

Bisphenol AF Is a Full Agonist for the Estrogen Receptor ER α but a Highly Specific Antagonist for ER β

Ayami Matsushima,¹ Xiaohui Liu,¹ Hiroyuki Okada,¹ Miki Shimohigashi,² and Yasuyuki Shimohigashi¹

¹Laboratory of Structure-Function Biochemistry, Department of Chemistry, Research-Education Centre of Risk Science, Faculty and Graduate School of Sciences, Kyushu University, Fukuoka, Japan; ²Division of Biology, Department of Earth System of Science, Faculty of Science, Fukuoka University, Fukuoka, Japan

BACKGROUND: Bisphenol AF has been acknowledged to be useful for the production of CF₃-containing polymers with improved chemical, thermal, and mechanical properties. Because of the lack of adequate toxicity data, bisphenol AF has been nominated for comprehensive toxicological characterization.

OBJECTIVES: We aimed to determine the relative preference of bisphenol AF for the human nuclear estrogenic receptors ER α and ER β and the bisphenol A-specific estrogen-related receptor ERR γ , and to clarify structural characteristics of receptors that influence bisphenol AF binding.

METHODS: We examined receptor-binding activities of bisphenol AF relative to [³H]17 β -estradiol (for ER α and ER β) and [³H]bisphenol A (for ERR γ). Functional luciferase reporter gene assays were performed to assess receptor activation in HeLa cells.

RESULTS: We found that bisphenol AF strongly and selectively binds to ERs over ERR γ . Furthermore, bisphenol AF receptor-binding activity was three times stronger for ER β [IC₅₀ (median inhibitory concentration) = 18.9 nM] than for ER α . When examined using a reporter gene assay, bisphenol AF was a full agonist for ER α . In contrast, it was almost completely inactive in stimulating the basal constitutive activity of ER β . Surprisingly, bisphenol AF acted as a distinct and strong antagonist against the activity of the endogenous ER β agonist 17 β -estradiol.

CONCLUSION: Our results suggest that bisphenol AF could function as an endocrine-disrupting chemical by acting as an agonist or antagonist to perturb physiological processes mediated through ER α and/or ER β .

KEY WORDS: bisphenol A, bisphenol AF, endocrine disruptor, estrogen receptors, receptor antagonist, receptor binding. *Environ Health Perspect* 118:1267–1272 (2010). doi:10.1289/ehp.0901819 [Online 28 April 2010]

Bisphenol AF (also referred to as hexafluoro-bisphenol A) is a homolog of bisphenol A (BPA) (Figure 1). Bisphenol AF has a symmetrical chemical structure of HO–C₆H₄–C(CF₃)₂–C₆H₄–OH and is designated as 1,1,1,3,3,3-hexafluoro-2,2-bis(4-hydroxyphenyl)propane by IUPAC (International Union of Pure and Applied Chemistry) nomenclature. Bisphenol AF-containing polymers such as polycarbonate copolymers, polyimides, polyamides, and polyesters are used in high-temperature composites, electronic materials, and gas-permeable membranes. Bisphenol AF is also used in many other specialty polymer applications, including plastic optical fibers and waveguides. Although industrial production of bisphenol AF seems to be increasing considerably, no data are available on annual production or concentrations of bisphenol AF in environmental substrates.

In 2008, the U.S. National Institute of Environmental Health Sciences nominated bisphenol AF for comprehensive toxicological characterization based on the lack of adequate toxicity data [National Toxicology Program (NTP) 2008a]. In this nomination report, the NTP noted concern regarding potential exposure of the general population to bisphenol AF. Structural dissimilarities between bisphenol AF and BPA are determined by the presence of a trifluoromethyl (CF₃) or methyl

(CH₃) group, respectively. The potential toxicity of bisphenol AF is of concern in part because its CF₃ group is much more electro-negative (and potentially reactive) than is the CH₃ group of BPA.

Various “low-dose effects” of BPA have recently been reported *in vivo* for reproductive organ tissues in mice and rats. For example, *in utero* exposures to very low levels of BPA have been shown to increase the size and weight of the fetal mouse prostate (Gupta 2000; Nagel et al. 1997), and low-dose exposures have also been reported to decrease daily sperm production and fertility in male mice (Gupta 2000; vom Saal et al. 1998). Many lines of evidence have recently indicated that low doses of BPA affect the central nervous system as well (vom Saal and Welshons 2005; Welshons et al. 2003, 2006). All of these low-dose effects of BPA have been attributed to effects on steroid hormone receptors such as estrogen receptor (ER) and androgen receptor (AR) (Welshons et al. 2003; Xu et al. 2005). In the report by the NTP (2008b) on the potential for BPA exposure to affect human reproduction or development, “some concern” was indicated as the level of concern for potential effects on the brain, behavior, and the prostate gland.

BPA exhibits extremely weak binding activity for ER and AR. Based on the idea that

BPA may interact with nuclear receptors (NRs) other than ER and AR, we screened a series of NRs and eventually discovered estrogen-related receptor γ (ERR γ) as the BPA target receptor (Takayanagi et al. 2006). BPA binds to ERR γ very strongly [dissociation constant (K_d) = 5.5 nM] with high constitutive basal activity (Liu et al. 2007; Okada et al. 2008; Takayanagi et al. 2006). Strong binding of BPA to ERR γ was further demonstrated by direct X-ray crystallographic analysis of this complex (Matsushima et al. 2007, 2008). Moreover, using real-time PCR (polymerase chain reaction), we recently demonstrated that human ERR γ mRNA is expressed abundantly in the placenta, prostate, and fetal brain (Takeda et al. 2009).

Our efforts to explore the target receptor of BPA suggested that it is essential to examine endocrine chemicals for interactions with all 48 human NRs. We previously reported that bisphenol AF binds to ER α more strongly than does BPA, and that the receptor selectivity of bisphenol AF is seven times higher for ER α than for ERR γ (Okada et al. 2008). There are two subtypes of estrogen receptors, ER α and ER β , with distinctly different physiological distributions and functions. Because effects of a number of chemicals have been reported to differ between ER α and ER β (Harris et al. 2003; Manas et al. 2004), it is important to examine the effects of bisphenol AF on both ERs. In the present study, we evaluated the binding activity and functional biological activity of bisphenol AF for ER β and found that bisphenol AF is a potent ligand that functions as an antagonist on ER β .

Address correspondence to Y. Shimohigashi, Laboratory of Structure-Function Biochemistry, Department of Chemistry, Research-Education Centre of Risk Science, Faculty of Sciences, Kyushu University, Fukuoka 812-8581, Japan. Telephone/fax: 81-92-642-2584. E-mail: shimo@chem.kyushu-univ.jp

This study was supported by grant 08062690 from Health and Labour Sciences Research Grants for Research on the Risk of Chemical Substances from the Ministry of Health, Labor and Welfare of Japan. This work was also supported in part by grant-in-aid 19201012 from the Ministry of Education, Science, Sports and Culture in Japan.

The authors declare they have no actual or potential competing financial interests.

Received 14 December 2009; accepted 28 April 2010.

Materials and Methods

Test compounds. We obtained 17 β -estradiol (CAS no. 50-28-2; 98.9%) from Research Biochemicals International (Natick, MA, USA), and BPA (CAS no. 80-05-7; purity 99%) and bisphenol AF (CAS no. 1478-61-1; purity 99%) from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 4-Hydroxytamoxifen (4-OHT; CAS no. 68047-06-3; purity 98%) and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Preparation of glutathione S-transferase-(GST)-fused NR ligand-binding domain (LBD) protein. cDNA clones of ER α and ER β were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). GST-fused receptor LBDs expressed in *Escherichia coli* BL21 α (GST-ER α -LBD, GST-ER β -LBD, and GST-ERR γ -LBD) were purified on an affinity column of glutathione-Sepharose 4B (GE Healthcare BioSciences Co., Piscataway, NJ, USA) followed by gel filtration on a Sephadex G-10 column (15 \times 10 mm; GE Healthcare BioSciences).

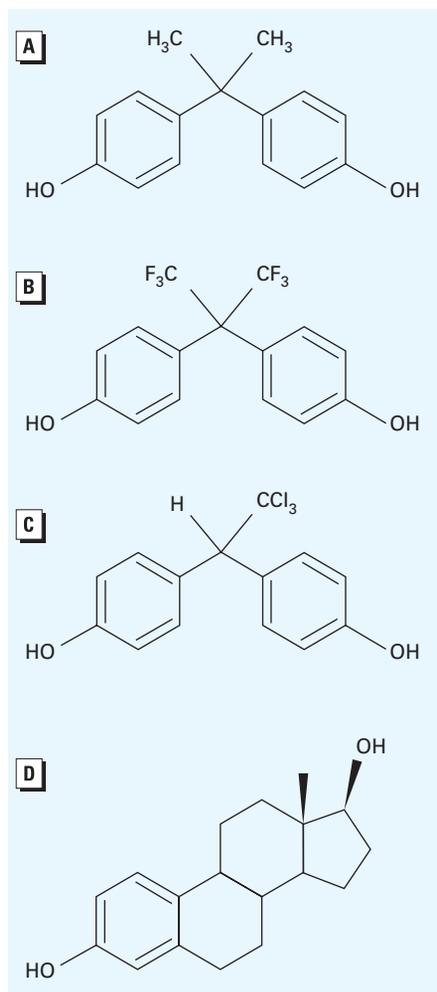


Figure 1. Chemical structures of (A) BPA, (B) bisphenol AF, (C) 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), and (D) 17 β -estradiol.

Radioligand binding assays for saturation binding. We conducted the saturation binding assays for ER α and ER β essentially as reported by Nakai et al. (1999) using tritium-labeled ligand [³H]17 β -estradiol (5.96 TBq/mmol; GE Healthcare UK Ltd., Buckinghamshire, UK). Receptor protein GST-ER α -LBD or GST-ER β -LBD (0.3 nM) was incubated with increasing concentrations of [³H]17 β -estradiol (0.1–30 nM) in a final volume of 100 μ L binding buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate(V), 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 10% glycerol; pH 7.4). Nonspecific binding was determined in a parallel set of incubations that included 10 μ M nonradiolabeled 17 β -estradiol. After incubation for 2 hr at 20°C, free radioligand was removed by incubation with 0.4% dextran-coated charcoal (Sigma-Aldrich Inc.) in phosphate-buffered saline (PBS; pH 7.4) for 10 min on ice and then centrifuged for 10 min at 15,000 rpm.

We performed the saturation binding assay for ERR γ as reported previously (Okada et al. (2008) using [³H]BPA (5.05 TBq/mmol; Moravek Biochemicals, Brea, CA, USA). Specific binding of tritium-labeled ligand was calculated by subtracting the nonspecific binding from the total binding. Receptor proteins that were expressed and purified were evaluated in a saturation binding assay to estimate K_d and

receptor density (B_{max}), and only good-quality preparations with appropriate K_d and B_{max} were used for competitive receptor-binding assays.

Radioligand binding assays for competitive binding. Bisphenol AF, BPA, 17 β -estradiol, and 4-OHT were dissolved in 0.3% DMSO in 1% bovine serum albumin (BSA; a blocker of nonspecific adsorption to the reaction vessels). HPTE was tested as a reference compound that acted as an ER α agonist and an ER β antagonist. These chemicals were examined for their ability to inhibit the binding of [³H]17 β -estradiol (5 nM in final) to GST-ER α -LBD (26 ng) and GST-ER β -LBD (26 ng). The reaction mixtures were incubated overnight at 4°C, and free radioligand was removed with 1% dextran-coated charcoal by filtration. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences Japan, Tokyo, Japan). We calculated the half-maximal inhibitory concentrations (IC₅₀) for 17 β -estradiol from dose–response curves obtained using the nonlinear analysis program ALLFIT (DeLean et al. 1978). Each assay was performed in duplicate and repeated at least five times. For reconfirmation, we also performed the binding assay for ERR γ using [³H]BPA (5 nM final concentration) and GST-ERR γ -LBD (26 ng).

Luciferase reporter gene assay. HeLa cells were maintained in Eagle's minimum essential medium (MEM; Nissui, Tokyo, Japan)

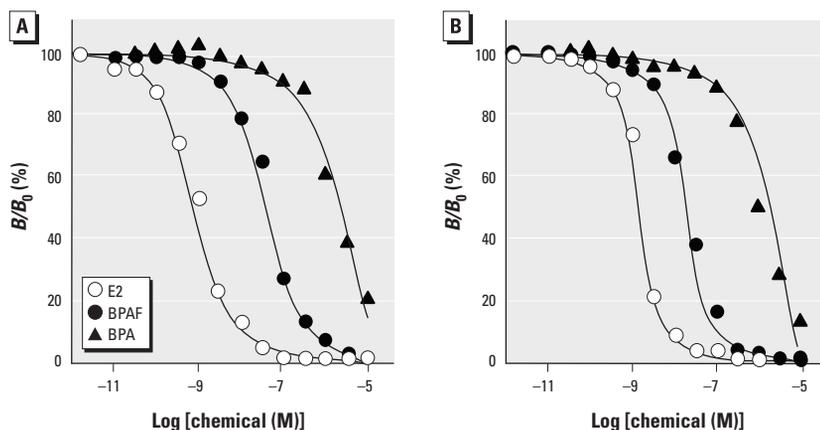


Figure 2. Radioligand receptor-binding assays of bisphenol AF (BPAF), BPA, and 17 β -estradiol (E2) to measure the ability of the compounds to displace [³H]17 β -estradiol in recombinant human ER α (A) and ER β (B). B/B_0 , sample bound/maximum binding. The representative dose-dependent binding curves show the IC₅₀ value closest to the mean IC₅₀ from at least five independent assays. The IC₅₀ values showed a between-experiment coefficient of variation of 5–12%.

Table 1. Receptor-binding characteristics of BPA and bisphenol AF for ER α , ER β , and ERR γ .

Compound	IC ₅₀ (nM)		
	ER α	ER β	ERR γ
17 β -estradiol	0.88 \pm 0.04	2.17 \pm 0.12	NB
4-OHT	2.88 \pm 0.15	3.17 \pm 0.24	10.3 \pm 0.8
BPA	1,030 \pm 70	900 \pm 70	9.70 \pm 0.59
Bisphenol AF	53.4 \pm 3.1	18.9 \pm 0.84	358 \pm 3.1
HPTE	59.1 \pm 1.5	18.1 \pm 1.9	36.4 \pm 4.4

Abbreviations: HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; NB, not bound (no significant receptor binding at 10 μ M, the highest concentration tested).

in the presence of 10% (vol/vol) fetal bovine serum at 37°C. For luciferase assays, HeLa cells were seeded at 5×10^5 cells per 6-cm dish for 24 hr and then transfected with 4 μ g reporter gene (pGL3/3xERE) and 3 μ g of ER α or ER β expression plasmid (pcDNA3/ERs) by Lipofectamine Plus reagent (Invitrogen Japan, Tokyo, Japan) according to the manufacturer's protocol. Approximately 24 hr after transfection, cells were harvested and plated into 96-well plates at 5×10^4 cells/well. The cells were then treated with varying doses of chemicals diluted with 1% BSA/PBS (vol/vol). After 24 hr, luciferase activity was measured with the appropriate reagent using a Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Light emissions were measured using a Wallace 1420 ARVox multilabel counter (PerkinElmer). Cells treated with 1% BSA/PBS were used as a vehicle control. Each assay was performed in triplicate and repeated at least three times. The assay for ERR γ was carried out as previously reported (Okada et al. 2008).

To measure the antagonistic activity of bisphenol AF for ER β , we examined four concentrations (0.01, 0.1, 1.0, and 10 μ M) of bisphenol AF for a serial concentration of 17 β -estradiol (10^{-12} to 10^{-5} M in the final solution). Also, a serial concentration of bisphenol AF (10^{-12} to 10^{-5} M in the final

solution) was assayed in the presence of 10 or 100 nM concentrations of 17 β -estradiol, which normally elicit full activation of ER β .

Results

Strong binding activity of bisphenol AF to ER β receptor. We selected receptor protein preparations suitable for the competitive receptor-binding assay based on Scatchard plot analyses of saturation-binding assays. Receptor populations with the appropriate dissociation constant (K_d) and receptor density (B_{max}) were used for each radioligand receptor-binding assay. Because all of the NRs are secreted protein preparations, observed B_{max} values were comparable with those calculated from their molecular weight.

BPA was a very weak ligand for ER α ($IC_{50} = 1,030$ nM) based on its ability to inhibit [3 H]17 β -estradiol binding (Figure 2A, Table 1), as we previously reported (Okada et al. 2008). In the present study, we confirmed that BPA is also a very weak ligand for ER β ($IC_{50} = 900$ nM; Figure 2B, Table 1), indicating comparable interactions of BPA with ER α and ER β despite the subtle structural differences between these ERs. In contrast, bisphenol AF was 20 times more potent than BPA as a ligand for ER α ($IC_{50} = 53.4$ nM; Figure 2A, Table 1) and was approximately 48 times more potent for ER β

($IC_{50} = 18.9$ nM; Figure 2B, Table 1). This high binding activity for ER β suggests that the binding pocket of ER β possesses specific structural elements that interact much more favorably with the CH $_3$ groups of bisphenol AF than with the CH $_3$ groups of BPA. We also assayed HPTE, an analog of BPA and bisphenol AF with the CCl $_3$ group. HPTE was almost equipotent to bisphenol AF in the assays for both ER α and ER β (Table 1), but approximately 10 times more potent than bisphenol AF for ERR γ .

Receptor-binding selectivity of bisphenol AF and BPA. We used the IC_{50} values shown in Table 1 (from the competitive receptor-binding assay for nuclear ER α , ER β , and ERR γ) to estimate receptor selectivity ratios for BPA and bisphenol AF (Table 2). The results indicate that BPA is exclusively selective for ERR γ , being 90–100 times more active for ERR γ than for ER α or ER β . In contrast, bisphenol AF receptor binding is much more selective for ER α and ER β than for ERR γ (6.70 times more selective for ER α than for ERR γ and 18.94 times more selective for ER β than for ERR γ ; Table 2). Bisphenol AF binding is also about three times more potent for ER β than for ER α .

Differential effects of bisphenol AF in the reporter gene assay. We next examined reporter gene activity after bisphenol AF exposure in HeLa cells transiently cotransfected with an ER α or ER β expression plasmid and an estrogen-response element (ERE)-luciferase reporter plasmid. Bisphenol AF fully activated ER α (increasing activity to ~ 7 times the baseline level) in a dose-dependent manner at concentrations of 10^{-10} to 10^{-5} M (Figure 3A). The half-maximal effective concentration (EC_{50}) of bisphenol AF was 58.7 nM.

When we compared potencies for ER α activation versus ER α binding to determine receptor activation potency [expressed as EC_{50} (nM)/ IC_{50} (nM)], we found a clear discrepancy between 17 β -estradiol and bisphenol AF. As shown in Table 3, we estimated the receptor activation potency for 17 β -estradiol to be 0.085 (0.075 nM/0.88 nM based on values from Figure 3A and Table 1, respectively). In contrast, the receptor activation potency of bisphenol AF [1.099 (58.7 nM/ 53.4 nM)] was approximately 13 times greater than that

Table 2. Receptor-binding selectivity of BPA and AF for ER α , ER β , and ERR γ .

Compound	Receptor-binding selectivity			Preferred receptor(s)
	ER α vs. ER β	ER α vs. ERR γ	ER β vs. ERR γ	
17 β -estradiol	2.47 ER α	(ER α) ^a	(ER β) ^a	ER α
4-OHT	1.10 ER α	3.58 ER α	3.25 ER β	ER α ~ ER β
BPA	1.14 ER β	106.18 ERR γ	92.78 ERR γ	ERR γ
Bisphenol AF	2.83 ER β	6.70 ER α	18.94 ER β	ER β
HPTE	3.27 ER β	1.63 ERR γ	2.01 ER β	ER β

HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane. Data are *n*-fold strength of the preferred receptor compared with the nonpreferred receptor; for example, "2.47 ER α " means that 17 β -estradiol binds to ER α 2.47 times more strongly than to ER β . ^aBecause of inactivity of 17 β -estradiol in ERR γ , 17 β -estradiol is active exclusively in ER α and ER β .

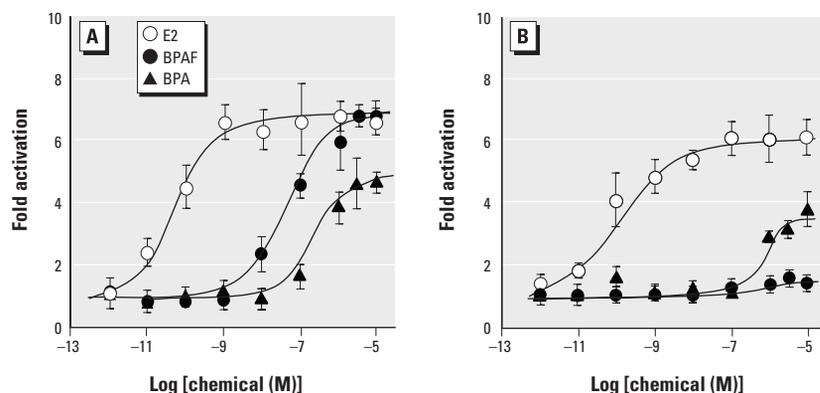


Figure 3. Luciferase-reporter gene assays of bisphenol AF (BPAF), BPA, and 17 β -estradiol (E2) for ER α and ER β using reporter gene (pGL3/3xERE) and either ER α or ER β expression plasmid (pcDNA3/ER α or pcDNA3/ER β) in HeLa cells. Concentration-dependent responses of 17 β -estradiol, bisphenol AF, and BPA in the luciferase-reporter gene assay for ER α (A) and ER β (B). For ER α , bisphenol AF displays full activation in a concentration-dependent manner, whereas for ER β it displays extremely weak activity. 17 β -Estradiol exhibits very strong activity, with approximately 4.5 times more activity induced at 10^{-14} to 10^{-9} M than at baseline.

Table 3. Binding affinities of 17 β -estradiol, BPA, and bisphenol AF relative to their potencies for stimulating reporter gene activity by ER α and ER β in HeLa cells.

Compound	EC_{50} (nM)/ IC_{50} (nM)	
	ER α	ER β
17 β -estradiol	0.085 (1.0)	0.041 (1.0)
BPA	0.308 (3.6)	0.770 (18.8)
Bisphenol AF	1.099 (12.9)	—

Values in the parentheses show the relative value of the EC_{50}/IC_{50} ratio (17 β -estradiol = 1.0).

of 17 β -estradiol (Table 3). This means that the concentration of 17 β -estradiol required to stimulate a 50% response is about 13 times lower than the concentration required to occupy 50% of receptors, whereas the concentration of bisphenol AF required to stimulate a 50% response is about the same as that required to occupy 50% of receptors. This suggests that the receptor conformation induced by bisphenol AF is not as conducive to receptor activation as that induced by 17 β -estradiol when measured in HeLa cells.

BPA was an extremely weak activator of both ER α (EC₅₀ = 317 nM) and ER β (EC₅₀ = 693 nM) based on the luciferase reporter gene assay. The receptor activation potencies of BPA for ER α (0.308) and ER β (0.770) were 3.6 and 18.8 times greater than the receptor activation potencies of 17 β -estradiol for ER α and ER β , respectively (Table 3). These suggest that, compared with 17 β -estradiol, the concentration of BPA required to stimulate a 50% response is much higher than the concentration required to occupy 50% of receptors. In addition, as shown in Figure 3B, BPA exhibited a reduced ability to bring about full activation of ER β (3.5 times greater activity relative to baseline in response to BPA vs. an increase to 6 times the baseline level in response to 17 β -estradiol). This difference in efficacy indicates that BPA does not have the same ability as 17 β -estradiol to induce activation conformation when measured in HeLa cells on this promoter.

Antagonist activity of bisphenol AF on ER β . For ER β , bisphenol AF was almost completely inactive, with very little increase in activity even at 10 μ M, the highest concentration tested (Figure 3B). Based on the strong receptor-binding activity of bisphenol AF for ER β (IC₅₀ = 18.9 nM; Table 1), we expected

that bisphenol AF would also have a high receptor activation potency for ER β . This unexpected inactivity in the reporter gene assay suggests that bisphenol AF binding disrupts the ER β -LBD activation conformation, in which the α -helix 12 (H12) of the receptor is normally positioned to recruit the coactivator protein conformation (Brzozowski et al. 1997; Ruff et al. 2000).

We therefore evaluated the antagonist activity of bisphenol AF against 17 β -estradiol. When we examined 17 β -estradiol, an endogenous agonist ligand of ER β , in the presence of 0.01, 0.1, 1.0, and 10 μ M bisphenol AF, its activity (EC₅₀ = 0.075 nM) was gradually weakened. As shown in Figure 4A, the dose-dependent curves of 17 β -estradiol shifted to the right with increasing concentrations of bisphenol AF, indicating that bisphenol AF effectively inhibits the interaction between 17 β -estradiol and ER β . When the results of Figure 4A were analyzed using a Schild plot, pA₂, a measure of affinity of the antagonist for receptor, was calculated to be 7.87 from the dissociation equilibrium constant ($K_B = 1.35 \times 10^{-8}$ M).

The antagonist activity of bisphenol AF for 17 β -estradiol/ER β was further evidenced by assays in which we added serial concentrations of bisphenol AF (10⁻¹² to 10⁻⁵ M) to a solution of 17 β -estradiol maintained at a constant concentration. When 1 $\times 10^{-8}$ M 17 β -estradiol was treated with bisphenol AF, the activity of 17 β -estradiol was reduced in a dose-dependent manner in response to bisphenol AF concentrations ranging from 10⁻¹⁰ to 10⁻⁵ M (Figure 4B). We obtained a similar result for 1 $\times 10^{-7}$ M 17 β -estradiol. These results demonstrate that bisphenol AF can antagonize the activity of 17 β -estradiol on the ER β receptor.

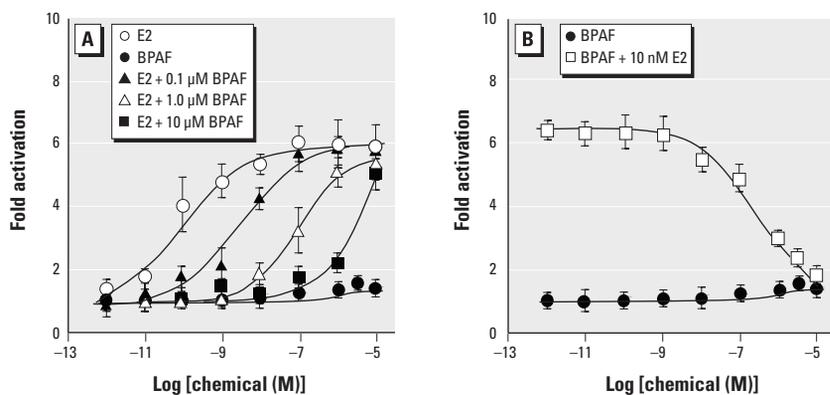


Figure 4. Effects of bisphenol AF (BPAF) on the agonist activity of 17 β -estradiol (E2) in the luciferase-reporter gene assays for ER β . (A) Concentration-dependent luciferase-reporter activities of 17 β -estradiol by fold activation in the presence and absence of bisphenol AF (0.1, 1, or 10 μ M); these concentrations of bisphenol AF clearly weaken the agonist activity of 17 β -estradiol for ER β . (B) Concentration-dependent effects of bisphenol AF on the agonist activity of 17 β -estradiol; the agonist activity of 10 nM 17 β -estradiol was clearly inhibited by bisphenol AF in a dose-dependent manner. Bisphenol AF itself sustained extremely weak activity for ER β . In these assays, the reporter gene (pGL3/3xERE) and ER β expression plasmid (pcDNA3/ER β) were measured in HeLa cells.

Discussion

Structural characteristics of bisphenols and ERs/ERR γ receptors. The differences in receptor selectivity between bisphenol AF and BPA are due to the CH₃ \leftrightarrow CF₃ substitution on the bisphenol backbone structure. Bisphenol AF is a hexafluoro derivative of BPA with the CH₃ \rightarrow CF₃ substitution on the backbone structure of 2,2-disubstituted propane CH₃-C-CH₃. BPA binds strongly to ERR γ , but bisphenol AF binds to ERR γ only weakly; we therefore judged that the binding pocket of ERR γ -LBD possesses structural elements unfavorable for interaction with the trifluoro groups. The molecular size of CF₃ is almost the same as that of CH₃, and thus there would be no structural repulsion or steric hindrance between these groups. However, because the CF₃ group is very electron rich, the structural elements standing face to face with CF₃ must also be electron rich, resulting in their electrostatic repulsion.

In our previous study (Matsushima et al. 2007, 2008), we found that the ERR γ binding sites for BPA CH₃ groups were Phe435 and Met306. Because the aromatic phenyl and S-CH₃ groups of Phe435 and Met306 are electron rich, conditions would be unfavorable for binding of bisphenol AF's electron-rich CF₃ groups. Corresponding receptor residues in ER α are Leu525 and Leu384, respectively. Apparently, there would be no electrostatic repulsion between the bisphenol AF's CF₃ groups and the Leu residues. Such a release in structural stress must be very favorable for receptor activity and the selectivity of bisphenol AF for ER α .

In the present study, we found bisphenol AF to be a strong ligand for both ER α and ER β receptors, although it shows a 3 times greater preference for ER β over ER α . A much more important finding is that bisphenol AF functions in a different way for ER α and ER β . Bisphenol AF is a full agonist for ER α but an antagonist for ER β . The LBDs of ER α and ER β share a high sequence identity (59%) and similar three-dimensional structures. We observed no obvious differences between ER α and ER β in the ERE transcriptional assays in the presence of 17 β -estradiol.

Among the amino acid residues lining the binding pockets of ER α and ER β , two residues differ significantly: Leu384 in α -helix 5 (H5) of ER α is replaced by Met336 in ER β , and Met421 in loop 6-7 of ER α is replaced by Ile373 in ER β . These two residues are most probably responsible for the discriminative affinity and reverse functional activity of bisphenol AF for ER α and ER β . Furthermore, because bisphenol AF is an ER β antagonist, the binding of bisphenol AF to the ER β ligand-binding pocket must damage the ER β -LBD activation conformation, in which the α -helix 12 (H12) in LBD is

positioned to recruit the coactivator proteins conformation (Brzozowski et al. 1997; Ruff et al. 2000). Bisphenol AF binding to LBDs of ER α and ER β are being analyzed in light of the crystal structures in studies in progress in our laboratory.

Bisphenol AF as a candidate of potential endocrine disruptor. Bisphenol AF is a potent estrogen agonist for ER α and a potent estrogen antagonist for ER β . ER α and ER β are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues. ER α is expressed primarily in the uterus, liver, kidneys, and heart (Couse and Korach 1999), whereas ER β is expressed primarily in the ovaries (Couse and Korach 1999), prostate (Couse and Korach 1999), lungs (Kuiper et al. 1997), and gastrointestinal tract and bladder (Nilsson et al. 2001). Coexpression of both receptors occurs in the mammary glands (Pettersson and Gustafsson 2001), epididymis (Pau et al. 1998), thyroid (Pau et al. 1998), adrenals (Pau et al. 1998), bone (Arts et al. 1997; Brandenberger et al. 1997), and certain regions of the brain (Couse and Korach 1999). [For additional information, see Nuclear Receptor Signaling Atlas (2010).] 17 β -Estradiol plays a critical role in many physiological processes in both females and males. These include normal growth, development, and cell-type-specific gene regulation in tissues of the reproductive tract, central nervous system, and skeleton (Couse and Korach 1999; Nilsson et al. 2001; Pettersson and Gustafsson 2001). Bisphenol AF is a potent binder of ER α and ER β and thus would perturb these physiological processes, perhaps providing significant adverse influences for the central and peripheral systems.

Effects of the bisphenol trihalogenated methyl group on receptor actions. Bisphenol AF is an agonist for ER α and an antagonist for ER β . Similar results have been reported for HPTE, a bisphenolic metabolite of methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane]. HPTE behaved as an ER α agonist and an ER β antagonist with estrogen-responsive promoters in HeLa cells (Gaido et al. 1999). We confirmed these results in our assay systems as well. HPTE was a strong binder of ER α with IC₅₀ = 59.1 nM and of ER β with IC₅₀ = 18.1 nM (Table 1). As reported previously by Gaido et al. (1999) and Nettles et al. (2004), HPTE acts as a full agonist for ER α but a strong antagonist for ER β . However, bisphenol AF and HPTE differ in their receptor preference for ERR γ . HPTE was approximately 10 times more potent than bisphenol AF for ERR γ binding, although both chemicals were most strongly bound to ER β (Tables 1, 2). As an antagonist for ER β , bisphenol AF (pA_2 = 7.87) was somewhat stronger than HPTE, the pA_2 of which

was reported to be 7.52 (Gaido et al. 1999). However, both bisphenol AF and HPTE are significantly potent as ER β antagonists.

Chemical structures of bisphenol AF and HPTE differ, with one of two CF₃ groups of bisphenol AF replaced by CCl₃ in HPTE, and the other by H (Figure 1). However, these compounds are similar in that both have trihalogenated methyl groups that may produce different activities for ER α and ER β via their interactions with the ligand-binding pockets of each ER, namely, Leu384 in H5 of ER α \leftrightarrow Met336 in ER β , and Met421 in loop 6–7 of ER α \leftrightarrow Ile373 in ER β .

Methoxychlor is a chlorinated hydrocarbon pesticide structurally similar to DDT (dichlorodiphenyltrichloroethane) and thus is sometimes referred to as dimethoxy or methoxy DDT. It had been used to some degree as a replacement for DDT to protect crops, ornamentals, livestock, and pets against various insects, because it was believed to be metabolized more quickly than DDT, thus reducing or preventing bioaccumulation (Kapoor et al. 1970). Methoxychlor is uterotrophic in the ovariectomized rat and can cause adverse developmental and reproductive effects in mice and rats (Alm et al. 1996; Cummings 1997; Hall et al. 1997). However, HPTE is approximately 100 times more active at ERs than is methoxychlor. To date, the use of methoxychlor has been banned in many countries, including the United States, Japan, and the European Union. All these issues clearly raise concerns that not only HPTE but also bisphenol AF may be a potential endocrine disruptor affecting either ER α or ER β , or both.

Conclusions

BPA binds strongly to ERR γ but very weakly to ER α and ER β . In contrast, bisphenol AF binds very weakly to ERR γ but strongly to ER α and ER β . These differences in receptor selectivity reflect subtle but distinct structural differences resulting from the CH₃ \leftrightarrow CF₃ substitution on the bisphenol backbone structure. The trifluoromethyl group is much more electronegative than the methyl group. These results suggest that apparently minor structural differences among chemicals and NRs may have pronounced effects on binding affinity and selectivity. Thus, the present study emphasizes the crucial importance of accurate evaluation of receptor responses to understanding interactions between endocrine-disrupting compounds and diverse human NRs. Taken together, these results clearly indicate the importance of examining the degree and ways in which bisphenol AF may influence the physiological roles of ER α and ER β . Given that bisphenol AF and BPA function as endocrine disruptors, these chemicals would work differently via different NRs.

REFERENCES

- Alm H, Tiemann U, Torner H. 1996. Influence of organochlorine pesticides on development of mouse embryos *in vitro*. *Reprod Toxicol* 10(4):321–326.
- Arts J, Kuiper GGJM, Janssen JMMF, Gustafsson J-Å, Löwik CWGM, Pols HAP, et al. 1997. Differential expression of estrogen receptors α and β mRNA during differentiation of human osteoblast SV-HFO cells. *Endocrinology* 138(11):5067–5070.
- Brandenberger AW, Tee MK, Lee JY, Chao V, Jaffe RB. 1997. Tissue distribution of estrogen receptors alpha (ER- α) and beta (ER- β) mRNA in the midgestational human fetus. *J Clin Endocrinol Metab* 82(10):3509–3512.
- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 389:753–758.
- Couse JF, Korach KS. 1999. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 20(3):358–417.
- Cummings AM. 1997. Methoxychlor as a model for environmental estrogens. *Crit Rev Toxicol* 27(4):367–379.
- DeLean A, Munson PJ, Rodbard D. 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* 235(2):E97–E102.
- Gaido KW, Leonard LS, Maness SC, Hall JM, McDonnell DP, Saville B, et al. 1999. Differential interaction of the methoxychlor metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors α and β . *Endocrinology* 140(12):5746–5753.
- Gupta C. 2000. Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. *Proc Soc Exp Biol Med* 224(2):61–68.
- Hall DL, Payne LA, Putnam JM, Huet-Hudson YM. 1997. Effect of methoxychlor on implantation and embryo development in the mouse. *Reprod Toxicol* 11(15):703–708.
- Harris HA, Albert LM, Leathurby Y, Malamas MS, Mewshaw RE, Miller CP, et al. 2003. Evaluation of an estrogen receptor- β agonist in animal models of human disease. *Endocrinology* 144(10):4241–4249.
- Kapoor IP, Metcalf RL, Nystrom RF, Sangha GK. 1970. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects, and in a model ecosystem. *J Agric Food Chem* 18(6):1145–1152.
- Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* 138(3):863–870.
- Liu X, Matsushima A, Okada H, Tokunaga T, Isozaki K, Shimohigashi Y. 2007. Receptor binding characteristic of the endocrine disruptor bisphenol A for the human nuclear estrogen-related receptor γ . Chief and corroborative hydrogen bonds of the bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues. *FEBS J* 274(24):6340–6351.
- Manas ES, Unwalla RJ, Xu ZB, Malamas MS, Miller CP, Harris HA, et al. 2004. Structure-based design of estrogen receptor-beta selective ligands. *J Am Chem Soc* 126(46):15106–15119.
- Matsushima A, Kakuta Y, Teramoto T, Koshiba T, Liu X, Okada H, et al. 2007. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR γ . *J Biochem* 142(4):517–524.
- Matsushima A, Teramoto T, Okada H, Liu X, Tokunaga T, Kakuta Y, et al. 2008. ERR γ tethers strongly bisphenol A and 4- α -cumylphenol in an induced-fit manner. *Biochem Biophys Res Commun* 373(3):408–413.
- Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. 1997. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect* 105:70–76.
- Nakai M, Tabira Y, Asai D, Yakabe Y, Shinmyozu T, Noguchi M, et al. 1999. Binding characteristics of dialkyl phthalates for the estrogen receptor. *Biochem Biophys Res Commun* 254(2):311–314.
- Nettles KW, Sun J, Radek JT, Sheng S, Rodriguez AL, Katzenellenbogen JA, et al. 2004. Allosteric control of ligand selectivity between estrogen receptors α and β : implications for other nuclear receptors. *Mol Cell* 13(3):317–327.
- Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. 2001. Mechanisms of estrogen action. *Physiol Rev* 81(4):1535–1565.
- NTP (National Toxicology Program). 2008a. Chemical

- Information Profile for Bisphenol AF [CAS No. 1478-61-1], Supporting Nomination for Toxicological Evaluation by the National Toxicology Program. Available: http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/BisphenolAF_093008_508.pdf [accessed 30 March 2010].
- NTP (National Toxicology Program). 2008b. NTP-CERHR Monograph on the Potential Human Reproductive Developmental Effects of Bisphenol A. NIH Publication No. 08-5994. Available: <http://cerhr.niehs.nih.gov/evals/bisphenol/bisphenol.pdf> [accessed 2 August 2010].
- Nuclear Receptor Signaling Atlas. 2010. Datasets: Tissue-Specific Expression Patterns of Nuclear Receptors. Available: www.nursa.org/10.1621/datasets.02001 [accessed 2 August 2010].
- Okada H, Tokunaga T, Liu X, Takayanagi S, Matsushima A, Shimohigashi Y. 2008. Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor- γ . *Environ Health Perspect* 116:32–38.
- Pau CY, Pau KY, Spies HG. 1998. Putative estrogen receptor beta and alpha mRNA expression in male and female rhesus macaques. *Mol Cell Endocr* 146(1–2):59–68.
- Pettersson K, Gustafsson J-Å. 2001. Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* 63:165–192.
- Ruff M, Gangloff M, Wurtz JM, Moras D. 2000. Estrogen receptor transcription and transactivation: structure-function relationship in DNA- and ligand-binding domains of estrogen receptors. *Breast Cancer Res* 2(2):353–359.
- Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y. 2006. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERR γ) with high constitutive activity. *Toxicol Lett* 167(2):95–105.
- Takeda Y, Liu X, Sumiyoshi M, Matsushima A, Shimohigashi M, Shimohigashi Y. 2009. Placenta expressing the greatest quantity of bisphenol A receptor ERR γ among the human reproductive tissues: predominant expression of type-1 ERR γ isoform. *J Biochem* 146(1):113–122.
- vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, et al. 1998. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol Ind Health* 14:239–260.
- vom Saal FS, Welshons WV. 2005. Large effects from small exposures. II. The importance of positive controls in low-dose research on bisphenol A. *Environ Res* 100:50–76.
- Welshons WV, Nagel SC, vom Saal FS. 2006. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology* 147(6 suppl):56–69.
- Welshons WV, Thayer KA, Judy BM, Taylor JA, vom Saal FS. 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ Health Perspect* 111:994–1006.
- Xu L-C, Sun H, Chen J-F, Bian Q, Qian J, Song L, et al. 2005. Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. *Toxicology* 216(2–3):197–203.