Estrogenic Effects of Organochlorine Pesticides on Uterine Leiomyoma Cells in Vitro

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Although benign, uterine leiomyomas occur with high frequency and significant morbidity in reproductive-age women, and they present a significant health problem. Leiomyomas develop in the uterine myometrium and are sensitive to ovarian hormones, making them potential target sites for endocrine disruptors. Here we utilize cell lines derived from rat uterine leiomyomas to determine if a panel of 7 organochlorine pesticides have potential agonist activity in myometrial cells using cellular and molecular in vitro assays. The organochlorine pesticides investigated have been previously characterized as having agonist activity in other hormonally responsive tissues, but their effects have not been studied in uterine myometrial cells. In Eker rat leiomyoma-derived cells, HPTE, kepone, and the α isomer of endosulfan stimulated proliferation, an effect dampened by the antiestrogen ICI 182,780. In addition, these compounds stimulated transcription of the vitellogenin estrogen-response element via the ER in a transcriptional reporter gene assay and induced the expression of an endogenous estrogen-responsive gene, the progesterone receptor (PR). This contrasted with the agonist profile of methoxychlor, dieldrin, toxaphene, and endosulfan-β. These compounds, unable to stimulate proliferation of uterine leiomyoma cells, did exhibit agonistic activity in these cells at the transcriptional level in the estrogen-sensitive reporter gene assay, and they were also able to upregulate PR message. These data demonstrate that organochlorine pesticides act as estrogen receptor agonists in Eker rat uterine myometrial cells, and they indicate a need for further investigation of the potential tissue-specific agonist activity of these pesticides and their role in the pathogenesis of uterine leiomyoma.

Key Words: uterine leiomyoma; uterine myometrium; endocrine disruptors; organochlorine pesticides; 17β-estradiol.

Uterine leiomyomas are benign neoplasms arising from the myometrial compartment of the uterus, which are found in upwards of 77% premenopausal women, making them the most common gynecological neoplasm in reproductive-age women (Cramer and Patel, 1990). Although benign, these tumors are a major health concern due to their high incidence, lack of satisfactory non-surgical treatments, and impact on the reproductive health of women. When symptomatic, uterine leiomyomas are associated with gynecological problems such as abnormal uterine bleeding, morbidity, and infertility (Buttram and Reiter, 1981). They are responsible for 200,000 hysterectomies annually in the U.S., making uterine leiomyomas the leading indication for hysterectomy for premenopausal women (Easterday et al., 1983).

Although the etiology of uterine leiomyomas is unknown, estrogen and downstream signaling from the estrogen receptor (ER) is generally believed to play a major role in the pathogenesis of these tumors in women. Leiomyomas develop during the reproductive years and decrease in size after menopause (Novak and Woodruff, 1979). Creation of a hypoestrogenic state, such as during GnRH agonist therapy, causes a reduction in tumor volume (Friedman et al., 1990). Increased ER levels along with an elevated transcriptional response to estrogen in leiomyoma compared to normal myometrium has been reported, suggesting that leiomyomas may be hypersensitive to estrogen (Andersen et al., 1995b; Rein et al., 1990). In addition, several estrogen-regulated genes, such as the progesterone receptor, and the growth factors IGF-I, EGF, and their receptors have been found to have elevated expression in uterine leiomyomas (reviewed in Andersen and Barbieri, 1995a). The hormone responsive nature of the normal uterus and the increased sensitivity of leiomyomas to estrogen make them potential targets of endocrine disruption by exogenous chemicals.

Organochlorine pesticides are in widespread use and even pesticides that have been banned in western countries for more than two decades are still detectable in these regions (Blais, 1998; Simonich and Hite, 1995) and in mammalian fat stores (Stellman et al., 1998). Many organochlorine pesticides have been associated with estrogenic activity both in vivo and in vitro. Exposure to the organochlorine pesticide kepone, previously banned in part for its estrogenic activity, produces persistent vaginal estrous and anovulation in rats treated neonatally (Gellert, 1978). Methoxychlor, a pesticide still in common use, exhibits multiple estrogenic effects in neonatally exposed mice such as precocious vaginal opening, persistent vaginal estrous, and alterations in initiating and/or maintaining pregnancy (Eroschenko and Cooke, 1990; Swartz and Eroschenko, 1998). Many organochlorine pesticides have potential agonist activity in other hormonally responsive tissues, but their effects have not been studied in uterine myometrial cells. In Eker rat leiomyoma-derived cells, HPTE, kepone, and the α isomer of endosulfan stimulated proliferation, an effect dampened by the antiestrogen ICI 182,780. In addition, these compounds stimulated transcription of the vitellogenin estrogen-response element via the ER in a transcriptional reporter gene assay and induced the expression of an endogenous estrogen-responsive gene, the progesterone receptor (PR). This contrasted with the agonist profile of methoxychlor, dieldrin, toxaphene, and endosulfan-β. These compounds, unable to stimulate proliferation of uterine leiomyoma cells, did exhibit agonistic activity in these cells at the transcriptional level in the estrogen-sensitive reporter gene assay, and they were also able to upregulate PR message. These data demonstrate that organochlorine pesticides act as estrogen receptor agonists in Eker rat uterine myometrial cells, and they indicate a need for further investigation of the potential tissue-specific agonist activity of these pesticides and their role in the pathogenesis of uterine leiomyoma.

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Methoxychlor and dieldrin were purchased from Aldrich (Milwaukee, WI). Chemicals containing 1% BSA (Sigma Chemical Co, St. Louis, MO) was used. A panel of 7 organochlorine pesticides were added to the cells, serum-free, phenol red-free DF8-basal medium as described (Howe et al., 1995a). For experiments in which test compounds were used: 10^(-2) to 30 h before the addition of chemicals. The following concentrations of DDT and its metabolites have been detected in the blood of women with leiomyoma (Khar, 1985) and also in leiomyomatous tissue (Saxena et al., 1987). These studies suggest the possibility of a link between the development of leiomyoma and exposure to organochlorine pesticides with estrogenic properties. However, studies to determine if these compounds have agonist activity in uterine myometrial cells are lacking.

As a first step toward addressing the possibility that organochlorine pesticides act as estrogen receptor agonists in the uterine myometrium, we tested a panel of 7 organochlorine pesticides for their ability to stimulate leiomyoma cell growth and induce an estrogenic response on the molecular level in this cell type. The cell lines used in these assays were derived from spontaneous uterine leiomyoma from the Eker rat and have been characterized previously for their estrogen responsiveness (Everett et al., 1995; Howe et al., 1995a,b). Several functional assays for estrogen activity were utilized: stimulation of cell growth in vitro, a transcriptional assay utilizing the vitellogenin estrogen response element (ERE), and induction of an endogenous estrogen-responsive gene, the progesterone receptor (PR). The results presented here indicate that all the pesticides examined act as estrogen receptor agonists at the molecular level in leiomyoma cells and several are fully functional agonists that stimulate uterine myometrial cell growth. Consequently, these results suggest that exposure to these compounds could contribute to the pathogenesis of uterine leiomyoma by altering endocrine signaling via the estrogen receptor.

**MATERIALS AND METHODS**

**Cell Lines**

ELT-3 and ELT-6 Eker rat uterine leiomyoma cell lines have been characterized previously and were maintained in DF8 medium supplemented with 10% fetal calf serum (HyClone Laboratories Inc., Logan UT) at 37°C, 5% CO₂, as described (Howe et al., 1995a). For experiments in which test compounds were added to the cells, serum-free, phenol red-free DF8-basal medium [containing 1% BSA (Sigma Chemical Co, St. Louis, MO)] was used.

**Chemicals**

Kepone and toxaphene were purchased from Supelco (Bellefonte, PA). Methoxychlor and dieldrin were purchased from Aldrich (Milwaukee, WI). 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTe) was a kind gift of Dr. Leo T. Burk. The endosulfan isomers α and β were purchased from Chem Service (West Chester, PA). 17β-Estradiol was purchased from Sigma. ICI 182,780 was a kind gift of Dr. Robin Fuchs-Young. DMSO was used as a solvent for toxaphene (TXP), kepone (KPN), methoxychlor (MXC), dieldrin (DLN), and HPTe. Ethanol was used as a solvent for 17β-estradiol (E2), ICI 182,780, and the endosulfan isomers α and β (Endo α, β). According to the manufacturers, the minimum chemical purity of each of the organochlorine pesticides is: MXC-95%, HPTe-98%, KPN-96%, DLN-90%, Endo α-99.5%, Endo β-98%. The purity of TXP is not available due to its complex mixture.

All solutions of chemicals used to treat cells were diluted 1:1000 in DF8-basal medium, unless otherwise stated.

**Proliferation Assays**

ELT 3 cells were plated into 24-well cell-culture dishes (Corning, Corning, NY) in DF8 medium and incubated 48 h at 37°C in 5% CO₂-humidified atmosphere. The wells were aspirated and rinsed twice with 1X phosphate-buffered saline (PBS), and triplicate wells were dosed with 1 ml of DF8-basal medium containing pesticide or control solutions. At each time point, triplicate wells were rinsed with 1X PBS and cells detached with 5X trypsin-EDTA (Gibco BRL, Grand Island, NY), resuspended in DF8 medium, and counted with a Coulter counter (Coulter Electronics, Hialeah, FL).

**Reporter Gene Assays**

ELT 3 cells were transiently transfected in 12-well plates with the vitellogenin ERE-tk-LUC6a reporter plasmid, human ER expression plasmid pRSVT7 (both described by Tzukerman et al., 1994) and a control plasmid containing a constitutive CMV promoter-driven β-galactosidase reporter (kind gift of Dr. Andrew Butler). Transfections were performed using the calcium phosphate method at a reporter:receptor:β-gal ratio of 9:1:1. The following day, cells were rinsed twice with 1X PBS, and were dosed with DF8-basal medium containing DMSO, 17β-estradiol, or test compounds. Two days later, cells were rinsed with 1X PBS and harvested and assayed for reporter-gene activity using Galactolight™ and Luciferase Assay Kit by Tropix™ (Bedford, MA) according to the manufacturer’s instructions. Luminescence was detected using a Dynex-MLX luminometer (Chantilly, VA), and luciferase values from triplicate wells were normalized to β-galactosidase values for the same wells, to control for transfection efficiency. The β-galactosidase values for 50 and 100 μM dieldrin were consistently lower than vehicle, suggesting some cytotoxicity at high doses for this compound.

**Quantitative RT-PCR**

The expression levels of PR, as well as of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were measured with a quantitative RT-PCR technique utilizing a competitive template. The competitor template was generated for each gene using the method of Celi (Celi et al., 1993) from rat cDNA. The primer sets for each gene included upstream (A), internal downstream (BC), and external downstream (C) primers. PR and GAPDH sequences were as follows: PR (A) 5'-CTGAATGAGCAGAGGTAGAA-3', (CB) 5'-GACCACCCCTTTTCTGTTAACCCTCCTTCTCCAATCGTG-3', (C) 5'-GACCACCCCTTTTCTGGTTT-3', and GAPDH (A) 5'-AAACCCATCCACATCTTC-CAG-3', (BC) 5'-AGGGGCCATCCACAGTCTTCTTTCCAGTGGCACAGAAGTGTGCA-3' and (C) 5'-AGGGGCCATCCACAGTCTTCTTC-3'. Primers were designed so that amplification of both endogenous and competitor targets could be performed using a single primer pair (A and C), with the competitor product being 36–38 bp shorter than the endogenous product. Each competitor template was amplified using the A and BC primers, purified by electrophoresis, quantified by spectrophotometry, serially diluted in H₂O, and stored at -20°C. ELT 3 cells were plated in DF8 medium and switched to DF8-basal medium 20 to 30 h before the addition of chemicals. The following concentrations of chemicals were used: 10 μM KPN, 10 nM MXC, 100 nM TXP, 1 μM HPTe, 1 μM ICI 182,780, 10 nM E2. These concentrations were chosen based on their ability to stimulate growth in the in vitro proliferation assay, or if no proliferation was observed, the highest dose that did not significantly inhibit proliferation was chosen. Three concentrations of DLN were used: 100 nM, 1 μM and 10 μM. Cells were harvested after 48 h from log phase cultures. Total RNA was isolated from ELT 3 cells by standard methods of cesium chloride isoalization. An RT reaction of 20 μg of total RNA was performed for 1 h at 37°C in a 100 μl reaction containing 50 nM KCl, 20 mM Tris–HCl (pH 8.4), 2.5 mM MgCl₂, 0.4 mM dNTPs, 5 μM/ml random hexamer primers, 400 U RNase inhibitor (Promega, Madison, WI), and 1000 U MMLV-reverse transcriptase.
doses of HPTE, KPN, and Endosulfan-α showed agonistic activity during log phase growth of uterine leiomyoma cells. Although these compounds stimulated growth over vehicle alone, except that 3 μl of 1:200 diluted cDNA and increasing amounts from 0 to 5 ng of GAPDH competitive template were used, due to higher expression levels.

RESULTS

HPTE, Kepone, and Endosulfan-α Stimulate Uterine Leiomyoma Cell Growth In Vitro

The Eker rat leiomyoma-derived cell line ELT 3 is estrogen receptor (ER)-positive, and 17β-estradiol (E2) dramatically promotes the growth of these cells in culture (Howe et al., 1995b). As shown in Figure 1, cell proliferation was induced in ELT 3 cells treated with several organochlorine pesticides from our panel. 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), kepone (KPN) and endosulfan-α (Endo-α) showed agonist activity during log phase growth of uterine leiomyoma cells. Although these compounds stimulated growth over vehicle alone, none of the doses tested stimulated growth as well as 10 nM E2. HPTE was the most potent chemical in our panel, stimulating cell growth at 100 nM. Proliferation of uterine leiomyoma cells was also observed for 1 μM (data not shown) and 10 μM of HPTE while 100 μM HPTE produced an inhibition of cell growth. Methoxychlor (MXC), the parent compound of the active metabolite HPTE, did not exhibit any agonistic activity. Several doses of KPN, 1 μM and 10 μM, stimulated uterine leiomyoma cell growth while 100 μM inhibited cell growth. Only one concentration tested of Endo-α, 10 μM, was agonistic for cell growth while the same concentration of the β isomer (Endo-β) was inhibitory to proliferation. Toxaphene (TXP) and dieldrin (DLN) showed no increase in proliferation and the highest concentrations of these compounds inhibited cell growth.

Next, we investigated whether HPTE, KPN, and Endo-α-induced proliferation was mediated via the ER. The compound ICI 182,780 has previously been shown to act as a potent antiestrogen in vitro (Wakeling et al., 1991). Proliferative doses of HPTE, KPN, and Endo-α were treated in combination with ICI 182,780 or vehicle alone (Fig. 2). By itself, ICI 182,780 caused no significant decrease in cell number compared to vehicle alone. However, ICI 182,780 dampened proliferation in ELT 3 cells treated with E2, Endo-α, HPTE, and KPN by 58.6%, 75%, 55.2%, and 98.6%, respectively. Furthermore, proliferation was not observed using proliferative doses of these compounds in ER-negative ELT 6 cells, also derived from Eker rat leiomyoma cells (data not shown). These data indicate that the stimulation of proliferation observed in uterine leiomyoma cells treated with HPTE, KPN, and Endo-α is mediated largely through the ER.

All 7 Organochlorine Pesticides Induced Transcription via AF-2 of the ER in ELT 3 Cells

To further characterize potential agonistic activity, a transcriptional assay was employed to test our panel of organochlorine pesticides for transactivational activity on a classical estrogen-responsive promoter element in a myometrial cell background. The construct used, ERE-tk-LUC6a, contains a single copy of the vitellogenin ERE, previously shown to require both AF-1 and AF-2 activation of the ER for transcriptional activity (Tzukerman et al., 1994). This construct also contains a luciferase reporter gene along with a minimal viral promoter, thymidine kinase. ELT 3 uterine leiomyoma-derived cells were transfected with ERE-tk-LUC6a, along with exogenous ERα, and treated with varying doses of organochlorine pesticides. Figure 3 shows the fold induction of luciferase activity over vehicle control. HPTE, KPN, MXC, TXP, DLN and Endo-α and Endo-β exhibited a greater than 3-fold increase over vehicle control for at least one concentration tested. Consistent with the proliferation assays, HPTE was the most potent of the organochlorine pesticides tested, demonstrating a 3.8-fold increase at 100 nM. In summary, all organochlorine pesticides in our panel induced the transcription of an AF2-requiring estrogen-inducible reporter gene in uterine leiomyoma cells.

Induction of an Endogenous Estrogen-Responsive Gene, the Progesterone Receptor (PR), by Organochlorine Pesticides

It has been appreciated that the ability of the ER to transactivate gene expression is dependent on the ligand, cell type, and promoter context (Tzukerman et al., 1994). The PR is a well-characterized endogenous estrogen-responsive gene in many cell types and its expression is increased in response to E2 and several xenoestrogens in ELT 3 cells (Hunter et al., 1999). In contrast to the transcriptional assay in which vitellogenin ERE is transiently expressed, the endogenous progesterone receptor is appropriately complexed with nucleosomes and is an integral part of the native chromatin. Quantitative RT-PCR, utilizing a competitive template (Celi et al., 1993), was employed to measure endogenous levels of PR mRNA transcripts in ELT 3 cells treated for 48 h with organochlorine pesticides, E2, ICI 182,780, or vehicles ethanol or DMSO. In addition to PR, the housekeeping gene GAPDH was amplified
FIG. 1. Organochlorine pesticides alter proliferation in ELT 3 uterine leiomyoma cells. Kinetics of cell proliferation was determined by counting cell number in triplicate wells of vehicle, 10 nM E2 (positive control) or test compound. Concentrations varying from 1–100 μM were used; however, only the most informative concentrations are shown. Cells were counted at several time points prior to confluence. Each graph represents one experiment that was independently repeated up to 3 times. Error bars represent SEM of triplicate wells. Asterisks (*) denote significant differences at $p \leq 0.05$ from vehicle control values determined by ANOVA.
from the same RT sample to normalize for total RNA in each sample. Figure 4A shows the PCR products from endogenous and competitor templates for PR and GAPDH. E2 upregulated PR mRNA levels 11.2-fold over vehicle while the antiestrogen ICI 182,780 did not produce any significant change (Fig. 4B). Endo-α, Endo-β, MXC, TXP, KPN, DLN, and HPTE induced PR mRNA from 2.8- to 6.4-fold over vehicle. Ten μM was the only concentration of DLN tested that was able to increase PR transcript levels, although this dose was inhibitory to cell growth. Neither 1 μM nor 100 nM DLN produced any significant change in PR levels, providing additional evidence for the estrogenic activity of these compounds in this cell type.

**DISCUSSION**

These are the first data to indicate that organochlorine pesticides may act as agonists in the uterine myometrium, a target tissue that has been overlooked in earlier investigations assessing the agonist activity of endocrine-active compounds. These experiments describe the modulation of cellular and molecular responses of myometrial cells to a panel of 7 organochlorine pesticides. Table 1 compares the effective doses of these compounds in proliferation and transcriptional assays and their ability to induce PR mRNA. HPTE, KPN, and Endo-α-stimulated cell growth, transactivated an estrogen-responsive promoter in a transcriptional reporter gene assay, and induced the expression of an endogenous estrogen-responsive gene, the PR. While MXC, TXP, DLN, and Endo-β were unable to stimulate the growth of uterine leiomyoma cells, these compounds did exhibit estrogenic activity in these cells at the molecular level in both an estrogen-sensitive reporter gene assay and via up-regulation of endogenous PR transcripts. Both MXC, the parent compound of HPTE, and KPN have been reported to have some tissue-specific non-ER-mediated agonist activity in vivo (Das et al., 1998, 1997; Ghosh et al., 1999). In our uterine leiomyoma cells, however, agonism at the level of cell proliferation was inhibited by ICI 182,780 for both these compounds, confirming that the agonistic effects on the cellular level are largely mediated by the estrogen receptor.

Currently, there is disagreement in the scientific community on how to best assess the estrogenic activity of potential endocrine disruptors. This is reflected by the variety of methods used by investigators for testing potential estrogen-like compounds. Our results indicate that there were distinct differences in the profiles of these organochlorine pesticides be-

![Figure 2](image1)  
**FIG. 2.** The antiestrogen ICI 182,780 dampens the proliferative effect of HPTE, KPN, and Endo-α. 100 nM ICI 182,780 was used in combination with vehicle or 10 nM E2, 10 μM Endo-α, 1 μM HPTE, or 10 μM KPN to treat ELT 3 cells for 6 days. Bars represent mean cell number counted from triplicate wells ± SEM. For each treatment group, the vehicle-treated cells (either ethanol or DMSO) are represented as solid bars to illustrate the increase in cell number over vehicle- and vehicle-plus-ICI 182,780-treated cells. There is no significant decrease in cell number due to ICI 182,780 treatment with either ethanol or DMSO used as vehicle. Asterisks (*) denote significant differences of ICI-treated conditions at *p* ≤ 0.05 compared to their non-ICI treated counterparts as determined by ANOVA.

![Figure 3](image2)  
**FIG. 3.** Reporter gene activity using vitERE-tk-Luc6a. Uterine leiomyoma-ELT 3 cells were transiently transfected with vitERE-tk-Luc6a along with plasmids for ERα and β-galactosidase. Cells were treated 24 h later with varying concentrations of test compound. Luciferase activity was normalized to β-galactosidase activity in triplicate wells and fold induction calculated as the mean normalized luciferase activity over the vehicle control. Results presented are the mean values of 3–6 separate experiments. The following are mean 10 nM E2 fold induction values over vehicle control for each treatment group: HPTE-23.38; KPN-12.62; MXC-10.55; TXP-10.06; DLN-16.87; Endo-α, and Endo-β -6.7.
TABLE 1

<table>
<thead>
<tr>
<th>Transactivation EC25</th>
<th>PR induction</th>
<th>Proliferative EC25</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPTE 100 nM</td>
<td>+</td>
<td>1 μM</td>
</tr>
<tr>
<td>MXC 1 μM</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>KPN 5 μM</td>
<td>+</td>
<td>1 μM</td>
</tr>
<tr>
<td>Endo-α 5 μM</td>
<td>+</td>
<td>10 μM</td>
</tr>
<tr>
<td>Endo-β 5 μM</td>
<td>+</td>
<td>None</td>
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<tr>
<td>TXP 10 μM</td>
<td>+</td>
<td>None</td>
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<td>DLN 100 μM</td>
<td>+</td>
<td>None</td>
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Note. Transactivation EC25, potency of test compound required to induce transactivation of the vitellogenin ERE reporter construct. Values indicate the lowest dose required to achieve 25% of the mean 10 nM E2 value used in all experiments in which the compound was tested. PR induction, significant increase in PR mRNA over vehicle control as determined by quantitative PCR. Proliferative EC25, potency of test compound required to produce proliferation. Values indicate the lowest dose achieving 25% of the mean 10 nM E2 value used in the same experiment and also observed in at least 2 independent experiments.

tween the estrogenic assays employed in these studies. The proliferation assay identified agonist activity for the least number of organochlorine pesticides in our panel, with only HPTE, KPN, and Endo-α showing activity with this assay. However, all the pesticides in the panel stimulated transcription in the reporter gene assay and also upregulated PR mRNA, suggesting that these compounds are ER agonists and may induce relevant estrogenic responses in exposed leiomyoma cells. Our in vitro results with MXC/HPTE and KPN correlate well with those observed by others in vivo in which these compounds functionally mimic the effects of E2 by increasing the expression of estrogen-induced genes in the whole uterus of treated immature rats (Cummings et al., 1994, 1995; Hammond et al., 1979).

The increased activity of metabolite HPTE over its parent compound, methoxychlor, in the proliferation assay illustrates that the metabolic products of organochlorine pesticides may be more estrogenic than the original chemical, most likely due to the absence of relevant metabolizing enzymes. Bulger et al. have shown that in whole rat uterine extracts methoxychlor is a proestrogen that requires metabolic activation by hepatic microsomes to induce translocation of the ER to the nucleus (Bulger et al., 1978a,b). In addition, certain isomers of a chemical may exhibit more estrogenic activity than other isomers as we have shown for endosulfan. Although the α and β isomers of endosulfan were estrogenic in the transcription assay and PR induction, only the α isomer showed agonistic activity for cell proliferation. While HPTE and KPN stimulated cell proliferation, higher doses were inhibitory to proliferation, most likely due to cytotoxicity. There may be a very fine line in cell signaling whereby the estrogenic response is overcome by toxic effects. A similar dose-effect has been observed by the phytoestrogen genistein on leiomyoma cells, with higher doses of genistein exhibiting non-ER-mediated inhibition (Hunter, et al., 1999). In addition, proliferative doses of HPTE, KPN and Endo-α are 10–1000 times higher than for 10nM E2, most likely due to the lower binding affinities of these compounds for ER (Bulger et al., 1978a; Kuiper et al., 1997).

The Eker rat model of spontaneous uterine leiomyoma closely models the human disease since they develop tumors with similar frequencies and proliferate in response to ovarian hormones. Ovariectomizing these animals results in virtual ablation of tumors (Walker et al., manuscript submitted), although the precise role of E2 and progesterone in tumor initiation and progression have not been elucidated. Uterine leiomyoma isolated from Eker rats exhibit an altered responsiveness to steroid hormones (Burroughs et al., manuscript submitted), exhibiting proliferation even in response to very low hormone levels. Uterine leiomyoma thus may be sensitive to very low levels of steroid hormones and the presence of even modest levels of xenoestrogens such as organochlorine pesticides could contribute to the growth of these tumors. These data, along with previous work by us correlating in vitro AF-2 transactivation with agonist activity in vivo (see below), indicate that the uterine myometrium, in addition to the more obvious hormone-responsive tissues, represent a potential target tissue of endocrine-active compounds. These in vitro assays can be used for not only screening potential endocrine disruptors that target the uterine myometrium, it also can be used to dissect mechanisms behind tissue-specific responses elicited by endocrine disruptors.

To consider xenoestrogens in a mass-action context in which their impact is relegated to the production of a hyperestrogenic state may be too limiting, since the ER-xenoestrogen complex may form conformations that are distinct from the ER-E2 complex. The receptor-ligand complex could potentially inter-
act with estrogen-responsive promoter regions in a manner that may produce a dose response to xenoestrogens that differs from the dose response seen to E2 (Stancel et al., 1995). The partial ER agonist tamoxifen produces a dose-response pattern distinct from E2 for regulating estrogen-responsive genes (Nephew et al., 1993). Furthermore, ER ligands have varying effects in hormonally responsive tissues, perhaps contributing to a unique response of the uterine myometrium to exogenous estrogens. For example, tamoxifen and its active metabolite 4-hydroxytamoxifen act as antagonists on Eker rat uterine leiomyoma-derived cells (Howe et al., 1995b) and decreases tumor incidence in vivo in this animal model (Walker et al., manuscript submitted). Tamoxifen also acts as an antagonist in the breast (Jordan, 1992). In contrast, tamoxifen is a partial agonist for the uterine endometrium, bone, and cardiovascular system (Sato et al., 1996). Thus, the cellular milieu of the uterine myometrium may be an important factor in determining whether this often overlooked, hormonally-sensitive tissue will be a target for agonist activity by endocrine disruptors.

The tissue and promoter specificity of ER ligands may be partly due to the ability of ligands to activate transcription via AF-1 alone or via both AF-1 and AF-2 domains of the ER. While activation of the AF-1 domain is generally considered a constitutive function of partial and full agonists, activation of the AF-2 domain of the ER has been shown to be ligand-, promoter-, and cell-type-specific (Berry et al., 1990). The vitellogenin ERE has been shown to require AF-2 for transcriptional activity and the partial agonist tamoxifen is unable to activate transcription from this construct (Tzukerman et al., 1994). The partial agonists tamoxifen and the raloxifene analogs LY117018 and LY317783, all antagonists for uterine leiomyoma cell growth in vivo, are unable to stimulate transcription from the vitellogenin ERE in a myometrial cell background. This is in contrast to the synthetic estrogen diethylstilbestrol (DES) which can stimulate transcription in vitro as well as proliferation in vivo (Hunter et al., 1999). These results led us to propose previously that AF-2 activation of the ER is necessary but not sufficient for full agonist activity in this cell type. All of the organochlorine pesticides in these experiments stimulated transcription of the reporter gene containing the vitellogenin ERE, suggesting that these endocrine active compounds can activate AF-2 and have the potential to act as agonists in the uterine myometrium in vivo. Given the tissue, cell, and promoter specificities of many ER ligands, it is important to consider each target tissue as unique and correlate in vitro data with agonist activity in vivo.

In the 3 in vitro assays used to determine agonist activity, the organochlorine pesticides examined in this study exhibited differential activity profiles depending on the assay employed. All compounds were able to induce expression of an endogenous estrogen-responsive gene, the PR, whereas only HPTE, KPN, and Endo-α were able to stimulate transcription from the vitellogenin ERE and induce cell proliferation. Previous in vitro and in vivo data suggest that the latter 2 assays, proliferation and transactivation, are the most predictive for agonist activity in this target tissue. We have examined other xenoestrogens, namely the pharmaceutical agents DES, tamoxifen, and two raloxifene analogs, LY117018 and LY317783, for activity in these same in vitro assays and for agonist activity in vivo. In vitro, only DES induces proliferation of ELT 3 cells and exhibits the ability to transactivate the vit-ERE; tamoxifen and the raloxifene analogs both fail to induce proliferation or vit-ERE transactivation, whereas all 3 compounds upregulate the progesterone receptor in vitro (Hunter et al., 1999, and unpublished data). In vivo, only DES induces proliferation in the uterine myometrium (Hunter et al., 1999), while treatment with tamoxifen or LY326315 reduces the incidence of uterine leiomyoma by ~50% (Walker et al., manuscript submitted). Therefore, transactivation of the vitellogenin ERE and stimulation of cell proliferation correlate best with in vivo agonist activity in uterine myometrial cells. In contrast, the induction of the PR appears to be a promiscuous response to estrogen receptor ligands in this cell type, possibly because it may require functional activation only of AF-1 of the ER. Consequently, induction of PR does not correlate well with in vivo agonism in uterine myometrial cells.

It has been suggested that the increased incidence of cancer in hormonally responsive tissues is linked to environmental factors (Colborn et al., 1993). However, there have been several conflicting reports correlating breast cancer and organochlorine exposure. Wolff et al. reported in 1993 that there are higher levels of the DDT metabolite DDE in the serum of women with breast cancer, while more recently Hunter et al. (1997) found no association between organochlorine pesticide levels in serum and breast cancer. A recent study by Hoyer et al. (1998) found no increase in the levels of DDT or its metabolites in the serum of breast cancer patients, but they did find a significantly higher level of dieldrin. Determining exposure levels may be more complex than just measuring serum levels, due to complex issues such as bioaccumulation and the developmental stage in which people are exposed to these compounds. They bioaccumulate in fat stores and are mobilized during lactation (Sonawane, 1995) and fasting (Bigsby et al., 1997). This mobilization could result in exposure levels that are several-fold higher than those originally encountered in the environment. The timing of exposure to endocrine disruptors is another important factor, since hormones play a pivotal role in many stages of the life cycle including development, puberty, pregnancy, lactation, and menopause. Exposure to xenoestrogens at a time when endogenous estrogens are low could potentially produce an inappropriate estrogenic response resulting in deleterious effects on the reproductive system. For example, neonatal treatment of rats to KPN results in persistent vaginal estrus and anovulation (Gellert, 1978).

In the present study, although only a subset of compounds (HPTE, KPN, and Endo-α) were able to stimulate cell proliferation, all the organochlorine pesticides in the panel exhibited agonistic activity on the molecular level. Even in the absence
of cell proliferation, an alteration in endocrine signaling could potentially impact the growth and/or development of uterine leiomyomas by perturbing the hormonal milieu or downstream signaling from the PR and/or ER. The increase in PR message produced by these compounds in uterine leiomyoma cells is of particular concern, especially since PR is found to be increased in leiomyomas compared to the normal myometrium (Brandon et al., 1993; Rein et al., 1990; Viville et al., 1997). The consequences of these molecular changes in uterine leiomyomas are unknown at present but further study of the impact of the pesticides in vivo will be necessary to determine if they constitute a novel mechanism of uterine leiomyoma pathogenesis due to altered endocrine signaling.

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REFERENCES


