0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 12
Printed in U.S.A.

Differential Interaction of the Methoxychlor Metabolite 2,2-Bis-(*p*-Hydroxyphenyl)-1,1,1-Trichloroethane with Estrogen Receptors α and β*

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ABSTRACT

Concern that some chemicals in our environment may affect human health by disrupting normal endocrine function has prompted research on interactions of environmental contaminants with steroid hormone receptors. We compared the activity of 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), an estrogenic metabolite of the organochlorine pesticide methoxychlor, at estrogen receptor α (ERα) and estrogen receptor β (ERβ). Human hepatoma cells (HepG2) were transiently transfected with either human or rat ERα or ERβ plus an estrogen-responsive, complement 3-luciferase construct containing a complement 3 gene promoter sequence linked to a luciferase reporter gene. After transfection, cells were treated with various concentrations of HPTE in the presence (for detecting antagonism) or absence (for detecting agonism) of 17β-estradiol. HPTE was a potent ERα agonist in HepG2 cells, with EC_{50} values of approximately 5×10^{-8} and 10^{-8} M for human and rat ERα, respectively. In contrast, HPTE had minimal agonist activity with either human or rat ERβ and almost completely abolished 17β-estradiol-induced ERβ-mediated activity. Moreover, HPTE behaved as an ERα agonist and an ERβ antagonist with other estrogen-responsive promoters (ERE-MMTV and vtERE) in HepG2 and HeLa cells. This study demonstrates the complexity involved in determining the mechanism of action of endocrine-active chemicals that may act as agonists or antagonists through one or more hormone receptors. (*Endocrinology* **140:** 5746–5753, 1999)

CONCERN OVER THE possible effects of environmental chemicals on human endocrine function has focused research on identifying and characterizing chemical interactions with steroid hormone receptors. It has been hypothesized that environmental chemicals that interact with steroid hormone receptors may disrupt normal endocrine function, leading to altered reproductive capacity, infertility, endometriosis, and cancers of the breast, uterus, and prostate (1–5). Much of the research effort has focused on chemical interactions with the estrogen receptor (ER). Environmental chemicals known to interact with the ER include natural products such as coumestrol and genistein, commercial products such as bisphenol A and *p*-nonylphenol, and pesticides such as dichloro-diphenyl-trichloroethane and methoxychlor (6–11).

Estrogenic activity is mediated by ligand binding to specific intracellular proteins known as ERs (12). Ligand binding induces conformational changes in the receptor, enabling the bound receptor complex to interact with specific sites on DNA. Once bound to DNA the ligand receptor complex alters expression of estrogen-responsive genes, resulting in tissue-specific estrogenic responses. Until recently it was thought that all estrogenic response occurred through a single receptor, now termed ERα. However, the identification of a second ER, ERβ (13), has prompted intensive research on the overlapping and differential roles of these two receptors in mediating estrogenic responses.

ERα and ERβ share a number of common physical and functional properties. The DNA- and ligand-binding domains are highly homologous between the two receptors (13–15). ERα and ERβ also demonstrate many similarities in ligand binding affinities and regulation of gene expression (14, 16–19). *Trans*-activation studies using 17β-estradiol (E_2)-responsive constructs transfected into mammalian cell lines have shown that ERβ, like ERα, mediates the effects of E_2 in a dose-dependent manner, and ERβ *trans*-activation is induced by most ERα agonists and blocked by ERα antagonists (14, 16, 17, 19).

There are some reported differences in the transcriptional activities of these two ER subtypes. Agonist activity of tamoxifen is selectively observed with ERα, but not ERβ, in transiently transfected MCF-7 cells (20). In addition, E_2 activates ERα-dependent transcription and inhibits ERβ-dependent transcription at activating protein-1 sites in transfected HeLa, Ishikawa, and MCF-7 cells (21). Together, these studies demonstrate some distinct differences in *trans*-activational mechanisms between ERα and ERβ. Thus, it is important to characterize interactions of hormonally active environmental chemicals with both ERα and ERβ when trying to determine their potential to modulate endocrine function.

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenol)-ethane] has been of interest in our laboratories because of its well characterized estrogenic effects both *in vitro* and *in vivo*.

Received April 7, 1999.
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* This work was supported by NIH Grants ES-09106, ES-04917, and DK-48807 and the Texas Agricultural Experiment Station.

Methoxychlor is a chlorinated hydrocarbon pesticide structurally similar to DDT that was introduced commercially for insect control in the 1950s and is still in use. Although it is structurally related to DDT, methoxychlor has an advantage in that it is more readily metabolized and excreted by mammalian systems and does not accumulate or bioconcentrate in fatty tissue (22). Like DDT, methoxychlor is estrogenic *in vivo*. Methoxychlor is uterotropic in the ovariectomized rat and can cause adverse developmental and reproductive effects in laboratory animals (23–28).

Methoxychlor is metabolized in the liver by *O*-demethylation to polar mono- and bis-phenolic metabolites (23). The bisphenolic metabolite of methoxychlor 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is approximately 100-fold more active at ERα than methoxychlor. HPTE competes with E_2 for binding to ERα and induces ornithine decarboxylase and uterotropic activity in ovariectomized rats (23, 29, 30). Conversion of methoxychlor to HPTE is generally considered to be a pathway for metabolic activation into a more potent estrogen. Although the interaction of HPTE with ERα has been characterized (30), no data on the interaction of this compound with ERβ have been reported.

In our studies we compared the activities of HPTE at ERα and ERβ and show that HPTE is primarily an ERα-specific agonist and an ERβ antagonist. Our results may lead not only to a better understanding of the mechanism of methoxychlor toxicity but also to the identification of additional ERα- and ERβ-specific agents.

Materials and Methods

Chemicals

HPTE was synthesized by dissolving 1 g methoxychlor (Aldrich Chemical Co., Inc., Milwaukee, WI) in 100 ml methylene chloride and then treating with excess boron tribromide in methylene chloride for 24 h. Water (5 ml) was carefully added, and crude HPTE was isolated in methylene chloride. The residue (0.8 g) was purified by preparative TLC. The resulting HPTE was more than 97% pure as determined by gas-liquid chromatography. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were 97% or more pure.

Plating and transfection

HepG2 human hepatoma cells (American Type Culture Collection, Manassas, VA) were plated in triplicate in 24-well plates (Falcon Plastics, Oxnard, CA) at a density of 10^5 cells/well in complete medium consisting of phenol red-free Eagle's MEM (Life Technologies, Inc./BRL, Grand Island, NY) supplemented with 10% resin-stripped FBS (HyClone Laboratories, Inc., Logan, UT), 2% L-glutamine, and 0.1% sodium pyruvate. Cells were incubated overnight at 37 C in a humidified atmosphere of 5% CO_2-air and then transfected following the SuperFect procedure (QIAGEN, Valencia, CA) with three plasmids (31): 1) various concentrations of receptor plasmid encoding either human or rat ERα (32, 33) or ERβ (16); 2) 405 ng/well complement 3-luciferase (C3-Luc), mouse mammary tumor virus (MMTV)-luc, or estrogen response element-thymidine kinase-luciferase (ERE-TK-luc) reporter plasmid (32, 34); and 3) 10 ng/well pCMVβ-gal plasmid (transfection control and for monitoring for toxicity; CMV, cytomegalovirus; β-gal, β-galactosidase) 32). Transfected cells were then rinsed with PBS and treated with various concentrations of test chemical or dimethylsulfoxide (vehicle control; Sigma Chemical Co.) in complete medium. After a 24-h incubation, treated cells were rinsed with PBS and lysed with 65 μl lysing buffer [25 mM Tris-phosphate (pH 7.8), 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 0.5% Triton X-100, and 2 mM dithiothreitol]. Lysate was divided into 2 96-well plates for luciferase and β-galactosidase determinations.

Luciferase assay

Luciferase assay reagent (100 μl; Promega Corp., Madison, WI) was added to 20 μl lysate, and luminescence was determined immediately using an ML3000 microtiter plate luminometer (Dynatech Corp., Chantilly, VA).

β-Galactosidase assay

Twenty microliters of a 4 mg/ml solution of chlorophenol red-β-D-galactopyranoside (Sigma Chemical Co.) and 150 μl chlorophenol red-β-D-galactopyranoside buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.8) were added to 30 μl lysate. Absorbance at 570 nm was determined over a 30-min period using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA).

Competitive binding assay

The assay was performed as previously described (33). Serial dilutions of E_2 were prepared in 10 mM Tris (pH 7.6), 0.3 M KCl, 5 mM dithiothreitol, and 1 mg/ml BSA. One hundred microliters of E_2 or HPTE ranging in concentration from 10^{-5}–10^{-10} M were transferred to a polystyrene tube. [^{3}H]E_2 (Amersham Pharmacia Biotech, Arlington Heights, IL) at a concentration of 5 nM was added to each tube. Recombinant human ERα or ERβ (PanVera Corp., Madison, WI) were added at 8 or 11 pmol/ml, respectively, to each tube. Optimal concentrations for each receptor were empirically determined. After an overnight incubation at 4 C, 100 μl of a 6% hydroxyapatite (HAP) slurry in dilution buffer [10 mM Tris (pH 7.6) and 5 mM dithiothreitol] were added to each tube. Tubes were then incubated at 4 C for 30 min and spun at 1000 × *g* for 10 min. HAP pellets were washed four times in dilution buffer containing 1% Triton X-100. Pellets were resuspended in 1 ml dilution buffer and transferred to scintillation vials. Radioactivity was measured on a Packard Tri-Carb 460 scintillation counter (Packard Instruments, Meriden, CT).

Statistical analysis

Unless otherwise noted, values presented in this study represent the mean ± SE resulting from at least three separate experiments with triplicate wells for each treatment dose. Dose-response data were analyzed using the sigmoidal dose-response function of the graphical and statistical program Prism (GraphPad Software, Inc., San Diego, CA).

Results

HPTE is an ERα agonist and ERβ competitive antagonist

We first compared the activities of HPTE in HepG2 cells cotransfected with either human or rat ERα or ERβ receptor plasmid along with the estrogen-responsive reporter plasmid C3-Luc (Fig. 1). HPTE was a complete agonist with both human and rat ERα (Fig. 1, A and C). EC_{50} values for induction of luciferase activity for E_2 and HPTE were 4×10^{-9} and 5.1×10^{-8} M, respectively, with human ERα and 10^{-9} and 10^{-8} M, respectively, with rat ERα. Maximal ERα activity was not affected when various concentrations of HPTE were combined with an inducing concentration of E_2 (10^{-7} M; Fig. 1, A and C). In contrast to results obtained with ERα, HPTE induced minimal activity with ERβ (Fig. 1, B and D). The maximum activity of 10^{-5} M HPTE with ERβ was only 13% of that obtained with 10^{-7} M E_2. In addition, HPTE effectively antagonized E_2-induced ERβ activity (Fig. 1, B and D).

We further characterized the ERβ antagonist activity of HPTE in HepG2 cells by determining the effects of various concentrations of HPTE across a complete E_2 dose-response range (Fig. 2A). HPTE caused a parallel shift in the E_2 dose-response curve, indicating that HPTE is an ERβ competitive antagonist. Schild regression analysis yielded a linear re-

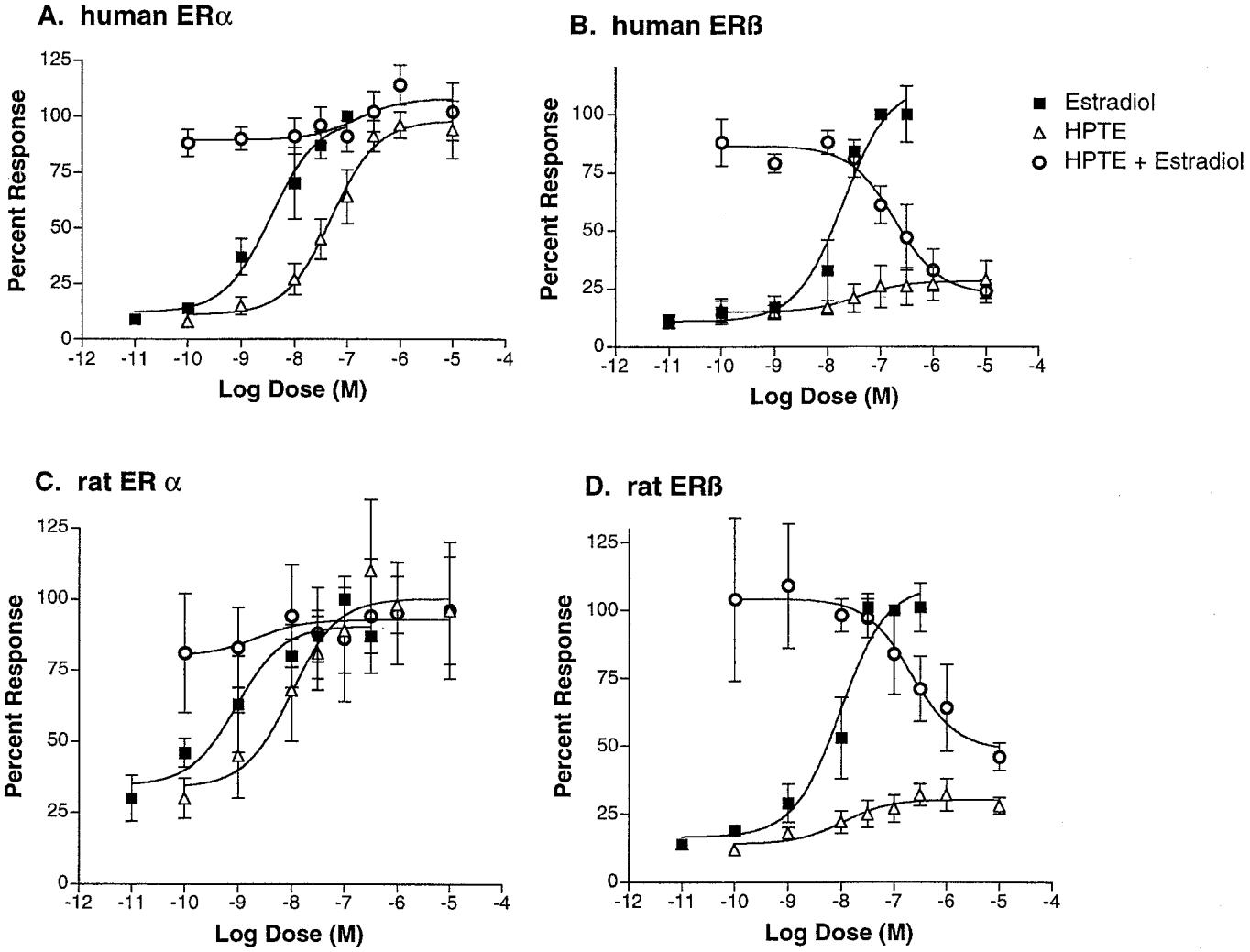

FIG. 1. Activities of E_2 and HPTE at human and rat ERα and ERβ. HepG2 cells were transiently transfected with expression plasmids for human ERα (A) or ERβ (B) or for rat ERα (C) or ERβ (D) plus C3-luciferase reporter plasmid (C3-Luc) and a constituitively active β-galactosidase expression plasmid (transfection control). Cells were treated with increasing concentrations of E_2 (■) or HPTE (△) or with HPTE plus 10 nM E_2 (○). After 24-h incubation, cultures were assayed for both luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity. Values represent the mean ± SE of three or four separate experiments and are presented as the percent response, with 100% activity defined as the activity achieved with 10^{-7} M E_2.

gression with a slope not significantly different from 1 (Fig. 2B). A negative logarithm of the equilibrium dissociation constant (pK_B) value of 7.5 was determined ($K_B = 3.05 \times 10^{-8}$ M) from the calculated *x*-intercept in Fig. 2B.

Competitive binding assays were performed to ensure that the antagonistic activity of HPTE with ERβ was due to competition with E_2 for ERβ receptor binding. HPTE caused a dose-dependent decrease in [^{3}H]E_2 binding to both ERα and ERβ (Fig. 3). The relative binding affinities of ERα and ERβ for HPTE were 0.004 and 0.02, respectively (E_2 = 1.0).

Differential activity of HPTE with ERα and ERβ in HepG2 and HeLa cells is not promoter specific

HepG2 and HeLa cells were transfected with alternate estrogen-responsive promoters to determine whether the differential activity of HPTE with ERα and ERβ was promoter specific. The estrogen-responsive MMTV-luciferase reporter (ERE-MMTV-Luc) had high background activity in HepG2 cells transfected with ERα (Fig. 4A). Despite this high background activity, both E_2 and HPTE demonstrated ERα agonist activity. ERE-MMTV-Luc had much lower background in HepG2 cells when cotransfected with ERβ (Fig. 4B), and HPTE exhibited clear ERβ antagonistic activity. HPTE behaved as a partial ERα agonist/antagonist and a complete ERβ antagonist in HepG2 (Fig. 4, C and D) and HeLa (Fig. 4 E and F) cells transfected with the estrogen-responsive, vitellogenin ERE-luciferase reporter gene (ERE-Luc; Fig. 4, C and D).

ERα activity predominates when ERα and ERβ are coexpressed

ERα and ERβ are coexpressed in some tissues *in vivo*, and therefore, HepG2 cells were cotransfected with various concentrations of ERα and ERβ expression plasmid to determine

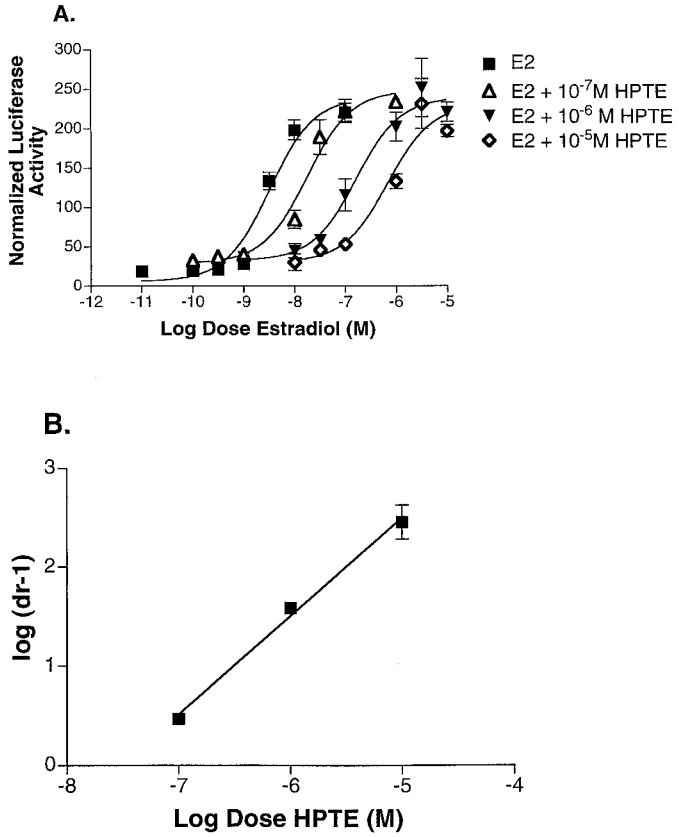

FIG. 2. Effects of various concentrations of HPTE on an E_2 dose-response curve with ERβ. A, Experiments were performed as described in Fig. 1 with 10^{-11}–10^{-5} M E_2, either alone (■) or in combination with 10^{-7} (△), 10^{-6} (▼), and 10^{-5} M HPTE (◇) in HepG2 cells transfected with expression plasmids for human ERβ, C3-Luc, and β-galactosidase. Values represent the mean ± SE normalized luciferase activity from three separate assays. B, Schild regression analysis of the data shown in A. The dose ratio (dr) is [A′] ÷ [A], where [A′] and [A] refer to equiactive concentrations of E_2 in the presence and absence of HPTE, respectively.

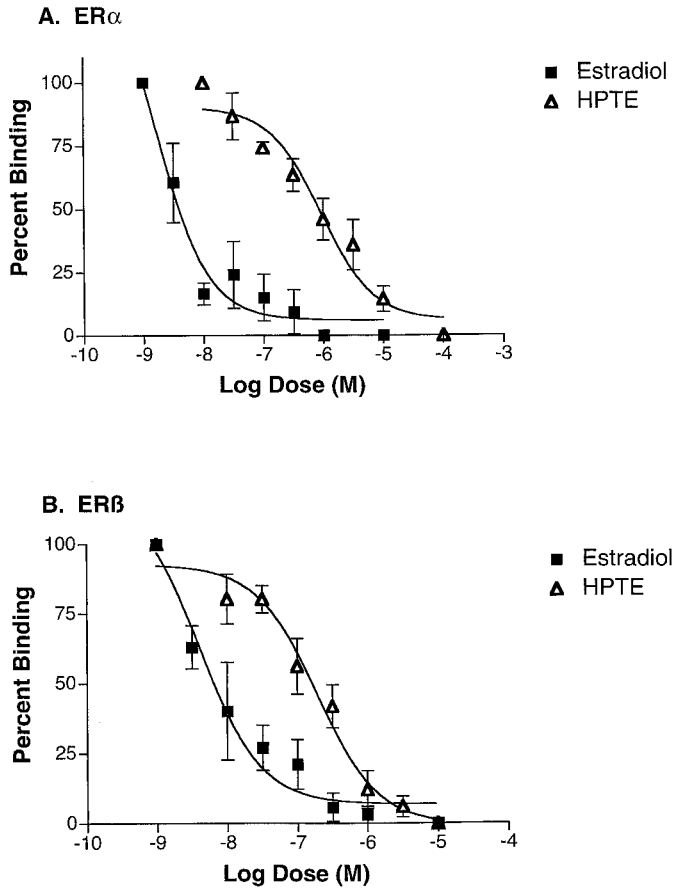

FIG. 3. Competitive binding of E_2 and HPTE to purified ERα and ERβ. E_2 (■) and HPTE (△) were mixed with 5 nM [^{3}H]E_2 plus recombinant ERα (A) or ERβ (B). After overnight incubation, the receptor complexes were precipitated with HAP, and pellet radioactivity was determined. Values represent the mean ± SE of three or four separate experiments and are presented as the percent response.

overall activity of HPTE when both ERα and ERβ are present (Fig. 5). A concentration-response curve for ERβ plasmid in the absence of ERα is presented in Fig. 5A. Transfection with increasing concentrations of ERβ plasmid into HepG2 cells enhanced peak luciferase activity, but did not alter the overall agonist-antagonist activity of E_2 or HPTE. The activities of E_2 and HPTE in the presence of a fixed concentration (40 ng/well) of ERα plasmid and various amounts of ERβ plasmid are presented in Fig. 5B. Addition of ERβ plasmid had no significant effect on peak E_2-induced luciferase activity, and HPTE behaved primarily as an agonist. Increasing concentrations of ERβ plasmid resulted in decreased activity of HPTE. However, even when ERβ/ERα plasmid ratios (by weight) were 10:1, HPTE still displayed agonist activity. ERα activity also predominated across a complete concentration-response curve of HPTE when ERα and ERβ were cotransfected at equal concentrations (80 ng/well; Fig. 5C). We have confirmed by Western analysis that ERα and ERβ are expressed at equivalent concentrations from their respective expression plasmids under the conditions of our assay and that transfection with increasing concentrations of either expression plasmid results in a corresponding increase in receptor protein (McDonnell, D. P., and J. M. Hall, unpublished observations).

Discussion

Interaction of HPTE with ER has been previously reported (23), and the results of this study demonstrate that HPTE has differential activity with ERα and ERβ. HPTE is an ERα agonist, although the efficacy of the response (complete or partial agonism) depends on the cell type and promoter. In contrast, HPTE is primarily an ERβ competitive antagonist.

Differential activity of chemicals with ERα and ERβ has been previously reported (14, 20, 21, 36, 37). E_2 selectively activates ERα and inhibits ERβ *trans*-activation at activating protein-1 sites (21). In addition, tamoxifen displays some agonist activity with ERα, but not ERβ, using ERE-dependent promoters (20). However, this differential activity of tamoxifen between ERα and ERβ is highly promoter and cell specific. Selective action of tamoxifen with ERα and ERβ may be due to differences in the activation function (AF1) region, which is only 30% homologous between the two receptors (14), and previous studies show that AF1 region is essential for the ERα agonist activity of tamoxifen (32, 35). A recent

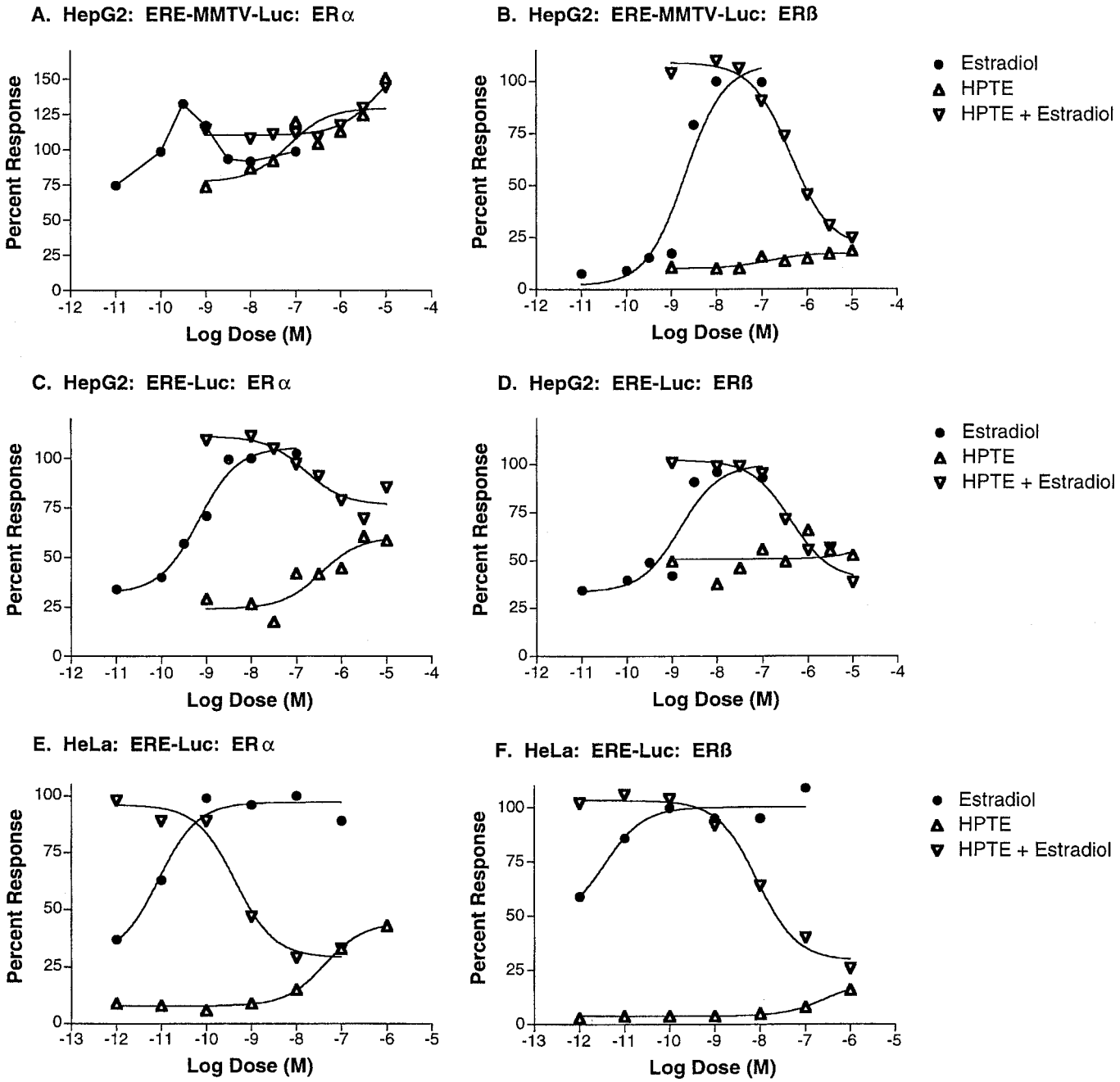

FIG. 4. Activities of E_2 and HPTE with estrogen-responsive MMTV and vt-ERE promoters. Experiments were performed as described in Fig. 1. HepG2 cells (A–D), and HeLa cells (E and F) were transfected with either human ERα or ERβ plus either ERE-MMTV-luciferase (ERE-MMTV-Luc) or vtERE-luciferase (ERE-Luc) reporter plasmids plus β-galactosidase expression plasmid. Values represent the mean of three or four separate experiments and are presented as the percent response, with 100% activity defined as the activity achieved with the plateau E_2 concentration.

report using chimeric ER containing AF2 of ERβ and AF1 of ERα confirms that differences in tamoxifen action between ER subtypes are AF1 dependent (36). The roles of various domains of ERα and ERβ in the action of HPTE are currently being investigated. In contrast to the cell-specific differences observed for tamoxifen as an ER agonist and antagonist, the differential activity of HPTE with ERα and ERβ is neither cell type nor promoter specific. HPTE exhibited primarily ERα-dependent agonist activity and ERβ-dependent antagonist activity in HepG2 and HeLa cells in this study and in three additional human cancer cell lines derived from three different tissues (data not shown). The *R,R*-enantiomer of tetrahydrochrysene has also recently been shown to have differential ERα and ERβ activity (37). Like HPTE, R,R-THC behaves as an ERα agonist and an ERβ antagonist. In contrast, the *S,S*-enantiomer is an agonist with both ERα and ERβ. Thus, it is likely that additional chemicals will be identified that have differential activity at ERα and ERβ.

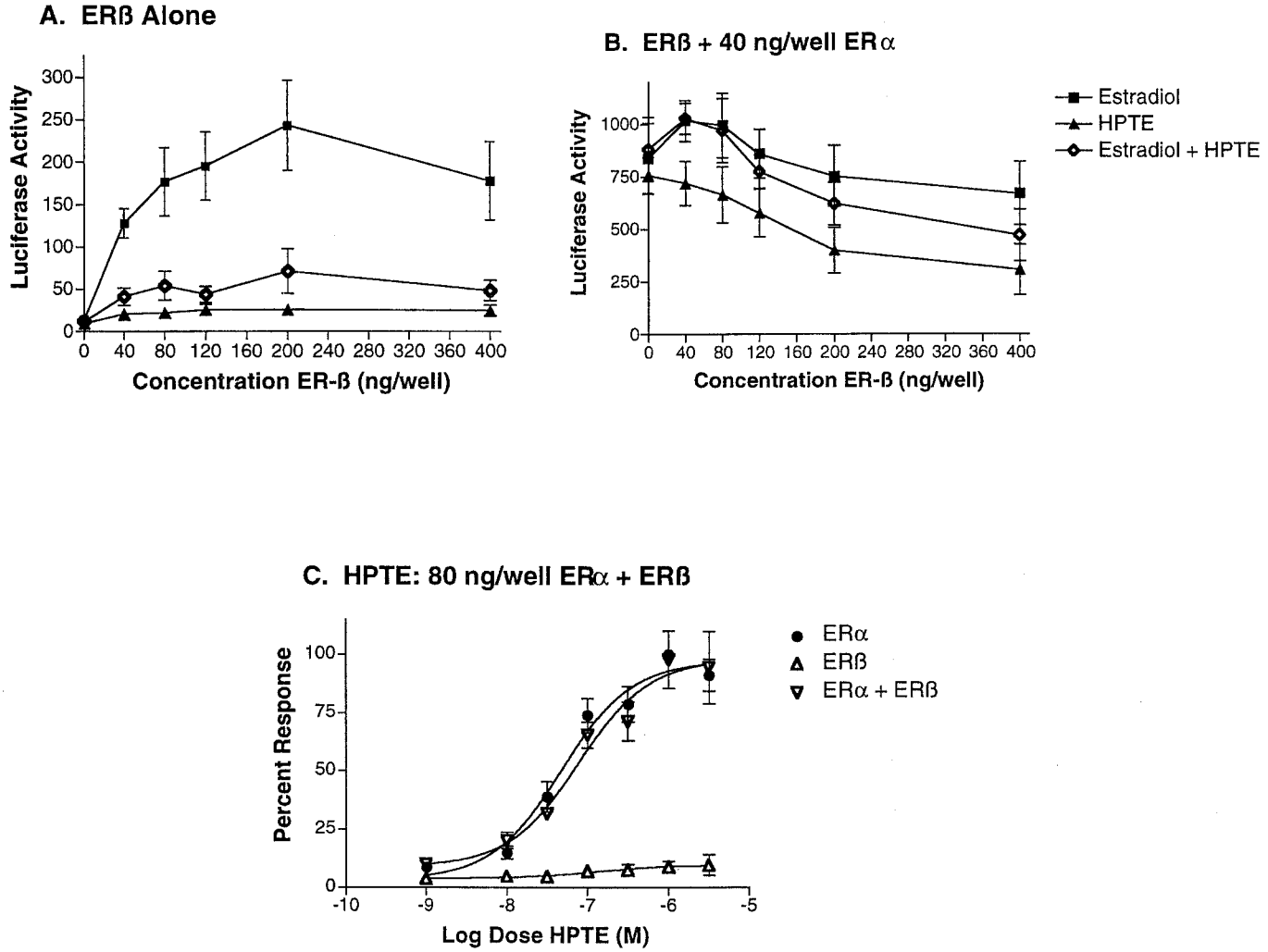

FIG. 5. Activities of E_2 and HPTE with coexpression of ERα and ERβ. Experiments were performed as described in Fig. 1. HepG2 cells were transfected with 0–400 ng/well human ERβ plasmid alone (A) or in combination with 40 ng/well ERα plasmid plus C3-Luc and β-galactosidase expression plasmids (B). C, Cells were cotransfected with 80 ng/well each of ERα and ERβ. Values represent the mean ± SE normalized luciferase activity from three separate experiments.

ERα and ERβ have different, but overlapping, patterns of expression *in vivo*. In some tissues, such as the rat uterus, both receptors are highly expressed (38, 39), whereas in other tissues, such as rat prostate epithelial cells, only one receptor subtype is expressed, (40). ERα and ERβ can form either homodimers or heterodimers depending on which subtypes are present within the cell (41–44). Our results indicate that there is not a simple stoichiometry when ERα and ERβ are cotransfected. ERα activity predominates in cotransfections instead of having an intermediate activity between that demonstrated by either receptor alone. Even a 10-fold excess of transfected ERβ did not significantly diminish activity. Thus, our results suggest that in tissues where both receptor types are present, HPTE would predominately act as an ER agonist. However, when only one receptor subtype is present, then HPTE will primarily act as either an agonist or an antagonist depending on the receptor subtype present. The mechanism for this higher than expected activity when both receptor subtypes are present as well as the tissue selectivity for this response remain to be determined. Additional studies show that under some conditions cotransfection of ERβ with ERα causes a shift to the right in the dose-response curve (Hall, J. M. and D. P. McDonnell, unpublished observations). Thus, under these conditions, low doses that were active with ERα alone would not be active with ERα plus ERβ.

HPTE is considered the primary metabolite responsible for the estrogenic effects associated with methoxychlor exposure. Like E_2, methoxychlor is uterotropic *in vivo* and induces a number of estrogen-dependent uterine responses (23, 30, 45–47). In addition, methoxychlor mimics the action of E_2 on induction of uterine epidermal growth factors, vaginal estrus, cyclicity, and alterations in sexual behavior in female rats (24, 48, 49). However, some differences in the *in vivo* activity of methoxychlor and E_2 have been reported. Unlike estrogen, methoxychlor does not increase FSH and LH levels in ovariectomized rats (48). Moreover, methoxychlor acts as an estrogen agonist in the uterus and an antagonist in the ovary (28). In addition, dissimilar translation products have been reported in neonatal mice exposed to E_2 or methoxychlor (50, 51). Antagonism of ERβ action by HPTE may play

a role in responses induced by methoxychlor that differ from those induced by E_2. For example, the ability of methoxychlor to act as an antagonist in the ovary may be due to the high level of ERβ expression relative to ERα in this tissue (38). HPTE has recently been shown by us and others to be an androgen receptor antagonist (52, 53), and this may also account for some of the effects associated with exposure to methoxychlor.

The estimated adult intake of methoxychlor is approximately 0.8 μg/day based on a recent FDA food basket survey that monitored pesticide residues on food. However, as methoxychlor is rapidly metabolized and does not bioaccumulate in fat (22), it is unlikely that HPTE levels would reach the concentrations in humans that would be sufficient to alter ERα, ERβ, or androgen receptor activity. HPTE remains a model xenohormone, however, for several reasons. First, it is a highly active metabolite of a relatively inactive compound, thus illustrating the importance of metabolic activation for some endocrine-active chemicals. Second, the physiological consequences of a chemical that is a relatively high affinity ERα agonist, ERβ antagonist, and androgen receptor antagonist are unknown, and HPTE can serve as a model for investigating the *in vivo* effects of an agent that modulates multiple endocrine pathways. Finally, our current studies with HPTE as well as our recent publication demonstrating the unique estrogenic activity of bisphenol A (33) illustrate the difficulty in labeling a chemical as an estrogen receptor agonist or an androgen receptor antagonist. As our current understanding of steroid hormone receptor function evolves, it is likely that other chemicals will be identified as selective hormone receptor modulators with a broad spectrum of activities that differ from endogenous hormones steroids, and therefore, a more detailed understanding of their mechanism of action will be required. Additional studies with HPTE and structural analogs may lead to further insights on ligand specificity for ERα and ERβ action and to a better understanding of the physiological roles of these two receptors.

Acknowledgments

We thank Dr. Jan-Ake Gustafsson (Karolinska Institute, Huddinge, Sweden) for providing rat ERβ and human ERβ.

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