterine leiomyoma, also known as fibroids. These tumors occur in as many as 77% of adult women (9), an incidence that is arguably higher than any other type of neoplasm. Despite the benign nature of these tumors, leiomyoma may result in abnormal bleeding, abdominal pain, and infertility, making them a frequent cause of gynecological problems (10). Leiomyomas are responsive to ovarian hormones and can be reduced in volume by the maintenance of a hypo-ovarian hormonal milieu using gonadotropin-releasing hormone agonists; however, this therapy rapidly results in adverse effects in other tissues, particularly a dramatic loss in bone density, that is not completely reversible (11). Surgical intervention, often necessitating hysterectomy, is generally the only treatment option. As a result, leiomyomas are the most common cause of hysterectomy in the United States, accounting for >250,000 surgeries annually.

Recent increases in the incidence of hormonally related cancers have stimulated research into a potential role for endocrine disruptors in...
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cancer development (12). The striking frequency with which leiomyomas are observed calls into question the influence of environmental factors, including exogenous ER ligands. Unanswered questions have emerged regarding whether estrogen agonists are potential promoters of leiomyoma growth and whether exposure to an exogenous ligand can either predispose to or protect against the development of leiomyoma. To date, the effects of xenoestrogens on the myometrium have not been evaluated.

Here, we have used the Eker rat model of spontaneous uterine leiomyoma (13) to investigate the effects of a panel of suspected ER modulators on the uterine myometrium. The nine compounds selected represent a variety of synthetic and naturally occurring chemicals, of varied structure, that have not been characterized for their effects on the myometrium. Some have been previously categorized as estrogen agonists or antagonists in other tissues (Table 1). Each compound was tested for its affinity for and ability to activate ERs in vitro, and its ability to induce proliferation in an estrogen-sensitive myometrial tumor-derived cell line. Additionally, compounds demonstrating ER-mediated transactivation were tested for their ability to induce expression of an endogenous estrogen-responsive gene, the PR, in myometrial cells. Finally, a prototypical agonist (DES) and a partial agonist/antagonist (tamoxifen) were evaluated for their effects on the normal myometrium in an in vivo assay. Six of nine xenoestrogens in this panel exhibited agonism in one or more of these assays. The mechanism of in vitro transactivation by these compounds was correlated to agonist activity to define the molecular determinants of estrogen agonism in myometrial tissue.

MATERIALS AND METHODS

Chemical Compounds

Test compounds were solubilized in either ethanol or DMSO. Sources of compounds were as follows: E2, OH-tam, naringenin, and DES were from Sigma Chemical Co. (St. Louis, MO); endosulfan isomers α and β were from Chem Service (West Chester, PA); genistein was from Aldrich Chemical Company (Milwaukee, WI); coumestrol was from Acros Organics (Fairlawn, NJ); and LY317783 and LY317783 were both gifts from Eli Lilly and Co. (Indianapolis, IN).

Cell Lines

ELT3 and ELT6 rat uterine leiomyoma cell lines (previously characterized; Ref. 24) were maintained in DF8 medium supplemented with 10% FCS (Hyclone Laboratories Inc., Logan, UT), as described previously (24), at 37°C and 5% CO2. Experiments in which cells were grown in the presence of test compounds were conducted using serum-free, phenol red-free DF8-Basal medium (containing 1% BSA; Sigma).

Ligand Binding Studies

Preparation of Receptor. Recombinant hER was produced in Sf9 insect cells and prepared as ammonium sulfate precipitates (25). Briefly, Sf9 cells were grown in Grace’s Insect Medium (Mediatech, Inc., Herndon, VA), supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). Confluent Sf9 cells in 150-cm2 flasks were infected with the hER baculovirus and incubated for 5 days at 25°C. Cells were then suspended in a buffer consisting of 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM NaPO4, 50 mM NaF, and 1 mM NaVO3, and cells were lysed with three rounds of freeze-thaw. The extracts were brought to 400 mM NaCl and 10% glycerol and incubated for 20 min on ice. The whole cell extract was prepared by centrifugation for 10 min at 15,000 × g. For preparation of ammonium sulfate precipitates, the whole-cell extract was brought to 40% ammonium sulfate and incubated on ice for 30–60 min. The precipitates were collected by centrifugation at 15,000 × g for 5 min and flash-frozen for later use.

Competition Binding Assays. Recombinant hER (in ammonium sulfate precipitates) was dissolved in binding buffer consisting of 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM NaPO4, 50 mM NaF, 1 mM NaVO3, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride, 30 mg/ml aprotinin, and 0.2 mM leupeptin. Reactions were incubated at 25°C for 1 h in the presence of 32 nM 17β-[3,4,6,7-3H]estradiol (84 Ci/mmol; DuPont-NEN Products, Boston, MA) and radiolabeled compounds or vehicle (DMSO). Reactions contained a final volume of 102 ml. Reactions were terminated by incubation with a 50% slurry of hydroxyapatite (Bio-Rad, Hercules, CA) for 10 min at 4°C and centrifugation for 1 min at 15,000 × g. Samples were washed three times in binding buffer plus 0.05% Triton X-100. After three washes, the samples were resuspended in 100% ethanol, and the bound [3H]E2 was measured using liquid scintillation counting. The data are representative of two independent experiments with three replicates.

Proliferation Assays

Cells were plated into 24-well plastic cell culture dishes (Corning, Corning, NY) at 2000–3000 cells per well in DF8 medium and allowed to proliferate for 48–72 h. The media was then aspirated, and the wells were washed twice with 1× PBS, followed by the addition of DF8-Balbas medium containing the test compounds. Either ethanol or DMSO was used as a vehicle control, depending on the solubility of the test compound. The relative volume of the vehicle plus test compound did not exceed 0.1% of the solution. At each time point, the medium was aspirated and the cells were rinsed twice with 1× PBS and detached with trypsin-EDTA (Life Technologies, Inc.). Cells were resuspended in DF8 medium and counted with a Coulter Counter (Coulter Electronics, Hialeah, FL).

Northern Analysis

Cells were plated in DF8 medium and switched to DF8-Basal medium 8–12 h before the addition of chemicals. The following concentrations of chemicals were administered in basal medium with 0.1% ethanol or DMSO: DES, 10 nM; 4-OH-tam, 1 nM; naringenin, 10 μM; LY317783, 1 mM; LY117018, 1 nM; genistein, 10 μM; coumestrol, 1 μM; E2, 10 nM; endosulfan-α, 100 mM; and endosulfan-β, 100 mM. These doses had previously produced stimulation in the proliferation assays; if no proliferative dose had been observed, the highest dose that did not significantly inhibit proliferation was chosen for this experiment. Cells were harvested after 24 h from logarithmic-phase cultures; RNA was extracted as described previously (26). Five μg of polyadenylated RNA were isolated by oligodeoxynucleotide cellulose chromatography and sepa-

Table 1 Test panel of xenoestrogens

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical characteristics</th>
<th>Estrogenic effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DES</td>
<td>Synthetic hydroxystilbene drug previously used to prevent miscarriages</td>
<td>Increased risk of adenocarcinoma of the vagina in women treated in utero</td>
<td>14, 15</td>
</tr>
<tr>
<td>OH-tam</td>
<td>Synthetic triphenylethylene; active form of therapeutic compound tamoxifen</td>
<td>Promotes structural and functional defects in the reproductive tracts of mice treated in utero</td>
<td>16</td>
</tr>
<tr>
<td>LY117018</td>
<td>Synthetic benzoithioephene; raloxifene analogue</td>
<td>Antiestrogenic in breast and endometrium</td>
<td>17</td>
</tr>
<tr>
<td>LY317783</td>
<td>Synthetic naphthalene; raloxifene analogue</td>
<td>Estrogenic in bone and cardiovascular system</td>
<td>18</td>
</tr>
<tr>
<td>Genistein</td>
<td>Natural isolavone present in soybeans</td>
<td>Same as LY117018</td>
<td>19</td>
</tr>
<tr>
<td>Naringenin</td>
<td>Natural hydrogenated flavone present in citrus fruits</td>
<td>Concentration-dependent effect on growth of breast cancer cells</td>
<td>20</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>Natural coumestan present in some grains</td>
<td>Inhibits proliferation of breast cancer cells</td>
<td>21</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>Chlorinated hydrocarbon present in some pesticides; α and β isomers</td>
<td>Inhibits E2-induced hypertrophy of uterine wet weight of immature rats</td>
<td>22</td>
</tr>
</tbody>
</table>

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RESULTS

ER Binding Affinities. To determine the ability of these xenoestrogens to bind ER, we performed competitive binding analysis using recombinant hERα produced in an Sf9 baculoviral system. Dissociation of radiolabeled E2 from the hER was used to accurately rank the relative binding affinities of compounds in the study (Fig. 1). IC50s (the concentration of compound necessary to displace 50% of bound E2) were determined from the displacement curves. OH-tam, DES, and LY317783 demonstrated affinities for the receptor that were 5–6-fold that of E2, whereas LY117018 had an affinity that was 2-fold that of E2. Genistein and coumestrol had ~100-fold less affinity, and naringenin had ~3000-fold less affinity for the receptor than E2. The IC50 could not be determined for the endosulfan isomers because displacement of E2 from the receptor was minimal at the limits of compound solubility. With this information, it was possible to rank order the panel of potential ER ligands (Table 2) based on receptor affinity as compared to E2, given an arbitrary value of 100.

In Vitro Proliferation. ELT3 cells have been shown previously to proliferate in response to E2 in culture (31). The ability of xenoestrogens to induce proliferation in this ER-positive cell line was assessed over logarithmic and semilogarithmic dose ranges. Results of proliferative assays are shown in Fig. 2. The effective concentrations for compounds inducing proliferation varied from picomolar to micromolar ranges, over which a dose response was generally observed until an inhibitory threshold was reached. The relative potency of proliferative compounds was assessed by determining the lowest dose with the ability to induce proliferation at a level that was ≥25% that of 10 nM E2 during logarithmic growth. By these criteria, DES, coumestrol, genistein, naringenin, and endosulfan-α exhibited agonist activity over the range of the means of two independent experiments with three replicates each.

Fig. 1. Inhibition of tritiated-E2 binding to Sf9 cell extract by radioinert chemicals. Recombinant hER was incubated with 32 nM [3H]E2 in the presence of increasing concentrations of radioinert chemicals. Data points, two independent experiments of three replicates each. Inset, IC50s represent the concentration of competitor that displaced 50% of receptor-bound radiolabeled E2 ± the range of the means of two independent experiments with three replicates each.

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Table 2 Comparison of proliferative stimulation, transactivation, and ER binding of affinitya

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proliferative EC25</th>
<th>Compound</th>
<th>Transactivation EC50</th>
<th>Compound</th>
<th>Relative binding affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>10 pm</td>
<td>E2</td>
<td>100 pm</td>
<td>LY317783</td>
<td>610</td>
</tr>
<tr>
<td>DES</td>
<td>100 pm</td>
<td>DES</td>
<td>1 nm</td>
<td>DES</td>
<td>550</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>10 nm</td>
<td>Coumestrol</td>
<td>100 nm</td>
<td>OH-tam</td>
<td>490</td>
</tr>
<tr>
<td>Genistein</td>
<td>100 nm</td>
<td>Genistein</td>
<td>1 μm</td>
<td>LY117018</td>
<td>200</td>
</tr>
<tr>
<td>Naringenin</td>
<td>10 μm</td>
<td>Naringenin</td>
<td>10 μm</td>
<td>E2</td>
<td>100</td>
</tr>
<tr>
<td>Endosulfan-α</td>
<td>10 μm</td>
<td>Endosulfan-α</td>
<td>10 μm</td>
<td>Coumestrol</td>
<td>2.4</td>
</tr>
<tr>
<td>Endosulfan-β</td>
<td>None</td>
<td>Endosulfan-β</td>
<td>10 μm</td>
<td>Genistein</td>
<td>0.8</td>
</tr>
<tr>
<td>OH-tam</td>
<td>None</td>
<td>OH-tam</td>
<td>None</td>
<td>Naringenin</td>
<td>0.036</td>
</tr>
<tr>
<td>LY317783</td>
<td>None</td>
<td>LY317783</td>
<td>None</td>
<td>Endosulfan-α</td>
<td>---</td>
</tr>
<tr>
<td>LY117018</td>
<td>None</td>
<td>LY117018</td>
<td>None</td>
<td>Endosulfan-β</td>
<td>---</td>
</tr>
</tbody>
</table>

a Compounds are shown in rank order of potency in each assay. Proliferative EC25 was the lowest dose achieving 25% of 10 nm E2 activity in proliferation assay, calculated from triplicate wells. Transactivation EC50 was the lowest dose achieving 50% of 10 nm E2 activity. (The mean 10 nm E2 was calculated from two to five experiments in which the test compound was analyzed.) Relative binding affinity was calculated from the displacement of [3H]E2 relative to unlabeled E2, which was arbitrarily designated as 100.

activity by inducing proliferation in ELT3 cells, and endosulfan-β, LY117018, LY317783, and OH-tam did not.

For genistein, which stimulated proliferation at concentrations of <10 μm, a dramatic antiproliferative effect was seen in the 40–100 μm concentration range (Fig. 2 and data not shown). A similar inhibition has been previously reported in MCF-7 cells (18). Treatment of ER-negative ELT6 cells with genistein (Fig. 3) revealed a similar growth suppression in this range of concentrations, but proliferative stimulation was not observed at any dose. These data support previous indications that agonist activity of genistein is ER mediated, whereas antiproliferative effects at the higher dose are independent of the ER and may be mediated, instead, by inhibition of tyrosine kinase or topoisomerase activity (18, 32–34).

**Reporter Gene Transactivation.** To determine whether xenoestrogens are able to directly activate gene transcription of a classical estrogen-responsive promoter element (the vitellogenin ERE) in a myometrial cell background, ELT3 cells were transiently transfected with a firefly luciferase reporter gene driven by a single vitellogenin ERE linked to a minimal viral promoter. This ERE has been previously characterized in other cell types as requiring the function of both activation domains of ERα (AF1 and AF2; Ref. 29). Transcriptional activation of most estrogen-responsive genes has been shown to require both of these transcription functions. Along with this reporter (vitERE-tk-LUC), plasmids containing hERα and β-gal (CMV-β-gal) under the control of constitutive promoters were cotransfected with the respective functions of increasing the measurable transcriptional response and normalizing for transfection efficiency. Normalized luciferase activity in treated cells was reported as fold induction over baseline activity of the reporter in untreated cells that were identically transfected. Activity was tested over increasing logarithmic and semilogarithmetic dose ranges, increasing until an inhibitory dose was reached or until the limits of compound solubility resulted in exceeding the upper bound of 0.1% vehicle. The results of multiple experiments are summarized in Fig. 4. DES and E2 were roughly equipotent at inducing transactivation of the reporter, at a range of doses 3 logs below that of coumestrol or genistein. Naringenin and the endosulfan isomers were able to activate the receptor at dose ranges that were ~100 times higher than coumestrol or genistein. Notably, although the effective doses were nearly identical for naringenin and the endosulfans, the maximum values for naringenin were consistently higher. Examination of the β-gal values (data not shown) for these treated cells revealed an inhibitory effect by endosulfans at doses of >5 × 10−5, indicating a general suppression of gene expression at this dose level. LY317783, LY117018, and OH-tam could not induce transactivation at any dose tested; a 30–90% inhibition of baseline luciferase activity was observed for each of these compounds, irrespective of dose. All xenoestrogens with the ability to activate vitERE-tk-LUC displayed agonist activity in the proliferation assay, with the exception of endosulfan-β, which inhibited proliferation at the high concentrations required to induce transactivation. Rank order of potency in transactivation (Table 2), which was determined by the average compound dose that produced a fold-induction at least 50% that of E2, coincided with the order of compounds as stimulators of proliferation.

Previous studies have shown that the ability of “partial” or “mixed” agonists, such as tamoxifen, to mediate ER transactivation is specific to both promoter context and cell type and is mediated by the AF1 domain of the ER (35, 36). A prototypical AF1-responsive promoter is the C3 (complement protein-3) promoter, which can be transcriptionally activated in vitro and in vivo in some cell types by OH-tam (37). To determine whether xenoestrogens could function as agonists in the context of C3 transactivation in a myometrial cell background, we cotransfected ELT3 cells with hERs and the C3-tk-LUC reporter plasmid and then treated them with varying concentrations of E2 and other ligands. E2 was minimally but reproducibly able to induce expression of this reporter in repeated assays (Fig. 5). In contrast, in control experiments, C3-tk-LUC could be induced 16-fold by E2 in Ishikawa cells derived from the human uterine endometrium (data not shown), confirming that the responsiveness of this promoter is generally low in the myometrial cell background. The ability of xenoestrogens to transactivate C3-tk-LUC is shown in Table 3, in order of concentration yielding the maximum response in a representative experiment, with E2 being most potent and endosulfan-β being the least potent. These data are compared to the fold-induction observed using the vitERE-tk-LUC reporter at the same concentration of test compound. Although the level of induction varied in magnitude, the concentrations of E2, coumestrol, naringenin, genistein, and the endosulfans that were required for maximal stimulation remained ordered roughly in the same range as activation of vitERE-tk-LUC. In repeated experiments, OH-tam, LY317783, and LY117018 were consistently able to induce C3-tk-LUC activity over basal levels and did not exhibit the suppression of activity observed with the AF2-requiring vitERE-tk-LUC, even at doses as high as 100 μm. The consistent ability of these xenoestrogens to induce C3-tk-LUC activity over basal levels contrasts with that of the pure antiestrogen ICI182780 that did not produce any increase in C3-tk-LUC reporter gene activity in ELT3 cells (data not shown). These results suggest that ER-mediated transcriptional activity of the C3 promoter in cells of myometrial origin is reduced relative to the endometrium and that agonists and nonagonists in this cell type are equally capable of stimulating transcription via this promoter. In this tissue, estrogen agonism could not be discriminated at the level of AF1 activity and was better predicted by transactivation via the AF2-requiring vitellogenin ERE.

**Induction of PR.** Compounds that induced transactivation of vitERE-tk-LUC were tested for their ability to up-regulate the endogenous PR to verify that transactivation could occur in the context of an endogenous promoter. PR induction reflects ER-mediated gene regulation, which is often but not always indicative of estrogen agonism.
ERT3 cells were incubated with the lowest dose of compound that consistently induced proliferation, and PR message levels in these cells were compared with that of cells grown in basal medium alone. The Northern blot shown in Fig. 6 is representative of repeated experiments showing increased levels of PR message by E2, DES, coumestrol, genistein, naringenin, and endosulfan after normalization to GAPDH. In repeated experiments (Fig. 6 and data not shown), densitometric quantitation and normalization of the 7.2- and 3.5-kb bands suggested increased message levels for each of these compounds, at levels varying between 2- and 20-fold, with the absolute level depending on which PR band was measured. These results confirm the up-regulation of an endogenous estrogen-responsive gene in myometrial cells by compounds capable of inducing vitERE-tk-LUC reporter gene activity.

**In Vivo Proliferation of Myometrium.** To determine whether the proliferative or antiproliferative activity of xenoestrogens observed in vitro could also occur in intact myometrial tissue, a prototypical agonist (DES) and partial agonist/antagonist (tamoxifen) were evaluated in an in vivo assay. Young, sexually mature female Eker rats were exposed to either tamoxifen (which is converted in vivo to OH-tam; Ref. 40), DES, or vehicle control via 60-day time release s.c. implant. At the conclusion of treatment, reproductive tract tissues were examined for changes in histology and for the presence of proliferating cells in the myometrial layers. In previous studies (41), we and others

have demonstrated that myometrial cells proliferate in untreated female rats only during proestrus, the period of highest levels of endogenous ovarian steroids (20 pg/ml E \(_2\); Ref. 42). Animals sacrificed during proestrus exhibit the uterine morphology that is typical during this stage: an enlarged lumen, columnar luminal epithelium, and a limited degree of myometrial proliferation. Controls sacrificed during other stages of the reproductive cycle are virtually devoid of proliferating cells in the myometrium. Uteri from DES- and tamoxifen-treated animals are shown in Fig. 7. As compared to controls, uteri from each of three tamoxifen-treated animals were unusually small, with an immature, quiescent appearance, and they exhibited a complete absence of proliferation in the myometrium. In contrast, uteri from four of five DES-treated animals were dramatically enlarged, with profound disruption of the normal tissue architecture. The appearance of the uterus in DES-treated animals was variable. In some regions, the myometrial and stromal layers of the uterine wall were reduced to a thickness of a few cell layers, concomitant with highly dilated lumen. In other areas, both circular and longitudinal layers of the myometrium were enlarged to several times their normal size, with only the stromal layer being diminished in thickness. In DES-treated animals, myometrium was highly proliferative, as evidenced by incorporation of BrdUrd. Although DES and OH-tam might be expected to interfere with normal cycling, all treated animals showed evidence of recent ovulation. Endogenous E \(_2\) levels in DES-treated animals were in the range of 8–9 ng/ml, well below the levels of 20–40 ng/ml, which occur during proestrus and are associated with luminal enlargement and myometrial proliferation in untreated animals (41), indicating that the observed proliferation in the myometrium was due to DES treatment rather than the influence of endogenous estrogens.

**DISCUSSION**

Estrogen agonist activity of DES, coumestrol, genistein, narigenin, and endosulfan-\(\alpha\) was demonstrated by the dose-dependent proliferation of ELT3 tumor-derived uterine myometrial cells. Additionally, each of these compounds was able to transactivate an AF2-requiring reporter gene and increase expression of endogenous PR in ELT3 cells, consistent with ER-mediated agonist activity in this cell type. Proliferation was not observed in response to the synthetic ligands OH-tam, LY117018, or LY317783. None of these three compounds activated the AF2-requiring, vitERE-tk-LUC reporter gene, but all could stimulate an AF1-requiring C3-tk-LUC reporter to some extent, suggesting that AF2-mediated transactivation is required for agonism in this cell type and is a better predictor of agonist activity in this tissue than transcriptional activation mediated by AF1.

Comparison of ER-binding affinity, stimulation of proliferation, and transactivation in estrogen-responsive leiomyoma cells showed that the rank order for potency of compounds as transactivators and mitogens was identical. Interestingly, ER transactivation was achieved by both endosulfan isomers, despite negligible receptor
binding. The magnitude of difference between effective concentrations was also preserved between the two functional assays, with the exception of endosulfan-β, which was transactivating but did not induce proliferation. Endosulfan β may have failed to elicit a proliferative response, due to a partial toxicity at the concentrations required to activate the ER. In most cases, proliferation was more sensitive to toxicity than transactivation in terms of dose, perhaps due to the longer incubation period with the test compounds in the proliferative assay. For compounds that could activate transcription via the ER, the relative binding affinities exhibited the same rank order, with one notable exception. DES, while having 5.5 times greater affinity for the receptor, was slightly less potent than E₂ in both stimulation of proliferation and transactivation. Additionally, for coumestrol, genistein, and the endosulfan isomers, although the rank order of affinity predicts potency, the magnitude of difference between binding affinities is inconsistent with that observed in the functional assays. Especially interesting are the endosulfans, each of which were positive in one or both of the functional assays, despite having a very limited affinity for the receptor. Other studies have showed similar results (43), and some investigators have proposed that these effects may not require interaction with the ER (44). In this investigation, in vitro transactivation by endosulfans suggests mediation by ERα and classical response elements, although an indirect mechanism could be involved. For example, it is possible that these compounds can increase the activity of ligand-free receptor; however, this type of mechanism is difficult to substantiate. Although reporter and proliferation assays measure the effects of xenoestrogens cultured cells, in vivo administration of two compounds, one that exhibited agonist activity in ELT3 cells (DES) and one that did not (tamoxifen), showed that the intact myometrium of a normal female rat could also be influenced by xenoestrogen exposure. These results imply that agonistic or antagonistic effects of exogenous ER ligands observed in assays using tumor-derived myometrial cells may also be relevant to the normal myometrium of an intact animal.

Together, these results suggest that exogenous ER ligands can act as estrogen agonists in myometrial cells. Although environmental estrogens have been implicated epidemiologically and experimentally as risk factors for both neoplasia and developmental disorders (45), no environmental compound has been firmly implicated in the etiology of uterine leiomyoma. Given the high incidence of these tumors, an increase in tumor frequency in an exposed population would be difficult to detect. Previous studies have shown, however, that women harboring uterine leiomyomas have higher serum burdens of some organochlorine pesticides than unaffected women and that tissue organochlorine levels are higher in leiomyomas than in normal myometrium (46). A common feature of organochlorines is their persistence and tendency to accumulate in body lipid, a characteristic that makes them arguably suspect as etiological agents in cancer of reproductive tissues. In contrast, dietary phytoestrogens are generally considered antitumorigenic, despite their well-demonstrated estrogenicity in tissues such as the breast (21, 47). Historically, phytoestrogen effects were first noted as a toxicity, resulting in infertility and developmental disorders in grazing livestock (reviewed in Ref. 48), and thus, the potential for adverse effects to mammals exposed to these compounds is well established. However, phytoestrogen-rich foods, such as soy, are statistically associated with a reduced risk of cancer (12). Although a mechanism for this protective effect is unknown, recent studies suggest that a suppressive role for phytoestrogens may rely on the developmental window of exposure (49, 50), an observation with vital implications for the role of endogenous hormones in tumor development.

Table 3 Reporter gene activity of test compounds using C3-tk-LUC compared to vitERE-tk-LUC

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Maximum fold induction, C3-tk-LUC</th>
<th>Fold induction at equivalent dose, vitERE-tk-LUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₂</td>
<td>1 nM</td>
<td>1.8</td>
<td>10.3</td>
</tr>
<tr>
<td>LY317783</td>
<td>10 nM</td>
<td>1.7</td>
<td>0.15</td>
</tr>
<tr>
<td>LY117018</td>
<td>10 nM</td>
<td>1.5</td>
<td>0.15</td>
</tr>
<tr>
<td>OH-tam</td>
<td>10 nM</td>
<td>1.4</td>
<td>0.50</td>
</tr>
<tr>
<td>DES</td>
<td>100 nM</td>
<td>2.4</td>
<td>13.9</td>
</tr>
<tr>
<td>Coumestrol</td>
<td>1 μM</td>
<td>2.2</td>
<td>10.1</td>
</tr>
<tr>
<td>Naringenin</td>
<td>10 μM</td>
<td>1.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Genistein</td>
<td>10 μM</td>
<td>3.5</td>
<td>18.9</td>
</tr>
<tr>
<td>Endosulfan-β</td>
<td>10 μM</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Endosulfan-β</td>
<td>20 μM</td>
<td>1.8</td>
<td>1.6*</td>
</tr>
</tbody>
</table>

*Maximum fold induction of luminescence was calculated for each compound using the C3-tk-LUC reporter in a representative experiment. Compounds are listed according to potency as determined by the concentration at which this maximum induction was observed. Induction of C3-tk-LUC is compared with increase or decrease in vitERE-tk-LUC activity over control at the same concentration of compound.

Concentration, 10 μM.
In terms of agonist activity, the profile of ER ligands in the uterine myometrium appears to more closely resemble that of mammary tissue than uterine endometrium. For example, the therapeutic compounds OH-tam and the two raloxifene analogues (LY117018 and LY317783) did not exhibit estrogen agonism in myometrial cells and, furthermore, were able to inhibit the baseline activity of the ER on the vitellogenin ERE in estrogen-free conditions. This inhibition was not observed using the C3 promoter, an observation that is consistent with the ability of tamoxifen and raloxifene to act as agonists in some cell types (51–53). Additionally, the proliferative potential of test compounds for estrogen-sensitive ELT3 cells was most closely predicted by the ability of the compounds to activate transcription via the AF2-mediated vitellogenin ERE rather than by the AF1-mediated C3 promoter. The C3 promoter was only weakly active in ELT3 cells,

Fig. 7. Myometrial proliferation in rats treated in vivo with vehicle, tamoxifen, or DES. A, example of a BrdUrd-stained uterine horn of vehicle control-treated rat during proestrus. (l) and (c), longitudinal and circular layers of the myometrium, respectively; endometrial stroma and luminal epithelium lie to the left of the myometrial layers. Arrows, BrdUrd-positive cells in the myometrium, some of which are associated with glandular structures or vasculature. Magnification, ×100. B, BrdUrd-stained uterine horn of vehicle control-treated rat during estrus. Myometrial tissue (at the periphery) is nearly devoid of BrdUrd-stained cells, although a few stained cells are visible in the stroma and immediately beneath the luminal epithelium. Magnification, ×40. C, BrdUrd-stained uterine horn of tamoxifen-treated rat. Note the small size of the horn in cross-section as compared to uterus from vehicle-treated rat and the absence of BrdUrd-stained cells. Magnification, ×40. D, H&E-stained uterine horn of DES-treated rat. (l) and (c), longitudinal and circular layers of the myometrium, respectively, with condensed stromal tissue and thickened luminal epithelium with numerous invaginations visible above the myometrial layers. Magnification, ×100. E, BrdUrd-stained uterine horn of DES-treated rat. Note BrdUrd-positive cells in the longitudinal layer and dramatic enlargement of the tissue as compared to untreated uterus at the same magnification. Arrows, BrdUrd-positive cells in the longitudinal myometrium. Magnification, ×100. F, BrdUrd-stained uterine horn of DES-treated rat. The two myometrial layers are greatly enlarged, with an abundance of BrdUrd-stained cells. The proximal boundary of the myometrium is adjoined by a very thin stromal layer and an effaced luminal surface. Magnification, ×100.
suggesting that AF1-mediated transactivation may be less important in the repertoire of the uterine myometrium than in the endometrium. Because the agonistic activity of tamoxifen is mediated via AF1 (35), this observation predicts that tamoxifen would not mimic estrogen function in this tissue, consistent with in vitro and in vivo evidence from this study. Thus, the myometrium responds to tamoxifen in a similar manner to mammary tissue, where tamoxifen acts as an antagonist, in contrast with the response of endometrial cells, in which both E2 and tamoxifen can stimulate the transcription of C3 and promote proliferation.

Controlled clinical studies have not clearly defined the effect of pharmacological ER modulators on the uterine myometrium. Here, OH-tam and the raloxifene analogues showed no evidence of estrogenicity in myometrial cells, a quality that could be clinically relevant, given increasing numbers of perimenopausal and postmenopausal women on treatment regimens for breast cancer chemotherapy or prophylaxis. In contrast, DES and each of the environmental compounds exhibited estrogenicity in multiple assays, with their order of potency predicted by receptor binding affinity relative to E2. It is notable that the estrogenic effects of compounds used here (excluding DES) were observed at concentrations 100 to 100,000 times greater than the equipotent concentration of E2. The probability of achieving concentrations of this magnitude from exposures other than pharmacological ones is an important factor for assessing biological effects, but this probability is not easily determined. Efforts to measure or estimate the circulating levels or tissue levels of exogenous ER ligands (54) have not satisfactorily resolved this issue. It could be argued that the impact of an exogenous estrogens with a receptor affinity in the micromolar range on the myometrium of a mature female with high levels of endogenous estrogens may be inconsequential due to competition for receptor binding. However, exposure to exogenous ER ligands may have a much more significant impact in individuals in which endogenous estrogen levels are very low; for example, children and postmenopausal women.

Our ability to predict whether the effects of ER ligand exposure are beneficial or hazardous, in the absence of empirical data, is still very poor but is improving as we discover the molecular mechanisms of tissue specificity. Some tissue-specific effects may reflect the participation of the recently discovered ERβ (55), although a role for this receptor is not expected in the myometrium, where ERβ expression is low to undetectable (56, 57). Additionally, whereas most mechanistic studies of ligand effects have been conducted with EREs, other studies have noted ER-mediated transactivation via other genetic elements, such as Sp-1 (58) and AP-1 (59), each of which carries the potential for its own pattern of ligand-specific effects. It has recently become apparent that the tissue-specific effects of ER ligands on receptor-mediated responses are governed by the participation of steroid co-activator and co-repressor proteins (60–64). The interaction and expression pattern of these co-activators is not fully understood but is likely to explain the ability of the ER to effect a wide variety of cellular responses and vary those responses to given ligands in a tissue-specific manner. These data and those of others suggest that the ability of a ligand to activate the ER in a particular cellular context can predict agonist activity in that tissue. Knowing more about the molecular determinants of this agonism will help improve our ability to modulate ER-mediated responses without unwanted side effects.

In summary, these experiments describe an in vitro/in vivo model system for determining the effects of ER ligands in uterine myometrium and identifying their mechanism of action. Six of the nine xenoestrogens in this panel (DES, genistein, coumestrol, naringenin, endosulfan-α, and endosulfan-β) exhibited evidence of agonist activity in myometrial cells of the Eker rat in multiple assays, demonstrating the potential for an impact of xenoestrogens on both transformed and normal myometrial tissues. In addition, the results of this study are consistent with the necessity for activation of the AF2 function of the ER for agonist activity in this tissue. These results suggest that the response of the myometrium to a mixed function ligand differs from that of other uterine compartments; this distinction is important and implies that each tissue must be independently evaluated for the effects of a potential estrogen or antiestrogen.

ACKNOWLEDGMENTS

We thank Kalyn Sowell and Dr. Lezlee Coghan for their contributions to the in vivo studies and Michelle Gardiner for secretarial assistance. We are also grateful to Dr. Kevin Burroughs for helpful insight and to Dr. Tim Zacharewski for critical review of the manuscript.

REFERENCES


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ER AGONISM OF XENOESTROGENS IN UTERINE MYOMETRIUM


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Deborah S. Hunter, Leslie C. Hodges, Peter M. Vonier, et al.


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