receptors. To this end, we prepared two expression vectors in which human ERα and ERRα cDNAs are cross-linked together via cDNA of FLAG peptide (DYKDDDDK), producing the cDNA conjugates ERα-FLAG-ERRα and ERRα-FLAG-ERα. These vectors were transiently transfected into the HeLa cells, and the expression of dimer protein was confirmed by the Western blotting analysis using anti-FLAG antibody. Intracellular localization of the heterodimers was also ascertained by fluorescence microscopy observation using anti-FLAG antibody. The heterodimers as well as ERα and ERRα were found both in the cytoplasm and nucleus (Fig. 1).

Functional activities of receptors were evaluated by the binding assay for specific ligand interaction and the luciferase reporter gene assay for biological activity. We first examined ERα by using its endogenous agonist ligand 17β-estradiol (E2). In the radio-labeled receptor binding assay, [^{3}H]E2 was found to bind strongly to the heterodimers as in the case of ERα, with the K_D value of about 2 nM (Fig. 2). We also analyzed the transcriptional activity of these heterodimers against ERE or ERRE by reporter gene assays. Transcriptional ability of the heterodimers was found to increase by addition of E2 in a dose-dependent manner. These results indicated that ERα in this heterodimer retains its original characteristics of free ERα. ERα in the heterodimer is probably the influencer of ERRα.

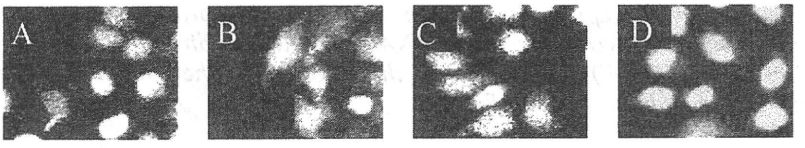

Fig. 1. Intracelluar localization of the nuclear receptor monomers and their heterodimers. ERα (A), ERRα (B), ERα-FLAG-ERRα (C), and ERRα-FLAG-ERα (D). All the receptors were stained by the indirect immunofluorescence method using fluorescein isothiocyanate (FITC)-conjugated secondary antibody with anti-FLAG antibody.

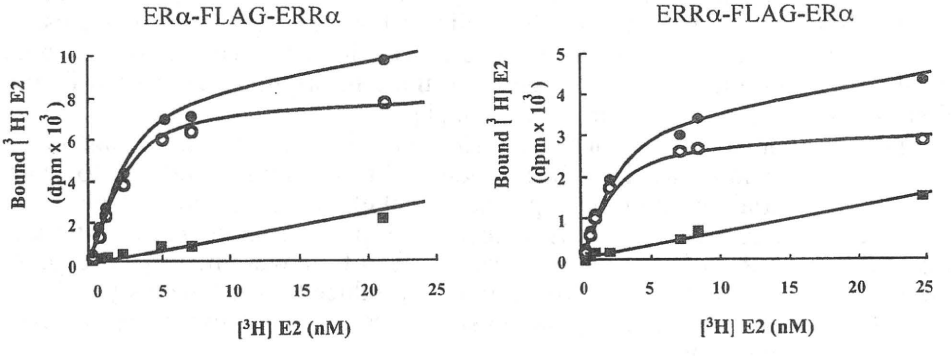

Fig. 2. The results of saturation binding assay for the nuclear receptor heterodimers.

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Bisphenol A-Specific Nuclear Receptor ERRγ: Structure-Function Analysis of the Two Novel Isoforms Lacking Vital Peptide Fragment in the Ligand Binding Domain

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We have demonstrated that ERRγ binds strongly bisphenol A (BPA), one of the nastiest endocrine disruptors, retaining ERRγ's high basal constitutive activity. In the present study, we found the two different LBD-wrecked ERRγ isoforms, which are abundant (about 20%) broadly in various human tissues. These ERRγ isoforms were suggested to affect the sound ERRγ as a regulatory element.

Keywords: bisphenol A, endocrine disruptor, ERRγ, isoforms, nuclear receptor

Introduction

Estrogen-related receptor γ (ERRγ), one of 48 human nuclear receptors, is in a fully activated conformation with no ligand. We have recently demonstrated that ERRγ binds strongly bisphenol A (BPA) with the high basal constitutive activity [1]. In the *in vivo* animal experiments, the low-dose effects of BPA have been evidenced, and thus the adverse effects of ERRγ are very much doubtful. Although we found that human placenta and brain stem express predominantly one of ERRγ mRNA variants, little is known about the intrinsic molecular mechanism of ERRγ functions. In the present study, we revealed the molecular multiplicity of ERRγ mRNAs and proteins, which must be correlated to the multiplicity of physiological functions.

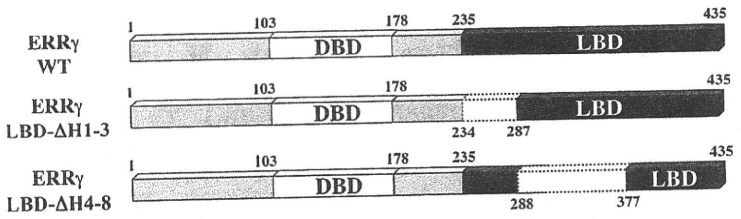

Fig. 1. The three different types of human ERRγ isoforms with wrecked LBD.

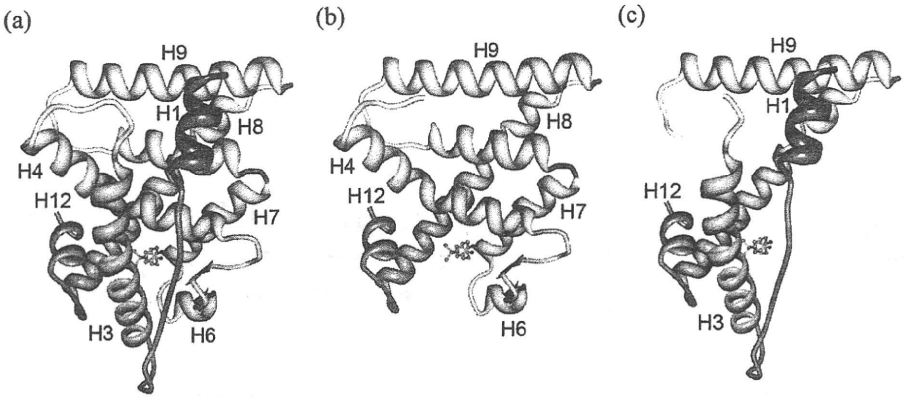

Fig. 2. 3D-Structure of the wild type ERRγ-LBD and its LBD-wrecked forms. (a) ERRγ LBD-WT, (b) ERRγ LBD-ΔH1-3, and (c) ERRγ LBD-ΔH4-8.

Results and Discussion

We eventually identified total 34 mRNA splicing variants from human tissues. As a result, these variants were established to produce 10 protein isoforms. Among these isoforms, two distinct isoforms have a wrecked or broken LBD due to the lack of a peptide fragment either 234-287 or 288-377 (Fig. 1). Those were designated as ERRγ LBD-ΔH1-3 and ERRγ LBD-ΔH4-8, respectively. By means of real-time PCR measurement, these LBD-wrecked isoforms were found to be considerably abundant (3-6% and 12-20%, respectively) broadly in human tissues, suggesting their physiological significance. It should be noted that those do not exhibit any constitute activity in the reporter gene assay.

The LBD of nuclear receptors is constructed by 12 highly conserved α-helix peptides (H1 - H12). Structural evidence for BPA binding to ERRγ-LBD revealed importance of the receptor residues such as Glu275, Arg316, Tyr326, and Asn346 in the ligand-binding pocket [2-4]. The polar amino acids Glu275, Arg316, and Asn346 play central role in forming the hydrogen bonding of two BPA-phenol-OH groups. Surrounding hydrophobic bonds, especially those by Tyr326, reinforce BPA's specific binding. These critical residues are located in H3 (Glu275), H4 (Arg316), and H7 (Asn346), respectively. Tyr326 is in a β-sheet structure located in H4 and H6. As shown in Fig. 2, ERRγ LBD-ΔH1-3 just wrecks H1 and H3, while ERRγ LBD-ΔH4-8 is devoid of the peptide fragment corresponding to H4 - H8. It was strongly suggested that these LBD-broken isoforms cannot bind BPA in their incomplete ligand pockets. Since both isoforms retain H10 essential for functional receptor dimerization, those are probably a regulatory element of sound ERRγ.

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Induced-fit Type Ligand Binding Guided by Free-rotatory Leu Residue Present in the 7th α-Helix Peptide in the Estrogen-related Receptor γ (ERRγ)

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4-α-Cumylphenol, which lacks an OH group from endocrine disruptor bisphenol A (BPA), binds to ERRγ very strongly and specifically. X-ray crystal structural analysis of the 4-α-cumylphenol/ERRγ complex was performed and the structure was compared in detail with the BPA/ERRγ complex. The result clearly indicated that the strong binding of 4-α-cumylphenol is due to the structural flexibility of Leu-side chain at position 345, resulting in a formation of the ligand binding site in an induced-fit manner.

Keywords: bisphenol A, nuclear receptor, X-ray crystal structural analysis

Introduction

Estrogen-related receptor γ (ERRγ) is an orphan nuclear receptor that belongs to the steroid hormone receptor family. This family contains the estrogen receptors α and β, and androgen receptor, considered to be the main targets of endocrine disruptors. Bisphenol A (BPA) has long been recognized as an estrogenic endocrine disruptor, but BPA binds to estrogen receptor very weakly. We have recently discovered that BPA binds to another nuclear receptor named ERRγ very strongly and specifically [1, 2].

Almost all of the nuclear receptors have a unique ligand binding domain, in which the ligand binding pocket is constructed with 12 α-helices (H1-H12) and 2 β-strands in a sheet (S1). These helices play extremely important role to select a specific ligand for the receptor activation. BPA was found to assemble the amino acid residues from H3, H5, H7, H11, H12, and S1 of ERRγ. BPA binds to ERRγ strongly because of the formation of three hydrogen bonds between the phenol-OH groups of BPA and Glu275 from H3, Arg316 from H5, and also Asn346 from H7, respectively. More recently, we found that, in spite of the lack of one of the two BPA-phenol-OH groups, 4-α-cumylphenol exhibits a strong binding ability to ERRγ as well as BPA [2]. In this study, we performed the X-ray crystal structural analysis of 4-α-cumylphenol/ERRγ complex, and compared in detail with the BPA/ERRγ complex.

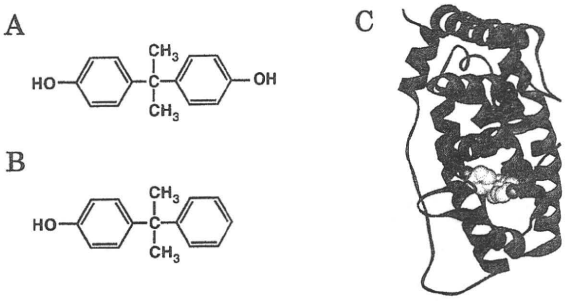

Fig. 1. Chemical structures of bisphenol A (A) and 4-α-cumylphenol(B). Overall structure of 4-α-cumylphenol/ERRγ complex (C).

Results and Discussion

ERRγ was expressed in *E. coli* BL21 as glutathione *S*-transferase (GST) fusion protein. The protein expressed was affinity-purified by using glutathione Sepharose resin, and GST was enzymatically cleaved. The resulting protein was crystallized in the presence of 4-α-cumylphenol. The X-ray crystal structural analysis was performed, and 4-α-cumylphenol/ERRγ complex structure was resolved by molecular replacement method using BPA/ERRγ complex as the searching model. The final model was refined at a 2.0 Å resolution.

The overall structure of 4-α-cumylphenol/ERRγ complex features 12 α-helices (H1-H12) and 2 β-strands in a sheet (S1) without any disordered amino acid residues, and the amino acid residues in the binding pocket were found to be almost the same as those in the BPA/ERRγ complex. Two hydrogen bonds were formed between the remaining OH group of 4-α-cumylphenol and Glu275 or Arg316. Another hydrogen bond of the second BPA-phenol-OH is formed with Asn346 of ERRγ-LBP. Since 4-α-cumylphenol lacks this phenol-OH groups, it should hold substitute residues for a strong receptor binding. When we superimposed the 4-α-cumylphenol/ERRγ-LBD complex with the BPA/ERRγ-LBD complex, we found that the Leu345-isobutyl group rotates 180° completely around the $_{\alpha}$C-$_{\beta}$C bond. ERRγ changes its conformation of Leu345 side chain by the back-and-forth rotation to receive either phenol (BPA) or phenyl (4-α-cumylphenol). Since the Leu conformation in the 4-α-cumylphenol complex was found to be the same as in the apo-form, it become evident that that Leu345 does made a rotation of about 180° to adopt the phenol group of BPA. The crystal structure of the ERRγ complex with (or without) chemicals clearly revealed that ERRγ's Leu345-β-isoprorpyl plays a role in the tight binding of 4-α-cumylphenol and BPA, rotating in a back-and-forth induced-fit manner.

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Direct Evidence Revealing Structural Elements Essential for the High Binding Ability of Bisphenol A to Human Estrogen-Related Receptor-γ

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Background: Various lines of evidence have shown that bisphenol A [BPA; HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH] acts as an endocrine disruptor when present in very low doses. We have recently demonstrated that BPA binds strongly to human estrogen-related receptor-γ (ERR-γ) in a binding assay using [^{3}H]4-hydroxytamoxifen ([^{3}H]4-OHT). We also demonstrated that BPA inhibits the deactivation activity of 4-OHT.

Objectives: In the present study, we intended to obtain direct evidence that BPA interacts with ERR-γ as a strong binder, and also to clarify the structural requirements of BPA for its binding to ERR-γ.

Methods: We examined [^{3}H]BPA in the saturation binding assay using the ligand binding domain of ERR-γ and analyzed the result using Scatchard plot analysis. A number of BPA derivatives were tested in the competitive binding assay using [^{3}H]BPA as a tracer and in the luciferase reporter gene assay.

Results: [^{3}H]BPA showed a K_D of 5.50 nM at a B_{max} of 14.4 nmol/mg. When we examined BPA derivatives to evaluate the structural essentials required for the binding of BPA to ERR-γ, we found that only one of the two phenol-hydroxyl groups was essential for the full binding. The maximal activity was attained when one of the methyl groups was removed. All of the potent BPA derivatives retained a high constitutive basal activity of ERR-γ in the luciferase reporter gene assay and exhibited a distinct inhibitory activity against 4-OHT.

Conclusion: These results indicate that the phenol derivatives are potent candidates for the endocrine disruptor that binds to ERR-γ.

Key words: bisphenol A, constitutive activity, endocrine disruptor, estrogen receptor, estrogen-related receptor-γ, inverse agonist, nuclear receptor. *Environ Health Perspect* 116:32–38 (2008). doi:10.1289/ehp.10587 available via *http://dx.doi.org/* [Online 5 October 2007]

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] has a symmetrical chemical structure of HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH. BPA is used mainly in the production of polycarbonate plastics and epoxy resins. Its worldwide manufacture is approximately 3.2 million metric tons/year. BPA has been acknowledged to be an estrogenic chemical able to interact with human estrogen receptors (ER) (Dodds and Lawson 1938; Krishnan et al. 1993; Olea et al. 1996), and many lines of evidence have revealed that BPA, at even low doses, acts as an endocrine disruptor (Gupta 2000; Nagel et al. 1997; vom Saal et al. 1998; Welshons et al. 2003). However, its binding to and hormonal interaction with ER are extremely weak, 2–3 orders of magnitude lower than those of natural hormones, and thus the intrinsic significance of these low-dose effects is rather intangible and obscure (Safe et al. 2002). These facts led us to hypothesize that BPA may interact with nuclear receptors (NRs) other than ER.

We have recently demonstrated that BPA binds strongly to estrogen-related receptor-γ (ERR-γ) with high constitutive activity (Takayanagi et al. 2006). ERR-γ is a member of the human NR family and the estrogen-related receptor (ERR) subfamily of orphan NRs, which are closely related to the ERs ER-α and ER-β (Giguère 2002; Horard and Vanacker 2003). The ERR family includes three members—ERR-α, ERR-β, and ERR-γ—with ERR-γ being the most recently identified (Eudy et al. 1998; Hong et al. 1999). The amino acid sequences are quite highly conserved among ERRs and ERs, but 17β-estradiol (E_2), a natural ligand of ERs, does not bind to any of the ERR family members. Our discovery that BPA binds strongly to ERR-γ, but not to ERs, indicates that the effects of the so-called endocrine disruptors should be examined for all NRs without delay.

ERR-γ is expressed in a tissue-restricted manner—for example, very strongly in the mammalian brain during development, and then in the brain, lung, and many other tissues during adulthood (Eudy et al. 1998; Heard et al. 2000; Lorke et al. 2000). Our preliminary results have shown that the highest expression is brought about in the placenta (Takeda Y, Sumiyoshi M, Liu X, Matsushima A, Shimohigashi M, Shimohigashi Y, unpublished data). Strong binding of BPA to ERR-γ would affect not only the physiologic functions but also the metabolism of this NR as a transcription-activating factor. Although the intrinsic physiologic functions of ERR-γ have not yet been clarified, it is crucial that a structure–function study be performed to clarify the structural requirements for the binding of BPA to ERR-γ.

In a previous study (Takayanagi et al. 2006), we used tritium (^{3}H)-labeled 4-hydroxytamoxifen (4-OHT) as a tracer in a receptor binding assay for ERR-γ. 4-OHT binds strongly to ERR-γ and deactivates it as an inverse agonist, decreasing the very high level of spontaneous constitutive activity (Coward et al. 2001). As a substitute for [^{3}H]4-OHT, BPA was found to be as potent as 4-OHT in this binding assay. Furthermore, BPA was found to retain or rescue ERR-γ's high basal constitutive activity in the reporter gene assay for ERR-γ using HeLa cells. These results indicated that BPA and 4-OHT bind to ERR-γ with equal strength, but have structural differences that affect their occupation of ERR-γ's ligand binding pocket. In the complex formed between 4-OHT and the ERR-γ–ligand binding domain (LBD), 4-OHT remained at the ligand binding pocket of ERR-γ–LBD, but the α-helix 12 of the receptor was repositioned from the activation conformation (Greschik et al. 2004; Wang et al. 2006). In contrast, BPA was suggested to bind to the pocket without changing the positioning of helix 12, and thus preserved the high receptor constitutive activity of ERR-γ.

It is evident that the binding ability of BPA to ERR-γ should be examined by means of tritium-labeled BPA. Fortunately, [^{3}H]BPA is now commercially available; thus, in the present study we performed the first saturation binding assay for direct exploration of the binding characteristics of BPA. We then established a competitive receptor binding assay in which chemicals were assessed for their ability to displace [^{3}H]BPA from the receptor binding pocket. In particular, industrial chemical products of BPA analogs were inspected structurally in order to better understand the

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The authors declare they have no competing financial interests.

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structural elements of BPA that are required for binding to the ERR. Here we describe the structural elements of BPA that are required for the binding to ERR-γ–LBD and for maintaining the receptor in an active conformation.

Materials and Methods

Chemicals. We purchased 2,2-bis(4-hydroxyphenyl)propane and 4,4-isopropylidenediphenol, both denoted as BPA, from Tokyo Kasei Kogyo Co. (Tokyo, Japan), Nakarai Tesque (Kyoto, Japan), Aldrich (Madison, WI, USA), Junsei Chemical (Tokyo, Japan), Acros (Geel, Belgium), Lancaster Synthesis (Windham, NH, USA), Merck (Darmstadt, Germany), and Fluka (Buchs, Switzerland). The purity designated on the labels varied from 95 to 99%. We also obtained the following analogs of BPA: bisphenol AF [2,2-bis(4-hydroxyphenyl)hexafluoropropane; Tokyo Kasei], bisphenol AP [4,4′-(1-phenylethylidene)bisphenol; Tokyo Kasei], bisphenol B [2,2-bis(4-hydroxyphenyl)butane; Tokyo Kasei], bisphenol E [2,2-bis(4-hydroxyphenyl)ethane; Aldrich], and bisphenol F [bis(4-hydroxyphenyl)methane; Tokyo Kasei].

4-α-Cumylphenol [2-(4-hydroxyphenyl)-2-phenylpropane], 4-*tert*-amylphenol, 4-*tert*-butylphenol, 4-isopropylphenol, and 4-ethylphenol were obtained from Tokyo Kasei. 2,2-Diphenyl propane, and 4-*tert*-octylphenol were obtained from Aldrich, and *p*-cresol and phenol from Kishida Chemical (Osaka, Japan).

Preparation of receptor protein GST-fused ERR-γ–LBD. ERR-γ–LBD was amplified from a human kidney cDNA library (Clontech Laboratories, Mountain View, CA, USA) by polymerase chain reaction (PCR) using gene-specific primers and cloned into pGEX6P-1 (Amersham Biosciences, Piscataway, NJ, USA). Glutathione *S*-transferase (GST)–fused receptor protein expressed in *Escherichia coli* BL21α was purified on an affinity column of glutathione-sepharose 4B (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) to obtain GST-ERR-γ–LBD. The glutathione used for elution of GST-ERR-γ–LBD from the column was removed by gel filtration on a column of Sephadex G-10 (15 × 100 mm; GE Healthcare Bio-Sciences Co.) equilibrated with 50 mM Tris-HCl (pH 8.0), and the protein content (506.24 μg/mL) was estimated by the Bradford method using a Protein Assay CBB Solution (Nakarai Tesque). Preparation of GST-fused ER-α–LBD was carried out as described previously (Takayanagi et al. 2006).

Radioligand binding assays for saturation binding. The saturation binding assay for GST-ERR-γ–LBD was conducted at 4°C using [^{3}H]BPA (5 Ci/mmol; Moravek Biochemicals, Brea, CA, USA) with or without BPA (10 μM in the final solution). Purified protein (0.32 μg/mL) was incubated with increasing concentrations of [^{3}H]BPA (2.1–24.3 nM) in a final volume of 100 μL of binding buffer [10 mM HEPES (pH 7.5), 50 mM sodium chloride, 2 mM magnesium chloride, 1 mM EDTA, 2 mM CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}, and 2 mg/mL γ-globulin]. Nonspecific binding was determined in a parallel set of incubations that included 10 μM nonradiolabeled BPA. After incubation for 2 hr at 4°C, all the fractions were filtered by the direct vacuum filtration method (MultiScreen$_{HTS}$ HV, 0.45 μm pore size; Millipore, Billerica, MA, USA) for the B/F separation (the separation of receptor-bound ligand from free ligand) (Nakai et al. 1999). Filtration was carried out on a multiscreen separation system (Millipore). Before filtration, 100 μL of 1% dextran-coated charcoal (DCC) (Sigma) in phosphate buffer (pH 7.4) was added to the assay vessels, and the mixture was incubated for 10 min on ice. The radioactivity of the filtered solution was counted on a liquid scintillation counter (LS6500; Beckman Coulter, Fullerton, CA, USA). The saturation assay was performed in triplicate. The specific binding of [^{3}H]BPA was calculated by subtracting the nonspecific binding from the total binding.

Radioligand binding assays for competitive binding. BPA and the BPA-related chemicals were dissolved in a binding buffer containing 0.3–1.0% *N,N*-dimethylsulfoxide (DMSO). These compounds were examined for their ability to inhibit the binding of [^{3}H]BPA (3 nM in the final solution) to GST-ERR-γ–LBD (0.32 μg/mL in the final solution). The reaction mixtures were incubated for 2 hr at 4°C and free radioligand was removed with 1% DCC by filtration as described above. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; PerkinElmer Life Sciences Tokyo, Japan). The IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose–response curves obtained using the nonlinear analysis program ALLFIT (De Lean et al. 1978). Each assay was performed in duplicate and repeated at least three times. The competitive binding assay for GST-ER-α–LBD was carried out as described above using [^{3}H]E_2 (5.74 TBq/mmol; Amersham Biosciences, Buckinghamshire, UK).

Cell culture and transient transfection assays. HeLa cells were maintained in Eagle's MEM (EMEM; Nissui, Tokyo, Japan) 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/6-cm dish for 24 hr and then transfected with 4 μg of reporter gene (pGL3/3×ERRE) and 3 μg of ERR-γ expression plasmids (pcDNA3/ERR-γ) 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% bovine serum albumin/phosphate-buffered saline (BSA/PBS, vol/vol). To measure the antagonistic activity, a fixed concentration of compounds (10^{-5} M to 10^{-10} M in the final solution) was added along with 4-OHT. 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 emission was measured using a Wallac 1420 ARVOsx 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.

Results and Discussion

Highly specific binding of BPA to ERR-γ. To demonstrate the direct binding of BPA to ERR-γ, we first attempted to establish a saturation receptor binding assay using radiolabeled BPA. We analyzed the saturation binding of [^{3}H]BPA against the recombinant ERR-γ–LBD protein, to which GST was fused at the N-terminus. In the actual receptor binding assay, we used [^{3}H]BPA (2.0–24 nM) against purified protein at a concentration of 0.32 μg/mL, which corresponds to a concentration of 6.3 nM. The removal of receptor-free [^{3}H]BPA was carried out with 1% DCC. In this procedure, DCC mixtures were transferred to a 96-well HV-plate with a filter (0.45-μm pore size) for direct vacuum.

As shown in Figure 1A, the binding of BPA to ERR-γ was specific and saturated. Specific binding of [^{3}H]BPA to ERR-γ was estimated to be approximately 80%, which we judged to be a very high value. In other words, the level of nonspecific binding of [^{3}H]BPA was very low (Figure 1A). The high level of specific binding of [^{3}H]BPA clearly demonstrated that BPA has no structural elements for nonspecific binding to the receptor protein and exclusively occupies the binding pocket of ERR-γ–LBD. GST did not bind [^{3}H]BPA at all. It should be noted that the specific binding of [^{3}H]4-OHT was only about 50% (Takayanagi et al. 2006).

The Scatchard plot analysis showed a distinct single binding mode (Figure 1B). From the slope, the binding affinity constant (K_D) was calculated to be 5.50 nM. The receptor density (B_{max}) was estimated to be 14.4 nmol/mg protein, which is roughly compatible with the calculated value of 18.9 nmol/mg protein. The B_{max} value of [^{3}H]4-OHT is much smaller than that of [^{3}H]BPA. These results further demonstrate that ERR-γ binds [^{3}H]BPA very specifically and exclusively.

Binding ability of BPA to ERR-γ. We performed the competitive receptor binding assay using [^{3}H]BPA (3 nM in the final solution) for GST–ERR-γ–LBD (0.32 μg/mL in the final solution). To confirm that BPA is a truly specific ligand for ERR-γ, we tested all nonradiolabeled BPA compounds available in Japan, which we obtained from seven different reagent companies. Because the compounds all had different levels of purity (95–99%), we adjusted their initial concentration, 1.0×10^{-2} M, based on the purity indicated on the label.

We found that BPA displaces [^{3}H]BPA in a dose-dependent manner. Its binding curve was sigmoidal in a single binding mode (slope = ~ 1), which afforded an average IC_{50} value of 9.78 nM. We found all BPA compounds purchased to be equally potent. These results clearly demonstrate that BPA binds very strongly to the NR ERR-γ.

4-OHT as a potent displacer of BPA in ERR-γ. 4-OHT has been reported to potently displace [^{3}H]4-OHT in the binding to ERR-γ (Greschik et al. 2004; Takayanagi et al. 2006). In the present study, 4-OHT very potently displaced [^{3}H]BPA (IC_{50} = 10.9 nM) (Table 1). BPA and 4-OHT yielded sigmoidal binding curves indistinguishable from each other (data not shown), indicating that the two are almost equipotent. These results obtained using the [^{3}H]BPA tracer were almost identical to those obtained by [^{3}H]4-OHT (Takayanagi et al. 2006).

BPA and 4-OHT share only a phenol group, and thus the phenol groups of these compounds are highly likely to occupy the same binding site in the ERR-γ receptor. Because the phenol group of 4-OHT is anchored by hydrogen bonds to Glu275 and Arg316 of ERR-γ (Greschik et al. 2004), the phenol group of BPA may also bind to these ERR-γ residues. Indeed, this has been proven by our recent X-ray crystal structure analysis of the complex between BPA and human ERR-γ–LBD (Matsushima et al. 2007). Hereafter, we designate the benzene ring of this phenol group of BPA as the A-ring and the additional benzene ring as the B-ring.

BPA-methyl as a structural requirement for binding to ERR-γ. We evaluated the role of the two methyl (CH_3) groups on the sp^3-C atom of BPA in binding to ERR-γ by a series of analogs of BPA, HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH. First, we examined the effect of incorporation of the methyl group on the binding affinity of BPA. When CH_3 was

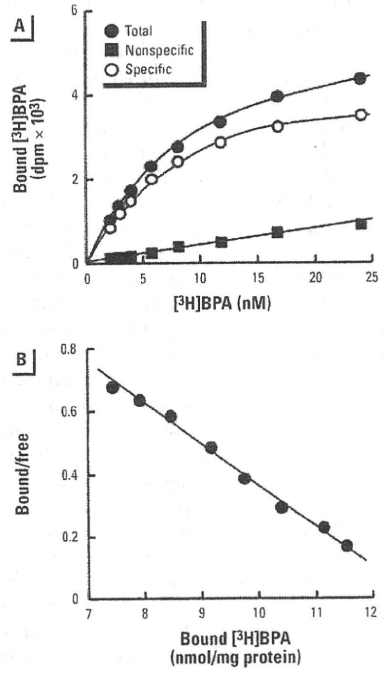

Figure 1. The saturation binding analysis of BPA for ERR-γ. (*A*) Saturation binding curve of [^{3}H]BPA for the recombinant human ERR-γ–LBD showing total, nonspecific, and specific binding. Determination of nonspecific binding was carried out by excess unlabeled BPA (10 μM). (*B*) Binding data analyzed by Scatchard plot analysis to estimate the dissociation constant (K_D) and the receptor density (B_{max}). The plot was linear, the K_D value was estimated to be 5.50 ± 0.87 nM, and B_{max} was 14.4 nmol/mg protein. The saturation binding analysis was performed in duplicate and repeated four times.

Table 1. Receptor binding affinity (mean ± SE) of BPA and its analogs, and 4-OHT for ERR-γ.

Chemical	Binding affinity (IC_{50}, nM)
BPA	9.78 ± 0.87
Bisphenol AF	358 ± 30.5
Bisphenol AP	123 ± 15.1
Bisphenol B	26.3 ± 2.65
Bisphenol E	8.14 ± 0.83
Bisphenol F	131 ± 17.9
4-OHT	10.9 ± 0.91

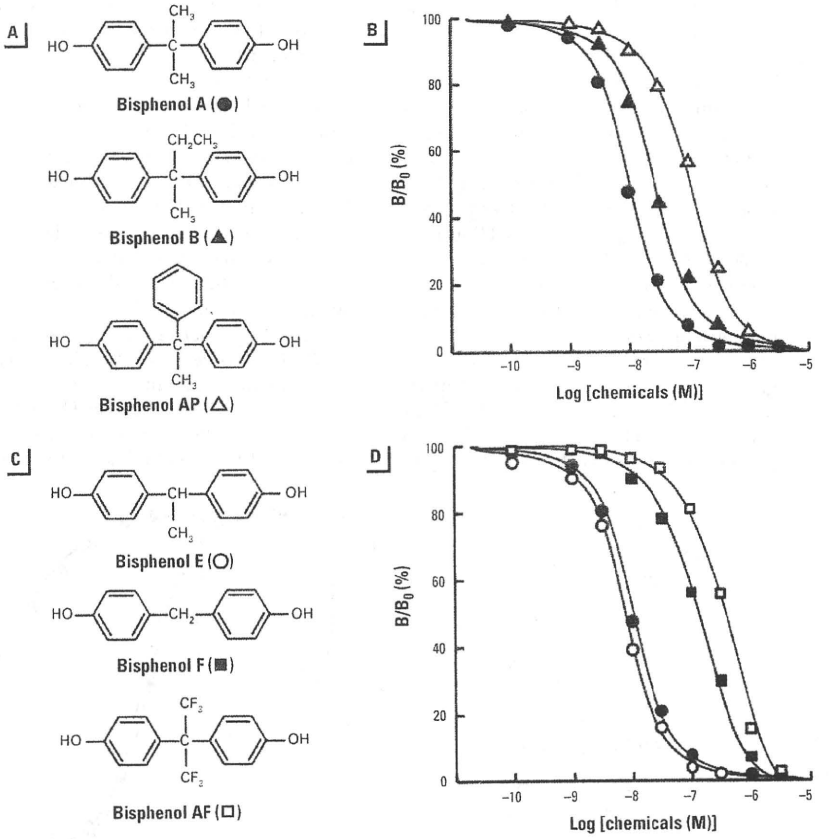

Figure 2. Chemical structure of BPA and its derivatives and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structures of BPA (two methyl groups) and its derivatives: bisphenol B (a methyl group and an ethyl group) and bisphenol AP (a methyl group and a phenyl group). (*B*) Binding activities of BPA, bisphenol B, and bisphenol AP examined by the competitive binding assay using [^{3}H]BPA and GST–ERR-γ–LBD. (*C*) Chemical structures of bisphenol E (one methyl group) and its derivatives, bisphenol F and bisphenol AF [two trifluoromethyl groups (CF_3)]. (*D*) Binding activities of BPA, bisphenol E, bisphenol F, and bisphenol AP examined by the competitive binding assay. (*B*) and (*D*) each show representative curves with the IC_{50} values closest to the mean IC_{50} from at least five independent assays for each compound. B/B_0 is the relative inhibitory activity estimated from the calculation of the percentage of displacement by the chemical tested (B) against the specific binding (B_0 = 100%) of [^{3}H]BPA.

incorporated into the parent methyl group to produce HO-C_6H_4-$C(CH_3)(CH_2CH_3)$-C_6H_4-OH (Figure 2A), we found the resulting bisphenol B to be approximately half as potent (IC_{50} = 26.3 nM) as BPA (Table 1). This result clearly indicates that a bulky group on the central sp^3-C atom is obviously disadvantageous in terms of the binding of BPA to ERR-γ's binding pocket.

On the other hand, an enhancement of activity was observed when one of the methyl groups was eliminated from BPA. The resulting bisphenol E [HO-C_6H_4-$CH(CH_3)$-C_6H_4-OH] (Figure 2C) exhibited slightly better binding activity (IC_{50} = 8.14 nM) than BPA (Table 1). Bisphenol E is indeed the most potent chemical to date for the NR ERR-γ (Figure 2D). The maximal activity was attained when one of the methyl groups was removed from BPA. Apparently, the concomitance of two methyl groups on the central sp^3-C atom of BPA is disadvantageous and unfavorable.

The fact that a single methyl group had the best fit for ERR-γ was further demonstrated by the diminished activity of bisphenol AP, which has a phenyl group in place of the hydrogen atom that is found in bisphenol E (Figure 2A). Bisphenol AP exhibited approximately 15-fold weaker binding affinity for ERR-γ than bisphenol E, with IC_{50} = 123 nM (Figure 2B, Table 1). Steric hindrance by the benzene ring, as well as its electron-rich characteristics, might be responsible for this drop in the receptor binding affinity of bisphenol AP.

The importance of the remaining methyl group in bisphenol E became evident from the drastically reduced activity of bisphenol F [HO-C_6H_4-CH_2-C_6H_4-OH]. This compound was approximately 16-fold less potent than bisphenol E, exhibiting an IC_{50} value of 131 nM (Table 1). All of these results clearly indicate that one of the two methyl groups is involved in the intermolecular interaction with the receptor residue(s). The interaction involving the CH_3 group is a kind of hydrophobic interaction, such as CH_3-alkyl and CH/π interactions.

The fundamental nature of this interaction involving the CH_3 group became rather apparent from the binding result of bisphenol AF [HO-C_6H_4-$C(CF_3)_2$-C_6H_4-OH]. The $CH_3 \rightarrow CF_3$ substitution in BPA creates this compound (Figure 2C), which has two electron-rich trifluoromethyl CF_3 groups instead of the rather electron-poor methyl CH_3 group. The molecular size of CF_3 is almost equal to that of CH_3. A drastically reduced activity of bisphenol AF, about 35-fold less potent (358 nM) than BPA (Table 1), thus demonstrates that the BPA's CH_3 group is in an electrostatic interaction with the electron-rich residue(s) of the receptor. Replacement of CH_3 with CF_3 is definitely disadvantageous, because CF_3 is very electron-rich and thus brings about a strong repulsion with such electron-rich residues of the receptor. One of the electron-rich candidates of the receptor is the aromatic ring of Phe, Tyr, His, and Trp. Based on the reported X-ray crystal structure of ERR-γ, feasible candidates are Phe-435 and Phe-450 (Greschik et al. 2002, 2004; Matsushima et al. 2007; Wang et al. 2006).

A single phenol-hydroxyl group is enough for BPA to bind to ERR-γ. BPA has a very simple symmetrical chemical structure of HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH (Figure 2A). When one of the phenol-hydroxyl groups (–OH) of BPA was eliminated, the resulting 4-α-cumylphenol (HO-C_6H_4-$C(CH_3)_2$-C_6H_5; Figure 3A) still bound very strongly to ERR-γ. 4-α-Cumylphenol was as potent as BPA (Figure 3B), having an IC_{50} value of 10.6 nM (Table 2). Contrary to the expectation that both of the phenol-hydroxyl groups of BPA would participate in the hydrogen bonds, this result indicates that the second hydroxyl group does not necessarily participate in the hydrogen bonding. Given that this hydroxyl group forms a hydrogen bond with the ERR-γ receptor residue, the bond would be considered extremely weak, as suggested by the X-ray crystal analysis of 4-α-cumylphenol–ERR-γ complex (Matsushima A, Teramoto T, Okada H, Liu X, Tokunaga T, Kakuta Y, Shimohigashi Y, unpublished data).

When both of the phenol-hydroxyl groups were eliminated from BPA, the resulting 2,2-diphenyl propane [C_6H_5-$C(CH_3)_2$-C_6H_5] was almost completely inactive (Figure 3B, Table 2). This compound elicits only about 30% inhibition of the binding of [^{3}H]BPA at the 1-μM concentration, whereas BPA almost completely inhibits the binding of [^{3}H]BPA at this concentration (Figure 3B). It is clear that one of the phenol-hydroxyl groups of BPA is indispensable for the interaction with a binding pocket of ERR-γ. These results, together with the fact that 4-α-cumylphenol and BPA are equipotent, emphasizes the significance of one of the two phenol groups in the interaction of BPA with ERR-γ. As described above, this hydroxyl group should be attached to the benzene A-ring. It became apparent that the phenol-hydroxyl group attached to another phenol-benzene ring (B-ring) is not necessarily required for binding of BPA to ERR-γ.

BPA-phenol as a structural requirement for binding to ERR-γ. As described above, 4-α-cumylphenol is as active as BPA. The importance of the benzene B-ring can be examined by replacing the B-ring with the alkyl groups. When the benzene B-ring of 4-α-cumylphenol was substituted with either methyl or ethyl, the resulting 4-*tert*-butylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_3] and

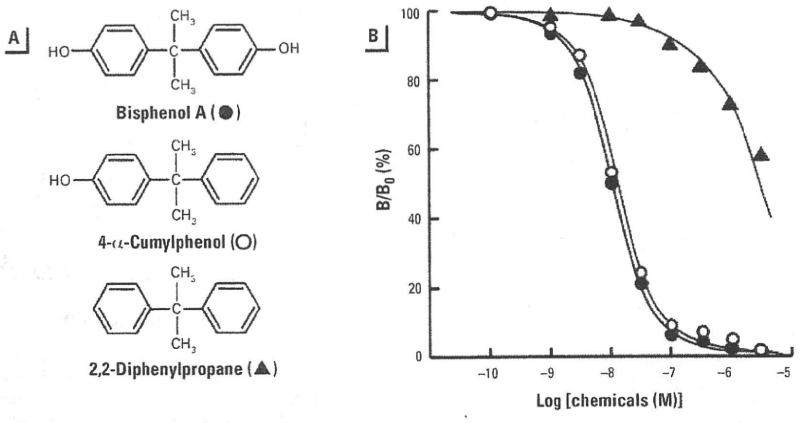

Figure 3. Chemical structure of BPA and its derivatives lacking the hydroxyl group(s) and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structure of BPA and its derivatives lacking the hydroxyl group(s): 4-α-cumylphenol (without one hydroxyl group from BPA), and 2,2-diphenylpropane (without either hydroxyl groups from BPA). (*B*) Binding activities of BPA, 4-α-cumylphenol, and 2,2-diphenylpropane examined by the competitive binding assay using [^{3}H]BPA and GST-ERR-γ–LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

Table 2. The receptor binding affinity (mean ± SE) of BPA and its derivatives lacking of the phenol group for human ERR-γ.

Chemical	Binding affinity (IC_{50}, nM)
BPA	9.78 ± 0.87
4-α-Cumylphenol	10.6 ± 0.87
2,2-Diphenylpropane	ND
4-*tert*-Butylphenol	26.1 ± 2.45
4-*tert*-Amylphenol	33.2 ± 2.85
4-Isopropylphenol	71.1 ± 7.73
4-*tert*-Octylphenol	238 ± 28.1
4-Ethylphenol	289 ± 45.9
p-Cresol	1,290 ± 72.5
Phenol	ND

ND, not determined (IC_{50} value could not be calculated because of extremely weak binding activity, even at a 10 μM concentration).

4-*tert*-amylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_2CH_3] (Figure 4A) were considerably potent (Figure 4B), with values of 26.1 nM and 33.2 nM, respectively (Table 2). This reveals that alkyl groups can be substituted for the aromatic benzene ring without affecting the basal binding capability.

However, because both 4-*tert*-butylphenol and 4-*tert*-amylphenol are still a few times less active than 4-α-cumylphenol, a specific binding site of ERR-γ appears to prefer the aromatic benzene ring to the alkyl groups. This suggests that BPA's second phenol-phenyl group (benzene B-ring) is in the π interaction with the receptor residue(s), that is, either a XH/π interaction (X = N, O, and C) or a π/π interaction. The most plausible candidate for the receptor residue in this interaction is the Tyr residue at position 326 of ERR-γ. Indeed, the phenol-hydroxyl group of this Tyr-326 was found in the OH/π interaction with the B-ring of BPA (Matsushima et al. 2007).

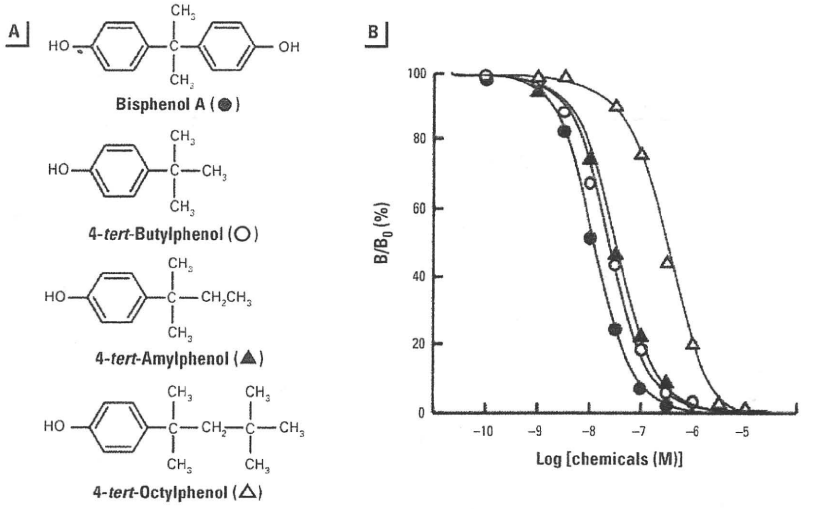

Figure 4. Chemical structure of BPA and its derivatives lacking the phenol group and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structure of BPA and its derivatives with the alkyl group at the position of phenol group: 4-*tert*-butylphenol (a methyl group); 4-*tert*-amylphenol (an ethyl group); and 4-*tert*-octylphenol (a *tert*-butyl methyl group). (*B*) Binding activities of BPA, 4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-*tert*-octylphenol examined by the competitive binding assay using [^{3}H]BPA and GST-ERR-γ–LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

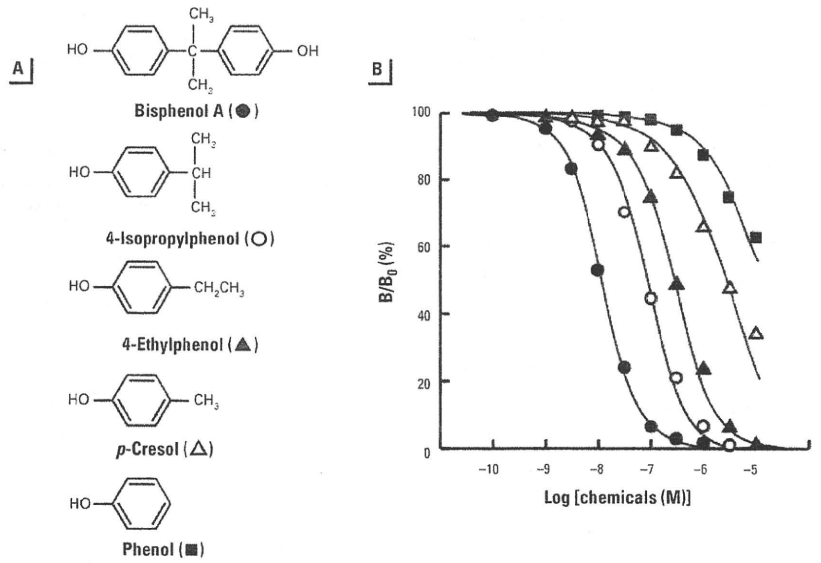

Figure 5. Chemical structure of BPA and a series of alkyl phenols and their dose–response curves in the radioligand receptor binding assay for ERR-γ. (*A*) Chemical structure of BPA and its derivatives with the alkyl group at the *para* position: 4-isopropylphenol (a 4-isopropyl group); 4-ethylphenol (an ethyl group); *p*-cresol (a methyl group); and phenol (a hydrogen atom). (*B*) Binding activities of BPA, 4-isopropylphenol, 4-ethylphenol, *p*-cresol, and phenol examined by the competitive binding assay using [^{3}H]BPA and GST-ERR-γ–LBD; representative curves indicate the IC_{50} value closest to the mean IC_{50} from at least five independent assays for each compound.

In a BPA molecule, two C_6H_4-OH (phenol) groups are connected to the sp^3 carbon atom (sp^3-C) together with two CH_3 (methyl) groups. The most simple structure–activity study is to compare the activity of compounds lacking one of these groups. The compound that lacks the phenol group is 4-isopropylphenol [HO-C_6H_4-$CH(CH_3)_2$] (Figure 5A), and this *para*-isopropyl phenol was fairly potent at displacing [^{3}H]BPA (Figure 5B), with an IC_{50} value of 71.1 nM (Table 2). However, 4-isopropylphenol was still approximately 7-fold less active than BPA, indicating that the phenol backbone structure is an essential structural element for the binding to ERR-γ.

When one of the two methyl groups was eliminated from 4-isopropylphenol, the resulting 4-ethylphenol [HO-C_6H_4-CH_2-CH_3] (Figure 5A) was found to be very weakly active (289 nM) (Table 2). Elimination of another methyl group still afforded a compound of inactive *p*-cresol [HO-C_6H_4-CH_3], but with the IC_{50} value being approximately 1.3 μM. Phenol [HO-C_6H_5] tended to bind to ERR-γ (Figure 5B). These results clearly indicate that the phenol group is a core structure for the attachment of BPA to ERR-γ.

4-Alkyl phenols as putative potent binders to ERR-γ. Attachment of the methyl group to 4-isopropylphenol [HO-C_6H_4-$CH(CH_3)_2$] to create 4-*tert*-butylphenol [HO-C_6H_4-$C(CH_3)_3$] considerably facilitates the binding of the phenol derivative to ERR-γ (Table 2). 4-*tert*-Amylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_2CH_3] is almost as active as 4-*tert*-butylphenol. However, 4-*tert*-octylphenol [HO-C_6H_4-$C(CH_3)_2$-CH_2-$C(CH_3)_3$] (Figure 4B) was significantly weaker (approximately 10 times less potent) than 4-*tert*-butylphenol (Table 2). Thus, the activities of HO-C_6H_4-$C(CH_3)_2$-$CH(CH_3)_2$, HO-C_6H_4-$C(CH_3)_2$-$CH(CH_3)_3$, HO-C_6H_4-$C(CH_3)_2$-CH_2-CH_2-CH_3, and HO-C_6H_4-$C(CH_3)_2$-CH_2-$CH(CH_3)_2$ are expected to be intermediate between those of 4-*tert*-amylphenol and 4-*tert*-octylphenol, although these molecules are not commercially available. It appears that, among the 4-alkylphenols of HO-C_6H_4-$C(CH_3)_2$-C_nH_{2n+1} (=R), 4-*tert*-butylphenol (R = CH_3) and 4-*tert*-amylphenol (R = CH_2-CH_3) show the maximum competitive activity with the binding of ERR-γ.

The structural comparison of HO-C_6H_4-$C(CH_3)_2$-CH_3 (4-*tert*-butylphenol), HO-C_6H_4-$C(CH_3)_2$-CH_2CH_3 (4-*tert*-amylphenol),

and BPA HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH clearly indicated that the R group should not be bulky for high receptor binding activity. A plain π electron-rich benzene aromatic ring is thus optimal for interaction with the receptor residue of ERR-γ-Tyr326.

Inhibitory activity of BPA derivatives for ERR-γ. We found that BPA retained a high constitutive basal activity of ERR-γ in the luciferase reporter gene assay (Figure 6A). ERR-γ is in a full activation with no ligand; it is one of the self-activated NRs and is deactivated by the so-called "inverse agonists" such as 4-OHT (Greschik et al. 2004; Takayanagi et al. 2006). Although BPA shows no apparent effect on the high basal activity of ERR-γ, BPA evidently antagonizes or inhibits the deactivation activity of 4-OHT in a dose-dependent manner (Figure 6B), as reported by Takayanagi et al. (2006). This neutral antagonist is a distinct inhibitor or suppressor of the inverse agonist, reversing the deactivation conformation to the activation conformation.

All of the potent BPA derivatives (i.e., bisphenol E, bisphenol AF, 4-α-cumylphenol, and 4-*tert*-butylphenol) were found, just like BPA, to retain a high constitutive basal activity of ERR-γ in the same luciferase reporter gene assay (Figure 6C). In addition, these compounds inhibited the inverse agonist activity of 4-OHT and thus were specific inhibitors against the inverse agonist 4-OHT. Their abilities to antagonize 4-OHT are approximately one order lower than their binding potencies to ERR-γ (Figure 6B,D). This discrepancy is probably caused by the inclusion of a number of co-effecter proteins for eliciting a gene expression in the luciferase reporter gene assay.

Receptor selectivity of BPA derivatives for ERR-γ over ER-α. We classified BPA and its derivatives into the four groups, depending on their receptor binding affinity for ERR-γ: that is, group A, BPA and chemicals as potent as BPA; group B, chemicals considerably potent; group C, chemicals moderately potent; and group D, inactive chemicals. All chemicals were then examined for their ability to bind to ER-α, and the affinity measured was compared respectively with that for ERR-γ (Table 3). As reported previously (Takayanagi et al. 2006), BPA is highly selective for ERR-γ. It binds to ER-α only weakly; we calculated BPA's receptor selectivity to be 105, which suggests that BPA prefers ERR-γ 105 times more strongly than ER-α. Other group A compounds, namely, bisphenol E and 4-α-cumylphenol, were also greatly selective for ERR-γ (Table 3). In particular, bisphenol E was found to be exclusively selective and specific for ERR-γ because it was almost completely inactive for ER-α.

para-Alkyl phenols in group B ($IC_{50}^{ERR\text{-}\gamma}$ = of 26–71 nM) were also almost completely inactive for ER-α (Table 3). Those include

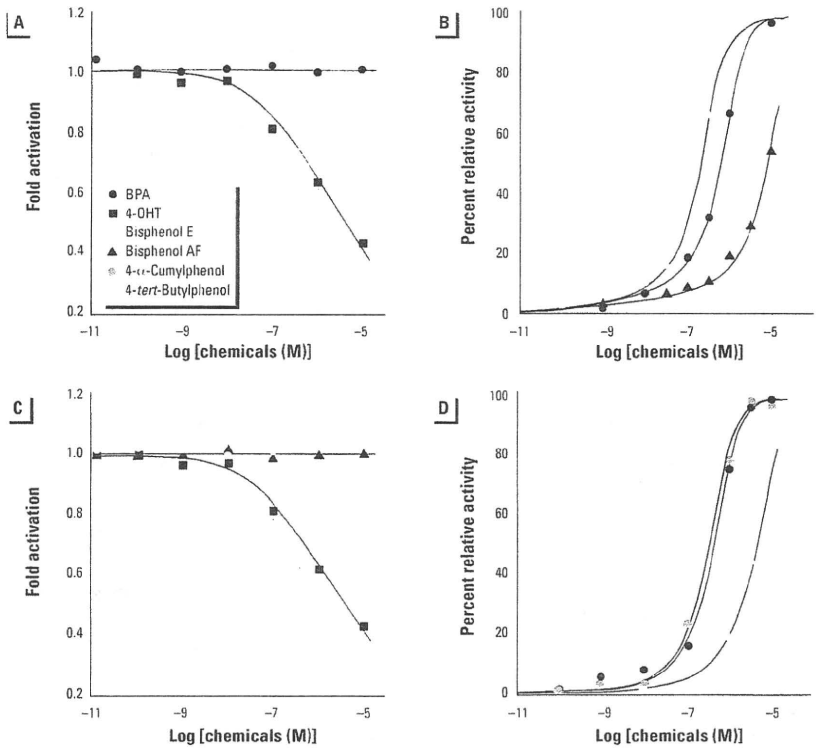

Figure 6. Luciferase-reporter gene assay of BPA and its derivatives for human ERR-γ. (*A*) Deactivation of the fully activated human ERR-γ by the inverse agonist 4-OHT and sustainment by BPA. (*B*) Reversing activity of BPA, bisphenol E, and bisphenol AF against the inverse agonist activity of 1.0 μM 4-OHT; 1.0 μM 4-OHT exhibited approximately 0.4-fold deactivation, and the inhibitory activities are shown by the percentage of relative activity. (*C*) Sustainment of the fully activated human ERR-γ by bisphenol E and bisphenol AF together with inverse agonist activity by 4-OHT. (*D*) Reversing activity of BPA, 4-α-cumylphenol, and 4-*tert*-butylphenol; the inverse agonist activity of 4-OHT was clearly reversed by all bisphenols tested in a dose-dependent manner. Data are from a single experiment performed in triplicate; two additional experiments gave similar results. High basal constitutive activity of ERR-γ was evaluated with the luciferase-reporter plasmid (pGL3/3 × ERRE), and the highest activity was estimated in a cell preparation of 1.0×10^5 HeLa cells/well.

Table 3. Receptor binding affinity (mean ± SE; $n = 3$) of BPA and its analogs for ER-α and their receptor selectivity for ERR-γ over ER-α.

Chemical	Binding affinity for ER-α (IC_{50}, nM)	ERR-γ receptor selectivity ER-α (IC_{50}, nM)/ERR-γ (IC_{50}, nM)
E_2	0.88 ± 0.13	Exclusively ER-α
Group A (chemicals as active as BPA for ERR-γ)		
Bisphenol E	ND	Exclusively ERR-γ
BPA	1,030 ± 146	105
4-α-Cumylphenol	4,770 ± 510	450
Group B (chemicals considerably potent for ERR-γ)		
Bisphenol B	246 ±29.7	9.46
4-*tert*-Butylphenol	ND	Exclusively ERR-γ
4-*tert*-Amylphenol	ND	Exclusively ERR-γ
4-Isopropylphenol	ND	Exclusively ERR-γ
Group C (chemicals moderately potent for ERR-γ)		
Bisphenol AP	361 ± 22.6	2.93
Bisphenol F	ND	Exclusively ERR-γ
4-*tert*-Octylphenol	925 ± 83.9	3.89
4-Ethylphenol	ND	Exclusively ERR-γ
Bisphenol AF	53.4 ± 7.28	0.15
Group D (chemicals extremely weak or inactive for ERR-γ)		
2,2-Diphenylpropane	ND	Inactive for both receptors
p-Cresol	ND	Almost inactive for both receptors
Phenol	ND	Inactive for both receptors

ND, not determined (IC_{50} value could not be calculated because of extremely weak binding activity even at a 10-μM concentration).

4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-isopropylphenol, and they were fully selective and specific for ERR-γ. In contrast, bisphenol B was very weakly active (246 nM) for ER-α, although it was still selective (about 9.5 times) for ERR-γ.

Among group C chemicals ($IC_{50}^{ERR\text{-}\gamma}$ = 120–350 nM), bisphenol F was almost completely inactive for ER-α, making it fully selective for ERR-γ (Table 3). This was also true for 4-ethylphenol. Bisphenol AP showed a weak binding affinity (361 nM) for ER-α, but it was still selective (about 3 times) for ERR-γ. However, bisphenol AF emerged as a ligand selective for ER-α with a selectivity ratio of 0.15 (Table 3). The reciprocal of 0.15 [i.e., ERR-γ (IC_{50})/ER-α (IC_{50}) = 6.67] denotes a selectivity ratio of bisphenol AF for ER-α.

The results clearly indicate that the alkyl groups on the central sp^3-C atom of bisphenol derivatives play a key role in selection of the NR ERR-γ and ER-α. When we checked the receptor binding activities of one series of bisphenol derivatives (i.e., bisphenol E, BPA, bisphenol B, bisphenol AP, and bisphenol AF), we found this line-up to be the order of compounds with increasing affinity to ER-α. At the same time, it was the order of compounds with decreasing affinity to ERR-γ. ERR-γ prefers the less bulky and less electrophilic alkyl groups, whereas ER-α appears to prefer the bulkier and more electrophilic alkyl groups.

4-*tert*-Octylphenol is a well-known endocrine disruptor candidate, but it was only moderately potent for ERR-γ (IC_{50} = 238 nM; Table 2). However, it was considerably weak for ER-α, with an IC_{50} of 925 nM; thus, we judged 4-*tert*-octylphenol to be somewhat selective (approximately 4 times) for ERR-γ. Another representative endocrine disruptor candidate is 4-nonylphenol, which was moderately active for ERR-γ (Takayanagi et al. 2006). Thus, 4-nonylphenol was slightly more selective for ERR-γ. However, some 4-alkyl phenols are distinctly more potent for ERR-γ than 4-*tert*-octylphenol and 4-nonylphenol: 4-*tert*-butylphenol, 4-*tert*-amylphenol, and 4-isopropylphenol. These 4-alkyl phenols are definitely novel candidates of the endocrine disruptor specific for ERR-γ.

Conclusion

In the present study we have shown that all the structural elements of BPA—the phenol and methyl groups and the phenyl group on the central sp^3-C atom—are prerequisite for binding to the NR ERR-γ. Furthermore, we have shown that the phenol derivatives are potent candidates for the endocrine disruptor that binds to ERR-γ. The binding affinity of [^{3}H]BPA to ERR-γ–LBD is extremely high, with a K_D value of 5.50 nM. Thus, it appears to be important to evaluate whether the previously reported effects of BPA at low doses are mediated through ERR-γ and its specific target gene(s).

At the same time, it is necessary to clarify the physiologic roles of ERR-γ and to examine the degree and ways in which BPA may influence these. This is particularly important because ERR-γ is expressed in a tissue-restricted manner—for example, it is expressed very strongly in the mammalian fetal brain and placenta—at sites that could have important outcomes for newborns. Recently, many lines of evidence have indicated that low doses of BPA affects the central nervous system (reviewed by vom Saal and Welshons 2005; Welshons et al. 2003, 2006). The molecular mechanism for these effects could involve, at least in part, the high affinity binding of BPA to ERR-γ. A similar phenomenon may be observed for other NRs, and the exploration of such chemical–receptor interactions requires a specific assay system or concept applicable to all the NRs.

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A strategy to explore the target receptor of endocrine disruptors: Estrogen-related receptor γ (ERRγ) as a genuine acceptor of Bisphenol A

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Abstract
Bisphenol A (BPA) has been acknowledged as an estrogenic chemical able to interact with an estrogen receptor (ER), and many lines of evidence have revealed that BPA has an impact as an endocrine disruptor functioning at very low doses. However, its binding to ER and consequent hormonal activity are extremely weak, making the intrinsic significance of low-dose effects intangible and obscure. Based on the idea that BPA is accessible to all 48 human nuclear receptors, we searched BPA receptor(s) and eventually discovered the estrogen-related receptor γ (ERRγ). BPA functions as an inverse antagonist of ERRγ, and this disruptor activity is expected to shed light on the molecular mechanisms of self-activated orphan ERRγ receptor. To elucidate the physiological functions of these mechanisms it will be important to undertake the sophisticated excision of *in vivo* mechanisms and a profound analysis of their molecular subtleties *in vitro*. In this paper we review the current understandings about ligand-receptor interaction modes of both the ligand-regulated nuclear receptors and the self-activated orphan nuclear receptors, and propose a sophisticated test scheme for comprehensive elucidation of their molecular mechanisms.

Keywords: bisphenol A, endocrine disruptor, estrogen-related receptor γ, inverse antagonist, nuclear receptors

1. Nuclear receptors as the target of endocrine disruptors

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane, has long been recognized as an estrogenic chemical that interacts with human estrogen receptor (ER). This xenoestrogen, BPA is used mainly in the production of polycarbonate plastics and epoxy resins and is found in many plastic products, including food can linings and dental sealants. Its annual worldwide manufacture is approximately 3.2 million metric tones. The widespread industrial and household use, economic importance, and near ubiquitous presence of BPA in the environment emphasize its prospective risk as an endocrine disruptor.

Bisphenol A has been acknowledged as one of the so-called endocrine disruptors, or endocrine disrupting chemicals (EDC), that are able to alter the normal functioning of the endocrine and reproductive systems by mimicking or inhibiting endogenous hormone action, thereby modulating the production of endogenous hormones, or altering hormone receptor populations. A major mechanism of endocrine disruption is the action of chemicals that act as receptor *agonists* or *antagonists* through direct interaction with hormone receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via ER have been the focus of research for the last two decades.

Bisphenol A has also been assumed to be an anti-androgen, or an antagonist that blocks the action of dihydrotestosterone at a human androgen receptor (AR). In addition, various so-called "low-dose effects" of BPA have recently been reported *in vivo* for many organ tissues and systems in mice and rat (Welshons *et al.*, 2003; vom Saal and Welshons, 2005; Welshons *et al.*, 2006). Since BPA's binding to ER and AR and its hormonal activity are extremely weak--1,000 to 10,000 times weaker than for natural hormones--it is unlikely that BPA interacts directly with ER and AR to achieve its effects at low doses.

All these circumstances prompted us to assume that the target of BPA may be nuclear receptor(s) (NR) other than ER and AR. Recently, with the completion of the human genome project, it became clear that nuclear receptors form a superfamily of proteins, which consists of forty-eight different NR proteins. This superfamily consists of seven families called respectively class-0, and 1 – 6. Within these, ER and

AR belong to the class-3 NR family that consists of nine steroid hormone receptors. We searched these class-3 NRs to explore an actual receptor to which BPA binds, and eventually found recently that BPA binds very strongly to the estrogen-related receptor γ (ERRγ).

In addition to the ligand-regulated NRs such as ER and AR, the class-3 NR family includes a number of orphan receptors the physiological ligands of which remain to be identified. This group, the estrogen-related receptors (ERRs) (Giguère, 2002; Horard and Vanacker, 2003), consist of three members α, β, and γ (ERRα, ERRβ, and ERRγ). ERRs and ERs show a considerable level of amino acid sequence similarity and identity in both the DNA binding domain and the ligand-binding domain.

2. Ligand-regulated nuclear receptors and self-stimulated nuclear receptors

ERRs are almost fully active with no ligands, and we refer to such orphan receptors as self-activated NRs. There are 13 self-activated NRs amongst 48 human NRs. 17β-Estradiol (E2), a natural *agonist* ligand of ERα and ERβ, does not bind to any of the ERRs. On the other hand, 4-hydroxytamoxifen (4-OHT), which is an *antagonist* of ERs, deactivates ERRs in, for example, the luciferase reporter gene assay. Such deactivating activity against the self-activated NRs is characterized as the activity of an *inverse agonist*. We found that BPA inhibits and reverses in a dose-dependent manner the inverse agonist activity of 4-OHT to the originally high basal activation state, and thus acts as an *inverse antagonist* of ERRγ.

We also achieved the crystallization and X-ray structural analysis of the BPA/ERRγ-LBD complex (Matsushima *et al.*, 2007). In the complex, a single molecule of BPA stays at the ligand-binding pocket of each ERRγ-LBD protein molecule, whose α-helix 12 (H12) is stabilized in an activation conformation (Fig. 1). The structural elements important for the ligand-receptor interactions were elucidated by us from studies to clarify the structure-activity relationships between BPA-related compounds and ERRγ-LBD mutated receptors (Liu *et al.*, 2007; Okada *et al.*, 2008).

3. Schematic discrimination of intrinsic types of ligand-receptor interactions

For endocrine disrupting chemicals, it is important for us to know and evaluate the type of interactions these have with NRs. All the chemicals should first be evaluated in a receptor-binding assay. Those chemicals that bind should then each have their biological activities examined to determine whether they have an *agonist* or *antagonist* action, when bound to the ligand-regulated NRs. In the case that the compound is neither an *agonist* nor *antagonist*, it should then be examined in the assay to determine whether it is an *inverse agonist* or *inverse antagonist*. In such cases, the NR is likely to be one of the self-activated NRs. Fig. 2 shows a scheme for exploring intrinsic types of interactions.

It should be noted that BPA is neither an *agonist* nor an *antagonist* of ER and AR. BPA binds extremely weakly to these receptors, and thus is almost completely inactive against them. BPA binds to ERRγ very strongly, on the other hand, but at this receptor is neither an *agonist* nor an *antagonist*. BPA is also not an *inverse agonist*, simply sustaining

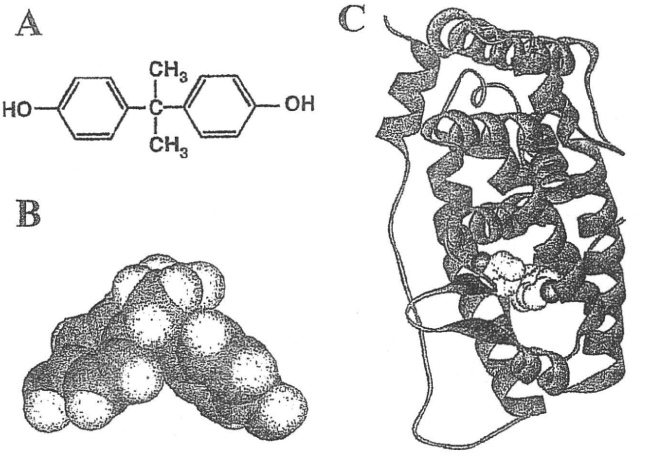

Fig. 1. Structure of bisphenol A (BPA) and its complex with estrogen-related receptor γ (ERRγ). (**A**) Chemical structure of bisphenol A, 2,2-bis(4-hydroxyphenyl)propane. (**B**) Space-filling three-dimensional structure of bisphenol A. (C) X-ray crystal structure of BPA/ERRγ-LBD complex.

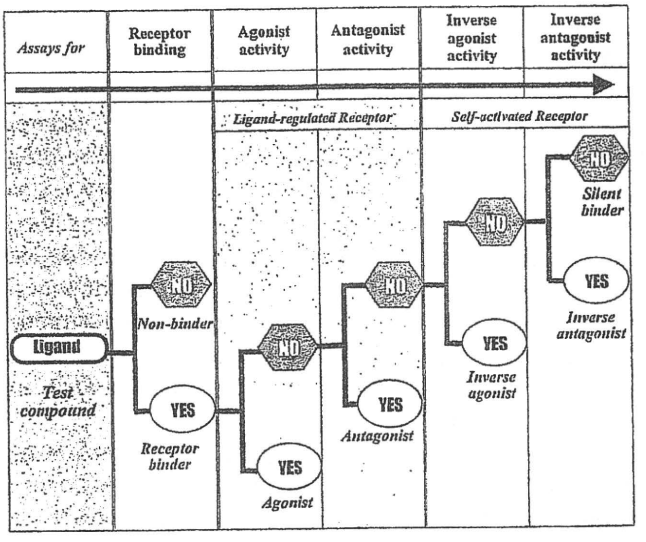

Fig. 2. Schematic flow diagram of receptor assays to discriminate the characteristic receptor responses. After initiating receptor-binding assay, the resulting binder should be assayed to test its either agonist or antagonist activity. If the compound is neither agonist nor antagonist, it should be assayed to evaluate its either inverse agonist or inverse antagonist activity.

ERRγ's high basal constitutive activity, but reverses 4-OHT's *inverse agonist* activity, thus showing very effective *inverse antagonist* activity. Consequently, BPA is a strong binder of ERRγ, but quite silent without the inverse agonist to reveal its action. This kind chemical poses a particular problem because it would be excluded from an assay or other test as simply an inactive or insensitive ligand, and thus it is very important to include an assay that examines the receptor binding capability in any assay scheme.

After these detailed *in vitro* examinations, it is definitely necessary to clarify what the physiological roles of ERRγ are, and to examine the degree and ways in which BPA may influence these. This is particularly important because ERRγ is expressed in a tissue-restricted manner—for example, very strongly in the mammalian placenta and fetal brain—at sites that in humans could have important outcomes for newborn children.

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Relationship between the results of *in vitro* receptor binding assay to human estrogen receptor α and *in vivo* uterotrophic assay: Comparative study with 65 selected chemicals

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Abstract

For screening chemicals possessing endocrine disrupting potencies, the uterotrophic assay has been placed in a higher level in the OECD testing framework than the ER binding assay to detect ER-mediated activities. However, there are no studies that can demonstrate a clear relationship between these assays. In order to clarify the relationship between the *in vitro* ER binding and *in vivo* uterotrophic assays and to determine meaningful binding potency from the ER binding assay, we compared the results from these assays for 65 chemicals spanning a variety of chemicals classes. Under the quantitative comparison between logRBAs (relative binding affinities) and logLEDs (lowest effective doses), the log RBA was well correlated with both logLEDs of estrogenic and anti-estrogenic compounds at $r^2 = 0.67$ ($n = 28$) and 0.79 ($n = 23$), respectively. The RBA of 0.00233% was found to be the lowest ER binding potency to elicit estrogenic or anti-estrogenic activities in the uterotrophic assay, accordingly this value is considered as the detection limit of estrogenic or anti-estrogenic activities in the uterotrophic assay. The usage of this value as cutoff provided the best concordance rate (82%). These findings are useful in a tiered approach for identifying chemicals that have potential to induce ER-mediated effects *in vivo*.

Keywords: Binding assay; Uterotrophic assay; Concordance

1. Introduction

After a variety of studies showed that certain chemicals might disrupt the sex hormonal systems of wildlife and humans, the Organization for Economic Cooperation and Development (OECD), European countries, the United States and Japan initiated efforts to develop and validate assays and screen chemicals for their potential to disrupt the endocrine system of human and wildlife (CEC, 2004; EPA, 1998; Gelbke et al., 2004). Under the OECD activity, "Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals" was developed. This framework has five components or levels each corresponding to a different level of biological complexity (OECD, 2002). The *in vitro* assays such as receptor binding assay are placed in level 2 in this framework to provide mechanistic information and serve for screening purposes. As a tool to detect hormonal effects mediated

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thorough specific hormone receptors, especially for estrogen receptors (ERs), the application of the ER binding assay has been investigated as a quick and cost-effective method. Furthermore, the rodent uterotrophic assay, which has been placed in level 3 to provide data about single endocrine mechanism and effects, has also been recognized as an *in vivo* assay to detect estrogen receptor mediated effects (Kanno et al., 2001; Owens and Koeter, 2003).

Since the uterotrophic assay and ER binding assay both detect ER-mediated activities, a clear relationship between the results from both two assays is expected. However, there are no studies that can demonstrate a clear relationship between two assays on a variety of chemical structures, although studies comparing at limited number of chemical structures has been available (Takemura et al., 2005; Hong et al., 2005; Legler et al., 2002; Yamasaki et al., 2004). Since the receptor binding assay is considered as one of the screening assays to determine if a chemical has potential to trigger receptor-mediated endocrine disruption such as ER and androgen receptor (AR). The results of receptor binding studies need to be related to biological relevance. The purpose of this study is to clarify the relationships between the results obtained in the *in vitro* ER binding assay and *in vivo* uterotrophic assay, and to determine the meaningful cutoff value from the ER binding assay. For this purpose, we compared the results of receptor binding assay using human ERα (hERα) with immature rat uterotrophic assay for 65 selected chemicals.

2. Materials and methods

2.1. Chemicals

The chemical names, CAS numbers and sources of 65 test chemicals subjected in this study are listed in Table 1. Parabenes, benzophenones, biphenyls, diphenylmethanes, diphenylethylenes, phthalates, phenols and other chemical structure classes were included. All test chemicals used in this study had more than 95% purity. Tris(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF) and 17β-Estradiol were purchased from Sigma-Aldrich. Dimethylsulfoxide (DMSO), leupeptin hemisulfate monohydrate, sodium metavanadate, ($NaVO_3$) dithiothreitol (DTT), glycerol and bovine serum albumin (BSA) were obtained from Wako Pure Chemical Industries, Ltd. and disodium salt dihydrate, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were from Dojindo Laboratories Inc.

2.2. In vitro ER binding assay

The receptor binding assay using recombinant human estrogen receptor α (hERα) was conducted by the method previously reported (Nakai et al., 1999; Yamasaki et al., 2004). Briefly, a recombinant hERα ligand binding domain (hERα-LBD) fused with glutathione-*S*-transferase (GST) was expressed in *E. coli* and purified using affinity chromatography technique. After the addition of sample solution (10 μL) of varied concentration (1×10^{-11} to 1×10^{-4} M as final concentrations) and [2,4,6,7,16,17-^{3}H] 17β-estradiol ([^{3}H]-E2, 10 μL; final conc. 0.5 nM, 155 Ci/mmol, Amarsham Biosciences Corp.) in Tris-HCl (pH 7.4, 50 μL) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 1 mM DTT, 10% glycerol, 10 mg/mL BSA, 0.5 mM PMSF, and 0.2 mM leupeptin, a solution of recombinant hERα-LBD (30 μL; final conc. 0.2 nM) was gently mixed. This mixture solution was incubated for 1 h at 25 °C. Free radiolabelled ligands were removed by incubation with 0.4% dextran-coated charcoal (Sigma) (100 μL) for 10 min at 4 °C followed by filtration. The radioactivity of residual radiolabelled ligands bound to receptors in filtrate were measured by liquid scintillation counting. The assay was repeated more than three times for each test chemical.

2.3. Immature rat uterotrophic assay

The chemicals listed in Table 1 were tested in the immature rat uterotrophic assay according to the OECD draft test guideline as previously reported (Yamasaki et al., 2004) in compliance with good laboratory practice (GLP).

Crj:CD (SD) IGS female rat pups (10-day old) purchased from Charles River Japan Inc. (Shiga, Japan) were weaned with their dams and individually housed until 19-days old. These immature female rats were weighted and weight-ranked to assign to each of the treated and control groups (6 rats/group). Three doses were used for each test chemical and the highest dose was set at the maximum tolerance dose based on the results of dose-range finding test. The limit dose was set at 1000 mg/kg/day. Each group of six immature, 20-days old female rats received subcutaneous injections of a test chemical into their back for three consecutive days (4 mL/kg/day) for evaluation of estrogenic activity. The vehicle control group treated with olive oil (s.c.) and the positive control group for estrogenic activity (s.c. 0.6 μg/kg/day of 17α-ethynylestradiol (EE, Sigma)) were concurrently run for each test chemical. To evaluate anti-estrogenic activity in another set of treatment groups, EE (0.6 μg/kg/day) was co-administered with the test chemical. The positive control group for anti-estrogenic activity received subcutaneous co-administration of 1 mg/kg/day of tamoxifen (TAM, Sigma) and 0.6 μg/kg/day of EE. The animals were sacrificed by bleeding from the abdominal vein under deep ether anesthesia 24 h after the final administration, and body weight and uterine weight of each animal were recorded.

2.4. Data analysis

The resulting data from the ER binding assay were analyzed using GraphPad Prism®, Version 4 (GraphPad Software, Inc.), and the IC_{50} value for each test chemical was obtained by logistic equation. The relative binding affinity

Table 1
List of test chemicals and summary results of ER binding and uterotrophic assays

Chemical Name	CAS No.	Source[a]	logRBA	logLED (μmol/kg/day)	
				Estrogenic	Anti-estrogenic
17β-Estradiol	50-28-2	NA	2.00	<−2.43[b]	N.A.
4-*n*-Amylphenol	14938-35-3	TCI	−2.49	3.69	–
p-Dodecyl-phenol	104-43-8	Kanto Chem.	−0.62	2.18	–
p-*tert*-Butylphenol	98-54-4	Wako	−2.63	2.82	3.30
p-(*tert*-Pentyl) phenol = *p*-(*tert*-Amyl) phenol	80-46-6	Wako	−1.76	3.09	3.09
4-Cyclohexylphenol	1131-60-8	TCI	−1.40	3.05	–
4-(1-Adamantyl)phenol	29799-07-3	Aldrich	0.23	1.54	–
2,4-Di-*tert*-butylphenol	96-76-4	TCI	−2.81	–	–
Pentachlorophenol	87-86-5	Wako	N.B.	–	–
2-Naphthol	135-19-3	Wako	−2.98	–	–
p-Hydroxybenzoic acid	99-96-7	Wako	N.B.	–	–
Ethyl-*p*-hydroxybenzoate	120-47-8	Wako	N.D.	–	–
2-Ethylhexyl-4-hydroxybenzoate	5153-25-3	Wako	−1.28	2.90	2.90
4,4′-Dimethoxybenzophenone	90-96-0	TCI	N.B.	–	–
4-Hydroxybenzophenone	1137-42-4	Sigma	−1.97	3.00	3.00
4,4′-Dihydroxybenzophenone	611-99-4	Wako	−1.77	2.97	2.97
2,4-Dihydroxybenzophenone	131-56-6	TCI	−1.86	2.67	3.15
2,4,4′-Trihydroxybenzophenone	1470-79-7	Aldrich	−1.13	2.24	2.24
2,2′,4,4′-Tetrahydroxybenzophenone	131-55-5	Wako	−1.03	2.91	2.21
4-Fluoro-4′-hydroxybenzophenone	25913-05-7	TCI	−2.50	2.67	–
2,3,4-Trihydroxybenzophenone	1143-72-2	Wako	−2.05	3.11	3.64
2,2-Bis(4-hydroxyphenyl)-4-methyl-n-pentane	6807-17-6	Wako	0.45	0.87	0.87
4,4′-Cyclohexylidenebisphenol	843-55-0	TCI	−0.67	2.05	2.05
4,4′-(Octahydro-4,7-methano-5H-inden-5-ylidene) bisphenol	1943-97-1	Acros	0.34	0.80	0.80
4,4′-(Hexafluoroisopropylidene)diphenol	1478-61-1	Aldrich	−0.11	1.08	1.08
4-(Phenylmethyl)-phenol	101-53-1	TCI	−1.65	3.04	3.04
4,4′-Dihydroxydiphenylmethane	620-92-8	TCI	−1.14	2.70	2.70
4,4′-Sulfonyldiphenol	80-09-1	TCI	−2.26	1.90	3.30
4,4′-Thiobis-phenol	2664-63-3	TCI	−0.61	0.96	1.66
Clomiphene citrate (*cis* and *trans* mixture)	50-41-9	ICN	1.57	0.52	0.52
4,4′-Dimethoxytriphenylmethane	7500-76-7	KKC	N.D.	–	–
3,3′-Dichlorobenzidine dihydrochloride	612-83-9	SIGMA	−3.36	–	–
4,4′-Biphenol	92-88-6	TCI	−1.05	2.51	2.51
4′-Hydroxy-4-biphenylcarbonitrile	19812-93-2	Wako	−2.84	–	–
3,3′,5,5′-Tetramethyl-(1,1′-bisphenyl)-4,4′-diol	2417-04-1	Aldrich	−2.39	–	–
Diethylphthalate	84-66-2	Kanto Chem.	N.B.	–	–
Di-*n*-propyl phthalate	131-16-8	TCI	N.D.	–	–
Di-*n*-pentyl phthalate	131-18-0	TCI	−2.78	–	–
Di-*n*-hexyl phthalate	84-75-3	TCI	−3.04	–	–
Diheptyl phthalate	3648-21-3	Aldrich	−2.95	–	–
Diisononyl phthalate	28553-12-0	Wako	−3.49	–	–
Diisodecyl phthalate	26761-40-0	TCI	−3.46	–	–
Di(2-ethylhexyl) phthalate	117-81-7	Wako	−1.15	–	–
Diallyl tetephthlate	1026-92-2	TCI	N.B.	–	–
Testosterone enanthate	315-37-7	Wako	N.B.	1.40	–
Methyltestosterone = 17α-Methyltestosterone	58-18-4	Wako	N.D.	1.52	–
N-Cyclohexyl-2-benzothiazolesulfenamide	95-33-0	TCI	−2.33	–	–
2,2′-Dibenzothiazolyl disulfide = 2,2′-Dithiobis[benzothiazole]	120-78-5	Wako	−1.89	–	–
2-Benzothiazolethiol = 2-Mercaptobenzothiazole	149-30-4	Wako	−2.78	–	–
4-*tert*-Butylpyrocatechol = 4-*tert*-Butylcatechol	98-29-3	Wako	−1.72	3.78	3.26
p-Dichlorobenzene	106-46-7	TCI	N.B.	–	–
Benzanthrone	82-05-3	Wako	N.B.	–	3.64
Flutamide	13311-84-7	SIGMA	N.B.	–	–
3-Amino-1,2,4-triazole	61-82-5	TCI	N.B.	–	–
Benomyl	17804-35-2	SIGMA	N.B.	–	–
Hexachlorocyclopentadiene	77-47-4	Wako	−1.97	–	–
Captafol; 1,2,3,6-Tetrahydro-*N*-(1,1,2,2-tetrachloroethylthio)phthalimide	2425-06-1	Wako	−1.34	–	–
Di (2-ethylhexyl) adipate= Bis(2-ethylhexyl)adipate	103-23-1	Wako	N.B.	–	–
Disulfiram	97-77-8	Wako	−1.34	–	2.53
4,4′-(1,3-Phenylendiisopropylidene)bisphenol	13595-25-0	Aldrich	−0.76	2.16	0.76
1,1,3-Tris(2-methyl-4-hydroxy-5-*tert*-butylphenyl)butane	1843-03-4	Wako	−1.67	–	–

(*continued on next page*)

Table 1 (*continued*)

Chemical Name	CAS No.	Source[a]	logRBA	logLED (μmol/kg/day)	
				Estrogenic	Anti-estrogenic
3,3,3′,3′-Tetramethyl-1,1′-spirobisindane-5, 5′,6,6′-tetrol	77-08-7	TCI	−1.00	-	3.37
Diphenyl-*p*-phenylenediamine	74-31-7	Wako	−1.87	2.58	-
Atrazine	1912-24-9	TCI	N.B.	-	2.97
4-Hydroxyazobenzene	1689-82-3	Wako	−1.13	2.30	-
4-Diethylaminobenzaldehyde	120-21-8	Wako	N.B.	3.05	-

N.A.: not available, N.B. (not bound): the maximum displacement of radiolabelled ligand was below 20%, N.D. (not determined): IC_{50} was not calculated and the maximum displacement of radiolabelled ligand was between 20–50%.

-: Significant increase or decrease of uterine weight was not observed in estrogenic or anti-estrogenic assay systems, respectively.

[a] TCI; Tokyo Chemical Industry Co., Ltd., Kanto Chem.; Kanto Chemical Co., Inc., Wako; Wako Pure Chemical Industries, Ltd., Acros; Acros Organics, ICN; KKC; Kankyo Kagaku Center Inc.

[b] The agonistic logLED of E2 was determined by the subcutaneous injection for three consecutive days to immature rat (from 23-days old, SD rat) conducted by Padilla-Banks et al., 2001). This value was not used for the quantitative and qualitative analyses in the study.

(RBA) of each test chemical was calculated using the following equation:

$$RBA = (IC_{50} \text{ for } E_2)/(IC_{50} \text{ for test chemical}) \times 100$$

When IC_{50} was not calculated and the maximum displacement of radiolabelled ligand was between 20% and 50%, the binding potency of test chemical was shown as "N.D. (not determined)". When the maximum displacement of radiolabelled ligand was below 20%, the binding potency of test chemical was shown as "N.B. (not bound)".

The Dunnet test was used to analyze the data from the uterotrophic assay. When the significant increase of uterine weights in agonism assay or significant decrease in antagonism assay were observed, the test chemical was evaluated as estrogenic or anti-estrogenic, respectively.

The lowest effective dose (LED, μmol/kg/day), the lowest dose showing a statistically significant effect in this assay, was employed as a quantitative parameter in this comparison study with logRBAs from the *in vitro* ER binding assay, and the correlation coefficients (r^2) and its P values were calculated by GraphPad Prism® version 4 (GraphPad Software, Inc.). Also, contingency analyses were performed to calculate concordance (the rate agreement of the results among assays), false-negative (the rate of negatives in the ER binding assay identified as positive in the uterotrophic assay) and false-negative rates (the rate of positives in the ER binding assay identified as negatives in the uterotrophic assay).

3. Results

3.1. In vitro ER binding assay

The results of the *in vitro* binding assay to hERα for 65 chemicals are shown in Table 1.

RBA values were obtained for 47 of 65 chemicals. The highest and lowest logRBAs were 1.57 of clomiphene citrate and −3.49 of diisononyl phthalate, respectively.

The remaining 18 chemicals were regarded as negatives (non-binders) in the concentration tested. Among them, although 4 chemicals, i.e., ethyl *p*-hydroxybenzoate, di(*n*-propyl)phthalate, 17α-methyltestosterone and 4,4'-dimethoxytriphenylmethane showed 20–50% displacement and they were regarded as negatives in this study.

3.2. Immature rat uterotrophic assay

Sixty-five chemicals were tested by immature rat uterotrophic assay in both estrogenic and anti-estrogenic assay systems. The test chemical was evaluated as estrogenic or anti-estrogenic if the uterine weights were significantly increased in the estrogenic assay or decreased in the anti-estrogenic assay. In such cases, the log lowest effective doses (logLED, μmol/kg/day) were shown in Table 1.

Based on this evaluation, 31 and 25 chemicals were identified as estrogenic and anti-estrogenic in immature rat uterotrophic assay, respectively. Twenty-one, including all diphenylmethanes tested, exhibited both estrogenic and anti-estrogenic responses. On the other hand, none of the phthalates tested in this study have either estrogenic nor anti-estrogenic.

3.3. In vitro ER binding assay vs. in vivo uterotrophic assay

3.3.1. Comparison between logRBA and logLED values

The logRBAs obtained from the *in vitro* ER binding assay were compared with logLEDs from the uterotrophic assay. As shown in Fig. 1, the logRBAs were well correlated with both logLEDs in estrogenic and anti-estrogenic assay systems at $r^2 = 0.67$ ($n = 28$, $P < 0.0001$) and 0.79 ($n = 23$, $P < 0.0001$), respectively.

The lowest logRBA that can detect estrogenic or anti-estrogenic response in the uterotrophic assay was −2.63 (RBA = 0.00233%) of *p-tert*-butylphenol, respectively.

3.3.2. Consistency between ER binding and uterotrophic assays for detecting estrogenicity/anti-estrogenicity

The results of ER binding and uterotrophic assays based on the evaluation of the ER related response as detectable (positive) or not (negative) are compared in Table 2-1. The rates of concordance, false negative and false positive for all chemicals tested was 66%, 14% and 57%, respectively.

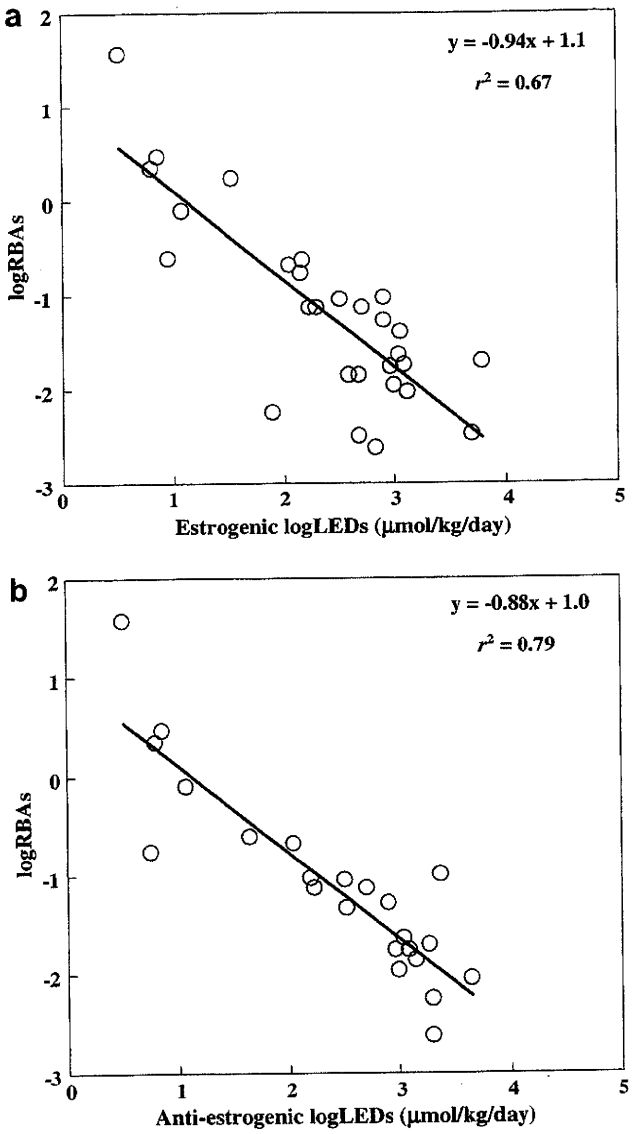

Fig. 1. Relationships between logLED and logRBA values. a, Relationship between estrogenic logLEDs and logRBAs b, Relationship between anti-estrogenic logLEDs and logRBAs.

Seventeen chemicals found to bind to the ER were neither estrogenic nor anti-estrogenic in the uterotrophic assay. Among these chemicals, 3 benzothiazoles and 6 phthalates were included, and the logRBAs of the remaining 8 chemicals were relatively weak ranging from −3.36 to −1.34. Five chemicals were classified as non-binders that showed estrogenic or anti-estrogenic responses in the uterotrophic assay. All 3 non-ER binder chemicals that showed estrogenicity in the uterotrophic assay were androgens (testosterone enanthate and 17α-methyltestosterone) and *p*-diethylaminobenzaldehyde. The other 2 non-ER binders that showed anti-estrogenic effects were atrazine and benzanthrone.

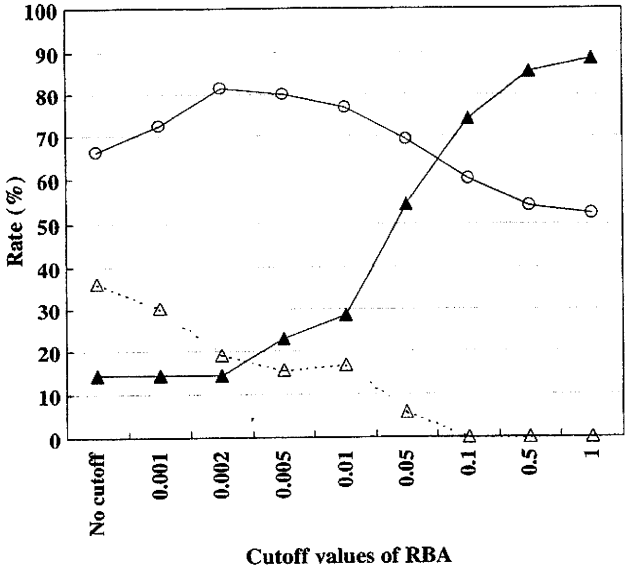

Fig. 2. Changes of indexes by contingency analysis depending on cutoff of RBA. The rates of concordance, false negative and false positive are shown as open circle with solid line, closed triangle with solid line and open triangle with dash line, respectively.

Based on the comparison above, the ER binding assay seemed to have higher sensitivity than immature rat uterotrophic assay. In order to determine the lowest biologically effective binding potency in the ER binding assay, the relationship between RBA cutoff value and the rates of concordance, false negative and false positive from contingency analysis were investigated (Fig. 2). As mentioned above, the lowest RBA that showed estrogenic or anti-estrogenic responses in the uterotrophic assay was −2.63 (RBA = 0.00233) of *p-tert*-butylphenol in this study. When this value is used as a cutoff value, the rates of concordance and false positive rate ratios were refined at 82% and 23%, respectively without increasing the false-negative rate

Table 2-1
Contingency table between ER binding and uterotrophic assays without applying cutoff

		ER-binding assay		Total	Index	Rate(%)
		P	N			
Uterotrophic assay	P/P	*21*	0	21	Concordance	66
Estrogenic	P/N	*7*	3	10		
/Anti-estrogenic	N/P	*2*	2	4	False negative	14
activities	N/N	17	*13*	30		
Total		47	18	65	False positive	57

P: positives, N: negatives, P/N: positives in estrogenic and negatives in anti-estrogenic activities, P/P: positives in both estrogenic and anti-estrogenic activities, N/P: negatives in estrogenic and positives in anti estrogenic activities and N/N: negatives in both estrogenic and anti-estrogenic activities.