Environmental Estrogens Induce Transcriptionally Active Estrogen Receptor Dimers in Yeast: Activity Potentiated by the Coactivator RIP140

Cameron Q. Sheeler, Mark W. Dudley,* and Sohaib A. Khan

Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

We used three yeast genetic systems to investigate the estrogen-like activity of octylphenol (OP), bisphenol-A (BPA), o,p'-DDT, and o,p'-DDE to induce human estrogen receptor (hER) dimerization and transcriptional activation. We have demonstrated that OP, BPA, and o,p'-DDT can induce hER ligand-dependent dimerization using a yeast two-hybrid assay. All three xenoestrogens, plus estradiol, enhanced estrogen response element (ERE)-dependent transcriptional activation of hER. In the presence of receptor interacting protein 140 (RIP140), ERE-dependent activity was dramatically amplified by 100-fold for estradiol, OP, BPA, and o,p'-DDT. A yeast whole-cell [^{3}H]estradiol binding assay was developed to determine the site of interaction on the hER. We determined nonspecific binding by parallel incubations run in the presence of 5 μM unlabelled estradiol in PCY2 yeast. At the concentrations tested, unlabeled estradiol, OP, and BPA displaced [^{3}H]estradiol in this binding assay, whereas the concentrations of o,p'-DDT and o,p'-DDE tested were insufficient to inhibit binding. Incubating yeast in the presence of increasing concentrations of estradiol and OP (1 μM) or BPA (1 μM) neither blocked nor altered the effect of estradiol on hER activity. We observed no agonistic activity of o,p'-DDE in any of the yeast models used. These results suggest that OP, BPA, and o,p'-DDT exert their estrogen-like activity through the ER in a manner similar to that of estradiol, and the coactivator RIP140 markedly potentiates this activity. *Key words:* environmental estrogens, estrogen receptor, ligand binding, receptor dimerization, RIP140, xenoestrogens. *Environ Health Perspect* 108:97–103 (2000). [Online 23 December 1999]

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Environmental estrogens are man-made chemicals that possess estrogen-like biologic activity. Some of these compounds, also called xenoestrogens, are associated with reproductive failure, abnormal feminization or masculinization, and altered immune function in wildlife (*1*). Many widely distributed xenoestrogens are industrial by-products. Octylphenol (OP), a nonionic surfactant, increases uterine weight in rats (*2*) and stimulates human breast cancer cell proliferation, trout vitellogenin gene expression, and other estrogen receptor-dependent gene transcription (*3*). o,p'-DDT and o,p'-DDE are components of commercial DDT that exhibit estrogen-like activity. o,p'-DDT can increase the weight, glycogen, water, and RNA content of mammalian and avian reproductive tracts (*4*). Furthermore, o,p'-DDT and o,p'-DDE have been reported to induce blastocyst implantation and maintain pregnancy in rats (*5*). Bisphenol A (BPA) is used in the manufacturing of polycarbonate and has been shown to bind to the estrogen receptor (ER), stimulate MCF-7 cell proliferation, and increase the expression of progesterone receptor (*6*). The majority of xenoestrogens are structurally distinct from estrogen, making it difficult to determine whether a compound is estrogenic on the basis of its chemical structure alone.

ER, a member of the ligand-dependent nuclear receptor superfamily of transcription factors (*7*), mediates the effects of estrogen. Estrogen enters the cell and binds to the ER, resulting in receptor dimerization. The dimer then binds to specific DNA sequences termed estrogen response elements (EREs) in the promoter region of ER-responsive genes. Once bound to the ERE, the ER influences gene expression through interactions with basal transcription factors. In conjunction with the basal transcription machinery, the ER associates with a group of novel nuclear proteins, co-modulators, to enhance (*8–11*) or suppress (*12*) transcriptional activity. One such co-modulator, receptor interacting protein 140 (RIP140), has been demonstrated to interact directly with the ER hormone-binding domain in the presence of estrogen and to amplify ER-dependent transcriptional activity (*13–15*). Recently, RIP140 has been shown to interact with other nuclear receptors and regulate gene expression (*14,16,17*). The complexing of receptor-interactive proteins with the ER serves as an additional level of selectivity and responsiveness in the regulation of estrogen-dependent gene expression.

The mechanism through which environmental estrogens exert their estrogen-like activity is unknown. We used three yeast genetic systems to determine whether several environmental estrogens affect transcriptional activation of human ER (hER). Also, it is unclear whether xenoestrogen-induced ER transactivation could also be influenced by the coactivator RIP140. Our data suggest that the estrogen-like activity of several environmental estrogens is mediated via the estrogen receptor, and that this activity is enhanced by RIP140. In addition, our binding data suggest that OP and BPA interact with the receptor through the ligand-binding site.

Materials and Methods

Materials. 4-*tert*-Octylphenol (OP) and bisphenol A (BPA) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). The DDT analogs 2-(4-chlorobenzene)-2-(2-chlorobenzene)-1,1,1-trichloroethane (o,p'-DDT) and 2-(2-chlorophenyl) 2-(4-chlorophenyl)-1,1-dichloroethylene (o,p'-DDE) were obtained from Supelco Inc. (Bellefonte, PA); [^{3}H]estradiol (84.1 Ci/mmol) was acquired from Dupont//NEN (Boston, MA). The complete supplement mixture (minus: leucine and tryptophan or leucine, tryptophan, and uracil) used for selection was purchased from BIO 101, Inc. (Vista, CA).

Yeast strains. We used two yeast strains to measure ligand-dependent hER dimerization or induction of hER-dependent transcription. The yeast strain PCY2 (MATα Δgal4 Δgal80 URA3::GAL1-*lacZ* *lys*2-801^{amber} *his*3-Δ200

$trp1$-$\Delta63$ $leu2$ $ade2$-101[ochre]) was used to measure ligand-dependent hER dimerization (18). The PCY2 yeast strain carries a genomic copy of the GAL4 binding site upstream of a $lacZ$ reporter. Induction of hER-dependent transcriptional activity was measured in the yeast strain RS188N (MATα $ade2$-1 $his3$-1 $leu2$-1 112 $trp1$-1 $ura3$-1) (19). Transformation of yeast with plasmid DNA was done using the lithium acetate method (20). Transformed yeast colonies were selected by culture on synthetic medium lacking uracil, tryptophan, and/or leucine.

cDNA and constructs. Construction of GAL4-hER fusion vectors involved cloning the full-length hER cDNA into the pPC62 (GAL4-DB) and pPC86 (GAL4-TA) fusion vectors, as previously described (20). hER cDNA, placed in the SalI site of pCMV-5, was digested with SalI and subcloned into the pBluescript II K+ at the SalI site such that its transcription was dependent on T7 polymerase (T7-hER). T7-hER was amplified with T3 primer and an oligonucleotide (5′-GGG GAT CGT CGA CTC GGT CTG CA-3′). This construct was designed to keep the correct reading frame intact in the GAL4-hER vector. The polymerase chain reaction (PCR) product was directly cloned into GAL4-DB and GAL4-TA at the SalI site such that, when expressed, the N-terminal end of the hER would be fused to the C-terminal end of DB and TA. GAL4-DB-hER and GAL4-TA-hER fusion cDNA were sequenced to confirm the correct reading frame before transforming yeast. The two expression vectors were cotransformed into the PCY2 yeast strain and used to assess ER dimerization

The RS188N yeast strain was cotransformed with an ERE-dependent reporter (YRpE2), hER cDNA (YEpE12), and an empty pPC62. YRpE2 contains two EREs from the *Xenopus* vitellogenin-A gene, upstream of a $lacZ$ gene. YEpE12 expresses hER fused to ubiquitin and is under the control of a yeast copper metallothionein promoter. The copper metallothionein promoter allows for regulated expression of hER in yeast by the addition of exogenous copper (19). The attachment of ubiquitin to the amino terminus of proteins such as hER improves both the quality and quantity of proteins expressed in yeast (19). Following expression in yeast, ubiquitin is cleaved from the fused product, releasing the desired protein. The cotransformation of an empty pPC62 vector in the RS188N is used as a baseline for comparing the effect of RIP140 on ER-dependent transcription. Yeast carrying an ERE$_2$-$lacZ$ reporter, hER, and pPC62 was used to assess ER-dependent transcription.

To construct the yeast RIP140 expression vector, we first amplified the coding region of RIP140 cDNA in pEF-BOS vector (15) by two oligonucleotides: an up-stream primer (5′-GCG TCG ACG CTT CTA TTG AAC ATG ACT CAT-3′) and a down-stream primer (5′-GGA CTA GTC CAA AAC TGG ATG GCA GGT-3′). The upstream oligonucleotide flanked the start codon (underlined in the up-stream primer), and the downstream primer was designed around the stop codon of RIP140 cDNA. The upstream primer included a SalI restriction site and the downstream primer contained a SpeI restriction site for further cloning. The RIP140 PCR product was then cloned into pBluescript II SK+ at SalI and SpeI. The RIP140 coding region, released from pBluescript II SK+ by SalI and SpeI, was cloned into GAL4-DB (pPC62) at the SalI and SpeI sites. The GAL4-DB–RIP140 fusion cDNA was sequenced to confirm correct reading frame before transforming yeast. pPC62 containing RIP140 cDNA was cotransformed, along with YEpE12 and YRpE2, into the RS188N yeast strain.

Dimerization assay using the yeast two-hybrid system. Ligand-dependent dimerization of the hER was determined by using the two domains of the yeast transcription factor GAL4, the DNA-binding (DB) domain and the transcription-activating (TA) domain (18,20). The functionality of GAL4 requires that the DB and the TA domains be juxtaposed. To exploit the use of these domains, we cloned hER into separate expression vectors (pPC62 and pPC86) such that hER was expressed as a fusion protein with DB and with TA. The two fusion plasmids were cotransformed into the PCY2 yeast strain.

The strategy behind these genetic manipulations was to design a recording system that could be used to quantitate hER dimerization. If ligand-dependent hER dimerization occurred, DB and TA fusion proteins would be brought in close proximity, reconstituting GAL4 activity. The reconstituted GAL4 increases β-galactosidase synthesis via the expression of a $lacZ$ reporter gene that is driven by the GAL1 promoter. Thus, the production of β-galactosidase confirms the ability of estrogenic ligands to induce hER dimerization.

Transactivation assay. We assessed the ability of environmental estrogens to promote transcriptional activity of hER in a genetically manipulated RS188N yeast strain. RS188N yeast was cotransformed with YEpE12, YEpE2, and an empty pPC62 vector (see "cDNA and Constructs") yielding an ERE-dependent yeast strain. We used β-galactosidase activity to quantitate hER-dependent gene transcription. We also used the yeast strain RS188N to determine the influence of RIP140 on xenoestrogen-induced ER transcription activity by cotransforming the yeast with YEpE12, YEpE2, and RIP140 cDNA in pPC62.

Ligand treatment. Stock cultures of the two-hybrid yeast or ERE-dependent yeast with or without RIP140 were grown in 20 ml synthetic supplemented medium lacking the appropriate amino acid selection markers; 1% (v/v) ethanol serves as the carbon source. Cultures were grown at 30°C until reaching an OD$_{600}$ (optical density at 600 nm) reading between 1.5 and 2.5 absorbance units (AU)/ml culture, after which the stock cultures were stored at 4°C for up to 1 month. For ligand treatment, we added a volume of the stock yeast culture to 1.0 ml of supplemented synthetic dextrose (SD) medium such that the initial OD$_{600}$ was between 0.1 and 0.15 AU/ml culture. PCY2 yeast were incubated in SD medium lacking leucine and tryptophan; RS188N yeast were incubated in SD medium lacking uracil, tryptophan, and leucine. Estradiol and test compound stock solutions were diluted in DMSO such that 1 μl of the test compound added to 1.0 ml of culture gave the desired final ligand concentration. Control cultures received an equal volume of DMSO. Expression of hER was induced in the ERE-dependent yeast by adding 100 μM CuSO$_4$. The cultures were then grown at 30°C in a shaking incubator (230 rpm) for 16–18 hr, after which the samples were removed and β-galactosidase was quantitated.

β-*Galactosidase assays.* The β-galactosidase assay was used to measure ligand-dependent dimerization in the two-hybrid yeast system and hER-dependent transcription activity in the ERE-dependent yeast system. Overnight yeast cultures were added to Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM β-mercaptoethanol, pH 7.0) containing sodium dodecyl sulfate (SDS; 0.027%), with a total volume of 1.1 ml. This was accomplished by adding 0.1 ml of PCY2 yeast to 0.7 ml Z buffer and 0.3 ml 0.1% SDS in Z buffer or 0.05 ml of RS188N yeast to 0.75 ml Z buffer and 0.3 ml 0.1% SDS in Z buffer. The reaction mixture was preincubated at 30°C for 10 min. The reaction, started by adding 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (ONPG; 4.0 mg/ml in Z buffer containing β-mercaptoethanol and lacking SDS), was incubated at 30°C for up to 1 hr and terminated by adding 0.5 ml of 1.0 M Na$_2$CO$_3$. β-Galactosidase activity was determined by the degree of ONPG hydrolysis at 420 nm. Total β-galactosidase units were calculated according to the following equation:

$$\text{RRU} = \frac{1,000 \times \left[\left(\text{OD}_{420} \right) - \left(1.75 \times \text{OD}_{550} \right) \right]}{t \times v \times \text{OD}_{600}},$$

where RRU = relative response units; t = ONPG reaction time (minutes); v = volume (milliliters) of yeast used in the reaction;

OD_{600} = cell density of yeast at the start of the assay; and OD_{550} = light scattering by yeast debris (20). The concentration of each ligand that gave 50% of its maximal β-galactosidase activity (EC_{50}) was determined by fitting the data to a four parameter logistic function, and analyzed by SigmaPlot software (SPSS Inc., Chicago, IL).

Receptor binding assays. We determined the affinities of the various test compounds and estradiol for hER in a whole yeast cell binding assay using [^{3}H]17β-estradiol (84.1 Ci/mmol). The yeast cultures used for the binding assays were prepared from stock two-hybrid cultures maintained at 4°C by growing fresh cultures in the appropriate volume of SD medium lacking tryptophan and leucine. The cultures were grown to an OD_{600} between 0.2 and 0.8 AU/ml culture. This density was found to give optimal specific binding.

Saturation analysis of [^{3}H]estradiol binding was performed in 96-well plates by incubating 0.08 ml of the yeast culture with 0.02 ml of various concentrations of [^{3}H]estradiol (0.1–10 nM) for 3 hr at room temperature. Total assay volume was 0.2 ml made up with yeast culture medium. We determined nonspecific binding at each concentration of ligand by parallel incubations run in the presence of 5 µM unlabeled estradiol. At the indicated time, we harvested the cells by filtration onto glass filter membranes using a 12-well cell harvester and measured the levels of bound radioactivity. The specific binding was analyzed by the method of Scatchard to obtain linear regression on a plot of bound/free versus bound (21). The apparent dissociation constant (K_d) was calculated from the slope of the regression line, and the number of binding sites per 1 million cells was calculated from the x-axis intercept.

We performed competition binding assays using the same protocol as above, except that cells were incubated in the presence of 1 nM [^{3}H]estradiol and various concentrations of test compounds (prepared in DMSO) at a concentration of 1.0 µl compound/200 µl incubation volume to give the correct final concentration (estradiol, 10^{-11}–10^{-7} M; OP, 10^{-9}–3×10^{-5} M; BPA, 10^{-7}–3×10^{-4} M; and o,p´-DDT and o,p´-DDE, 10^{-8}–1.4×10^{-4} M). Control cells were incubated under identical conditions with an equivalent volume of DMSO. We determined the concentration of unlabeled ligand required to inhibit 50% binding of [^{3}H]estradiol (IC_{50}) by fitting the data to a four parameter logistic function,

$$f(x) = \frac{(a-d)}{\left[1+(x/c)^b\right]+d},$$

(where a = asymptotic maximum, b = slope parameter, c = value at inflexion point, and d = asymptotic minimum). We used SigmaPlot software (SPSS) to analyze the results. The inhibition constant (K_i) for each compound was calculated using the method of Cheng and Prusoff (22).

Results

Induction of hER dimerization by environmental estrogens. The hER was expressed in PCY2 yeast as GAL4-DB-hER and GAL4-TA-hER fusion proteins. Ligand-dependent hER dimerization reconstitutes functional GAL4 activity, resulting in the expression of a *lacZ* reporter gene driven by a GAL1 promoter (20). This provides a powerful means to investigate ligand-induced protein–protein interaction. Yeast were grown overnight in selection medium with varying concentrations of estradiol, OP, BPA, o,p´-DDT, or o,p´-DDE. Estradiol generated a sigmoidal dose–response curve, with β-galactosidase activity first being detected at 30 pM and plateauing at 10 nM; no further stimulation was observed at higher concentrations (Figure 1). The EC_{50} value of estradiol was 0.12 ± 0.01 nM (mean ± SE). Similar shaped dose–response curves were observed for OP, BPA, and o,p´-DDT. The concentrations of
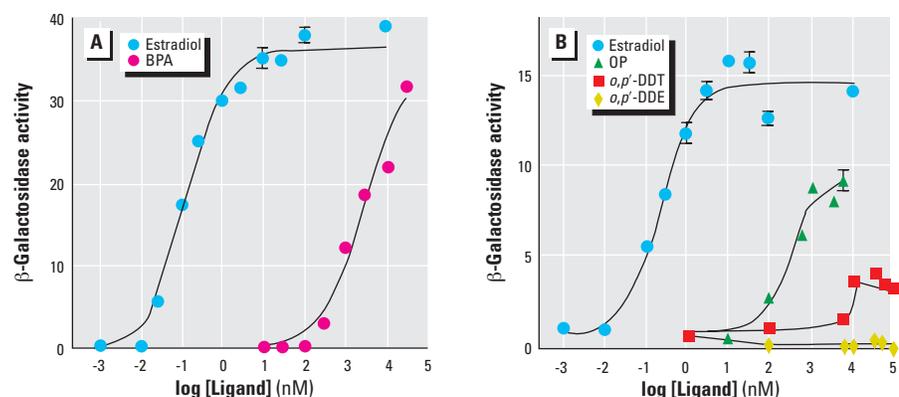
Figure 1. Dose response for estradiol, OP, BPA, o,p´-DDT, and o,p´-DDE in hER two-hybrid yeast. (*A*) Dose response for estradiol (EC_{50} = 0.12 ± 0.01 nM) and BPA (EC_{50} = 3.09 ± 0.05 µM). (*B*) Dose response for estradiol, OP (EC_{50} = 0.43 ± 0.06 µM), o,p´-DDT (EC_{50} = 6.47 ± 0.39 µM), and o,p´-DDE. The curves represent three to four independent experiments. Values are expressed as mean ± SE of individual samples done in triplicate. The EC_{50} values are the mean ± SE of all trials.

Table 1. Summary of the effects of estradiol, OP, BPA, o,p´-DDT, and o,p´-DDE on hER.

Compound	hER two-hybrid yeast				ERE-dependent yeast (plus copper) β-Galactosidase		ERE-dependent yeast with RIP140 (minus copper) β-Galactosidase		K_i values from literature[a]
	β-Galactosidase		Receptor binding						
	EC_{50}	Percent of max	IC_{50}	K_i	EC_{50}	Percent of max	EC_{50}	Percent of max	
Estradiol (nM)	0.12 ± 0.01	100	5.03 ± 0.84	3.98 ± 0.63	1.02 ± 0.31	100	1.28 ± 0.04	100	1.6 (34), 0.4 (30,31,40), 0.8 (32), 28 (26), 11 (26), 0.05 (33)
OP (µM)	0.43 ± 0.06	52.6 ± 5.7	1.72 ± 0.57	1.29 ± 0.41	0.70 ± 0.02	74.3 ± 6.7	0.67 ± 0.01	81.9 ± 4.9	2 (30), 0.4 (31), 0.11 (3)
BPA (µM)	3.09 ± 0.05	101.2 ± 8.5	71.0 ± 2.07	53.38 ± 1.55	2.15 ± 0.25	62.4 ± 0.1	12.16 ± 1.50	53.1 ± 1.2	2 (30), 6.4 (31), 76 (6)
o,p´-DDT (µM)	6.47 ± 0.39	16.3 ± 2.5	>100	>100	23.8 ± 8.74	16.6 ± 3.1	8.17 ± 0.70	16.3 ± 1.6	69 (34), 0.4 (33), 0.3 (41), 12 (32), 120 (26), 13 (26)
o,p´-DDE (µM)	No effect	0.0	>100	>100	No effect	—	No effect	—	10 (41)

max, maximum.
Percent of maximum is the ratio of the maximal β-galactosidase stimulated by each compound divided by the maximal β-galactosidase activity induced by estradiol. Values are expressed as the mean ± SE of at least three independent experiments done in triplicate at each concentration.
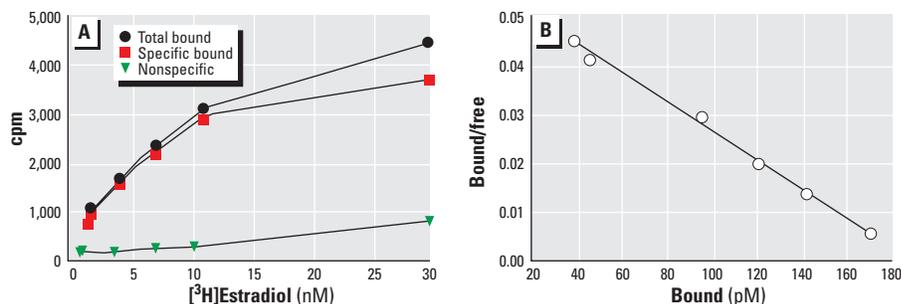[a]When a K_i value was not reported in the cited literature, one was calculated from available IC_{50} data using the Cheng and Prusoff equation (22) and our estradiol K_d value.
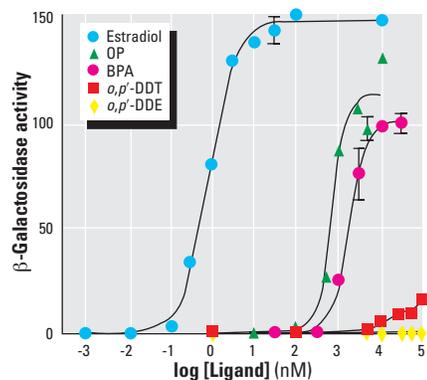
Figure 2. Dose response for estradiol, OP, BPA, *o,p´*-DDT, and *o,p´*-DDE in the ERE-dependent yeast grown in the presence of copper. The curves represent three to four independent experiments, with each point done in triplicate. The EC_{50} values (mean ± SE) are as follows: estradiol (1.02 ± 0.31 nM), OP (0.70 ± 0.02 µM), BPA (2.15 ± 0.25 µM), and *o,p´*-DDT (23.80 ± 0.39 µM).


Figure 3. Saturation binding of [³H]estradiol in hER two-hybrid yeast. cpm, counts per minute. (*A*) Saturation binding study. (*B*) Scatchard analysis. The ligand binding parameters were determined as described in "Materials and Methods." The K_d (3.00 ± 0.24 nM) and B_{max} (9.93 ± 3.50 fmoles/10⁶ cells) values (mean ± SE) represent three separate experiments. Each concentration of [³H]estradiol was run in quadruplicate.

xenoestrogens required to initiate the reporter gene were > 3,000 times that of estradiol. BPA began stimulating levels of β-galactosidase at approximately 100 nM, with maximal gene expression at 30 µM. OP began inducing the reporter gene between 10 and 100 nM, with maximal induction at approximately 1 µM. *o,p´*-DDT first induced hER dimerization at 5 µM, with full induction at approximately 10 µM. The EC_{50} values for OP, BPA, and *o,p´*-DDT were 0.43 ± 0.06 µM, 3.09 ± 0.05 µM, and 6.47 ± 0.39 µM, respectively (Table 1). BPA induced β-galactosidase activity to a level equal to that induced by estradiol, whereas maximal induction by OP or *o,p´*-DDT was 52.6% and 16.3%, respectively, of the estradiol maximum (Table 1). No β-galactosidase activity was observed in the presence of *o,p´*-DDE. Also, when GAL4-DB-hER was expressed in the yeast in the absence of GAL4-TA-hER, no β-galactosidase activity was detected (*13,20*).

Activation of an ERE-lacZ reporter in yeast by environmental estrogens. After determining that xenoestrogens can promote ER–ER dimerization, we sought to investigate whether such a protein–protein interaction is accompanied by ERE-dependent transcriptional activity in the RS188N yeast. This yeast expresses hER under the control of a yeast copper metallothionein promoter and an ERE-dependent *lacZ* reporter. The copper metallothionein promoter allows for regulated expression of hER in yeast by the addition of exogenous copper (*19*). Estradiol initiated *lacZ* production at 100 pM, with maximal induction reached at 10 nM. The EC_{50} value of estradiol was 1.02 ± 0.31 nM. OP, BPA, and *o,p´*-DDT also stimulated hER-dependent gene transcription (Figure 2). OP (EC_{50} = 0.70 ± 0.02 µM) increased β-galactosidase

activity beginning at approximately 100 nM and plateauing at approximately 3 µM. BPA (EC_{50} = 2.15 ± 0.25 µM) stimulated the reporter gene at 300 nM, with maximal stimulation at approximately 10 µM. *o,p´*-DDT (EC_{50} = 23.8 ± 8.74 µM) also stimulated the reporter gene in a dose–dependent manner. At 5 µM, *o,p´*-DDT stimulated β-galactosidase activity, with maximal activity reached between 50 µM and 100 µM. Of the xenoestrogens tested, OP stimulated the highest levels of β-galactosidase activity (74.3% of the maximal estradiol response). As compared to maximal estradiol activity, BPA and *o,p´*-DDT stimulated transcriptional activity to 62.4% and 16.6%, respectively (Table 1), whereas *o,p´*-DDE failed to promote ER-dependent gene transcription (Figure 2). Although OP was the most potent of the xenoestrogens tested, OP was 686 times less potent than estradiol at inducing the ERE-dependent reporter gene. As compared to estradiol stimulation of β-galactosidase, BPA and *o,p´*-DDT were 2,108 and 23,333 times less potent, respectively.

Inhibition of [³H]estradiol binding to hER by environmental estrogens in whole yeast cells. The experiments in the two-hybrid yeast and ERE-dependent yeast systems demonstrated that xenoestrogens could induce an active hER dimer. However, the exact site of binding to the receptor remained unknown. To investigate whether the environmental estrogens interacted with the ligand-binding site of the hER or at another site, a whole cell competition binding assay for yeast was developed using [³H]estradiol and the hER two-hybrid yeast. After determining the optimal assay conditions, we measured the specific binding of [³H]estradiol to hER and performed saturation analysis. Saturation analysis using [³H]estradiol gave a K_d of 3.00 ± 0.24 nM, with a B_{max} (maximum specific binding) of 9.39 ± 3.5 fmoles/10⁶ cells (Figure 3), showing that estradiol can transverse the yeast cell wall and bind to the receptor at physiologic conditions. The K_i values for estradiol and the xenoestrogens were obtained

in the presence of 1 nM [³H]estradiol. OP (K_i value = 1.29 ± 0.41 µM) and BPA (K_i value = 53.38 ± 1.55 µM) were able to compete for the labeled ligand (Figure 4), whereas neither *o,p´*-DDT nor *o,p´*-DDE displaced [³H]estradiol at any concentration tested (up to 140 µM; data not shown). Estradiol had a K_i value of 3.98 ± 0.63 nM. The rank order of ligand binding affinities, highest to lowest, was estradiol >> OP > BPA. This rank order corresponds with their observed potency for stimulating an active hER dimer (Table 1).
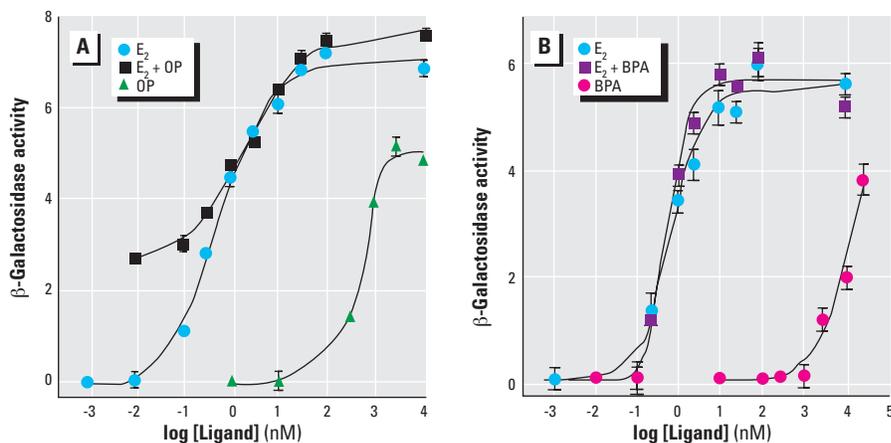
Environmental estrogens do not affect estradiol-induced hER transcriptional activity. The previous experiments have shown that both OP and BPA could, in the absence of estradiol, stimulate ER-dependent gene transcription via binding to the ligand-binding site of the receptor. However, both OP and BPA may be present simultaneously with estradiol in cells. The effect on ERE-regulated gene transcription in the presence of estradiol and OP or BPA is unknown. To address this issue, RS188N yeast were grown in medium lacking exogenous copper, thus limiting the production of hER and maximizing the ability to detect antagonistic or additive effects on the estradiol dose–response curve. The concentration-dependent response of estradiol in RS188N yeast (in the absence of copper) was similar to the response seen when copper was present (compare Figure 5 to Figure 2). We observed a copper-dependent increase in ERE-*lacZ* activity in RS188N yeast (data not shown). The EC_{50} values for estradiol were 0.71 ± 0.07 nM in the absence of copper and 1.02 ± 0.31 nM in the presence of copper. In the absence of exogenous copper, estradiol-induced levels of β-galactosidase were 6–8 RRU, as compared to 100–150 RRU in the presence of copper. In the absence of added copper, EC_{50} values for OP (Figure 5A) and BPA (Figure 5B) were similar, as were their values in the presence of copper (compare Figure 5 to Figure 2). Both OP and BPA stimulated β-galactosidase activity in the absence or presence of exogenous copper to approximately the same percent of maximal
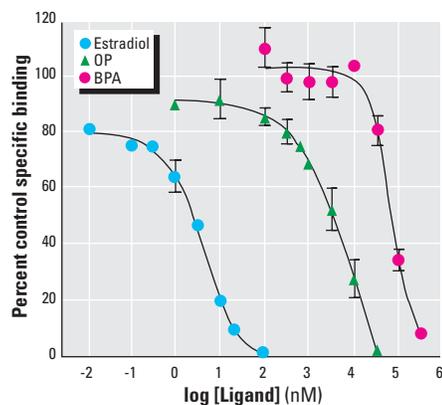
Figure 4. Competition binding studies. Error bars indicate SE. The curves represent three to five independent experiments, with ligand concentrations run in triplicate or quadruplicate. The K_i values (mean ± SE) for estradiol (3.98 ± 0.63 nM), OP (1.29 ± 0.41 μM), and BPA (53.38 ± 1.55 μM) represent three to five separate experiments.

Figure 5. The effect of 1 μM OP (*A*) or 1 μM BPA (*B*) on estradiol (E$_2$) dose response in ERE-dependent yeast grown in the absence of copper. (*A*) Comparison of the OP (1 μM) + E$_2$ curve (EC$_{50}$ = 2.19 ± 0.20 nM) with the E$_2$ curve (EC$_{50}$ = 0.71 ± 0.7 nM) and the OP curve (EC$_{50}$ = 0.44 ± 0.07 μM). (*B*) Comparison of the BPA (1 μM) + E$_2$ curve (EC$_{50}$ = 0.52 ± 0.07 nM) with the E$_2$ curve and the BPA curve (EC$_{50}$ = 13.45 ± 1.94 μM). Values represent three independent experiments, with each concentration run in triplicate. Error bars indicate SE. The EC$_{50}$ values (mean ± SE) represent three independent experiments, except the EC$_{50}$ value for estradiol, which was from seven experiments.

estradiol activity (66.1 ± 4.0% for OP and 73.2 ± 2.9% for BPA in the absence of copper; 74.3 ± 6.7% for OP and 62.4 ± 0.1% for BPA in the presence of copper). Therefore, copper does not appear to affect hER transactivation. When the xenoestrogens OP (1 μM) or BPA (1 μM) were tested in combination with estradiol, no changes in the maximal β-galactosidase activity or the EC$_{50}$ values were observed (Figure 5).

RIP140 amplifies xenoestrogen-induced hER activity. Because xenoestrogens show weak estrogenic activity, we sought to determine if nuclear receptor coactivators would influence ER-mediated transcriptional activity of hER. We chose to determine the effect of RIP140 on xenoestrogen-induced ERE-dependent gene transcription. RIP140 has been shown to interact with the ER in the presence of estradiol, increasing ERE-dependent gene transcription (*13,15*). To determine if RIP140 also can increase xenoestrogen-stimulated hER-dependent gene transcription, we expressed RIP140 in RS188N yeast. Although the basic transcription factors are conserved between yeast and mammalian cells, yeast appear to lack steroid receptor co-modulator proteins (*14*). Therefore, yeast are ideal for studying the effect co-modulators might have on nuclear receptor-mediated transcription. As mentioned above, the ERE-dependent yeast expressing RIP140 were grown in medium without added copper. When RIP140 was coexpressed with hER, the maximum β-galactosidase activity stimulated by estradiol was increased approximately 100-fold over yeast without RIP140 (compare Figure 5 to Figure 6). To investigate if RIP140 could affect xenoestrogen-induced hER transactivation, we repeated the experiment using OP, BPA, and *o,p'*-DDT. As with

estradiol, RIP140 increased the maximum β-galactosidase activity of OP, BPA, and *o,p'*-DDT by 100-fold over yeast without RIP140. The same concentrations of estradiol, OP, BPA, and *o,p'*-DDT are required to initiate and maximally stimulate the reporter gene in the presence or absence of RIP140 (compare Figure 2 to Figure 6). RIP140 did not appear to alter the EC$_{50}$ values for estradiol or OP and thus did not alter the potency of the ligands. However, in the presence of RIP140, the dose–response curve of BPA shifted to the right (compare Figure 2 to Figure 6), resulting in an approximate 6-fold difference between EC$_{50}$ values (Table 1). The EC$_{50}$ for *o,p'*-DDT decreased by approximately 3-fold in the presence of RIP140 (Table 1). In the absence of ligand, RIP140 did not induce β-galactosidase activity. Also, no induction of β-galactosidase activity was observed in the presence of *o,p*-DDE.

Discussion

The objective of this study was to characterize the mechanism by which several man-made nonsteroidal compounds emulate estrogen *in vivo*. Using three yeast genetic systems, we demonstrated that the xenoestrogens OP, BPA, and *o,p'*-DDT exert their estrogen-like activity through the ER.

In the yeast two-hybrid system, all three compounds were able to induce β-galactosidase activity. This required dimerization of the two hER fusion proteins to reconstitute the GAL4 DNA binding protein. These results support the hypothesis that ER dimer formation is ligand dependent (*20,23*). The results with the RS188N yeast showed that the ER dimers formed in the presence of these compounds were able to induce transcription of an ERE-dependent gene. In
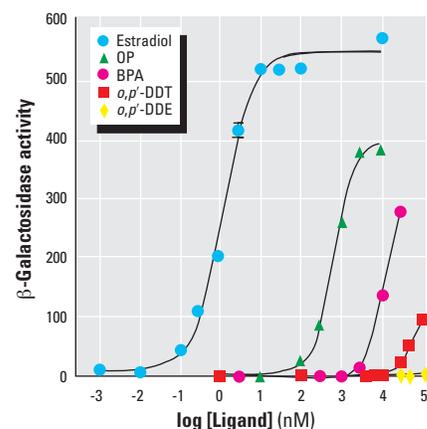
Figure 6. The effect of RIP140 on ligand-induced β-galactosidase activity for estradiol, OP, BPA, *o,p´*-DDT, and *o,p´*-DDE in the absence of copper. The curves represent three independent experiments. Values are expressed as the mean ± SE of each concentration run in triplicate. The EC$_{50}$ values (mean ± SE) are as follows: estradiol (1.28 ± 0.04 nM), OP (0.67 ± 0.01 μM), BPA (12.16 ± 1.50 μM), and *o,p´*-DDT (8.17 ± 0.70 μM).

both studies, OP, BPA, and *o,p'*-DDT induced β-galactosidase activities at concentrations much higher than those required by estradiol for a similar level of enzyme activity. These data are in agreement with *in vitro* and *in vivo* studies which also show that OP, BPA, and *o,p'*-DDT possess only weak estrogen-like activity, including activity in another yeast genetic system (*3,6,24–28*). We also used the RS188N yeast system to determine what effect OP and BPA would have on an estrogen-induced gene, given the possibility that xenoestrogens and estrogen can simultaneously be present in a cell. In our study, neither OP nor BPA had any effect on an estradiol-induced reporter gene.

Similar results were reported by Milligan et al. (29). These investigators measured uterine vascular permeability of ovariectomized mice and found that neither OP nor BPA altered the stimulatory effect of estradiol on vascular permeability.

Because the concentrations of OP, BPA, and o,p'-DDT required to promote hER dimerization and transcription were more than 3,000-times higher than estradiol, we classified these compounds as weak ER agonists. Another explanation for their weak estrogen-like activity could be their inability to cross the yeast cell wall. To address this issue we performed a whole yeast cell receptor binding assay, which showed that OP and BPA do bind to the hormone binding site of the ER. A comparison of K_i values for these compounds from the whole yeast cell-binding assay with K_i values from in vitro binding assays showed the values for estradiol, OP, and BPA to be of a similar magnitude (3,6,30,31). Thus, our results support the idea that OP and BPA are indeed weak ER agonists, and the yeast cell wall does not inhibit these compounds from entering the yeast. Although sufficient concentrations of o,p'-DDT enter our yeast to induce ER dimerization and ERE-dependent gene transcription, our whole yeast cell-binding assay may not be sensitive enough to detect competition between o,p'-DDT and [^{3}H]estradiol for ER binding. This is based on previous cell-free binding studies which demonstrate that o,p'-DDT can displace [^{3}H]estradiol from the ER (26,32–35). Furthermore, it has been demonstrated that o,p'-DDT can transcriptionally activate ER expressed in yeast (25,26,28). As with our o,p'-DDT binding results, there is at least one in vitro study which used spotted seatrout ER that also failed to show any competition of o,p'-DDT with [^{3}H]estradiol (36).

The concentrations of the xenoestrogens required to elicit a response in the yeast genetic systems appear to be difficult to achieve in vivo. However, several reports suggest circumstances under which the local concentrations of xenoestrogens could be increased. Many xenoestrogens are hydrophobic and can accumulate in fatty tissue, reaching physiologically relevant concentrations (37). Therefore, adipose tissue might serve as a xenoestrogen reservoir from which xenoestrogens could be released in concentrations sufficient to stimulate the ER (37). In addition, data suggest that environmental estrogens may not be bound to serum proteins as effectively as estradiol, thus increasing their bioavailibility and relative concentrations (28,31,38,39). Xenoestrogens could have a much greater effect on ER-dependent gene transcription than originally thought in the presence of coactivators such as RIP140.

In our yeast system in which hER and RIP140 are coexpressed, the coactivator dramatically increases ER-dependent gene transcription. The presence of RIP140 increases 17β-estradiol, OP, BPA, and o,p'-DDT induction of ER-dependent gene transcription by 100-fold. Also, there is the possibility that RIP140 may slightly alter the potency of some xenoestrogens such as BPA and o,p'-DDT. As in yeast lacking RIP140, o,p'-DDE did not induce ER-dependent gene transcription in the presence of RIP140. Previously reported data from our laboratory clearly demonstrated that RIP140 directly interacts with the ligand-binding domain of hER in the presence of estradiol (13). Additionally, the F-domain of the hER was shown to play an important regulatory role in the association of RIP140 with ER and in ER homodimerization (13). Using the yeast two-hybrid assay, Nishikawa et al. (40) reported that the xenoestrogen BPA can induce an interaction between the ligand-binding domain of rat ER and the receptor-interacting domain of RIP140. They observed BPA-induced activity with a concentration range and ratio similar to maximum estradiol response, as reported in this study. The ratio of RIP140 to ER has been reported to be critical for the coactivator to influence ER transactivation (14,15,17). Therefore, the effect of xenoestrogens on ER-dependent gene transcription could be much greater in cells expressing coactivators than originally anticipated.

In conclusion, the data presented in this report indicate that the environmental estrogens OP, BPA, and o,p'-DDT posses ER agonist activity. These compounds were able to induce the formation of a transcriptionally active hER dimer, whose activity was further enhanced in the presence of the ER coactivator RIP140. OP and BPA appear to activate the ER through interaction at the estradiol binding site. As a result, exposure to OP, BPA, or o,p'-DDT at sufficient concentrations or in the presence of an ER coactivator could have deleterious effect on normal cell function due to the untimely activation of estrogen-regulated genes.

REFERENCES AND NOTES

1. Tyler CR, Jobling S, Sumpter JP. Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28:319–361 (1998).

2. Bicknell RJ, Herbison AE, Sumpter JP. Oestrogenic activity of an environmentally persistent alkylphenol in the reproductive tract but not the brain of rodents. J Steroid Biochem Mol Biol 54:7–9 (1995).

3. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182 (1994).

4. Bitman J, Cecil HC, Harris SJ, Fries GF. Estrogenic activity of o,p′-DDT in the mammalian uterus and avian oviduct. Science 162:371–372 (1968).

5. Johnson DC, Sen M, Dey SK. Differential effects of dichlorodiphenylltrichloroethane analogs, chlordecone, and 2,3,7,8-tetrachlorodibenzo-p-dioxin on establishment of pregnancy in the hypophysectomized rat. Proc Soc Exp Biol Med 199:42–48 (1992).

6. Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279–2286 (1993).

7. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 240:889–895 (1988).

8. Li H, Chen JD. The receptor-associated coactivator 3 activates transcription through CREB-binding protein recruitment and autoregulation. J Biol Chem 273:5948–5954 (1998).

9. Kalkhoven E, Valentine JE, Heery DM, Parker MG. Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. EMBO J 17:232–243 (1998).

10. Glass CK, Rose DW, Rosenfeld MG. Nuclear receptor coactivators. Curr Opin Cell Biol 9:222–232 (1997).

11. Onate SA, Tsai SY, Tsai M-J, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354–1357 (1995).

12. Nagy L, Kao H-Y, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 89:373–380 (1997).

13. Peters GA, Khan SA. Estrogen receptor domains E and F: role in dimerization and interaction with coactivator RIP-140. Mol Endocrinol 13:286–296 (1999).

14. Joyeux A, Cavaillès V, Balaguer P, Nicolas JC. RIP 140 enhances nuclear receptor-dependent transcription in vivo in yeast. Mol Endocrinol 11:193–202 (1997).

15. Cavaillès V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J 14:3741–3751 (1995).

16. Treuter E, Albrektsen T, Johansson L, Leers J, Gustafsson J-Å. A regulatory role for RIP140 in nuclear receptor activation. Mol Endocrinol 12:864–881 (1998).

17. Harnish DC, Evans MJ, Scicchitano MS, Bhat RA, Karathanasis SK. Estrogen regulation of the apolipoprotein AI gene promoter through transcription cofactor sharing. J Biol Chem 273:9270–9278 (1998).

18. Chevray PM, Nathans D. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc Natl Acad Sci USA 89:5789–5793 (1992).

19. Lyttle CR, Damian-Matsumura P, Juul H, Butt TR. Human estrogen receptor regulation in a yeast model system and studies on receptor agonist and antagonists. J Steroid Biochem Mol Biol 42:677–685 (1992).

20. Wang H, Peters GA, Zeng X, Tang M, Ip W, Khan SA. Yeast two-hybrid system demonstrates that estrogen receptor dimerization is ligand-dependent in vivo. J Biol Chem 270:23322–23329 (1995).

21. Bylund DB, Yamamura HI. Methods for receptor binding. In: Methods in Neurotransmitter Receptor Analysis (Yamamura HI, Enna SJ, Kuhar MJ, eds). New York:Raven Press, 1990;1-35.

22. Cheng YC, Prusoff WH. Relationship between the inhibition constant (K_I) and the concentration of an inhibitor that causes a 50% inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108 (1973).

23. Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145–156 (1988).

24. Abraham EJ, Frawley LS. Octylphenol (OP), an environmental estrogen, stimulates prolactin (PRL) gene expression. Life Sci 60:1457–1465 (1997).

25. Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, McDonnell DP. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 143:205–212 (1997).

26. Chen CW, Hurd C, Vorojeikina DP, Arnold SF, Notides AC. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem Pharmacol 53:1161–1172 (1997).

27. Routledge EJ, Sumpter JP. Structural features of alkylphenolic chemicals associated with estrogenic activity. J Biol Chem 272:3280–3288 (1997).

28. Arnold SF, Robinson MK, Notides AC, Guillette LJ Jr, McLachlan JA. A yeast estrogen screen for examining

the relative exposure of cells to natural and xenoestrogens. Environ Health Perspect 104:544–548 (1996).

29. Milligan SR, Balasubramanian AV, Kalita JC. Relative potency of xenobiotic estrogens in an acute *in vivo* mammalian assay. Environ Health Perspect 106:23–26 (1998).

30. Dodge JA, Glasebrook AL, Magee DE, Phillips DL, Sato M, Short LL, Bryant HU. Environmental estrogens: effects on cholesterol lowering and bone in the ovariectomized rat. J Steroid Biochem Mol Biol 59:155–161 (1996).

31. Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 105:70–76 (1997).

32. Danzo BJ. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ Health Perspect 105:294–301 (1997).

33. McBlain WA. The levo enantiomer of o,p´-DDT inhibits the binding of 17β-estradiol to the estrogen receptor. Life Sci 40:215–221 (1987).

34. Hammond B, Katzenellenbogen BS, Krauthammer N, McConnell J. Estrogenic activity of the insecticide chlordecone (kepone) and interaction with uterine estrogen receptors. Proc Natl Acad Sci USA 76:6641–6645 (1979).

35. Forster MS, Wilder EL, Heinrichs WL. Estrogenic behavior of 2(o-chlorophenyl)-2-(p-chlorophenyl)-1,1,1-trichloroethane and its homologues. Biochem Pharmacol 24:1777–1780 (1975).

36. Thomas P, Smith J. Binding of xenobiotics to the estrogen receptor of spotted seatrout: a screening for potential estrogenic effects. Mar Environ Res 35:147–151 (1993).

37. Bigsby RM, Caperell-Grant A, Madhukar BV. Xenobiotics released from fat during fasting produce estrogenic effects in ovariectomized mice. Cancer Res 57:865–869 (1997).

38. Crain DA, Noriega N, Vonier PM, Arnold SF, McLachlan JA, Guillette LJ Jr. Cellular bioavailability of natural hormones and environmental contaminants as a function of serum and cytosolic binding factors. Toxicol Ind Health 14:261–273 (1998).

39. Nagel SC, vom Saal FS, Welshons WV. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies estrogenic activity. Proc Soc Exp Biol Med 217:300–309 (1998).

40. Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol Appl Pharmacol 154:76–83 (1999).

41. Nelson JA. Effects of dichlorodiphenyltrichloroethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17β-[³H]estradiol binding to rat uterine receptor. Biochem Pharmacol 23:447–451 (1974).