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# Bisphenol A interacts with the estrogen receptor $\alpha$ in a distinct manner from estradiol

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**Abstract**

We investigated the interaction of bisphenol A (BPA, an estrogenic environmental contaminant used in the manufacture of plastics) with the estrogen receptor alpha (ER$\alpha$) transfected into the human HepG2 hepatoma cell line and expanded the study in vivo to examine the effect of BPA on the immature rat uterus. Bisphenol A was 26-fold less potent in activating ER-WT and was a partial agonist with the ER$\alpha$ compared to $E_2$. The use of ER$\alpha$ mutants in which the AF1 or AF2 regions were inactivated has permitted the classification of ER ligands into mechanistically distinct groups. The pattern of activity of BPA with the ER$\alpha$ mutants differed from the activity observed with weak estrogens (estrone and estriol), partial ER$\alpha$ agonists (raloxifene or 4-OH-tamoxifen), or a pure antagonist (ICI 182, 780). Intact immature female Sprague–Dawley rats were exposed to BPA alone or with $E_2$ for 3 days. Unlike $E_2$, BPA had no effect on uterine weight; however, like $E_2$, both peroxidase activity and PR levels were elevated, though not to the level induced by $E_2$. Following simultaneous administration, BPA antagonized the $E_2$ stimulatory effects on both peroxidase activity and PR levels but did not inhibit $E_2$-induced increases of uterine weight. These results demonstrate that BPA is not merely a weak estrogen mimic but exhibits a distinct mechanism of action at the ER$\alpha$. 

*Keywords:* Estrogen; Anti-estrogen; Receptor; Bisphenol A; HepG2 cells; Uterotrophic assay

## 1. Introduction

Much recent public and scientific interest has been focused on environmental chemicals capable of interacting with the estrogen receptor (ER). These 'environmental estrogens' are hypothesized to mimic the actions of natural estrogens in humans and thereby disrupt normal endocrine function leading to reduced fertility, congenital malformations of the reproductive tract, and increased incidence of cancer in estrogen-responsive tissues (Colborn et al., 1993; Davis et al., 1993). One such chemical with estrogenic activity is bisphenol A (BPA). Bisphenol A is a monomer used in the manufacture of polycarbonate, epoxy, and polyester-styrene resins. Trace levels of BPA leach from the linings of food cans and dental resins (Brotons et al., 1995; Olea et al., 1996). High doses were reported to cause reproductive toxicity and affect cellular development in rats and mice (Reel et al., 1985; Morrissey et al., 1987). In in vitro systems, BPA competes with estradiol ($E_2$) for binding with the ER$\alpha$ (one of the two ER sub-types) and induces expression of progesterone receptor (PR) and proliferation of MCF-7 human breast cancer cells (Krishnan et al., 1993; Soto et al., 1995; Villalobos et al., 1995). These results demonstrate that BPA mimics

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some of the actions of estrogen in vitro. However, the effects of BPA on ERα transcriptional activity in vivo have yet to be determined.

ERα and other members of the steroid receptor superfamily exhibit a common domain structure with distinct regions responsible for ligand binding, dimer formation, DNA binding, and transcriptional activation (Kumar et al., 1987). Upon binding estrogen, the ER–estrogen complex is translocated into the nucleus, where it binds to estrogen-responsive elements (ERE) located in the 5′ flanking region of estrogen-responsive genes. The estrogen-occupied receptor then interacts with additional transcription factors and components of the transcription initiation complex to modulate gene transcription.

Although two sub types of the ER, ERα and ERβ, have been identified, extensive studies have investigated the molecular activation of the ERα. The ERα exhibits two distinct gene transactivating regions, AF1 in the amino terminus and AF2 in the carboxyl terminus (formerly designated as TAF1 and TAF2) (Tora et al., 1989). These sequences function in a cell- and promoter-specific manner to mediate ERα function (Berry et al., 1990; Tzukerman et al., 1994). In human hepatoma HepG2 cells transiently transfected with the ERα-wild type or mutants in the AF1 or AF2 regions and a reporter gene with a human complement factor 3 (C3)-ERE promoter, ER ligands have been divided into mechanistically distinct groups based upon their relative ability to activate AF1/AF2 regions ($E_2$, pure agonist; tamoxifen or raloxifene, partial agonists; ICI 182, 780, pure antagonist) (Tzukerman et al., 1994; McDonnell et al., 1995; Willson et al., 1997). These mechanistically distinct groups manifest unique biology in vivo. Tamoxifen was reported to exhibit anti-estrogenic activity in mammary tissue, but full ER agonist responses were observed in the uterus and bone (Robertson et al., 1982; Jordan et al., 1987; Love et al., 1992). Raloxifene ER agonist activity has only been observed in the bone, and ICI 182, 780 is a pure ER antagonist in all estrogen-responsive tissues (Wakeling et al., 1991; Evans et al., 1993; Black et al., 1994). Although the crystal structure of ERα is unknown, analysis of ERα structure using in vitro biochemical techniques in the presence of various ligands has revealed that the interaction of these compounds with the ER is not the same (McDonnell et al., 1995; Potthoff et al., 1996), indicating a link between receptor structure and biological activity. This hypothesis reflects an evolution in our understanding of the role of the ligand in ER function, suggesting that the ligand is not merely a switch but an integral component of the 'activated ERα'. Thus, binding affinity alone is only one property of a ligand that may predict biological activity. Consequently, classification of ERα ligands into mechanistically distinct groups and a determination of the in vivo response to members of the group will aid in the evaluation of these compounds as potential pharmaceuticals or toxicants. A useful model to classify different ER ligands, the HepG2 assay, was established utilizing the ERα. As observed in the present study and others (Robertson et al., 1982; Jordan et al., 1987; Wakeling et al., 1991; Love et al., 1992; Evans et al., 1993; Black et al., 1994; Tzukerman et al., 1994; McDonnell et al., 1995; Willson et al., 1997), this classification correlates to activity observed in vivo. Although ERβ likely plays an important role in the activity of estrogens in vivo, the molecular determinants of ERβ activity have yet to be characterized.

In this study, we investigated the interaction of BPA with the ERα transfected into the human HepG2 hepatoma cell line and compared the actions of BPA with $E_2$ and other ER agonist/antagonists (estriol, estrone, tamoxifen and raloxifene). We demonstrate that BPA displays distinct activities with the ER-WT, ER-AF1, and ER-AF2 mutants that differ from the activities of other known classes of ER ligands. Our results in a rat uterotrophic assay support this unique activity of BPA. Thus, BPA is not merely an estrogen mimic but likely interacts with the ERα in a unique manner to produce its own spectrum of activity.

## 2. Materials and methods

### 2.1. Chemicals

Chemicals were purchased from Fisher (Pittsburgh, PA), Sigma (St. Louis, MO) or Boehringer-Mannheim (Indianapolis, IN) unless otherwise noted. Raloxifene and ICI 182, 780 were a gift from Dr A.E. Wakeling (Zeneca Pharmaceuticals, UK). Cell culture solutions and chemicals were purchased from Gibco/BRL (Grand Island, NY). Human HepG2 cells (obtained from ATCC) were routinely maintained in minimal essential medium with phenol red and 9% fetal bovine serum (Hyclone, Logan, UT), 2% L-glutamine, 0.1% sodium pyruvate and 0.3% nystatin.

### 2.2. HepG2 transfection and ERα assay

The human wild-type (ER-WT), and mutant ERα (ER-AF1, ER-AF2, and ER-null) and the constitutively active β-gal plasmid were described previously (Tzukerman et al., 1994). The rat wild-type ERα (RSV Rc ER or ER-RAT) was a generous gift from Dr Richard Day (University of Virginia, Charlottesville, VA). It was prepared by replacing the β-globin sequence of RSV β-globin (Gorman et al., 1982) with the pRcER6 *Eco*R1 insert from the λ gt10 clone isolated by Koike and coworkers (Koike et al., 1987). The clone consists of 225 bp 5′UT, the complete rat ERα coding sequence, and 86 bp of 3′UT sequence. All plasmids contain a

RSV promoter, except the ERE-C3-Luc reporter plasmid, which contains an estrogen-responsive human complement factor 3 (C3) promoter. The method for transfection and the ERα assay were performed as described previously (Tzukerman et al., 1994). Briefly, cells were seeded for 18 h in 24-well plates in phenol red-free Eagle's minimal essential medium with non-essential amino acids (Gibco/BRL) containing 9% resin stripped fetal bovine serum, 2% L-glutamine and 0.1% sodium pyruvate at a density of $2.0 \times 10^5$ cells per well. The cells were transfected with three plasmids: receptor plasmid, C3-LUC reporter plasmid, and β-gal plasmid (transfection control) in Lipofectin and OptiMEM to a final volume of 300 μl for 3 h. Due to improved stability, Superfect (Qiagen, Santa Clarita, CA) and complete MEM were used at a final volume of 200 μl for 3 h in the weak estrogen experiments. After rinsing with phosphate-buffered saline, cells were treated with $10^{-11}$–$10^{-6}$ M 4-OH-tamoxifen (Research Biomedicals International, Natwick, MA), $10^{-11}$–$10^{-6}$ M raloxifene, $10^{-8}$–$10^{-5}$ M BPA, $10^{-5}$ M ICI 182, 780 and/or $10^{-11}$–$10^{-6}$ M $E_2$ (Aldrich, Milwaukee, WI), or vehicle (methanol at 0.1 or 0.2% in BPA or $E_2$ and ICI 182, 780 antagonist assays) in complete medium. For the weak agonist experiments, $10^{-10}$–$10^{-5}$ M estriol (Aldrich, Milwaukee, WI) or estrone or $10^{-11}$–$10^{-6}$ M $E_2$ was dissolved in DMSO (final concentration equalled 0.1% DMSO). After a 24 h incubation, cells were lysed with buffer (25 mM Tris, pH 7.8, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*,*N*-tetraacetic acid, 10% glycerol, 0.5% Triton X, 2 mM DTT). Lysate was divided for luciferase and β-galactosidase activity determination into two 96-well plates. For luciferase activity evaluation, 100 μl of luciferase assay reagent (Promega, Madison, WI) was added to lysate, and luminescence was determined using a Microlite ML3000 microtiter plate luminometer (Dynatech Laboratories, Chantilly, VA). For β-galactosidase activity determination, 20 μl of 4 mg/ml chlorophenol red-β-D-galactopyranoside (CPRG) and 150 μl CPRG buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KC1, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.8) was added to lysate. The absorbance rate at 570 nm was determined immediately on a kinetic microplate reader (Molecular Devices, Menlo Park, CA). Estrogen receptor activity was normalized to β-galactosidase activity, and the methanol blank activity was subtracted. Data were plotted and the $EC_{50}$ and $V_{max}$ determined using a Hill function:

$$\text{Response} = \frac{(V \cdot (\text{dose})^N)}{((10^{KK})^N + (\text{dose})^N)}$$

where $N$ = Hill coefficient, $V = V_{max}$ or maximal normalized luciferase activity, and $KK$ = the concentration at which normalized luciferase activity is 50% of maximum ($EC_{50}$). To normalize activity between experiments, data from multiple experiments was displayed as a percentage of the $V_{max}$ induced by $E_2$. The normalized $V_{max}$ and $EC_{50}$ values for $E_2$ and BPA for each receptor were compared using a *t*-test (JMP, SAS Institute, Cary, NC).

### 2.3. 17β-estradiol binding competition assay

The assay was performed as previously described by Tzukerman and colleagues (Tzukerman et al., 1994) with slight modifications (Obourn et al., 1993). Serial dilutions of $E_2$ ($10^{-11}$–$10^{-5}$ M) and BPA ($10^{-8}$–$3 \times 10^{-5}$ M) were prepared in 10 mM Tris (pH 7.6), 0.3 M potassium chloride, 5 mM dithiothreitol, and 1 mg/ml bovine serum albumin. A concentration of $5 \times 10^{-8}$ M 17β-[$^3$H]estradiol (Amersham, Arlington Heights, IL) was added to each tube. Recombinant human ERα or the mutated receptor containing an intact AF1 region isolated from yeast was added at 600 μg protein per tube. After an overnight incubation at 4°C, 100 μl of a 6% hydroxyapatite (HAP) slurry in dilution buffer (10 mM Tris (pH 7.6) and 5 mM DTT) were added. The tubes were incubated at 4°C for 30 min. The tubes were centrifuged at $1000 \times g$ for 5 min. HAP pellets were washed three times in dilution buffer containing 1% Triton X-100. The pellets were resuspended in 1 ml of dilution buffer and transferred to scintillation vials. Scintillation fluid, Ecolume (ICN, Costa Mesa, CA), was added, vortexed, and radioactivity was measured on a Packard Tri-carb 460 scintillation counter (Packard Instruments).

### 2.4. Animals

Animals procedures were conducted in accordance with all local and federal laws and followed the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). A total of 19 day-old female Sprague–Dawley rats were obtained from Harlan Sprague–Dawley (Houston, TX) and housed four to a cage with ad libitum access to food and water. Bisphenol A was dissolved in corn oil. Groups of female Sprague–Dawley rats ($n = 4$ or 5) received 0.2 ml of 5.0, 10, 25, 50, 100 or 150 mg BPA/kg or vehicle control p.o. for 3 days beginning on day 21. Due to solubility limitations, 150 mg BPA/kg was the highest dose administered. Bisphenol A was given orally because this route of exposure is more relevant to human exposure. Some groups also received 0.5 μg/day of $E_2$ in corn oil by i.p. injection of the same 3 treatment days (PND 21–23), and control rats received corn oil alone. Animals were killed by carbon dioxide asphyxiation 20 h after the last treatment; and the uteri were quickly removed, cleaned of connected tissue, weighed, and placed in ice-cold buffer.

### 2.5. Progesterone receptor measurement

The uterine bisections of each treatment group were pooled in an ice-cold TESHMo buffer (10 mM Tris–HCl, pH 7.4; 1.5 mM EDTA; 15 mM thioglycerol; 10 mM sodium molybdate), 1 ml/50 mg tissue. Uteri were homogenized with three 8 s bursts using a Brinkman/Polytron tissue grinder. Samples were then centrifuged for 45 min at $105000 \times g$, and the clear supernatant, constituting the cytosol for this experiment, was carefully decanted and immediately used for receptor assays. Progesterone receptor levels were determined with competitive binding assays. Cytosolic fractions described above were incubated with 20 nM [$^3$H]R5020 with or without 2 $\mu$M unlabeled progesterone at 4°C. After 16–18 h, incubations were placed on ice and treated with 0.1 volume dextran-coated charcoal suspension (0.5% dextran: 5% charcoal, v/v) for 10 min. Samples were then centrifuged at $5000 \times g$ for 10 min, and the radioactivity of the entire supernatant was measured by liquid scintillation counting. Progesterone receptor levels are reported in femtomol per uterus. Results are expressed as means $\pm$ SEM, and statistical differences were determined by Analysis of variance and the Duncan new multiple range test.

### 2.6. Uterine peroxidase assay

Uterine bisections were pooled into treatment groups and homogenized as described above. Homogenates were centrifuged at $39000 \times g$ at 2°C for 45 min, and the resultant pellet was washed and resuspended in 10 mM Tris–HC1 buffer containing 0.5 M $CaCl_2$ using a Brinkman homogenizer. The extract was clarified by centrifugation of the sample for 45 min at $30000 \times g$ at 2°C. The assay mixture (3.0 ml total) contained 13 mM guaiacol and 0.3 mM $H_2O_2$ in the extraction buffer. The reaction was started by addition of 1.0 ml of the $CaCl_2$ extract. The initial rate (1 min) of guaiacol oxidation was monitored on a Beckman spectrophotometer using a time drive program at 470 nM. An enzyme unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per minute under the assay conditions described. Enzyme activity is expressed per milligram uterine protein. Results are expressed as means $\pm$ SEM, and statistical differences were determined by Analysis of variance and the Duncan new multiple range test.

## 3. Results

Maximal luciferase activity after being normalized for transfection efficiency, was determined using a Hill function. Within each experiment, variability in triplicate wells from the vehicle-treated HepG2 cells transfected with the ER-WT was $13.4 \pm 3.1$ normalized luciferase activity units ($\pm$ SEM). To control for variation in peak luciferase activity between experiments and to allow for combining experiments for statistical purposes, each experiment was normalized to peak $E_2$ activity from that experiment. The maximal luciferase activity induced by $E_2$ was $769 \pm 455$ normalized luciferase activity units ($\pm$ SEM; $n = 6$). Variability represents differences in peak luciferase activity from experiment to experiment. Relative potency and peak activity between $E_2$ and BPA did not vary significantly (despite this variation in peak luciferase activity between experiments). Student's $t$-test was performed on combined experiments to determine statistical differences between maximal activity or potency of $E_2$ and BPA. Estradiol increased luciferase activity in a dose dependent manner in human HepG2 hepatoma cells cotransfected with the human wild-type ER$\alpha$ (ER-WT) and a luciferase reporter gene (Fig. 1A). Half-maximal transcriptional response (effective concentration; $EC_{50}$) to $E_2$ occurred at a concentration of $9.9 \pm 2.7$ nM. The $EC_{50}$ values represent the concentration required to achieve half-maximal luciferase activity for that chemical. Bisphenol A induced a maximal luciferase activity that was $70.7 \pm 6.5\%$ of that induced by $E_2$ (Fig. 1A). In addition, BPA was significantly less potent than $E_2$ with an $EC_{50}$ of $218 \pm 25$ nM.

We next used an established paradigm utilizing human ER$\alpha$ mutants in the AF1 or AF2 regions to investigate ER$\alpha$-ligand interactions (Tzukerman et al., 1994; McDonnell et al., 1995). The mutant human ER$\alpha$ containing an inactive transcriptional AF2 region, ER-AF1, was investigated for the ability of BPA to bind to the ER$\alpha$ and activate transcription. The maximal luciferase activity induced by $E_2$ with the ER-AF1 mutant was $482 \pm 169$ normalized luciferase activity units ($\pm$ SEM). Estradiol and BPA activated the ER-AF1 in a dose-dependent manner (Fig. 1B). Bisphenol A had partial agonist activity with the ER-AF1, inducing $40 \pm 4.0\%$ of the activity observed with $E_2$.

The activity of BPA with the ER-AF2 (containing a truncated AF1 region) was also determined. Maximal transcriptional response observed after $E_2$ treatment was only $24.9 \pm 1.4$ normalized luciferase activity units ($\pm$ SEM), $10.3 \pm 2.1\%$ of that obtained with the ER-WT. Bisphenol A again acted as a partial agonist with the ER-AF2 relative to $E_2$, inducing only $69.8 \pm 4.4\%$ of the activity induced by $E_2$ (Fig. 1C).

The ER-null, a transcriptionally inactive receptor containing mutations in both the AF1 and AF2 regions was employed as a negative control. No luciferase activity was observed after treatment of ER-null transfected HepG2 cells with $E_2$ or BPA (Fig. 1A). Background luciferase activity in vehicle-treated cells transfected with ER-WT was $14.5 \pm 3.6\%$ above that observed with ER-null. Both ER-AF1 and ER-AF2

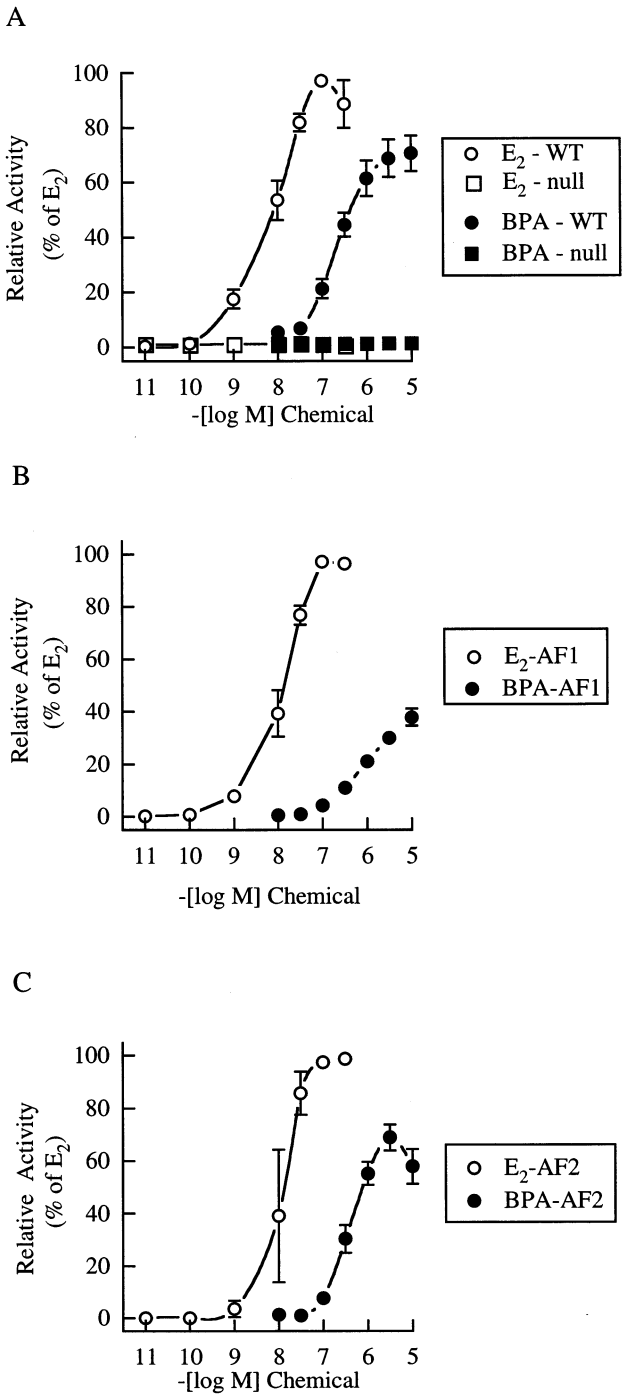


Fig. 1. Partial agonist activation of the human wild-type (ER-WT) (A) and estrogen receptors mutant in the AF1 (B) or AF2 (C) regions by bisphenol A compared to estradiol. HepG2 cells were transiently cotransfected with 270 ng of ER-WT (A), ER-null (A), ER-AF1 (B) or AF2 (C) plasmid, 405 ng pC3-luc reporter plasmid and 135 ng pRSV-$\beta$-gal plasmid. Cells were treated with increasing concentrations of $E_2$ or BPA, or vehicle (methanol) for 24 h and assayed for luciferase activity (luciferase activity was normalized to $\beta$-galactosidase activity which is indicative of transfection efficiency). Mean maximal luciferase activity induced by $E_2$ with the ER-AF1 and ER-AF2 was $58.3 \pm 4.1$ and $24.9 \pm 1.4\%$, respectively, of the activity induced with the ER-WT. Luciferase activity is shown as the mean of the percent activity of the maximal $E_2$ induced activity. Estradiol was run in parallel with BPA for each individual experiment. Data for ER-WT and ER-null represent the combined data from six experiments, ER-AF1 data represent the combined data from ten experiments, and ER-AF2 data represent the combined data from three experiments (each dose and receptor in triplicate). The data represent the mean $\pm$ SEM of all replicate experiments for each chemical and receptor.

mutants when transfected into HepG2 cells had background activity equivalent to the level observed with ER-null (data not shown). Background luciferase activity is probably due to ligand-independent ER$\alpha$ activation (McDonnell et al., 1995). A pure ER antagonist, ICI 182, 780, was used to emphasize that estrogenic effects of BPA occurred through the ER$\alpha$. ICI 182, 780 decreased background luciferase activity with the ER-WT to levels observed with ER-null (Fig. 2). In HepG2 cells transfected with ER-WT or ER-AF1, ICI 182, 780 ($10^{-6}$ M) completely blocked $E_2$-induced ($3 \times 10^{-7}$ M) luciferase activity (Fig. 2). Similarly, BPA-induced ($10^{-6}$ M) luciferase activity with the ER-WT and ER-AF1 was inhibited by ICI 182, 780 ($10^{-6}$ M) (Fig. 2). Together, these results emphasize that the estrogenic effects of BPA are occurring through the ER$\alpha$.

Bisphenol A has been shown previously to compete with $E_2$ for binding to the ER$\alpha$ (Olea et al., 1996). However, the partial agonist activity of BPA with the ER-AF1 mutant could be due to decreased binding affinity for this mutant ER$\alpha$. Therefore, the binding of $E_2$ and BPA with purified ER-WT or ER-AF1 was examined. As was observed with $E_2$, the binding affinity of BPA was the same for both receptors, ER-AF1 and ER-WT, (Fig. 3).

We compared the activity of BPA to other ER$\alpha$ ligand classes in ER$\alpha$-transfected HepG2 cells represented by raloxifene (type III) and 4-OH-tamoxifen (4OH-T) (type IV) (McDonnell et al., 1995). Estradiol and BPA induced activity with ER-WT, ER-AF1, and ER-AF2 was discussed above (Figs. 1 and 4). Luciferase activity induced by 4OH-T with the ER-WT, and ER-AF1 was $27.8 \pm 2.1$ and $10.9 \pm 3.7\%$, respectively, compared to $E_2$ (Fig. 4A,4B). Raloxifene decreased basal ER-WT activity (Fig. 4A) in a manner similar to that observed with ICI 182, 780 (Fig. 2A); however, raloxifene was a partial agonist with the ER-AF1 reaching $14.5 \pm 2.5\%$ of $E_2$-induced activity (Fig. 4B). Neither 4OH-T nor raloxifene induced luciferase activity in HepG2 cells transfected with ER- AF2 (Fig. 4C). The pattern of responses for 4OH-T and raloxifene were similar to what had been previously reported (McDonnell et al., 1995; Willson et al., 1997). However, BPA had a distinct fingerprint with this paradigm compared to other classes of ER ligands.

Endogenous weak estrogens were examined in the HepG2 assay. Estriol and estrone had the same maximal luciferase activity and similar potency with the ER-WT, ER-AF1, and ER-AF2 as that observed with $E_2$ (Fig. 5), indicating weak estrogens have the same pattern as $E_2$ in the HepG2 paradigm.

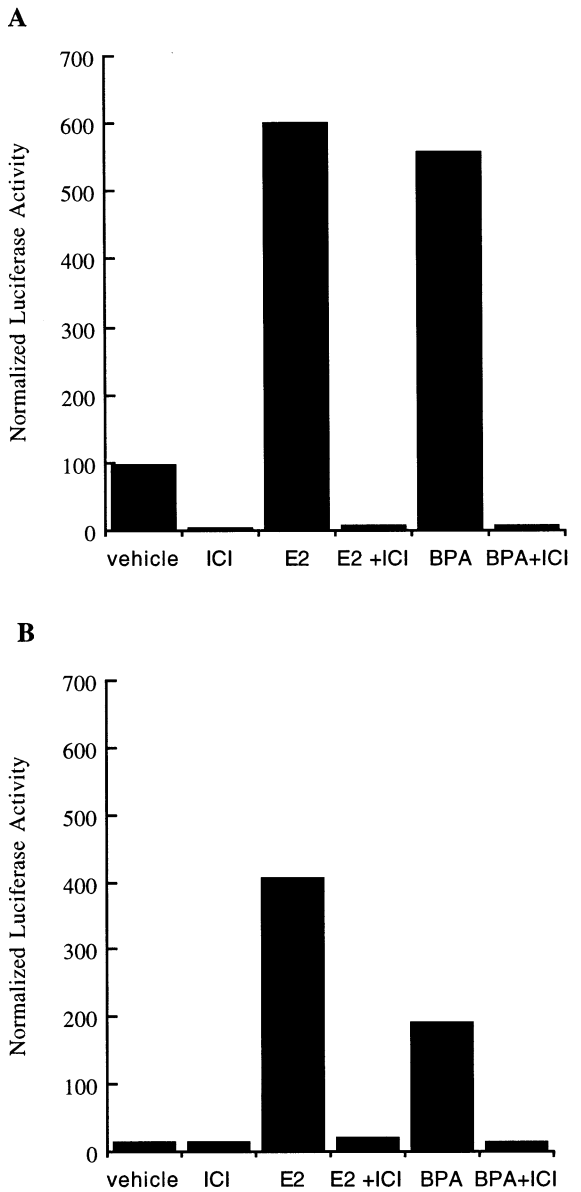


Fig. 2. ICI-182, 780 inhibits activation of the human wild-type estrogen receptor (ER-WT) (A) or Mutant Estrogen Receptor (ER-AF1) (B) induced by estradiol and bisphenol A. HepG2 cells were transiently cotransfected with 270 ng of ER-WT or ER-AF1 plasmid, 405 ng pC3-luc reporter plasmid, and 135 ng pRSV-$\beta$-gal plasmid. Cells were treated with vehicle (methanol and ethanol), $10^{-6}$ M ICI 182, 780, $3 \times 10^{-7}$ $\mu$M $E_2$, ICI 182, 780 and $E_2$, $10^{-6}$ M BPA, or ICI 182, 780 and BPA for 24 h and assayed for luciferase activity (luciferase activity was normalized to $\beta$-galactosidase activity which is indicative of transfection efficiency). Experiments were repeated three times, each dose and receptor in triplicate. The data above are one representative experiment.

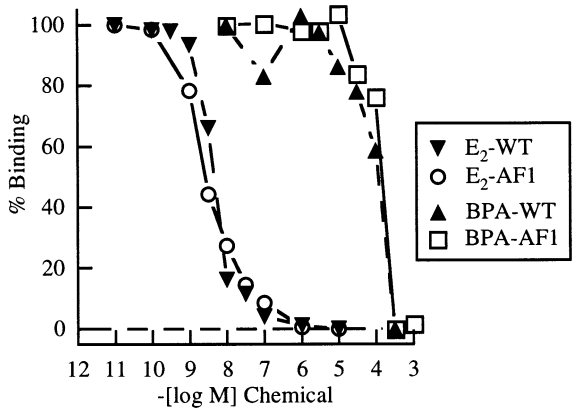


Fig. 3. Competitive binding of estradiol and bisphenol A to the purified human estrogen receptor or mutant estrogen receptor (ER-AF1). $E_2$ or BPA was mixed with 5 nM 17$\beta$-[$^3$H]estradiol and recombinant human ER, or purified ER-AF1 isolated from yeast. After an overnight incubation, the receptor complex was precipitated with a HAP solution. The HAP pellets were washed three times in dilution buffer containing 1% Triton X-100. The radioactivity in the pellets was measured. The data represent one experiment.

We transfected wild type rat ER$\alpha$ into HepG2 cells in order to compare species differences between the human and rat ER$\alpha$ in vitro. In contrast to the partial activity observed with BPA and the human ER$\alpha$ relative to $E_2$, BPA induced $29 \pm 10\%$ greater calculated maximal luciferase activity than $E_2$ (Fig. 6) with the rat ER$\alpha$. The $EC_{50}$ for $E_2$ and BPA were $6.5 \pm 2.6$ and $210 \pm 14$ nM, respectively.

The responsiveness of the rat uterus to BPA was examined to assess the in vivo estrogenic potential of BPA. A dose range of BPA (5, 10, 25, 50, 100 or 150 mg BPA/kg) was administered to immature female rats in a standard uterotrophic assay. Uterine wet weight and two estrogen-responsive proteins, peroxidase activity and progesterone receptors (PR) levels, were assessed (Table 1). Bisphenol A had no effect on uterine weight and increased peroxidase activity by at least 50% at the two highest dose levels (100 and 150 mg BPA/kg per day). A reduction in peroxidase activity was observed after treatment with the lowest dose treatment of BPA (5 mg BPA/kg per day). In contrast, PR levels were elevated at least 75% in all dose groups. The BPA effects observed on both peroxidase activity and PR levels were less than those observed with $E_2$ treatment.

Estradiol (0.5 $\mu$g/rat) and various doses of BPA were administered simultaneously to immature female rats; and uterine wet weight, peroxidase activity, and PR levels were determined (Table 2). Bisphenol A had no effect on $E_2$-induced uterine wet weight. However, BPA inhibited the $E_2$-stimulated peroxidase activity by at least 43% in the rats treated with 100 and 150 mg BPA/kg per day and inhibited the $E_2$-stimulated PR levels up to 55% at all dose levels of BPA.

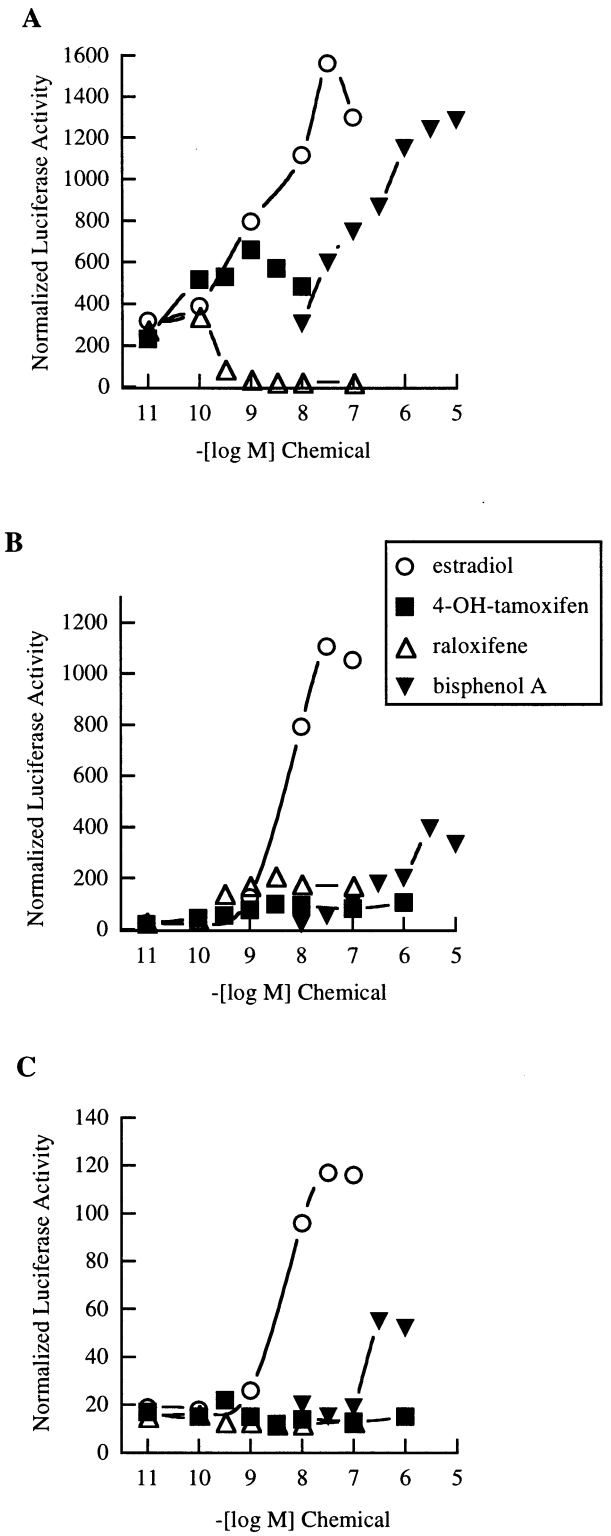


Fig. 4. A comparison of the activation of the human estrogen receptor (ER-WT) (A), mutant estrogen receptor ER-AF1 (B) or ER-AF2 (C) by estradiol, 4-OH-tamoxifen, raloxifene, or bisphenol A. HepG2 cells were transiently cotransfected with 40 ng of human ER-WT, ER-AF1, or ER-AF2 plasmid, 405 ng pC3-luc reporter plasmid and 110 ng pRSV-$\beta$-gal plasmid. Cells were treated with vehicle (methanol) or increasing concentrations of $E_2$, 4OH-T, raloxifene, or BPA for 24 h and assayed for luciferase activity (luciferase activity was normalized to $\beta$-galactosidase activity which is indicative of transfection efficiency). Each individual experiment consisted of $E_2$, BPA, 4OH-T, and raloxifene performed in parallel. Experiments were repeated four times (ER-WT and ER-AF2) and six times (ER-AF1), each dose and receptor in triplicate. The data above are one representative experiment.

## 4. Discussion

Estrogenic compounds have been classified based on their ability to induce a selective response with the ER$\alpha$ (Katzenellenbogen et al., 1996). In the present study we have used an established ER$\alpha$ paradigm (Tzukerman et al., 1994; McDonnell et al., 1995; Willson et al., 1997) in HepG2 cells to demonstrate that BPA interacts with the ER$\alpha$ in a unique manner that differs from other known classes of ER ligands. This unique interaction with the ER$\alpha$ may explain, in part, differences in the effects of BPA as compared with $E_2$ in vivo.

The ability of an estrogen–ER$\alpha$ complex to induce transcription is dependent upon the two transcription activation domains of the ER$\alpha$, AF1 and AF2 (Tasset et al., 1990). The receptor undergoes a conformational change upon estrogen binding, allowing these domains to interact with cellular cofactors that enhance DNA binding and transcription (Renaud et al., 1995; McInerney et al., 1996). ER ligands have been divided into mechanistically distinct groups based upon their relative ability to activate AF1/AF2 regions (McDonnell et al., 1995) which is thought to be due to the ability of these different classes to induce distinct conformations of the ER$\alpha$. At least four different structural classes of partial ER$\alpha$ agonists/antagonists have been described, and these include $E_2$ (pure agonist), ICI 182, 780 (Type II, pure antagonist), raloxifene (Type III, anti-estrogen), and 4OH-T (Type IV, anti-estrogen). These mechanistically distinct groups manifest unique biology in vitro (Fig. 4) and in vivo (Jordan et al., 1987; Wakeling et al., 1991; McDonnell et al., 1995).

Bisphenol A defines a new class of ER$\alpha$-interactive compound that has actions distinct from other ER$\alpha$ agonist/antagonist compounds. Although the partial agonist efficacy of BPA was almost that of $E_2$ in HepG2 cells transfected with the ER-WT, a marked decrease in maximal luciferase activity relative to $E_2$ was observed with the ER-AF1 and ER-AF2 mutants (Figs. 1 and 4). This in vitro activity of BPA was dissimilar from that of $E_2$, ICI 182, 780, raloxifene, and 4OH-T (Figs. 1, 2 and 4). The distinct activity of BPA is most likely due to an induction of a conformation of the activated ER$\alpha$ by BPA that differs from these other known classes of ER ligands. Thus, it is likely that BPA will induce a unique subset of ER$\alpha$-responsive genes in

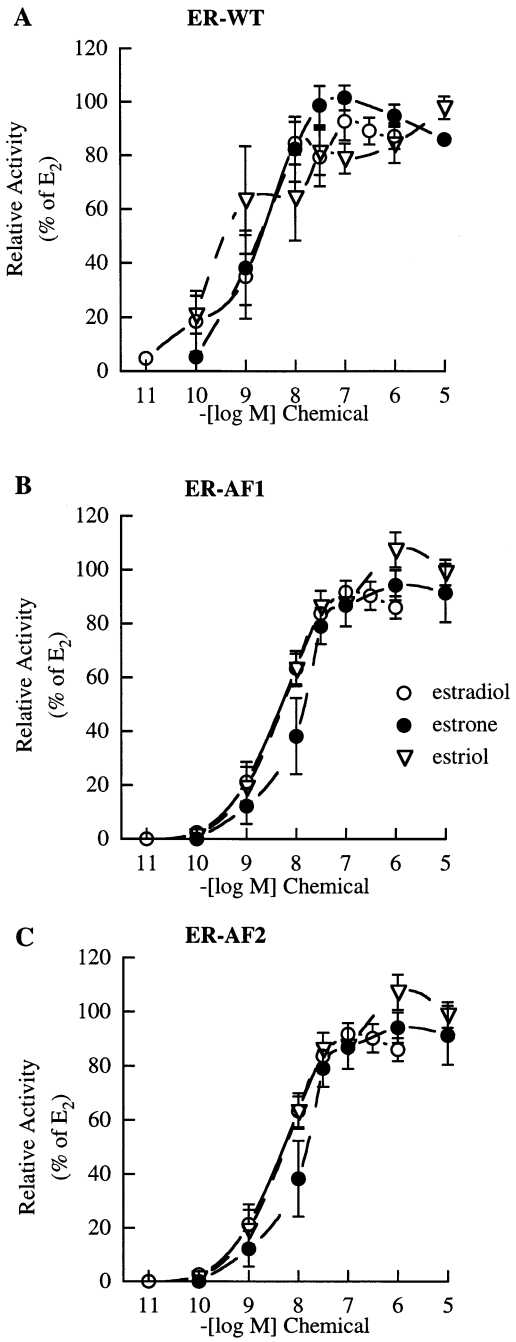


Fig. 5. A comparison of the activation of the human estrogen receptor (ER-WT) (A), mutant estrogen receptor ER-AF1 (B) or ER-AF2 (C) by Estradiol, Estrone, and Estriol. HepG2 cells were transiently cotransfected with 40 ng of human ER-WT, ER-AF1, or ER-AF2 plasmid, 405 ng pC3-luc reporter plasmid and 110 ng pRSV-$\beta$-gal plasmid. Cells were treated with vehicle (methanol) or increasing concentrations of $E_2$, estrone or estriol for 24 h and assayed for luciferase activity (luciferase activity was normalized to $\beta$-galactosidase activity which is indicative of transfection efficiency). Each individual experiment consisted of $E_2$, estrone and estriol performed in parallel. Experiments were repeated four times (ER-WT) and five times (ER-AF1 and ER-AF2), each dose and receptor in triplicate. The data represent the mean $\pm$ SEM of all replicate experiments for each chemical and receptor.

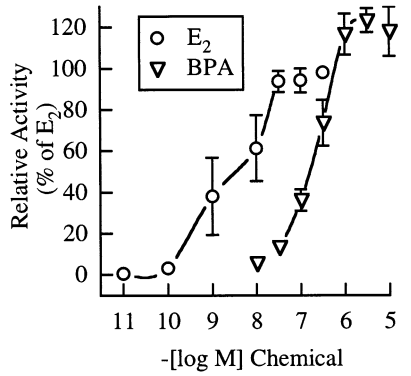


Fig. 6. Activation of the rat wild-type (ER-RAT) by estradiol and bisphenol A. HepG2 cells were transiently cotransfected with 16 ng of ER-RAT plasmid, 405 ng pC3-luc reporter plasmid and 135 ng pRSV-$\beta$-gal plasmid. Cells were treated with vehicle (methanol) or increasing concentrations of $E_2$ or BPA for 24 h and assayed for luciferase activity (luciferase activity was normalized to $\beta$-galactosidase activity which is indicative of transfection efficiency). Luciferase activity is shown as the mean of the percent activity of the maximal $E_2$ induced activity. Estradiol was run in parallel with BPA for each individual experiment. Data for ER-RAT represent the combined data from three experiments, each dose and receptor in triplicate. The data represent the mean $\pm$ SEM of all replicate experiments for each chemical.

vivo resulting in a biological response which differs from the known classes of ER ligands. In addition, we showed that the activity of BPA could not be explained just because it was a weak estrogen, since the activity of BPA also did not resemble that of other known weak ER agonists such as estriol and estrone. Using HepG2 cells transfected with human wild-type and mutant ER$\alpha$s, estrone or estriol had the same profile as $E_2$ (Fig. 5).

Our studies in vivo support the hypothesis that the activity of BPA is unique compared to other ER ligands. While BPA had no uterotrophic effects in immature female rat, uterine estrogen-responsive proteins were induced, although not to the extent observed for $E_2$ (Table 1). In addition, BPA antagonized induction of uterine responsive proteins by $E_2$ (Table 2). Together, these results indicate that BPA is acting as a partial agonist for these two responses. 4OH-T was reported to exhibit anti-estrogenic activity in mammary tissue, but full ER$\alpha$ agonist responses were observed in the uterus and bone (Robertson et al., 1982; Jordan et al., 1987; Love et al., 1992). Raloxifene ER$\alpha$ agonist activity has only been observed in the bone, and ICI 182, 780 is a pure ER$\alpha$ antagonist in all estrogen-responsive tissues (Wakeling et al., 1991; Evans et al., 1993; Black et al., 1994). Thus, BPA appears to have a unique profile of responses. Steinmetz and colleagues also observed that BPA stimulated some, but not all $E_2$-inducible responses in the F344 rat pituitary (Steinmetz et al., 1997). The lack of effect on uterine weight

Table 1
Effects of bisphenol A on estrogenic responses in the immature Sprague–Dawley rat uterus

| Treatment (mg/kg per day) | Uterine wet wt. (mg) | Peroxidase activity (activity/mg protein)[a] | Progesterone receptor (fmol/uterus)[b] |
|---|---|---|---|
| Control | 40 ± 8 | 0.36 ± 0.04 | 454 ± 37 |
| Estradiol 0.5 μg | 190 ± 80 | 2.94 ± 0.05 | 2786 ± 247* |
| Bisphenol A (mg/kg) | | | |
| 5.0 | 38 ± 3 | 0.12 ± 0.04* | 612 ± 17* |
| 10 | 33 ± 3 | 0.40 ± 0.05 | 683 ± 38* |
| 25 | 35 ± 3 | 0.21 ± 0.04 | 599 ± 19* |
| 50 | 35 ± 5 | 0.24 ± 0.03 | 627 ± 10* |
| 100 | 34 ± 5 | 0.54 ± 0.03* | 608 ± 49* |
| 150 | 40 ± 4 | 0.75* | 801 ± 36* |

[a] A total of 21 day old rats were treated with $E_2$ or different doses of BPA, and animals were killed 20 h after the last treatment. The pooled uterus was homogenized and purified by centrifugation. The uterine peroxidase assay monitored the initial rate of guaiacol oxidation at 470 nM. An enzyme unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per minute under the assay conditions described. Enzyme activity is reported as the mean (activity per mg uterine protein) ± SEM.
[b] A total of 21 day old rats were treated as described above. The pooled uterus was homogenized and the cytosolic fraction was obtained. Progesterone receptor levels were determined by competitive binding of [$^3$H]R5020 with or without unlabeled progesterone. Dextran-coated charcoal suspension was used to separate free hormone, and the radioactivity of the supernatant was measured. Progesterone receptor levels are reported as the means (fmol per uterus) ± SEM.
* Significant difference from control animals ($P<0.05$) was determined by Analysis of Variance and the Duncan new multiple range test.

was unexpected considering reports to the contrary described intraperitoneal injections of BPA increased uterine weight in a uterotrophic assay (Bond et al., 1980; Papaconstantinou et al., 1998). We recognize that a number of other factors such as absorption, differential metabolism and alternate signaling pathways also play a role in any in vivo response. These factors may account for some of the effects observed in the rat uterus (i.e. uterotrophic assay, stimulation of PR). Some effects of BPA may also occur via alternate pathways in addition to ERα mediated induction of these $E_2$ responsive proteins. For example, tumor necrosis factor-α can regulate PR via a ERα independent mechanism (Kalkhoven et al., 1996). The effects of BPA on these alternate pathways has not been investigated.

Estrogen receptor activation can also occur through pathways other than through classical ERα activation pathway. Epidermal growth factor, dopamine, insulin like growth factor-I, and transforming growth factor-α have been reported to induce estrogen-like responses and these responses were inhibited by ICI 164, 384, a pure ERα antagonist (Ignar-Trowbridge et al., 1992; Chalbos et al., 1993; Ignar-Trowbridge et al., 1993; Smith et al., 1993) These effects are believed to be through alternate pathways involving an unliganded receptor, cAMP, and/or mitogen-activated protein kinase (Kato et al., 1995; Bunone et al., 1996). However, studies suggest that unliganded activation of the ERα is AF1 dependent (; Ignar-Trowbridge et al., 1993, 1996). Point mutations in the AF1 region of the ERα obliterate ligand independent ERα activation (McDonnell, unpublished results). In HepG2 cells, BPA did induce luciferase activity with the ER-AF2 (Fig. 1) which does not mediate unliganded ERα activity, indicating that BPA activates the ERα through a ligand dependent mechanism.

The rat is a species that continues to be used extensively as a model for human exposure to xenobiotics and in mechanistic studies. In the present study, we examined both human and rat ERα. Both $E_2$ and BPA displayed similar potencies for human and rat ERα (Figs. 1 and 6). Interestingly, the rat ERα had higher activity relative to the human ERα in HepG2 cells. This difference in peak activity was not due to differences in the concentration of human and rat ERα in the cells since the promoter was the same for both plasmids and since varying the level of ERα plasmid transfected into the cells did not alter peak activity (data not shown). Instead, these results indicate that the rat ERα may interact with greater transcriptional efficiency in human HepG2 cells. In in vivo studies, however, BPA acted as a partial agonist in the rat uterus (Tables 1 and 2), and this response was similar to that observed for BPA in HepG2 cells transfected with human ERα (Fig. 1). These results indicate that ligand-ERα interactions observed in humans and rats are comparable. Further studies characterizing the rat AF1 and AF2 regions will be helpful in understanding differences between the human and rat ERα. Understanding these differences will allow for better interpretation of rodent studies and their implications for human exposure to endocrine toxicants.

Previous reports in vitro have shown that BPA behaved as an agonist, giving the impression that BPA behaves soley as a weak $E_2$ mimic (Krishnan et al., 1993; Brotons et al., 1995; Olea et al., 1996; Gaido et al., 1997). Our results clearly indicate that BPA inter-

Table 2
Effects of estradiol plus bisphenol A on estrogenic responses in the immature Sprague–Dawley rat uterus

| Treatment (mg/kg per day) | Uterine wet wt. (mg) | Peroxidase activity (activity/mg protein)[a] | Progesterone receptor (fmol/uterus)[b] |
|---|---|---|---|
| Control | 39 ± 4* | 0.04 ± 0.25* | 672 ± 215* |
| Estradiol 0.5 μg | 158 ± 46 | 3.48 ± 0.02 | 3882 ± 379 |
| Bisphenol A + 0.5 μg estradiol | | | |
| 5.0 mg/kg | 149 ± 83 | 2.72 ± 0.01* | 2827 ± 386* |
| 10 mg/kg | 174 ± 68 | 3.24 ± 0.01 | 2847 ± 384* |
| 25 mg/kg | 164 ± 35 | 3.58 ± 0.00 | 1765 ± 102* |
| 50 mg/kg | 153 ± 24 | 3.39 ± 0.02 | 2019 ± 204* |
| 100 mg/kg | 128 ± 26 | 1.98 ± 0.02* | 2365 ± 334* |
| 150 mg/kg | 141 ± 25 | 1.89 ± 0.01[c] | 1670 ± 444* |

[a] See Table 1 for description of peroxidase assay.
[b] See Table 1 for description of PR determination.
* Significant difference from $E_2$-treated animals ($P < 0.05$) was determined by Analysis of Variance and the Duncan new multiple range test.

acts with the ERα in a unique manner capable of inducing responses that differ from $E_2$ both in vitro and in vivo. Other chemicals such as organochlorine pesticides, phthalates, nonylphenol, phytoestrogens, and polychlorinated biphenyls have also been identified as compounds that competitively bind to ERα and are potential modulators of the endocrine system (Sharpe et al., 1995; Villalobos et al., 1995; Arnold et al., 1996; Olea et al., 1996; Gaido et al., 1997). Many of these chemicals may also interact with the ERα in a distinct manner to induce a unique spectrum of responses that are dependent on compound-specific induced changes in the conformation of the ERα and subsequent interactions with tissue and species-specific transcriptional machinery. While the molecular determinants of ERβ have yet to be determined, many of theses chemicals have also been shown to competitively bind ERβ (Kuiper et al., 1997) and it is likely that these compounds may also interact with ERβ in a unique manner. Therefore, a complete understanding of the interaction of a chemical with ERα as well as ERβ at the level of the tissue, cell, and gene both in vivo and in vitro is necessary to define the estrogenic potential of environmental estrogens.

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