DDT and Its Metabolites Alter Gene Expression in Human Uterine Cell Lines through Estrogen Receptor-Independent Mechanisms

Daniel E. Frigo,[1,2] Matthew E. Burow,[2,3] Kamron A. Mitchell,[2,4] Tung-Chin Chiang,[2] and John A. McLachlan[2,5]

[1]Molecular and Cellular Biology Program, [2]Center for Bioenvironmental Research, [3]Department of Medicine, [4]School of Public Health and Tropical Medicine, and [5]Department of Pharmacology, Tulane University Health Science Center, New Orleans, Louisiana, USA

Endocrine-disrupting organochlorines, such as the pesticide dichlorodiphenyltrichloroethane (DDT), bind to and activate estrogen receptors (ERs), thereby eliciting estrogen-like effects. Although ERs function predominantly through activation of transcription via estrogen-responsive elements, both ERs, α and β, can interact with various transcription factors such as activator protein-1 (AP-1). Additionally, estrogens may regulate early signaling events, suggesting that the biological effects of environmental estrogens may not be mediated through classic ER (α and β) activity alone. We hypothesized that known environmental estrogens, such as DDT and its metabolites, activate AP-1–mediated gene transactivation through both ER-dependent and ER-independent means. Using two Ishikawa human endometrial adenocarcinoma cell line variants that we confirmed to be estrogen responsive [Ishikawa(+)] and estrogen unresponsive [Ishikawa(–)], we generated stably transfected AP-1 luciferase cell lines to identify the role of an estrogen-responsive mechanism in AP-1–mediated gene expression by various stimuli. Our results demonstrate that DDT and dichlorodiphenyldichloroethane (DDD) were the most potent activators of AP-1 activity; 2,2-bis(*p*-chlorophenyl) acetic acid failed to activate. Although stimulated in both Ishikawa(+) and Ishikawa(–) cells by DDT and its congeners, AP-1 activation was more pronounced in the estrogen-unresponsive Ishikawa(–) cells. In addition, DDT, DDD, and dichlorodiphenyldichloroethylene (DDE) could also stimulate AP-1 activity in the estrogen-unresponsive human embryonic kidney 293 cells using a different promoter context. Thus, our data demonstrate that DDT and its metabolites activate the AP-1 transcription factor independent of ER (α or β) status. *Key words:* **AP-1, DDT, early signaling, estrogen receptor, Ishikawa cell line, organochlorines.** *Environ Health Perspect* **110:1239–1245 (2002). [Online 28 October 2002]**
http://ehpnet1.niehs.nih.gov/docs/2002/110p1239-1245frigo/abstract.html

Extracellular signals such as growth factors, hormones, and environmental compounds elicit an array of effects on a cell that include proliferation, differentiation, stress, and death (*1–3*). In many cases, external stimuli signal a cell, resulting in the activation or inhibition of transcription factors such as activator protein-1 (AP-1) and nuclear factor κB (NFκB), which form complexes and regulate gene expression (*4,5*). The resultant change in gene expression leads to an overall effect on the cell, dictating its final interpretation of the external cue. Determining what signals target transcription factors and how they do so is key to understanding the specificity of gene expression and resultant biological responses.

AP-1 is a generic term used to describe transcription factors that bind specifically to a DNA enhancer sequence [TGA(G/C)TCA] called the 12-*O*-tetradecanylphorbol-13-acetate responsive element (TRE), which is also referred to as the AP-1 site. In addition, AP-1 components can bind and potentiate transcription from AP-1–related DNA elements (e.g., cAMP response element) (*6*). Members of the Jun and Fos families of proteins dimerize to preferentially bind AP-1 sites with high affinity; hence, each dimer combination makes up an AP-1 protein. Upon stimulation, Jun and Fos proteins recruit p300/CBP coactivators that recruit other coactivators such as the p160s, which directly bind nuclear hormone receptors like the estrogen receptor (ER) (*7*). p300/CBP helps stimulate gene expression by two major mechanisms: making direct interactions with core RNA polymerase machinery and using their intrinsic histone acetylase activity to unravel chromatin structure. Both mechanisms work toward ultimately recruiting the RNA polymerase complex to the sites of AP-1–regulated genes (*8*). Conversely, AP-1–mediated gene expression is ablated when Jun or Fos family members associate with co-repressors such as silencing mediator of retinoic acid and thyroid hormone receptor, which is known to interact with Sin3 and recruit histone deacetylases, condensing chromatin and terminating transcription (*9*). AP-1 is a ubiquitous protein that can be induced by multiple stimuli, leading to diverse cellular effects. For example, proliferation, differentiation, cellular stress, and death have all been associated with elevated AP-1 activity (*5,10–15*). Hence, AP-1 appears to play a diverse role in the regulation of the cell cycle. Cell type, promoter context, associative proteins, and stimuli are factors that cumulatively will determine the effect AP-1 has on a cell.

Endocrine disruptors represent a class of both natural and synthetic compounds that exhibit hormonal activity. Organochlorines, such as the pesticide dichlorodiphenyltrichloroethane (DDT) and its metabolites, have been shown to mimic estrogen, binding to and activating the ERs, thereby often producing estrogen-like effects (*2,16–21*). DDT and its metabolites have displayed harmful feminizing effects on wildlife such as birds (*22,23*), fish (*22,24*), and reptiles (*22,25–27*), altering endogenous hormone levels (*25,26*), elevating estrogen-regulated protein levels (*24*), and reducing male phallus size (*25,26*). These reports in wildlife have led to the suggestion that organochlorines may alter a number of harmful estrogen-regulated health effects in humans such as breast cancer (*1,28–30*), endometriosis (*1,31–33*), spontaneous abortion (*34*), reduced bone mineral density (*35*), and decreased sperm counts (*36*). However, many of these reports are still controversial.

Although estrogen-like molecules are thought to function predominantly through ER-mediated activation of transcription via estrogen-responsive elements (EREs), both ERs, α and β, can interact with various cell-cycle transcription factors such as AP-1. The nature of the ER–AP-1 interaction depends on the ligand, ER subtype, and AP-1 components (*37–39*). Additionally, the ER can regulate AP-1 at the level of its gene expression by promoting transcription of the c-*fos* gene in the presence of the major endogenous estrogen, 17β-estradiol (E_2) (*40–44*). The cross-talk between the pathways modulating the ER and those modulating the AP-1 transcription factor introduces another level of complexity in determining the effects of hormonally active compounds.

Several environmental compounds that have estrogenic activity affect various signal transduction pathways. For example, at low concentrations the isoflavone genistein can

Address correspondence to J.A. McLachlan, Center for Bioenvironmental Research, Tulane University, 1430 Tulane Avenue SL-3, New Orleans, LA 70112 USA. Telephone: (504) 585-6910. Fax: (504) 585-6428. E-mail: john.mclachlan@tulane.edu

We thank J.-Å. Gustafsson for generously proviiding the pSG5-hERβ plasmid and D.M. Klotz for providing the Ishikawa(+) cells. We also thank C.C. Gulledge for critically reading the manuscript.

This work was supported by U.S. Department of Energy grant DE-FC26-00NT40843 (J.A.M.), Office of Naval Research grant N00014-99-1-0763 (J.A.M., M.E.B.), and the Cancer Association of Greater New Orleans (D.E.F.).

Received 6 December 2001; accepted 17 May 2002.

bind to and activate ERs, but at high concentrations it binds to and inhibits receptor tyrosine kinase activity (*2*). Organochlorine compounds, such as DDT, the polychlorinated biphenyls 2′,4′,6′-trichloro- and 2′,3′,4′,5′-tetrachloro-4-biphenylol, β-hexachlorohexane, and heptachlor, activate multiple kinase pathways (*45–52*). The weakly estrogenic polychlorinated biphenyl Arochlor 1254 has been reported to induce AP-1 activity (*53,54*). In neurons, the endocrine disruptor β-hexachlorohexane up-regulates expression of c-*fos* (*55*). Methoxychlor (a member of the DDT family), catechol estrogens, and the organochlorine kepone can stimulate expression of estrogen-responsive genes in ERα-knockout mice treated with ICI 182,780 (*56,57*). Additionally, catechol estrogens promote the formation of breast tumors in ERα-knockout mice that do not express ERβ in the mammary tissue, indicating the involvement of a non-ER (α or β) mechanism (*58*). Thus, compounds that can induce estrogenic responses may alter kinase signal transduction pathways via ER-dependent and/or ER-independent mechanisms, working through multiple mitogenic pathways. Hence, endocrine disruptors may cause adverse health effects due primarily to their pleiotropic nature rather than a single endocrine-altering event.

Given the ability of the ER to interact with AP-1 signaling and estrogenic compounds to elicit early signaling events, we hypothesized that known estrogenic organochlorines, such as DDT and its metabolites, could induce AP-1–mediated gene expression. To do this, we wanted to develop a cell culture system that would enable us to quickly and efficiently determine what role, if any, the ER has in AP-1–mediated signaling. This would allow us to explore whether the estrogenicity of certain compounds was responsible for their ability to regulate AP-1 activity or ER-independent mechanisms were additionally involved.

Here, we show that the persistent pesticide DDT, along with its metabolites, activates AP-1–mediated gene expression at environmentally relevant concentrations (i.e., 10–50 μM) (*59–61*). Using a novel, stable-reporter gene–cell system developed in our laboratory that includes the use of estrogen-responsive and estrogen-unresponsive cell variants, we show the activation is independent of classic ER-mediated mechanisms. This effect is also seen in other cell lines under different AP-1 promoter contexts. Collectively, this demonstrates another level of complexity regarding the nuclear signaling of environmental chemicals. Although hormonal activity may explain some of the adverse health effects of xenobiotics, the total effect of these exogenous chemicals will likely be the end result of multiple signaling pathways that are stimulated due to the pleiotropic nature of many environmental compounds.

Materials and Methods

Chemicals. 2,2-bis(*o,p*-dichlorophenyl)-1,1,1-trichloroethane (*o,p′-DDT*), 2,2-bis(*p,p*-chlorophenyl)-1,1,1-trichloroethane (*p,p′-DDT*), 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p′-DDD*), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′-DDD*), 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-phenyl)ethylene (*o,p′-DDE*), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p′-DDE*), and 2,2-bis(*p*-chlorophenyl) acetic acid (*p,p′-DDA*) were purchased from AccuStandard (New Haven, CT). All DDT metabolites were dissolved in DMSO. E_2 and tetradecanoyl-13-phorbol acetate (PMA) were purchased from Sigma (St. Louis, MO) and dissolved in DMSO or Dulbecco's Modified Eagle's Medium (DMEM), respectively. ICI 182,780 was purchased from TOCRIS (Ballwin, MO) and dissolved in DMSO.

RT-PCR analysis of ERα and ERβ expression. Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed as previously described (*62*). Briefly, RNA was extracted using an Ultraspec RNA isolation kit (Biotecx Lab, Houston, TX). Frozen cell pellets (1×10^6 cells) were lysed in 1 mL Ultraspec RNA solution. Then 0.2 mL chloroform was added to the cell lysate and shaken. After centrifugation, the colorless upper aqueous phase was transferred to a new tube. An equal volume of isopropanol was added, and the RNA was precipitated by centrifugation. The RNA was washed with 75% ethanol and recovered in water treated with diethylene pyrocarbonate. We checked RNA purity by gel electrophoresis and optical density ratio (data not shown). RT-PCR was done using a Perkin-Elmer AmpliTaq Gold with GeneAmp RNA PCR kit following the manufacturer's instructions. Primers were synthesized from Invitrogen (Carlsbad, CA). Primers used to amplify the ERα were: 5′-TGC CAA GGA GAC TCG CTA-3′ (nucleotides 894–912) and 5′-TCA ACA TTC TCC CTC CTC-3′ (nucleotides 1139–1157), giving an amplified product of 263 bp. For ERβ, primer sequences were: 5′-TTC CCA GCA ATG TCA CTA ACT-3′(nucleotides 33–53) and 5′-TCT CTG TCT CCG CAC AAG G-3′ (nucleotides 539–558), giving an amplified product of 525 bp. Fragments of glyceraldehyde phosphate-3-dehydrogenase (GADPH) were amplified in parallel to serve as an internal control. The primers for GADPH were: 5′-TC ACC ATC TTC CAG GAG C-3′ and 5′-CAA GAA GGT GGT GAA GCA G-3′, giving a PCR product of 571 bp. PCR reactions were run for 30 cycles, which were in the linear range of the reaction (data not shown). PCR products were verified by subcloning and sequencing (data not shown).

Cell culture, transient transfection, and reporter gene assay. Ishikawa endometrial adenocarcinoma cells were grown in Iscove's Modified Dulbecco's Medium: Ham's F12 (1:1) supplemented with 10% fetal bovine serum (FBS), Basal Medium Eagle (BME) amino acids, Minimum Essential Medium Eagle (MEM) nonessential amino acids, sodium pyruvate, penicillin–streptomycin, and 1×10^{-10} M insulin. Human embryonic kidney (HEK) 293 cells were grown as previously reported in DMEM supplemented with 10% FBS, BME amino acids, MEM nonessential amino acids, sodium pyruvate, penicillin-streptomycin, and 1×10^{-10}M insulin (*63*). Cultures of cells were transferred to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS), BME amino acids, MEM nonessential amino acids, sodium pyruvate, and penicillin-streptomycin for 48–72 hr before plating. Cells were plated at a density of 2×10^6 cells/well and maintained for an additional 24 hr in DMEM with DCC-FBS. For estrogen dose–response assays, cells were then transfected for 5 hr with 200 ng of pERE2x-luciferase plasmid with or without 50 ng of pcDNA3.1-hERα or pcDNA3.1-hERβ (485 bp) using Effectene transfection reagent (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. For all luciferase assays, we then incubated cells for 18–24 hr in DMEM with DCC-FBS in the presence of vehicle or various chemicals as previously described (*64*). When double treatments were performed, E_2 or *o,p′-DDT* was added 20 min after the ICI 182,780 treatment, or various concentrations of DDT metabolites were treated 20 min before adding 20 ng/mL PMA. We used PMA as a positive control because we have previously shown that 20 ng/mL PMA activates protein kinase C, downstream mitogen-activated protein kinases, and AP-1 (*65*). The concentration of ICI 182,780 used inhibits E_2-stimulated gene expression (*66*). In our results we have shown the data from treatments using 10–50 μM DDT and its metabolites, which gave significant activity and are environmentally relevant concentrations (*59–61*). Cells were then harvested and luciferase activity was measured using 30 μL of cell extract and 100 μL of luciferase assay substrate (Promega, Madison, WI) in a monolight 2010 luminometer. The data shown are an average of at least three independent experiments with 2–4 replicates.

Generation of stably transfected cell lines. Cells were transfected for 5 hr with either 10 μg of pAP-1(PMA)-luciferase (Clontech, Palo Alto, CA; Ishikawa cell variants) or with pAP-1-luciferase (Stratagene, La Jolla, CA;

HEK 293 cells) along with 1 μg of the neomycin-resistance gene expression vector pcDNA3.1 plasmid using Lipofectamine lipofection reagent (Life Technologies, Gaithersburg, MD) in 75-cm^2 flasks. Forty-eight hours after transfection, we switched cells to a medium containing 400–1,200 μg/mL G418 (Mediatech, Herndon, VA) and maintained them in this medium for 3 weeks until the disappearance of all cells in a control, nontransfected flask and appearance of colonies in the transfected flasks. Colonies were pooled, grown, and tested for luciferase activity by incubation with 20 ng/mL PMA for 24 hr.

Statistical analysis. We analyzed data using one-way analysis of variance and post-hoc Tukey's multiple comparisons with GraphPad Prism, version 3.02 (GraphPad Software, Inc., San Diego, CA). We separated the data of each Ishikawa stable cell line into two groups to compare fold activation over negative control (DMSO) and potentiation/inhibition of PMA-induced activity to explore the effects of organochlorines on a tumor promoter-stimulated cascade. Because of large differences in activation in some cases, we used a natural log transformation to obtain normal distribution. Statistically significant changes were determined at the $p < 0.05$ or < 0.001 level as indicated for each figure or table.

Results

Ishikawa endometrial cell variants show difference in ERα expression. To determine the difference between the two cell variants, we examined endogenous ERα and ERβ expression. RT-PCR analysis revealed that ERα was detected in Ishikawa(+) cells, but not in Ishikawa(–) cells (Figure 1, lanes 5 and 6). This effect was evident even after 45 cycles of PCR (i.e., the plateau stage; data not shown). ERβ was detected at low levels in both cell variants (Figure 1, lanes 10 and 11). For a biological control comparison, ERα and ERβ expression was demonstrated in other cell lines previously described by our lab and others [e.g., ERα+, ERβ– (MCF-7L) (*62,67*) and ERα–, ERβ+ (MDA-MB-231) (*62,67–69*), (MCF-7ADR) (*62,67*)].

Ishikawa(+) cells, unlike Ishikawa(–) cells, demonstrate estrogen-mediated gene expression. The difference in estrogen response was measured using a reporter gene assay in which an ERE-luciferase reporter plasmid was transiently transfected into the two Ishikawa cell variants alone or in combination with an hER(α or β) expression plasmid (Figure 2). Increasing concentrations of E_2 stimulated luciferase activity in Ishikawa(+) cells (Figure 2A), but not in Ishikawa(–) cells, even at 10 nM (Figure 2B). In all experiments where E_2 stimulated luciferase activity, the pure antiestrogen ICI 182,780 (100 nM) inhibited the effects of 10 nM E_2 (data not shown). The responsiveness of the Ishikawa(–) cells could be restored by cotransfection with either an hERα or hERβ expression plasmid, although, overexpression with hERβ consistently produced a lower activity profile. On the other hand, hERβ expression slightly inhibited the endogenous response of Ishikawa(+) cells (Figure 2A). This phenomenon is consistent with what other laboratories have reported and is a result of the difference in endogenous ER expression (*70–73*). The endogenous low ERβ expression was unable to transmit an estrogenic response (Figure 2B). Hence, the difference in estrogen response between the two Ishikawa cell variants is caused by a loss of ERα expression.

DDT and its metabolites stimulate AP-1–mediated gene expression in both Ishikawa cell variants. To examine the effects of the ER on AP-1–mediated gene expression by environmental compounds, we generated from our two Ishikawa cell variants stable cell lines containing an AP-1–responsive promoter linked to a luciferase reporter gene. This allowed us to quickly and efficiently test related cells that varied in their response to estrogen for AP-1–induced activity by various stimuli. Treatment of the estrogen-responsive Ishikawa(AP-1)+ stable cells produced a significant induction of AP-1 activity by all the DDT, DDD, and DDE compounds in a dose-dependent manner at the environmentally relevant concentrations 25 μM and 50 μM (Figure 3A) with 50 μM *o,p′-DDT* being the most potent (4.2-fold). In addition, ICI 182,780 (100 μM) potentiated *o,p′-DDT* (50 μM)-induced AP-1 activity 2.6 ± 0.97-fold in Ishikawa(AP-1)+ cells (data not shown), consistent with the findings that ICI 182,780 potentiates AP-1–mediated gene expression (*38*). Surprisingly, however, when the estrogen-unresponsive Ishikawa(AP-1)– cells were treated with *o,p′-DDT* and *o,p′-DDD*, we observed a potent activation of AP-1 of roughly 15.5-fold (Figure 3B). This result and the fact that *o,p′-DDD* has virtually no ER binding activity (*19,21*), indicates a non-ER–mediated AP-1 signaling mechanism. *p,p′-DDA*, a metabolite found in humans, had no effect on AP-1 activity. When organochlorine compounds were administered in conjunction with the phorbol ester PMA and compared to percent activation by PMA, potentiation of AP-1 activity was seen in both cell variants, with the Ishikawa(AP-1)– cells once again demonstrating higher-fold stimulation (Figure 3C, D). *o,p′*DDD in conjuction with PMA gave the greatest AP-1 stimulation at 50 μM in both the Ishikawa(AP-1)+ (7.6-fold) and Ishikawa(AP-1)– (38.1-fold) variants. Again, *p,p′-DDA* did not significantly increase AP-1 activity.

DDT and its metabolites stimulate AP-1–mediated gene expression in HEK 293 cells under a different AP-1–responsive promoter context. Next, we further tested this effect in an additional estrogen-unresponsive cell line using a different AP-1–responsive promoter context. HEK 293 cells, which have been shown to be estrogen unresponsive (*63,64,74*)

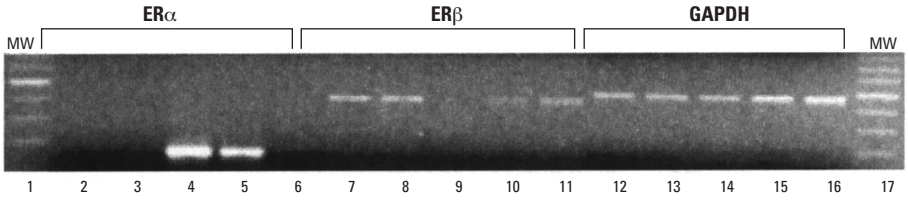

Figure 1. RT-PCR characterization of cell types. MBA-MD-231 (lanes 2, 7, and 12), MCF-7ADR (lanes 3, 8, and 13), MCF-7L (lanes 4, 9, and 14), Ishikawa(+) (lanes 5, 10, and 15), and Ishikawa(–) (lanes 6, 11, and 16) were subjected to RT-PCR using primers specific for hERα (lanes 2–6), hERβ (lanes 7–11), or GAPDH (lanes 12–16). Lanes 1 and 17 are size standards.

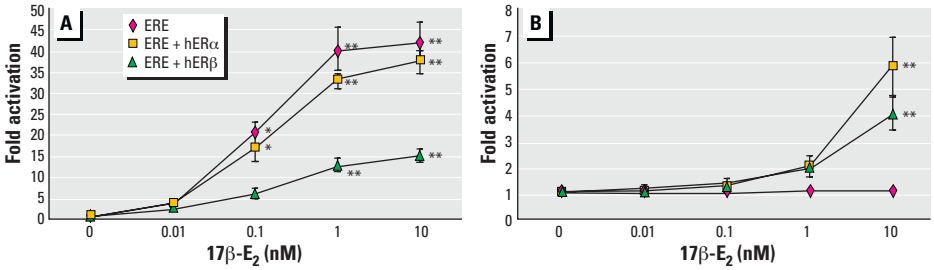

Figure 2. Characterization of estrogen-responsive [Ishikawa(+)] and estrogen-unresponsive [Ishikawa(–)]] cell variants. (*A*) Ishikawa(+) or (*B*) Ishikawa(–) cells were transiently transfected with 200 ng ERE-luciferase reporter plasmid alone or ERE-luciferase reporter plus 50 ng hER(α or β) expression plasmid. Cells were then treated with increasing concentrations of E_2. Luciferase activity was measured as described in "Materials and Methods." Results are expressed as fold activation over vehicle. The data shown are the results of at least three experiments with two replicates each.
*$p < 0.05$, **$p < 0.001$; significant increases from vehicle.

were used to make stable cell lines with a different AP-1-luciferase reporter construct integrated into the genome as confirmed above. As with both Ishikawa cell lines, the DDT, DDD, and DDE metabolites in conjunction with PMA all stimulated AP-1 significantly versus control, whereas *p,p´-DDA* could not induce an effect (Figure 4). Additionally, significant increases were detected at 10 µM for all activating compounds tested, raising the possibility that further testing of multiple cell lines or AP-1–responsive promoters could reveal effects of these compounds at yet lower concentrations. Unlike the Ishikawa cell variants, *o,p´-DDD* displayed lower activity as compared to both *o,p´-DDT* and *p,p´-DDE* in combination with PMA (542, 1,449, and 978% vehicle plus PMA, respectively). A summary of DDT metabolite regulation of AP-1 is shown in Table 1. DDT and its metabolites DDD and DDE are able to consistently activate an AP-1–responsive promoter and potentiate PMA-induced AP-1 activity in multiple cell lines independent of ER status and under different promoter contexts.

Discussion

Organochlorines represent a class of environmental compounds characterized by a chlorinated hydrocarbon backbone motif. These chemicals are found in pesticides, plastics, and industrial wastes and are ubiquitous environmental pollutants (*1,2*). DDT, one of most widely used pesticides until 1973, was banned because of its adverse effects on wildlife (*1,75*). However, this chemical is still used in many developing countries today and exists at high concentrations along with its metabolites and contaminants throughout the world, including the United States, because of long half-lives in soil, water, and the adipose tissue of animals (*24,28,30,75–77*).

Although DDT and some of its metabolites bind ERs (*16,19,21*), induce egg-shell thinning in raptors (*78,79*), and induce estrogen-like effects in exposed animals and humans (*1,22,25*), the mechanisms of action of this class of synthetic compounds is still not completely understood. Here we have shown that DDT can directly affect the activity of AP-1, an important cell regulatory factor.

To determine whether DDT and its metabolites induced AP-1 activation through an ER-mediated mechanism, we created human endometrial adenocarcinoma Ishikawa stable cell lines containing an AP-1–responsive promoter linked to a luciferase reporter gene. This novel cell system allowed us to evaluate rapidly the signaling effects of a number of environmental compounds. Our Ishikawa variants provide us with the cell culture equivalent of a comparison system similar to the wild-type versus ER-knockout mice. Treatment of the Ishikawa(AP-1)+ cells revealed that the DDTs, DDDs, and DDEs could all significantly stimulate AP-1 activity, with the DDTs and DDDs having the greatest activity on average. Although the DDTs have been demonstrated to have estrogenic activity (*1,19,21*), and hence, may work though an ER–AP-1–dependent pathway, the nonestrogenic DDDs also stimulated AP-1–mediated gene expression, indicating an alternative signaling mechanism for this class of compounds. Also, not only did treatment of the Ishikawa(AP-1)– cells activate AP-1, but the DDTs appeared to be more potent in this cell line, indicating that DDT and its metabolites can signal AP-1 in an ER-independent mechanism. Again, DDTs and DDDs appeared most potent, whereas in both cell lines the metabolite *p,p´-DDA* gave no significant induction. *p,p´-DDA*, unlike the DDT, DDD, and DDE isoforms, possesses no central chlorines but only a carboxylic acid motif, suggesting the chlorine residues are necessary.

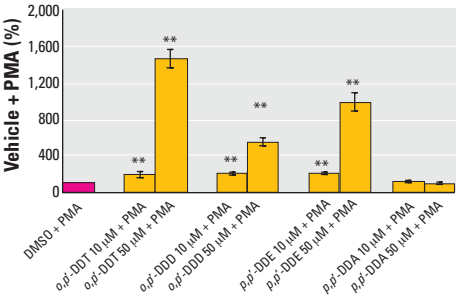

Figure 4. AP-1 induction by DDT metabolites in HEK 293 cells. HEK 293 cells containing a stably integrated AP-1-Luc response element were treated for 18–24 hr with vehicle (DMSO) or various DDT metabolites with 20 ng/mL PMA. Luciferase activity was measured as described in "Materials and Methods." Results are expressed as percent DMSO + PMA activity. The data shown are the results of at least three experiments with two replicates each.
**$p < 0.001$; significant increases from control.

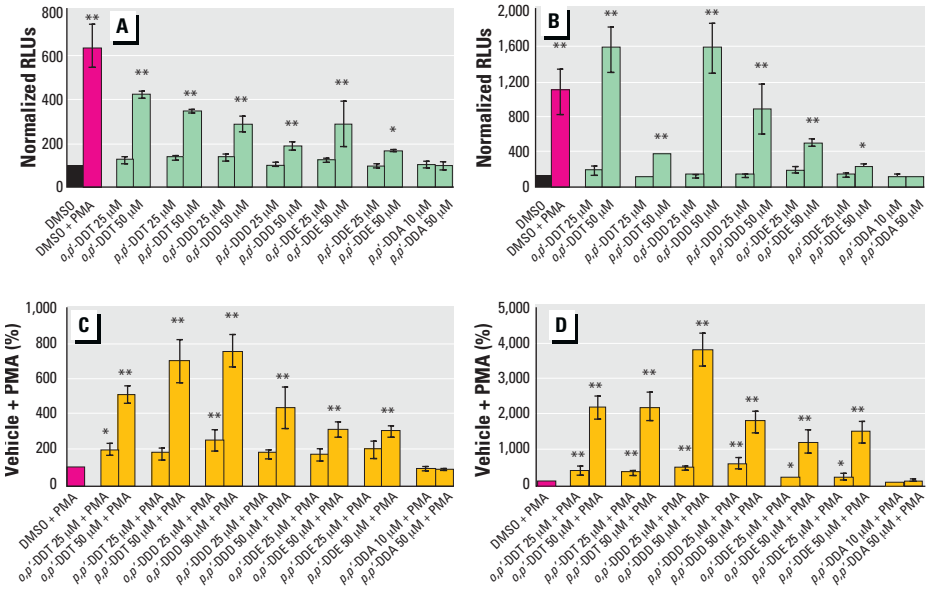

Figure 3. AP-1 induction by DDT metabolites in Ishikawa cells. (*A,C*) Ishikawa(+) and (*B,D*) Ishikawa(–) cells containing a stably integrated AP-1-Luc response element were treated for 18–24 hr with vehicle (DMSO) or various DDT metabolites with (*C,D*) or without (*A,B*) 20 ng/mL PMA. Luciferase activity was measured as described in "Materials and Methods." Results are expressed as relative light units (RLUs) normalized to vehicle (*A,B*) or percent DMSO + PMA activity (*C,D*). The data shown are the results of at least three experiments with two replicates each.
*$p < 0.05$, **$p < 0.001$; significant increases from control.

Table 1. Comparison of AP-1 activation by DDT metabolites.

Treatment	Ishikawa(+)	Ishikawa(–)
DMSO	22.9#	17.0#
o,p´-DDT	102.7*	71.3*
p,p´-DDT	100.0*	38.7
o,p´-DDD	77.7*	126.3*
p,p´-DDD	60.7*	91.0*
o,p´-DDE	72.3*	21.7#
p,p´-DDE	41.3*#	14.5#
p,p´-DDA	23.5#	13.5#
PMA	100.0*	100.0*
o,p´-DDT + PMA	511.3*#	2176.0*#
p,p´-DDT + PMA	701.7*#	2200.3*#
o,p´-DDD + PMA	759.3*#	3810.7*#
p,p´-DDD + PMA	434.0*#	1765.7*#
o,p´-DDE + PMA	312.0*#	1195.0*#
p,p´-DDE + PMA	303.7*#	1506.3*#
p,p´-DDA + PMA	79.7*	106.0*

Chemicals were tested for induction of AP-1 activity with or without 20 ng/mL PMA. Concentrations shown are 50 µM for all DDT metabolites. Data shown are the means from at least three different experiments with two replicates each and are represented as percent PMA activity.
*Significantly different from control (DMSO) ($p < 0.05$).
#Significantly different from 20 ng/mL PMA treatment ($p < 0.05$).

Often, late-stage endocrine cancers grow in the absence of hormones (*80–82*), indicating the potential up-regulation of some other cell survival/cell signaling mechanisms. Because our estrogen-unresponsive cells give a stronger AP-1 activation, it is possible that the AP-1 signaling mechanisms have been up-regulated to compensate for the loss of responsiveness to estrogen. Regardless of which variant is tested, treatment of cells in combination with the tumor promoter PMA revealed that DDT and its metabolites potentiate AP-1 activity well beyond PMA alone. This effect could also be seen in HEK 293 cells using a different AP-1–responsive promoter, indicating that DDTs additionally stimulate AP-1 in both other estrogen-unresponsive cell lines and under different promoter contexts, demonstrating the prevalence and diversity of this environmental signaling process. Significant increases in AP-1 activity were seen at lower concentrations in the HEK 293 cells, suggesting that different cell or AP-1 promoter contexts would reveal that the DDTs could signal AP-1 at more widespread concentrations. The potentiation could signify the involvement of a DDT–AP-1 signaling cascade different from tumor promoter-induced AP-1 activation. Future experiments will determine the kind of signal (i.e., proliferation, stress, etc.) these organochlorines are sending to the cell and how they are signaling AP-1.

Recently, compounds of the DDT family have been reported to affect a number of AP-1 regulated genes *in vivo*. Diel et al. (*83*) reported that ovariectomized rats treated with *o,p′-DDT* reduced the expression of clusterin, which plays an important role in apoptosis, similar to AP-1. The promoter region of the clusterin gene contains two functional AP-1 sites (*84*). In this situation, *o,p′-DDT* could potentiate members of the Jun and Fos families of proteins that have more inhibitory effects toward AP-1–regulated gene expression (e.g., JunB, Fra-1, Fra-2) and hence inhibit expression of the clusterin gene, leading to a more proliferative phenotype. This would help explain the results demonstrating the ability of *o,p′-DDT* to significantly increase uterine growth similar to E_2, despite poorly binding to the ER and displaying a different gene expression pattern in the uterus than E_2 (*83,85*). Ghosh et al. (*56*) demonstrated in ERα-knockout mice that methoxychlor, but not E_2, could still stimulate estrogen responsive lactoferrin and glucose-6-phosphate dehydrogenase mRNAs in the mouse uterus despite the addition of the pure antiestrogen ICI 182,780, indicating a non-ER (α or β) mechanism. In addition, Das et al. (*86*) demonstrated that mice treated with *p,p′-DDD*, which has no ER-binding ability, also increases lactoferrin expression, regardless of the presence of ICI 182,780. Both the lactoferrin and glucose-6-phosphate dehydrogenase genes contain complex promoters that are responsive to multiple stimuli, such as epidermal growth factor, PMA, transforming growth factor-α, that also potentiate AP-1 activity (*87*). In fact, the yeast homologue of the human c-*Jun*, YAP-1/PAR1, potentiates glucose-6-phosphate dehydrogenase activity (*88*). Further support for this comes from the fact that the lactoferrin promoter contains an AP-1–like element that has been shown to bind the AP-1 protein (*6*). Collectively, these *in vivo* observations combined with our *in vitro* data strongly suggest that organochlorine compounds such as DDT regulate gene expression through multiple elements present in the promoter regions of various genes.

AP-1 activation has become a marker for a number of effects elicited initially at the plasma membrane. External stimuli such as growth factors, hormones, ultraviolet irradiation, heavy metals, and even mechanical stress have been implicated in the induction of AP-1 (*5,7,89–91*). For example, epidermal growth factor binds to its tyrosine kinase receptor located at the plasma membrane, initiating a signaling cascade that results in the up-regulation of AP-1 activity (*3*). Estrogen has been implicated in a number of early signaling effects including activation of G-protein–coupled receptors and Ca^{2+} spikes (*92–94*). Cadmium stimulates AP-1–mediated gene expression through an ER-associated process (*95,96*), further hinting at the role of AP-1 as a potential environmental sensor. Additionally, treatment with cadmium, or other environmental estrogens such as bisphenol A and diethylstilbestrol, has been linked to alterations at the membrane level, leading to activation of specific signaling cascades (*97,98*). These responses are thought to be mediated through plasma membrane receptors. The effect we have reported here suggests that organochlorine compounds, such as DDT and its metabolites, may stimulate early membrane responses, similar to other extracellular stresses, leading to gene expression.

Here we demonstrated a novel effect of the DDTs using a new, stable-cell reporter-gene system. The pleiotropic nature of these organochlorines is revealed in their ability to stimulate expression from a classic ERE promoter element (*2,19*), activate receptor tyrosine kinases as well as multiple kinase pathways (*45,47,48,50,51*), inhibit L-type Ca^{2+} channels (*99,100*) and, as shown here, their capacity to stimulate AP-1 activity through non-ER–mediated mechanisms. Thus, just as the ER appears to be a convergent point for multiple cellular signals such as ligands (*38,101*), growth factors (*102–104*), and cell survival signals (*105*), AP-1 may function as a transcriptional mediator of various signaling pathways. Environmental compounds may adversely affect the physiology of a cell by mimicking aspects of its molecular endocrine system. Likewise, compounds may co-opt the cell's physiology by altering other cell signal regulators. Collectively, this may represent a new approach to environmental toxicology in which we go beyond cell damage to molecular modulators of cell responses.

References and Notes

1. Ahlborg UG, Lipworth L, Titus-Ernstoff L, Hsieh CC, Hanberg A, Baron J, Trichopoulos D, Adami HO. Organochlorine compounds in relation to breast cancer, endometrial cancer, and endometriosis: an assessment of the biological and epidemiological evidence. Crit Rev Toxicol 25:463–531 (1995).
2. Korach KS, Davis VL, Curtis SW, Bocchinfuso WP. Xenoestrogens and estrogen receptor action. In: Endocrine Toxicology (Thomas JA, Colby HD, eds). London:Taylor & Francis, 1997;181–211.
3. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 103:211–225 (2000).
4. Hsu TC, Young MR, Cmarik J, Colburn NH. Activator protein 1 (AP-1)- and nuclear factor kappaB (NF-kappaB)-dependent transcriptional events in carcinogenesis. Free Radic Biol Med 28:1338–1348 (2000).
5. Wisdom R. AP-1: one switch for many signals. Exp Cell Res 253:180–185 (1999).
6. Shi H, Teng CT. Characterization of a mitogen-response unit in the mouse lactoferrin gene promoter. J Biol Chem 269:12973–12980 (1994).
7. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311–317 (2000).
8. Giordano A, Avantaggiati ML. p300 and CBP: partners for life and death. J Cell Physiol 181:218–230 (1999).
9. Lee SK, Kim JH, Lee YC, Cheong J, Lee JW. Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. J Biol Chem 275:12470–12474 (2000).
10. Wisdom R, Johnson RS, Moore C. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. Embo J 18:188–197 (1999).
11. Brown JR, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, Pestell RG, Greenberg ME. Fos family members induce cell cycle entry by activating cyclin D1. Mol Cell Biol 18:5609–5619 (1998).
12. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 7:2135–2148 (1993).
13. Li XR, Chong AS, Wu J, Roebuck KA, Kumar A, Parrillo JE, Rapp UR, Kimberly RP, Williams JW, Xu X. Transcriptional regulation of Fas gene expression by GA-binding protein and AP-1 in T cell antigen receptor.CD3 complex-stimulated T cells. J Biol Chem 274:35203–35210 (1999).
14. Sawai H, Okazaki T, Yamamoto H, Okano H, Takeda Y, Tashima M, Sawada H, Okuma M, Ishikura H, Umehara H, et al. Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. J Biol Chem 270:27326–27331 (1995).
15. Wang Q, Ji Y, Wang X, Evers BM. Isolation and molecular characterization of the 5′-upstream region of the human TRAIL gene. Biochem Biophys Res Commun 276:466–471 (2000).
16. Kupfer D, Bulger WH. Interactions of chlorinated hydrocarbons with steroid hormones. Fed Proc 35:2603–2608 (1976).
17. Crews D, Willingham E, Skipper JK. Endocrine disruptors: present issues, future directions. Q Rev Biol 75:243–260 (2000).
18. Matthews J, Celius T, Halgren R, Zacharewski T. Differential estrogen receptor binding of estrogenic substances: a species comparison. J Steroid Biochem Mol Biol 74:223–234 (2000).
19. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263 (1998).
20. Jaga K. What are the implications of the interaction

between DDT and estrogen receptors in the body? Med Hypotheses 54:18–25 (2000).
21. Klotz DM, Beckman BS, Hill SM, McLachlan JA, Walters MR, Arnold SF. Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. Environ Health Perspect 104:1084–1089 (1996).
22. Gulledge CC, Burow ME, McLachlan JA. Endocrine disruption in sexual differentiation and puberty. What do pseudohermaphroditic polar bears have to do with the practice of pediatrics? Pediatr Clin N Am 48:1223–1240 (2001).
23. Fry DM, Toone CK. DDT-induced feminization of gull embryos. Science 213:922–924 (1981).
24. Tyler CR, Jobling S, Sumpter JP. Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28:319–361 (1998).
25. Guillette LJ Jr, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect 102:680–688 (1994).
26. Guillette LJ Jr, Woodward AR, Crain DA, Pickford DB, Rooney AA, Percival HF. Plasma steroid concentrations and male phallus size in juvenile alligators from seven Florida lakes. Gen Comp Endocrinol 116:356–372 (1999).
27. Willingham E, Crews D. Sex reversal effects of environmentally relevant xenobiotic concentrations on the red-eared slider turtle, a species with temperature-dependent sex determination. Gen Comp Endocrinol 113:429–435 (1999).
28. Wolff MS, Toniolo PG, Lee EW, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 85:648–652 (1993).
29. Safe SH, Zacharewski T. Organochlorine exposure and risk for breast cancer. Prog Clin Biol Res 396:133–145 (1997).
30. Cocco P, Kazerouni N, Zahm SH. Cancer mortality and environmental exposure to DDE in the United States. Environ Health Perspect 108:1–4 (2000).
31. Hruska KS, Furth PA, Seifer DB, Sharara FI, Flaws JA. Environmental factors in infertility. Clin Obstet Gynecol 43:821–829 (2000).
32. Vandelac L, Bacon MH. Will we be taught ethics by our clones? The mutations of the living, from endocrine disruptors to genetics. Baillieres Best Pract Res Clin Obstet Gynaecol 13:571–592 (1999).
33. Rier SE, Martin DC, Bowman RE, Dmowski WP, Becker JL. Endometriosis in rhesus monkeys (*Macaca mulatta*) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Fundam Appl Toxicol 21:433–441 (1993).
34. Korrick SA, Chen C, Damokosh AI, Ni J, Liu X, Cho SI, Altshul L, Ryan L, Xu X. Association of DDT with spontaneous abortion: a case-control study. Ann Epidemiol 11:491–496 (2001).
35. Beard J, Marshall S, Jong K, Newton R, Triplett-McBride T, Humphries B, Bronks R. 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)-ethane (DDT) and reduced bone mineral density. Arch Environ Health 55:177–180 (2000).
36. Skakkebaek NE, Rajpert-De Meyts E, Jorgensen N, Carlsen E, Petersen PM, Giwercman A, Andersen AG, Jensen TK, Andersson AM, Muller J. Germ cell cancer and disorders of spermatogenesis: an environmental connection? Apmis 106:3–11 (1998).
37. Metz R, Kouzarides T, Bravo R. A C-terminal domain in FosB, absent in FosB/SF and Fra-1, which is able to interact with the TATA binding protein, is required for altered cell growth. Embo J 13:3832–3842 (1994).
38. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277:1508–1510 (1997).
39. Webb P, Nguyen P, Valentine C, Lopez GN, Kwok GR, McInerney E, Katzenellenbogen BS, Enmark E, Gustafsson JA, Nilsson S, et al. The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13:1672–1685 (1999).
40. Duan R, Porter W, Safe S. Estrogen-induced c-*fos* protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. Endocrinology 139:1981–1990 (1998).
41. Duan R, Xie W, Safe S. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J Biol Chem 276:11590–11598 (2001).
42. Boettger-Tong HL, Murthy L, Stancel GM. Cellular pattern of c-*fos* induction by estradiol in the immature rat uterus. Biol Reprod 53:1398–1406 (1995).
43. Iguchi T, Fukazawa Y, Bern HA. Effects of sex hormones on oncogene expression in the vagina and on development of sexual dimorphism of the pelvis and anococcygeus muscle in the mouse. Environ Health Perspect 103(suppl 7):79–82 (1995).
44. Makela S, Santti R, Salo L, McLachlan JA. Phytoestrogens are partial estrogen agonists in the adult male mouse. Environ Health Perspect 103(suppl 7):123–127 (1995).
45. Tessier DM, Matsumura F. Increased ErbB-2 tyrosine kinase activity, MAPK phosphorylation, and cell proliferation in the prostate cancer cell line LNCaP following treatment by select pesticides. Toxicol Sci 60:38–43 (2001).
46. Ramamoorthy K, Vyhlidal C, Wang F, Chen I, Safe S, McDonnell DP, Leonard LS, Gaido KW. Additive estrogenic activities of a binary mixture of 2′,4′,6′-trichloro- and 2′,3′,4′,5′-tetrachloro-4-biphenylol. Toxicol Appl Pharmacol 147:93–100 (1997).
47. Hatakeyama M, Matsumura F. Correlation between the activation of Neu tyrosine kinase and promotion of foci formation induced by selected organochlorine compounds in the MCF-7 model system. J Biochem Mol Toxicol 13:296–302 (1999).
48. Enan E, Matsumura F. Activation of c-Neu tyrosine kinase by *o,p′*-DDT and beta-HCH in cell-free and intact cell preparations from MCF-7 human breast cancer cells. J Biochem Mol Toxicol 12:83–92 (1998).
49. Chuang LF, Chuang RY. Heptachlor and the mitogen-activated protein kinase module in human lymphocytes. Toxicology 128:17–23 (1998).
50. Shen K, Novak RF. DDT stimulates c-erbB2, c-met, and STATS tyrosine phosphorylation, Grb2-Sos association, MAPK phosphorylation, and proliferation of human breast epithelial cells. Biochem Biophys Res Commun 231:17–21 (1997).
51. Shen K, Novak RF. Differential effects of aroclors and DDT on growth factor gene expression and receptor tyrosine kinase activity in human breast epithelial cells. Adv Exp Med Biol 407:295–302 (1997).
52. Bagchi D, Bagchi M, Tang L, Stohs SJ. Comparative *in vitro* and *in vivo* protein kinase C activation by selected pesticides and transition metal salts. Toxicol Lett 91:31–37 (1997).
53. Shekhar PV, Werdell J, Basrur VS. Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J Natl Cancer Inst 89:1774–1782 (1997).
54. Mousa MA, Ganey PE, Quensen JF III, Madhukar BV, Chou K, Giesy JP, Fischer LJ, Boyd SA. Altered biologic activities of commercial polychlorinated biphenyl mixtures after microbial reductive dechlorination. Environ Health Perspect 106(suppl 6):1409–1418 (1998).
55. Barron S, Serratosa J, Tusell JM. Regulation of c-*fos* expression by convulsants and hexachlorocyclohexane isomers in primary cultures of cortical neurons. J Neurochem 64:1708–1714 (1995).
56. Ghosh D, Taylor JA, Green JA, Lubahn DB. Methoxychlor stimulates estrogen-responsive messenger ribonucleic acids in mouse uterus through a non-estrogen receptor (non-ER) alpha and non-ER beta mechanism. Endocrinology 140:3526–3533 (1999).
57. Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB. Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci USA 94:12786–12791 (1997).
58. Devanesan P, Santen RJ, Bocchinfuso WP, Korach KS, Rogan EG, Cavalieri E. Catechol estrogen metabolites and conjugates in mammary tumors and hyperplastic tissue from estrogen receptor-alpha knock-out (ERKO)/Wnt-1 mice: implications for initiation of mammary tumors. Carcinogenesis 22:1573–1576 (2001).
59. Falconer RL, Bidleman TF, Szeto SY. Chiral pesticides in soils of the Fraser Valley, British Columbia. J Agric Food Chem 45:1946–1951 (1997).
60. Aigner EJ, Leone AD, Falconer RL. Concentrations and enantiomeric ratios of organochlorine pesticides in soils from the U.S. corn belt. Environ Sci Technol 32:1162–1168 (1998).
61. U.S.G.S. Summary Statistics for Organochlorine Compounds in Whole Fish, Vols 1992–1998. Washington, DC:U.S. Geological Survey, 2001.
62. Burow ME, Weldon CB, Chiang TC, Tang Y, Collins-Burow BM, Rolfe K, Li S, McLachlan JA, Beckman BS. Differences in protein kinase C and estrogen receptor alpha, beta expression and signaling correlate with apoptotic sensitivity of MCF-7 breast cancer cell variants. Int J Oncol 16:1179–1187 (2000).
63. Collins-Burow BM, Burow ME, Duong BN, McLachlan JA. Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. Nutr Cancer 38:229–244 (2000).
64. Burow ME, Boue SM, Collins-Burow BM, Melnik LI, Duong BN, Carter-Wientjes CH, Li S, Wiese TE, Cleveland TE, McLachlan JA. Phytochemical glyceollins, isolated from soy, mediate antihormonal effects through estrogen receptor alpha and beta. J Clin Endocrinol Metab 86:1750–1758 (2001).
65. Burow ME, Weldon CB, Collins-Burow BM, Ramsey N, McKee A, Klippel A, McLachlan JA, Clejan S, Beckman BS. Cross-talk between phosphatidylinositol 3-kinase and sphingomyelinase pathways as a mechanism for cell survival/death decisions. J Biol Chem 275:9628–9635 (2000).
66. Burow ME, Tang Y, Collins-Burow BM, Krajewski S, Reed JC, McLachlan JA, Beckman BS. Effects of environmental estrogens on tumor necrosis factor alpha-mediated apoptosis in MCF-7 cells. Carcinogenesis 20:2057–2061 (1999).
67. Thompson EW, Paik S, Brunner N, Sommers CL, Zugmaier G, Clarke R, Shima TB, Torri J, Donahue S, Lippman ME, et al. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 150:534–544 (1992).
68. MacGregor Schafer J, Liu H, Levenson AS, Horiguchi J, Chen Z, Jordan VC. Estrogen receptor alpha mediated induction of the transforming growth factor alpha gene by estradiol and 4-hydroxytamoxifen in MDA-MB-231 breast cancer cells. J Steroid Biochem Mol Biol 78:41–50 (2001).
69. Yoon K, Pallaroni L, Stoner M, Gaido K, Safe S. Differential activation of wild-type and variant forms of estrogen receptor alpha by synthetic and natural estrogenic compounds using a promoter containing three estrogen-responsive elements. J Steroid Biochem Mol Biol 78:25–32 (2001).
70. Tyulmenkov VV, Jernigan SC, Klinge CM. Comparison of transcriptional synergy of estrogen receptors alpha and beta from multiple tandem estrogen response elements. Mol Cell Endocrinol 165:151–161 (2000).
71. Tyulmenkov VV, Klinge CM. Estrogen receptors alpha and beta exhibit different estradiol and estrogen response element binding in the presence of nonspecific DNA. Arch Biochem Biophys 390:253–264 (2001).
72. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566–5578 (1999).
73. McInerney EM, Weis KE, Sun J, Mosselman S, Katzenellenbogen BS. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology 139:4513–4522 (1998).
74. Cerillo G, Rees A, Manchanda N, Reilly C, Brogan I, White A, Needham M. The oestrogen receptor regulates NFkappaB and AP-1 activity in a cell-specific manner. J Steroid Biochem Mol Biol 67:79–88 (1998).
75. Ware GW. An Introduction to Insecticides. Minneapolis, MN:University of Minnesota Press, 2001.
76. Mitra J, Raghu K. Long-term DDT pollution in tropical soils: effect of DDT and degradation products on soil microbial activities leading to soil fertility. Bull Environ Contam Toxicol 60:585–591 (1998).
77. ATSDR. Toxicological Profile for 4,4′-DDT, 4,4′-DDE, 4,4′-DDD. Atlanta, GA:Agency for Toxic Substances and Disease Registry, 1992.
78. Sundlof SF, Forrester DJ, Thompson NP, Collopy MW. Residues of chlorinated hydrocarbons in tissues of raptors in Florida. J Wildl Dis 22:71–82 (1986).
79. Provini A, Galassi S. Polychlorinated biphenyls and chlorinated pesticides in bird eggs from Calabria (southern Italy). Ecotoxicol Environ Saf 43:91–97 (1999).
80. Flanagan L, Van Weelden K, Ammerman C, Ethier SP, Welsh J. SUM-159PT cells: a novel estrogen independent human breast cancer model system. Breast Cancer Res Treat 58:193–204 (1999).
81. Jenster G. Ligand-independent activation of the androgen receptor in prostate cancer by growth factors and cytokines. J Pathol 191:227–228 (2000).

82. Huang C, Ma WY, Young MR, Colburn N, Dong Z. Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. Proc Natl Acad Sci USA 95:156–161 (1998).
83. Diel P, Schulz T, Smolnikar K, Strunck E, Vollmer G, Michna H. Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity. J Steroid Biochem Mol Biol 73:1–10 (2000).
84. Wunsche W, Tenniswood MP, Schneider MR, Vollmer G. Estrogenic regulation of clusterin mRNA in normal and malignant endometrial tissue. Int J Cancer 76:684–688 (1998).
85. Strunck E, Stemmann N, Hopert A, Wunsche W, Frank K, Vollmer G. Relative binding affinity does not predict biological response to xenoestrogens in rat endometrial adenocarcinoma cells. J Steroid Biochem Mol Biol 74:73–81 (2000).
86. Das SK, Tan J, Johnson DC, Dey SK. Differential spatiotemporal regulation of lactoferrin and progesterone receptor genes in the mouse uterus by primary estrogen, catechol estrogen, and xenoestrogen. Endocrinology 139:2905–2915 (1998).
87. Shi H, Teng C. Promoter-specific activation of mouse lactoferrin gene by epidermal growth factor involves two adjacent regulatory elements. Mol Endocrinol 10:732–741 (1996).
88. Schnell N, Krems B, Entian KD. The *PAR1* (*YAP1/SNQ3*) gene of *Saccharomyces cerevisiae*, a *c-jun* homologue, is involved in oxygen metabolism. Curr Genet 21:269–273 (1992).
89. Galan A, Garcia-Bermejo L, Vilaboa NE, de Blas E, Aller P. Uncoupling of apoptosis and Jun/AP-1 activity in human promonocytic cells treated with DNA-damaging and stress-inducing agents. Eur J Cell Biol 79:1–9 (2000).
90. Shukla GS, Shukla A, Potts RJ, Osier M, Hart BA, Chiu JF. Cadmium-mediated oxidative stress in alveolar epithelial cells induces the expression of gamma-glutamylcysteine synthetase catalytic subunit and glutathione *S*-transferase alpha and pi isoforms: potential role of activator protein-1. Cell Biol Toxicol 16:347–362 (2000).
91. Peverali FA, Basdra EK, Papavassiliou AG. Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. Mol Med 7:68–78 (2001).
92. Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14:1649–1660 (2000).
93. Caldwell JD, O'Rourke ST, Morris M, Walker CH, Carr RB, Faggin BM, Mason GA. Steroid effects at the membrane level on oxytocin systems. Ann N Y Acad Sci 814:282–286 (1997).
94. Improta-Brears T, Whorton AR, Codazzi F, York JD, Meyer T, McDonnell DP. Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci USA 96:4686–4991 (1999).
95. Stoica A, Katzenellenbogen BS, Martin MB. Activation of estrogen receptor-alpha by the heavy metal cadmium. Mol Endocrinol 14:545–553 (2000).
96. Alam J. Multiple elements within the 5′ distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. J Biol Chem 269:25049–25056 (1994).
97. Hague F, Matifat F, Louvet L, Brule G, Collin T. The carcinogen Cd(2+) activates InsP(3)-mediated Ca(2+) release through a specific metal ion receptor in Xenopus oocyte. Cell Signal 12:419–424 (2000).
98. Nadal A, Ropero AB, Laribi O, Maillet M, Fuentes E, Soria B. Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta. Proc Natl Acad Sci USA 97:11603–11608 (2000).
99. Kelly MJ, Levin ER. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab 12:152–156 (2001).
100. Ruehlmann DO, Steinert JR, Valverde MA, Jacob R, Mann GE. Environmental estrogenic pollutants induce acute vascular relaxation by inhibiting L-type Ca^{2+} channels in smooth muscle cells. Faseb J 12:613–619 (1998).
101. Muramatsu M, Inoue S. Estrogen receptors: how do they control reproductive and nonreproductive functions? Biochem Biophys Res Commun 270:1–10 (2000).
102. Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivo SE, Stoica A. A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. Endocrinology 141:4503–4511 (2000).
103. Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS. Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci USA 89:4658–4662 (1992).
104. Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA, Korach KS. Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. Endocrinology 137:1735–1744 (1996).
105. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 276:9817–9824 (2001).