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In Vitro and in Vivo Interactions of Bisphenol A and Its Metabolite, Bisphenol A Glucuronide, with Estrogen Receptors α and β

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The estrogenic activities of bisphenol A (BPA) and its major metabolite BPA glucuronide (BPA-G) were assessed in a number of in vitro and in vivo assays. BPA competed with [^{3}H]-17β-estradiol (E2) for binding to mouse uterine cytosol ER, a glutathione *S*-transferase (GST)−human ER D, E, and F domain fusion protein (GST−hERαdef) and full-length recombinant hERβ. The IC_{50} values for E2 were similar for all three receptor preparations, whereas BPA competed more effectively for binding to hERβ (0.96 μM) than to either mouse uterine cytosol ER (26 μM) or GST−hERαdef (36 μM). In contrast, BPA-G did not competitively displace [^{3}H]E2 from any of the ER preparations. In MCF-7 cells transiently transfected with Gal4-hERαdef or Gal4-hERβdef, BPA induced reporter gene activity with comparable EC_{50} values (71 and 39 μM, respectively). No significant induction of reporter gene activity was seen for BPA-G. Cotreatment studies showed that concentrations of (10 μM) BPA and BPA-G did not antagonize E2-induced luciferase mediated through either Gal4-hERαdef or Gal4-hERβdef. In vivo, the uterotropic effect of gavage or subcutaneous (sc) administration of 0.002−800 mg of BPA/kg of body weight/day for three consecutive days was examined in immature rats. Dose-related estrogenic effects on the rat uterus were observed at oral doses of 200 and 800 mg/kg and at sc doses of 10, 100, and 800 mg/kg. These results demonstrate that BPA competes more effectively for binding to ERβ, but induces ERα- and ERβ-mediated gene expression with comparable efficacy. In contrast, BPA-G did not exhibit any in vitro estrogenic activity. In addition, there was a clear route dependency on the ability of BPA to induce estrogenic responses in vivo.

Introduction

Accumulating evidence suggests that exposure to natural and synthetic chemicals that mimic the activity of 17β-estradiol (E2) may adversely affect wildlife and human health. Although controversial (*1*), there have been reports of decreases in sperm production and seminal volume in humans during the past half-century (*2*−*4*). In wildlife, field studies indicate there are increases in reproductive abnormalities in mammals (*5*), reptiles (*6*), birds (*7*), and several fish species (*8*) following exposure to estrogenic environmental contaminants. Many of these effects are thought to occur through an estrogen receptor (ER)-mediated mechanism of action.

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The ER is a member of the nuclear receptor superfamily, a family of nuclear proteins that function as transcription factors to modulate gene expression in a ligand-dependent manner (*9*). The two subtypes, ERα and ER*β*, are products of distinct genes and differ in their tissue distribution, and in their ligand preferences (*10*, *11*). For example, in rat, ERα is expressed at higher levels in the uterus, kidney, and epididymis, while ER*β* levels are higher in the prostate, ovaries, and lung (*12*). As with other members of the nuclear receptor superfamily, the ER has a modular structure consisting of six domains (A−F) (*9*, *13*). The highly conserved DNA-binding domain (C domain) separates a highly variable NH_2-terminal region (A and B domains), which contains a ligand-independent activation region (AF-1), and a COOH-terminal region, which includes a hinge region (D domain), the ligand binding domain (E domain), and a variable F domain. The ER E domain also harbors a nuclear localization signal, a dimerization region, and a ligand-dependent activation region (AF-2) as well as residues which interact with heat shock protein 90 (hsp90) (*14*−*16*). When estrogen binds, the ER undergoes a conformational change, which allows it to bind to its cognate DNA target site, termed estrogen responsive elements (EREs), located in the regulatory region of estrogen-inducible genes, thereby modulating the expression of estrogen responsive genes.

Bisphenol A [4,4′-isopropylidene-2-diphenol (BPA)], a monomer used in the production of polycarbonate and epoxy resins, has been shown to elicit ER-mediated activity in a number of in vitro and in vivo assays. BPA has been shown to compete with [^{3}H]E2 for binding to ERα and ER*β* (*11*), and induce a number of in vitro effects, including ERα- and ER*β*-mediated reporter gene activity (*11*), MCF-7 human breast cancer cell proliferation, progesterone receptor expression (*17*), vitellogenin expression in carp hepatocytes (*18*), and prolactin release in a pituitary tumor cell line (*19*).

High doses of BPA have been reported to elicit reproductive toxicity and abnormal cellular development in rodents (*20*). However, unlike E2, BPA had no effect on uterine weight in exposed rats at doses as high as 150 mg/kg of body weight, but induced peroxidase activity and elevated progesterone receptor (PR) levels similar to that of E2 (*21*). Furthermore, in cotreatment studies, BPA antagonized the E2 stimulatory effects on both peroxidase activity and PR levels but did not inhibit E2-induced increases in uterine weight, suggesting that BPA may use a mechanism of action distinct from that of E2 (*21*).

BPA is readily metabolized in vivo through glucuronidation to BPA glucuronide (BPA-G), and subsequently excreted in the feces and urine (*22*, *23*). Pharmacokinetic studies have shown that there is a clear route dependency in the bioavailability of parent BPA, with considerably higher systemic blood concentrations of BPA after sc and intraperitoneal administration than after oral administration (*23*).

There has been increasing concern regarding the estrogenic activities of BPA as a result of reports that trace levels can leach from the lining of food cans, polycarbonate plastic ware, and dental resins (*24*, *25*). Consequently, the estrogenic activities of BPA and BPA-G were investigated for their ability to compete with [^{3}H]E2 for binding to the recombinantly expressed glutathione *S*-tranferase (GST)−estrogen receptor α (ERα) fusion protein, consisting of the D−F domains of hERα linked to GST, and to full-length ER*β* proteins, to induce and antagonize ERα- and ER*β*-mediated reporter gene expression in MCF-7 cells transfected with Gal4-hERαdef or Gal-hER*β*def and the Gal4-regulated luciferase reporter gene (17m5-G-Luc) in vitro. To account for pharmacodynamic and pharmacokinetic interactions that may affect in vivo estrogenic activity, the ability of BPA to induce a variety of uterine responses was investigated in immature Sprague-Dawley rats using two different routes of exposure.

Experimental Procedures

Chemicals. Bisphenol A (99.9% pure, Bayer AG) and bisphenol A glucuronide were provided by the BPA Global Industry Group. Dimethyl sulfoxide (DMSO) and 17*β*-estradiol (E2) used in the in vitro studies were obtained from Sigma (St. Louis, MO), and E2 used in the in vivo studies was from Sigma (Poole, Dorset, U.K.). [2,4,6,7,16,17-^{3}H]E2 (123 Ci/mmol) was purchased from New England Nuclear (Boston, MA). MicroScint 20 was obtained from Packard Instruments (Meriden, CT). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA), and restriction enzymes were obtained from Roche/Boehringer Mannheim (Indianapolis, IN). Phenol red-free Dulbecco's modified Eagle's medium (D-MEM) and medium supplements were from Life Technologies (Gaithesburg, MD). Fetal bovine serum (FBS) and D-luciferin were purchased from Intergen (Purchase, NY) and Molecular Probes (Eugene, OR), respectively. All other chemicals and biochemicals were of the highest quality available from commercial sources.

Animals. Immature female Alpk:APfSD rats (21−22 days old) with body weights of 38−53 g were obtained from the breeding unit at AstraZeneca (Alderley Park, U.K.). Animals were housed in multiple rat racks suitable for animals of this strain and the weight range expected during the course of the study. Humidity was controlled, and a 12 h light/dark cycle was maintained. Animals were weaned on Rat and Mouse No. 3 (RM3) breeding diet (Special Diets Services Ltd., Witham, Essex, U.K.) until they were transferred to AstraZeneca Central Toxicology Laboratory (CTL) and were then maintained on Rat and Mouse No. 1 (RM1) maintenance diet, as previously recommended (*26*). All animals were acclimatized to the laboratory for at least 18 h before being treated.

Construction of Plasmids. The plasmid pGEX-hERαdef was constructed as previously described (*27*). The plasmid pGal4-hER*β*def (amino acids 204−530) was constructed by PCR amplification of the hER*β* (kindly provided by L. Murphy, University of Manitoba, Winnipeg, MB), using the primers pr-hf (5′-caaactcgagcctgccgacttcggaagtgttacga-3′) and pr-hr (5′-caaaggatcctcactgagactgtgggttctgg-3′). The fragment was digested with *Xho*I and *Bam*HI and ligated into the similarly digested eukaryotic expression vector containing the DNA binding domain of the yeast transcription factor Gal4, pG4MpolyII (kindy provided by P. Chambon, IGBMC CNRS-LGME, Illkirch Cedex C. U. de Strasbourg, France). PCR amplification was performed essentially as previously described (*28*) using Vent DNA polymerase in a reaction mixture containing Thermopol buffer, 200 μM dNTPs, 1 mM $MgSO_4$, 500 nM primer, and 1.25 units of polymerase, which was heated to 94 °C for 5 min followed by 35 rounds at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 105 s. The sequence of each construct was confirmed with restriction enzyme digestion and ABI/Prism automated sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA).

Expression and Purification of the GST−ER Fusion Protein. Expression, purification, and characterization of the GST−hERαdef fusion protein were carried out as previously described (*27*).

Receptor Binding Assays. The partially purified GST−hERαdef fusion protein or recombinant hER*β* (PanVera, Madison, WI) was diluted in TEGD buffer [10 mM Tris (pH 7.6), 1.5 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] containing 1 mg/mL bovine serum albumin (BSA) as a carrier protein. For

mouse uterine cytosol preparation, immature female CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in a controlled environment (40−60% humidity; 20−22 °C ambient temperature) on a 12 h light/dark cycle. Harlen Teklad 22/5 Rodent Diet 8640 (Madison, WI) and tap water were provided ad libitum. Uterine tissue from 21-day-old CD-1 mice was excised, trimmed of excess fat and connective tissue, weighed, and homogenized in 1.0 mL of TEGD [10 mM Tris base, 1.5 mM EDTA, 10% glycerol, and 1.0 mM DTT (pH 7.6)] per 50 mg of uterine tissue with 3 × 20 s bursts using a Brinkman Polytron homogenizer at 50% output. Samples were centrifuged at 3000*g* for 10 min at 4 °C. The supernatant (cytosol) was centrifuged at 105000*g* for 1 h at 4 °C. The protein concentration of the cytosol was adjusted to 2.0 mg/mL and the protein stored at −80 °C.

Protein preparations and the mouse uterine cytosol were incubated at 4 °C for 24 h and at 30 °C for 2 h, respectively, with 2.5 nM [^{3}H]E2 and increasing concentrations of unlabeled competitor in 1 mL glass tubes arranged in a 96-well format (Marsh Scientific, Rochester, NY). Protein preparations were diluted to ensure 10 000 dpm of total binding (dilutions were 500-fold hERβ and 1500-fold GST−hERαdef). Binding assays were initiated by adding 240 μL of the protein preparation to glass tubes containing 5 μL of DMSO and 5 μL of [^{3}H]E2; thus, the solvent concentration did not exceed 4%. The amount of nonspecific binding was determined in the presence of a 400-fold excess of unlabeled E2. Bound [^{3}H]E2 was separated from free [^{3}H]E2 using a 96-well filter plate and vacuum pump harvester (Packard Instruments). Filter plates containing the protein were washed with 3 × 50 mL of TEG [10 mM Tris buffer (pH 7.6), 1.5 mM EDTA, and 10% (v/v) glycerol] and allowed to dry under continued suction for 30 s. After drying, the undersides of the filter plates were sealed and 50 μL of MicroScint 20 scintillation cocktail was added to each well. The amount of bound [^{3}H]E2 was measured using a TopCount luminescense and scintillation counter (Packard Instruments).

Each treatment was performed in quadruplicate, and results are expressed as the percent specific binding of [^{3}H]E2 versus the log of the competitor concentration. The reported IC_{50} values represent the concentration of test compound required to displace 50% of the [^{3}H]E2 from the ER preparation as compared to the 50% displacement of [^{3}H]E2 achieved by unlabeled E2. These analyses were performed using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA).

Cell Culture. MCF-7 human breast cancer estrogen receptor positive cells (obtained from L. Murphy, University of Manitoba, at passage 32−35) were maintained with phenol red-free D-MEM supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 500 μg/mL gentamicin, 2.5 μg/mL amphotericin B, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Cells were cultured in a humidified environment at 37 °C with 5% CO_2.

Transfection and Reporter Gene Assays. Transient transfections and gene transcription assays were performed essentially as previously described (*29*, *30*). Briefly, MCF-7 cells were seeded at approximately 50% confluency in six-well tissue culture plates in medium supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and allowed to settle for 7 h. Cells were transiently transfected by the calcium phosphate coprecipitation method (*31*) with 2.5 μg of 17m5-G-Luc, 0.2 μg of Gal4-hERαdef, or 0.2 μg Gal4-hERβdef, along with 0.01 μg of pCMV-lacZ (β-galactosidase expression vector). Cells were washed 16 h later with sterile phosphate-buffered saline (PBS), and then fresh medium was added to each well.

Transiently transfected MCF-7 cells were exposed to final concentrations ranging (i) from 1 nM to 10 μM for BPA or BPA-G and (ii) from 1 pM to 10 nM for E2 or (iii) DMSO (solvent) alone. Final concentrations were obtained by adding 2 μL of the test chemical to 2 mL of the medium. Following incubation with the sample for 24 h, cells were harvested and assayed for luciferase activity according to the method described previously (*32*).

In the transiently transfected MCF-7 cells, the reference plasmid pCMV-lacZ was cotransfected as an internal control to correct for differences in transfection efficiencies and extraction differences. β-Galactosidase activity was measured according to standard protocols (*31*). Each treatment was carried out in duplicate, and two samples were taken from each replicate. Each experiment was repeated three times. Values are reported as a percentage relative to the maximum induction observed with 10 nM E2.

Uterotrophic Assay. The protocol for the uterotrophic assay has been previously described (*26*, *33*). Animals were treated by either gavage or sc injection with BPA suspended in arachis (peanut) oil. Clinical observations and body weights were recorded daily. The dosing volume used in both studies was 5 mL/kg of body weight, calculated on the weight of the animal immediately prior to administration. Animals were treated with BPA for three consecutive days and were killed by an overdose of halothane Ph Eur vapor approximately 24 h after the final dose. The dose levels for the oral gavage study were 0.002, 0.02, 0.2, 1.0, 10, 100, 200, and 800 mg of BPA/kg/day with 10 animals per treatment group. The dose levels for the sc injection study were 0.002, 0.02, 0.2, 1.0, 10, 100, and 800 mg of BPA/kg/day with 10 animals per treatment group. The high dose levels for both studies were based on preliminary work which established that oral and subcutaneous doses of up to 800 mg of BPA/kg/day did not induce more than mild toxicity in the experimental animals. The remaining dose levels were chosen by the BPA Global Industry Group to cover a wide range of exposure to the experimental animal. E2 was used as the positive control for both studies, using a dose level of 0.4 mg/kg of body weight/day, as recommended previously (*26*).

At necropsy, the uteri were excised, trimmed free of fat and any adhering nonuterine tissue, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horn and the ovaries. The uterus was then weighed (wet weight), prior to bisection of the uterus at the junction of the uterine horns. One uterine horn was reweighed (wet) and then dried in a preweighed glass vial for a minimum of 24 h in an oven at a temperature of 70 °C. After being dried, the sample was removed and left to cool in a desiccator for a minimum of 1 h and the uterine dry weight recorded. The other section of the uterus was preserved in 10% neutral buffered formol saline and processed for histopathological examination. Liver weight and plasma alanine aminotransferase and aspartate aminotransferase levels were recorded, but the data are not presented in this paper.

Statistical analysis was conducted using the SAS (1989) package as follows: body weights, by analysis of covariance on initial body weight; and uterus weight, by analysis of variance and analysis of covariance on terminal body weights.

Results

Competitive Binding Ability of BPA and BPA-G. The ability of BPA and BPA-G to compete with [^{3}H]E2 for binding to mouse uterine ER, GST−hERαdef, and commercially available baculovirus-expressed full-length hERβ (Figure 1) preparations was investigated in vitro using a semi-high-throughput competitive binding assay. BPA competed for binding to each of the ER preparations; however, the IC_{50} values varied among preparations (Table 1). BPA bound to hERβ approximately 27- and 38-fold better than GST−hERαdef and mouse uterine cytosol, respectively (Table 1). BPA-G did not competitively displace [^{3}H]E2 from any of the ER preparations (Figure 1) at the concentrations that were examined (1 nM to 100 μM). Concentrations of >100 μM were not examined due to possible artifactual results from competitor ligand precipitation.

Ability of BPA and BPA-G To Induce ERα- and ERβ-Mediated Gene Expression. BPA and BPA-G

Table 1. Summary of the IC_{50} Values of the Competitive Binding of 17β-Estradiol, Bisphenol A, and Bisphenol A Glucuronide to GST−hERαdef, Recombinant hERβ, and Mouse Uterine Cytosol

chemical	IC_{50} (M)[a]		
	GST−hERαdef	hERβ	mouse cytosol
17β-estradiol	$(2.9 \pm 0.5) \times 10^{-9}$	$(3.6 \pm 0.4) \times 10^{-9}$	$(3.0 \pm 0.2) \times 10^{-9}$
bisphenol A	$(3.6 \pm 1.6) \times 10^{-5}$	$(9.6 \pm 2.3) \times 10^{-7}$	$(2.6 \pm 1.1) \times 10^{-5}$
bisphenol A glucuronide	nb[b]	nb	nb

[a] IC_{50} values represent the means and standard deviations from three independent experiments. [b] nb denotes nonbinder since this chemical did not displace more than 10% of the [^{3}H]E2 at the highest concentration that was examined (100 μM).

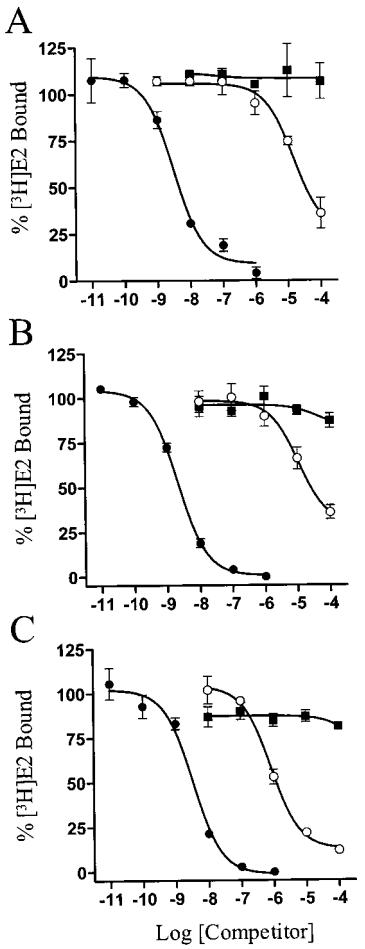

Figure 1. Competitive binding of BPA and BPA glucuronide to (A) the mouse uterine ER, (B) GST−hERαdef, and (C) recombinant hERβ. Protein preparations were incubated with 2.5 nM [^{3}H]E2 and increasing concentrations of unlabeled (●) E2, (○) BPA, or (■) BPA-G. The competitive displacement of radioligand was assessed using a vacuum pump filteration method as described in Experimental Procedures. Displacement curves were obtained for E2 and BPA. No significant displacement was observed with BPA-G. The data are the results from a representative experiment that was repeated three times.

induction of ER-mediated gene expression was assessed by measuring luciferase activity using MCF-7 cells cotransfected with Gal4-hERαdef or Gal4-hERβdef and the Gal4-regulated luciferase reporter gene, 17m5-G-Luc. The results in Figure 2 show that E2 treatment of transiently transfected MCF-7 cells with either Gal4-hERαdef or Gal4-hERβdef caused a concentration-dependent increase in luciferase activity. The EC_{50} values for this response were similar for both chimeric receptors (Table 2). MCF-7 cells transiently transfected with Gal4-hERαdef and Gal4-hERβdef exhibited a maximum induction response of approximately 30- and 20-fold, respectively, following treatment with 10 nM E2.

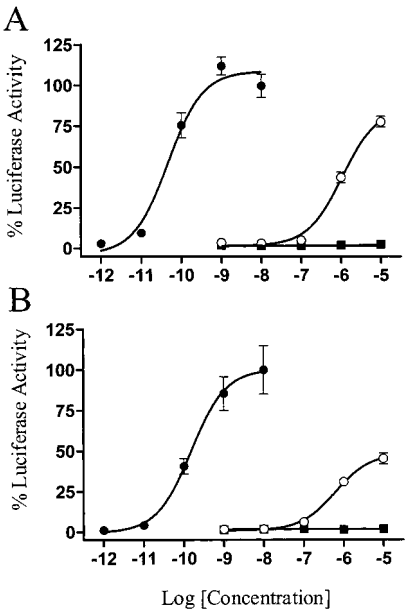

Figure 2. Effect of BPA and BPA glucuronide on reporter gene expression. MCF-7 cells transiently transfected with Gal4-human estrogen receptor α (Gal4-hERαdef) or Gal4-human estrogen receptor β (Gal4-hERβdef) and the Gal4-regulated reporter gene (17m-5-G-Luc) were treated with (●) E2, (○) BPA, or (■) BPA-G. Luciferase activity was measured 24 h after treatment as described in Experimental Procedures. The data are the results from a representative experiment that was repeated three times.

Table 2. Summary of the Ability of 17β-estradiol, Bisphenol A, and Bisphenol A Glucuronide to Induce Estrogen Receptor-mediated Gene Expression

chemical	EC_{50} (M)[a]	
	Gal4-hERαdef	Gal4-hERβdef
17β-estradiol	$(5.3 \pm 2.1) \times 10^{-11}$	$(8.3 \pm 2.9) \times 10^{-11}$
bisphenol A	$(7.1 \pm 2.9) \times 10^{-7}$	$(4.5 \pm 1.8) \times 10^{-7}$
bisphenol A glucuronide	ni[b]	ni

[a] EC_{50} values represent the means and standard deviations from three independent experiments. [b] ni denotes no induction.

BPA caused a dose-dependent increase in luciferase activity in MCF-7 cells transiently transfected with either Gal4-hERαdef or Gal4-hERβdef (Figure 2). The EC_{50} values for the response were similar for both ERα and ERβ (Table 2). Concentrations of >10 μM were not examined due to visible precipitation in the medium. BPA-G did not significantly induce ERα- or ERβ-mediated luciferase activity at the highest concentration that was examined (10 μM) (Figure 2). Similar results were observed using a Gal4-mERβdef consutruct (data not shown). Induction of luciferase activity by BPA was absent in transiently transfected MCF-7 cells if the chimeric construct, Gal4-hERαdef or Gal4-hERβdef, was not cotransfected (data not shown).

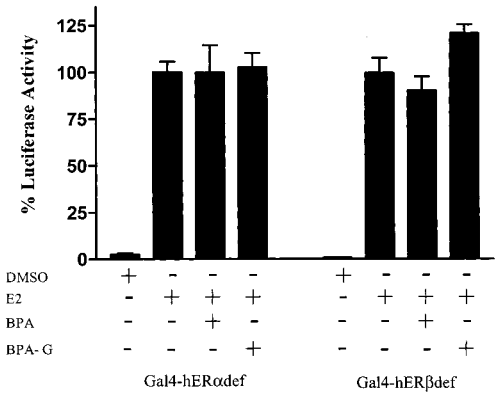

Figure 3. Effect of BPA and BPA-G cotreatment on E2 induction of reporter gene activity. MCF-7 cells transiently transfected with Gal4-human estrogen receptor α (Gal4-hERαdef) or Gal4-human estrogen receptor β (Gal4-hERβdef) and the Gal4-regulated reporter gene (17m-5-G-Luc) were cotreated with 10 nM E2 and 10 μM BPA or 10 μM BPA-G. The data are the results from a representative experiment that was repeated two times.

Figure 3 illustrates that cotreatment of MCF-7 cells with 10 μM BPA or BPA-G did not result in significant changes in the E2 induction of luciferase activity in MCF-7 cells transiently transfected with either Gal4-hERαdef or Gal4-hERβdef.

Uterine Effects following Oral Administration of BPA. There was a significant increase in mean uterine wet and dry weight in the groups treated with 200 and 800 mg of BPA/kg/day in comparison with the vehicle control group (Table 3). As expected, the uterine wet and dry weights in the positive control group were significantly increased compared with those of the vehicle control group.

Minimal to moderate endometrial hypertrophy/hyperplasia was observed in eight of the females treated with 800 mg of BPA/kg/day (Table 4). This effect was similar to, but less pronounced than, that of the positive control group (treated with 0.4 mg of E2/kg/day), where all the females exhibited moderate or marked change. Dose-related increases in severity of endometrial glandular epithelial apoptosis and the number of stromal neutrophils were observed in females treated with 200 or 800 mg of BPA/kg/day and also in the positive control group animals, where the increases were greater than those observed in the BPA-treated animals. An increased incidence and severity of lumenal epithelial apoptosis was also observed in females treated with 800 mg of BPA/kg/day, although this was not observed in the positive control group. There were no treatment-related microscopic changes observed in the uteri of the animals from the remaining groups.

One female in the group treated with 800 mg of BPA/kg/day was killed humanely due to adverse clinical signs, although there were no abnormalities observed at necropsy that could explain these signs. There was no effect of treatment on body weight gain.

Uterine Effects following Subcutaneous Injection of BPA. There was a slight but significant reduction in normalized body weight over the treatment period in the

Table 3. Effect of Oral Gavage of Bisphenol A on Blotted and Dry Uterine Weight in Rats

	dose level (mg/kg/day)	no. of animals	terminal body weight (g) (mean ± SD)	uterus weight (mg) (mean ± SD)	$^1/_2$ uterus blotted weight (mg) (mean ± SD)	$^1/_2$ uterus dry weight (mg) (mean ± SD)	$^1/_2$ uterus percent loss on drying (mean ± SD)
arachis oil	0	10	61.7 ± 4.5	29 ± 9	13 ± 3	3.4 ± 0.04	73.3 ± 3.8
bisphenol A	0.002	10	61.2 ± 6.1	34 ± 8	16 ± 3	3.8 ± 0.09	75.9 ± 2.2
bisphenol A	0.02	10	59.8 ± 6.3	31 ± 9	15 ± 3	3.8 ± 0.06	73.4 ± 3.6
bisphenol A	0.2	10	60.5 ± 8.4	31 ± 9	15 ± 4	3.7 ± 0.12	75.4 ± 3.1
bisphenol A	1	10	61.2 ± 6.6	33 ± 9	16 ± 4[a]	4.3[a] ± 0.11	73.7 ± 5.2
bisphenol A	10	10	61.8 ± 6.3	30 ± 11	13 ± 3	3.7 ± 0.09	71.8 ± 5.5
bisphenol A	100	10	60.6 ± 6.9	29 ± 6	14 ± 3	3.6 ± 0.08	74.0 ± 3.0
bisphenol A	200	10	61.8 ± 5.5	36 ± 8[a]	17 ± 4[a]	4.3 ± 0.07[a]	74.4 ± 2.2
bisphenol A	800	9	60.7 ± 6.7	58 ± 14[b]	28 ± 6[b]	6.0 ± 0.08[b]	78.1 ± 2.6[b]
17β-estradiol	0.4	10	61.4 ± 5.1	109 ± 19[b]	52 ± 11[b]	10.3 ± 0.18[b]	80.0 ± 1.1[b]

[a] $p < 0.05$ with a Student's t test (two-sided). [b] $p < 0.01$ with a Student's t test (two-sided).

Table 4. Microscopic Changes in the Uterus following Oral Gavage of Bisphenol A

	0[a]	0.002[a]	0.02[a]	0.2[a]	1[a]	10[a]	100[a]	200[a]	800[a]	0.4[b]
no. of animals	10	10	10	10	10	10	10	10	9	10
endometrial hypertrophy/hyperplasia										
minimal	0	0	0	0	0	0	0	2	4	0
slight	0	0	0	0	0	0	0	0	3	0
moderate	0	0	0	0	0	0	0	0	1	8
marked	0	0	0	0	0	0	0	0	0	2
endometrial epithelial apoptosis (lumen)										
minimal	1	0	0	1	0	0	1	0	3	3
slight	0	0	0	0	0	0	0	1	2	0
moderate	0	0	0	0	0	0	0	0	1	0
endometrial epithelial apoptosis (glands)										
minimal	7	5	4	8	4	5	6	6	3	0
slight	0	0	0	0	0	0	0	2	1	1
moderate	0	0	0	0	0	0	0	0	3	8
increased stromal neutrophils										
minimal	0	0	0	0	0	0	0	2	4	0
slight	0	0	0	0	0	0	0	0	4	2
moderate	0	0	0	0	0	0	0	0	0	8

[a] Milligrams of BPA per kilogram of body weight per day. [b] Milligram of E2 per kilogram of body weight per day.

Table 5. Effect of Subcutaneous Administration of Bisphenol A on Blotted and Dry Uterine Weight in Rats

	dose level (mg/kg/day)	no. of animals	terminal body weight (g) (mean ± SD)	uterus weight (mg) (mean ± SD)	1/2 uterus blotted weight (mg) (mean ± SD)	1/2 uterus dry weight (mg) (mean ± SD)	1/2 uterus percent loss on drying (mean ± SD)
arachis oil	0	10	63.3 ± 2.4	42 ± 8	19 ± 5.1	2.9 ± 1.7	85.3 ± 6.7
bisphenol A	0.002	10	61.9 ± 4.5	41 ± 14	19 ± 5.6	3.0 ± 2.7	85.2 ± 13.4
bisphenol A	0.02	10	61.4 ± 3.4	48 ± 21	22 ± 11	3.3 ± 4.7	88.7 ± 10.5
bisphenol A	0.2	10	63.4 ± 6.4	37 ± 8	16 ± 3.3	1.6 ± 1.4	90.1 ± 7.8
bisphenol A	1	10	66.5 ± 3.5	43 ± 10	21 ± 4.2	3.0 ± 2.4	86.7 ± 9.1
bisphenol A	10	10	66.4 ± 1.7	44 ± 10	19 ± 4.9	2.0 ± 2.1	89.5 ± 12.8
bisphenol A	100	10	64.4 ± 2.4	51 ± 5	22 ± 3.7	1.2 ± 1.2	94.9 ± 4.7[a]
bisphenol A	800	10	62.6 ± 4.0	91 ± 33[b]	42 ± 14	4.3 ± 3.7	90.7 ± 6.0
17β-estradiol	0.4	10	62.4 ± 5.8	135 ± 14[b]	60 ± 6.4[b]	7.7 ± 2.4[b]	87.5 ± 3.4

[a] $p < 0.05$ with a Student's t test (two-sided). [b] $p < 0.01$ with a Student's t test (two-sided).

Table 6. Microscopic Changes in the Uterus following Subcutaneous Administration of Bisphenol A

	0[a]	0.002[a]	0.02[a]	0.2[a]	1[a]	10[a]	100[a]	800[a]	0.4[b]
no. of animals	10	10	10	10	10	10	10	10	10
endometrial hypertrophy/hyperplasia									
minimal	0	0	0	0	0	0	8	0	0
slight	0	0	0	0	0	0	0	1	0
moderate	0	0	0	0	0	0	0	8	2
marked	0	0	0	0	0	0	0	1	8
endometrial epithelial apoptosis (lumen)									
minimal	0	0	0	0	0	6	1	4	0
slight	0	0	0	0	0	0	9	0	0
moderate	0	0	0	0	0	0	0	2	0
endometrial epithelial apoptosis (glands)									
minimal	0	0	0	0	0	6	1	1	3
slight	0	0	0	0	0	0	9	3	3
moderate	0	0	0	0	0	0	0	5	1
increased stromal neutrophils									
minimal	0	0	0	0	0	0	10	2	0
slight	0	0	0	0	0	0	0	8	1
moderate	0	0	0	0	0	0	0	0	9
vacuolar degeneration of the endometrial epithelium									
minimal	0	0	0	0	0	0	0	0	5
slight	0	0	0	0	0	0	0	0	2
mononuclear cell infiltration (total)	0	0	0	0	0	0	1	0	0
minimal	0	0	0	0	0	0	1	0	0

[a] Milligrams of BPA per kilogram of body weight per day. [b] Milligram of E2 per kilogram of body weight per day.

group treated with 800 mg of BPA/kg/day and in the positive control group in comparison with the control group (data not shown).

There was a significant increase in mean uterine wet and dry weights in the group treated with 800 mg of BPA/kg/day, compared with those in the vehicle control group (Table 5). The mean uterine wet weight for the group treated with 100 mg of BPA/kg/day was also increased, but was not statistically significance. As expected, the uterine wet and dry weights in the positive control group were significantly increased compared with those of the vehicle control group.

Slight to marked endometrial hypertrophy/hyperplasia was observed in all females treated with 800 mg of BPA/kg/day (Table 6). This effect was similar to, but less pronounced than, that of the positive control group (treated with 0.4 mg of E2/kg/day). At 100 mg of BPA/kg/day, eight females were observed with minimal change. Increases in the severity of endometrial glandular epithelial apoptosis were observed in females treated with 10, 100, or 800 mg of BPA/kg/day. These findings were also observed in the positive control group animals, where the increase and severity were lower than those observed in the animals treated with 800 mg of BPA. Increases in the number of stromal neutrophils were observed in females treated with 100 or 800 mg of BPA/kg/day. These findings were also observed in the positive control group animals, where the increases were higher than those observed in the animals treated with 800 mg of BPA. Luminal epithelial apoptosis was observed in females treated with 10, 100, and 800 mg of BPA/kg/day, with the highest incidence occurring in the group treated with 100 mg of BPA, although this was not observed in the positive control group. There were no treatment-related microscopic changes observed in the uteri of the animals from the remaining groups. There were no clinical signs observed during the study that were considered to be related to treatment.

Discussion

The ligand binding affinities of ERα and ERβ proteins for physiological ligands, such as E2, are quite similar (*12*). However, differences in ligand preference and relative binding affinity between subtypes for other estrogenic chemicals and phytoestrogens have been demonstrated (*11*, *34*, *35*) and were observed in this study. BPA exhibited a 38- and 27-fold greater ability to compete for binding to ERβ than to GST−hERαdef and mouse uterine cytosol, respectively. This differential binding is comparable to previously reported IC_{50} values (*12*), although lower values have been reported for other ERβ preparations (*11*). The reported differences may be due to the use of a baculovirus/SF9-expressed ERβ preparation in the current study that included an ad-

ditional 53 amino acid residues at the N-terminus (*36*), which was absent from earlier clones (*10*, *37*) and not present in the ERβ protein preparations used in other binding studies (*11*, *12*). Nevertheless, the amino acid sequences within the respective ligand binding domains are identical in all of the competitive binding studies. Differences in reported ERβ IC_{50} values for the same ligand among studies may also be due to variation in assay protocols. In contrast, BPA-G did not compete with [^{3}H]E2 for binding to either protein preparation.

Other studies have also reported differences in the relative binding affinity between ERα and ERβ for the same compound. For example, an aryl-substituted pyrazole exhibited higher binding affinity for ERα and 120-fold greater potency in transactivational activity than ERβ (*35*). In addition, genistein has been shown to preferentially bind with 30-fold greater potency to hERβ than to hERα (*12*). The overall structure of the ERβ ligand binding pocket is similar to that of ERα with ligand binding being achieved by a combination of specific hydrogen bonding interactions and the hydrophobic nature of the binding pocket (*38*−*40*). It has been suggested that the preference for genistein may be attributed to two conservative mutations within the binding pocket that further stabilize the hERβ−genistein complex (*38*).

BPA has been shown to exhibit mixed agonist and antagonistic effects through ERα while behaving solely as an agonist through ERβ (*41*). However, in the current study, no antagonistic effects were observed. Despite the preferential binding for hERβ, BPA exhibited an activity in Gal4-hERβdef reporter gene induction that was <2-fold greater than the activity in Gal4-hERαdef. The lack of correlation between binding affinity and transactivational activity may be due, in part, to alterations in the ability of the liganded ER complex to bind DNA (*42*, *43*). Differential interactions between ERα and ERβ with cellular proteins, such as coactivators, may also be a contributing factor (*44*). Crystal structure data for ERβ complexed with genistein indicate that helix 12, which interacts with coactivators, is positioned along a cleft rather than over the binding cavity as seen ERα−E2 and ERα−diethylstilbestrol structures (*39*, *45*). Therefore, the ligand-dependent positioning of helix 12 may account for the difference in the ability of genistein to induce ERα- and ERβ-mediated gene expression, despite the greater affinity for ERβ. To date, no ERα−genistein structure has been reported to support this hypothesis.

In vivo effects of BPA on uterine weight and uterine microscopic changes were examined in immature rats treated by gavage to mimic the primary route of exposure, and by sc to bypass hepatic first-pass elimination and subsequent metabolism to BPA-G. Increases in wet and dry uterine weight were observed at doses of 800 mg of BPA/kg/day for administration by both routes of exposure and also at a dose of 200 mg of BPA/kg/day for oral administration. The effects on uterine weight are consistent with other recent reports (*46*, *47*). However, rat strain differences in vaginal responses to BPA have been reported (*48*). In addition, sc administration of BPA has been shown to increase uterine wet weight in B6C3F1 mice at doses ranging from 0.02 to 8 mg/day (*49*). EC_{50} values of 0.72 mg of BPA/day and 19.4 ng of E2/day have been estimated for this response (*49*), although it has been argued that these values do not represent true potency comparisons, since BPA-induced a <2-fold increase in uterine weight compared to a >5-fold increase induced by E2 (*50*). Tinwell and co-workers (*51*) were unable to demonstrate that BPA is reproducibly active in the mouse uterotrophic assay in which AP mice were treated with either by sc or gavage with doses ranging from 0.02 μg/kg to 300 mg/kg of BPA. This is also consistent with the work of Coldham et al. (*52*), where sc injection of BPA failed to increase uterine weight at dose levels of 3, 33, and 330 mg/kg.

Increases in the incidence of endometrial hypertrophy, endometrial epithelial apoptosis in the lumen, and endometrial and stromal neutrophils were observed in gavaged rats treated with 200 and 800 mg of BPA/kg/day. Subcutaneous injections of 10, 100, and 800 mg of BPA/kg/day also increased the incidence of changes in the microscopic structure of the uterus. These microscopic findings are similar to effects induced by other weak estrogen agonists (*53*) and are consistent with vaginal cornification results (*47*).

BPA has also been shown to induce molecular, cellular, and tissue level effects in rats at doses that did not induce uterine weight. BPA induced progesterone receptor expression in rats treated with 5−150 mg/kg (*21*). Similarly, BPA and the phytoestrogen, diadzin, were weak stimulators of uterine growth in rat, but were able to alter the expression of the androgen receptor, the estrogen receptor, and complement 3 (*54*). Therefore, weak estrogenic chemicals can induce changes at the gene expression and cellular levels without affecting uterine weight. However, the toxicological significance of these changes is unclear.

At low doses that do not induce uterine wet weight, gestational exposure of mice to BPA has been reported to affect accessory reproductive organ weights and daily sperm production in male offspring (*55*, *56*). In contrast, other rodent studies indicate that 0.2−200 μg of BPA/kg/day in drinking water does not affect reproductive organ development or sperm counts (*57*, *58*). These contradictory results observed in similar assays make the assessment of BPA and other xenoestrogens a contentious issue that needs to be resolved (*50*).

The results of this study demonstrate that BPA exhibits a greater ability to compete for binding to ERβ than to ERα. However, the ability of BPA to induce reporter gene expression mediated by either isotype is comparable. In addition, these results demonstrate that there is a clear route of administration dependency on the ability of BPA to induce uterine responses, as observed in Long Evans rats (*59*). This route dependency supports results from competitive binding and reporter gene assays that demonstrate the BPA-G does not exhibit significant estrogenic activity in the uterus, since BPA is more rapidly metabolized to BPA-G when administered by gavage than when administered by sc injection (*23*).

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