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Structure-Activity Studies on Nociceptin and Its Receptor ORL1

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A novel diagram of receptor assays was established to discriminate the characteristic receptor responses, exemplifying the antagonists of nociceptin ORL1 receptor and nuclear receptor ERRγ. In particular, the novel idea of inverse-type of agonist/antagonist was introduced for constitutively active receptors.

Keywords: agonist; antagonist; bisphenol A; inverse agonist; nociceptin

Introduction

Nociceptin or orphanin FQ, a heptadecapeptide FGGFTGARKSARKLANQ, is the endogenous ligand of opioid receptor-like 1 (ORL1) receptor. The nociceptin receptor is a G protein-coupled receptor and involved in the regulation of numerous brain activities, particularly instinctive and emotional behaviors. We have performed a series of structure-activity studies on these neuropeptide and its target receptor ORL1. Those include (1) development of superagonists, (2) development of potent pure antagonists, (3) exploration of ORL1 receptor binding sites of superagonists, (4) identification of receptor residues essential for activation, and (5) identification of receptor residues essential for interactions between transmembrane domains.

In the present study, we concentrated the research scheme on the principal diagram to evaluate or design receptor antagonists. As shown in Fig. 1, there are two different types of receptor antagonist: *i.e.*, a regular receptor antagonist for ordinary receptors and an inverse-type antagonist for constitutively active receptors. We attempted to obtain a pure antagonist of nociceptin ORL1 receptor, and we have discussed its activity profile in comparison with bisphenol A, inverse antagonist of nuclear receptor ERRγ.

Results and Discussion

Nociceptin ORL1 receptor possesses a considerably low constitutive activity, although some mutants exhibit higher levels of activity. Thus, ORL1 receptor antagonist is to be regular-type to inhibit nociceptin. In order to pursue a pure antagonist, a series of analogs were prepared for parental Ac-Arg-Arg-Tyr-Arg-Ile-Lys-NH_2 (Ac-RRYRIK-NH_2), a partial agonist of ORL1. Eventually we reached a conclusion that the most important structural element for antagonism is the N-terminal acetyl group, and then several highly potent antagonists were designed and synthesized [1]. As a pure antagonist, for example, isovaleryl-RRYRIK-NH_2 was found with no agonist activity [2].

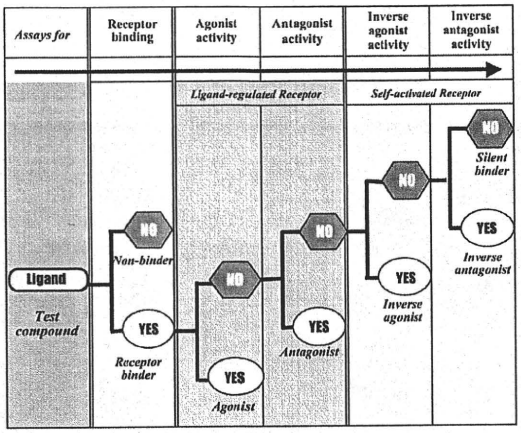

Fig. 1. Schematic flow diagram of receptor assays to discriminate the characteristic receptor responses.

It is not unusual to have a compound with no agonist activity and simultaneously with no antagonist activity. In this case, the target receptor must be constitutively active. Constitutively active receptors possess inverse-type agonists and their neutral antagonists. We have recently discovered bisphenol A as an inverse-type neutral antagonist of human nuclear receptor ERRγ [3]. Bisphenol A was found to reverse the deactivation activity of inverse agonist 4-hydroxytamoxifen in ERRγ [4].

For receptors, there are two different types, namely, ligand-activated receptors and self-activated constitutively active receptors (Fig. 1). The ordinary antagonist is a compound that inhibits the agonist activity in ligand-activated receptors, whereas the inverse antagonist is a compound that inhibits the inverse-agonist activity in self-activated receptors. Thus, the antagonist should be designed, depending upon the receptor type to which a ligand exhibits either activation or deactivation activity.

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Drosophila Neuropeptide hugγ Present in the Clock Cells Important for Circadian Rhythm Oscillation

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We prepared polyclonal antibodies against *Drosophila* neuropeptides hugγ and PK2. Prepared anti-hugγ-pAb stained dorsal lateral neuron in a time-dependent manner. Since this neuron works to generate an evening activity, hugγ is expected as a neurotransmitter candidate to emit an evening locomotor activity.

Keywords: *Drosophila melanogaster*; neuropeptide; circadian rhythm; hugγ; PK2

Introduction

The *Drosophila* gene *hugin* encodes two different neuropeptides named hugγ (QLQSNGEPAYRVRTPRL-NH_2) and PK2 (SVPFKPRL-NH_2). These peptides having C-terminal PRL-NH_2 were said to modulate feeding behaviors [1]. A mammalian homolog of this *hugin* is the gene encoding neuromedin U (NMU), having C-terminal PRN-NH_2 structure. It should be noted that NMU is involved in the neurotransmission of circadian rhythm [2]. Thus, hugγ and/or PK2 are likely to play a similar role of NMU in *Drosophila*. In the present study, we preformed immunostaining examination with specific antibodies for hugγ and PK2, respectively, to confirm the relations of these peptides with the circadian clock system.

Results and Discussion

Anti-hugγ polyclonal antibodies (anti-hugγ-pAb) and anti-PK2 polyclonal antibodies (anti-PK2-pAb) were prepared by immunizing KLH-conjugated antigen peptides into New Zealand white rabbits. The serum was treated for purification by the immunoprecipitation followed by affinity chromatography. The specificity of antibodies was determined by ELISA, and found not to interact with other PRX-NH_2 peptides such as PK1 (*Drosophila* PBAN like peptide), ETH1, ETH2, CAPA1, and CAPA2.

When brains of the fruit fly *Drosophila melanogaster* was examined, the cells stained by both antibodies were found in the subesophageal ganglion (SOG), and also dorsal lateral neurons (LNds) were stained (Fig. 1). This immunostaining of LNds was confirmed by co-expression of TIMELESS for brains of transgenic fly *tim-gal4/UAS-GFP* genotype. It should be noted that LNds are clock neurons that drive evening activity of *Drosophila* behavior [3].

Given the production of hugγ and PK2 peptides was involved in a circadian rhythmicity, these peptides were undoubtedly circadian neurotransmitter. Peptide expressionprofiles were evaluated by measuring the strength of immunostaining with anti-hugγ-pAb and anti-PK2-pAb at 4-hr intervals. It was found that the strength of hugγ-immunofluoresence exhibits clearly a circadian rhythmicity. In the LD/DD condition, the profiling of hugγ-immunofluoresence intensities in LNd cells peaked at 0-4 hr and reached to the lowest levels at 16-20 hr. On the other hand, the levels of PK2-immunofluoresence intensities have retained in almost a constant level.

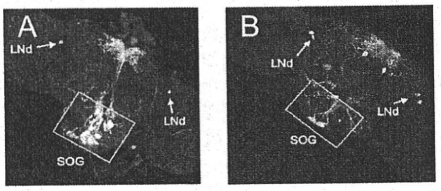

Fig. 1. Immunohistochemistry of anti-hugγ-pAb (A) and anti-PK2-pAb (B), in Drosophila *brain. Immunoreactive cells were found in the dorsal lateral neurons (LNd) (white arrows) and the subesophageal ganglion (SOG) (white box).*

In order to examine whether or not hugγ expression is regulated in the clock gene feedback system, we tested four clock mutant flies. Those include *period*-null mutant fly (per^{01}), *timeless*-null mutant fly (tim^{01}), *clock*-null mutant fly (clk^{Jrk}), and *pdf*-null mutant fly (pdf^{01}). The per^{01} mutant fly exhibited decrease in immunofluoresence at Zeitgeber time (ZT) 4 as compared with the wild type fly. However, similar reduction was not observed for any other null mutant flies. These results implied that hugγ production is regulated in the clock gene feedback system, again suggesting that hugγ is a neurotransmitter peptide to emit an evening locomotor activity in the *Drosophila* circadian clock.

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Bisphenol AF Is a Full Agonist for the Estrogen Receptor ERα but a Highly Specific Antagonist for ERβ

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BACKGROUND: Bisphenol AF has been acknowledged to be useful for the production of CF_3-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 ERα and ERβ and the bisphenol A–specific estrogen-related receptor ERRγ, and to clarify structural characteristics of receptors that influence bisphenol AF binding.

METHODS: We examined receptor-binding activities of bisphenol AF relative to [^{3}H]17β-estradiol (for ERα and ERβ) and [^{3}H]bisphenol A (for ERRγ). 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 ERRγ. Furthermore, bisphenol AF receptor-binding activity was three times stronger for ERβ [IC_{50} (median inhibitory concentration) = 18.9 nM] than for ERα. When examined using a reporter gene assay, bisphenol AF was a full agonist for ERα. In contrast, it was almost completely inactive in stimulating the basal constitutive activity of ERβ. Surprisingly, bisphenol AF acted as a distinct and strong antagonist against the activity of the endogenous ERβ agonist 17β-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 ERα and/or ERβ.

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 hexafluorobisphenol A) is a homolog of bisphenol A (BPA) (Figure 1). Bisphenol AF has a symmetrical chemical structure of HO–C_6H_4–$C(CF_3)_2$–C_6H_4–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 (CF_3) or methyl (CH_3) group, respectively. The potential toxicity of bisphenol AF is of concern in part because its CF_3 group is much more electronegative (and potentially reactive) than is the CH_3 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 (ERRγ) as the BPA target receptor (Takayanagi et al. 2006). BPA binds to ERRγ 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 ERRγ 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 *ERRγ* 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 ERα more strongly than does BPA, and that the receptor selectivity of bisphenol AF is seven times higher for ERα than for ERRγ (Okada et al. 2008). There are two subtypes of estrogen receptors, ERα and ERβ, with distinctly different physiological distributions and functions. Because effects of a number of chemicals have been reported to differ between ERα and ERβ (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 ERβ and found that bisphenol AF is a potent ligand that functions as an antagonist on ERβ.

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

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Materials and Methods

Test compounds. We obtained 17β-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 ERα and ERβ were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). GST-fused receptor LBDs expressed in *Escherichia coli* BL21α (GST-ERα-LBD, GST-ERβ-LBD, and GST-ERRγ-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 × 10 mm; GE Healthcare BioSciences).

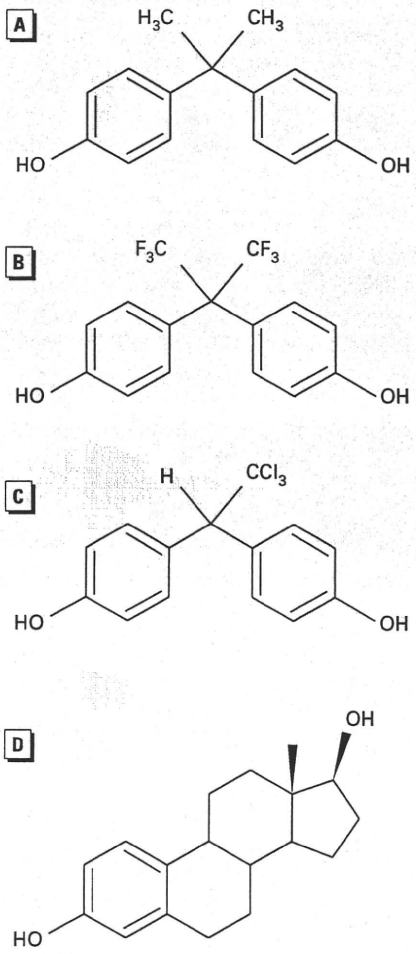

Figure 1. Chemical structures of (*A*) BPA, (*B*) bisphenol AF, (*C*) 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), and (*D*) 17β-estradiol.

Radioligand binding assays for saturation binding. We conducted the saturation binding assays for ERα and ERβ essentially as reported by Nakai et al. (1999) using tritium-labeled ligand [^{3}H]17β-estradiol (5.96 TBq/mmol; GE Healthcare UK Ltd., Buckinghamshire, UK). Receptor protein GST-ERα-LBD or GST-ERβ-LBD (0.3 nM) was incubated with increasing concentrations of [^{3}H]17β-estradiol (0.1–30 nM) in a final volume of 100 μL 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 μM nonradiolabeled 17β-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 ERRγ as reported previously (Okada et al. (2008) using [^{3}H]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, 17β-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 ERα agonist and an ERβ antagonist. These chemicals were examined for their ability to inhibit the binding of [^{3}H]17β-estradiol (5 nM in final) to GST-ERα-LBD (26 ng) and GST-ERβ-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 (IC_{50}) for 17β-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 ERRγ using [^{3}H]BPA (5 nM final concentration) and GST-ERRγ-LBD (26 ng).

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

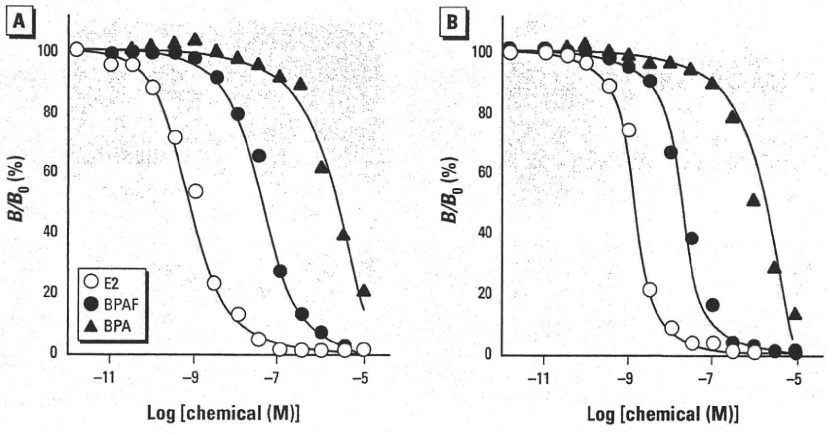

Figure 2. Radioligand receptor-binding assays of bisphenol AF (BPAF), BPA, and 17β-estradiol (E2) to measure the ability of the compounds to displace [^{3}H]17β-estradiol in recombinant human ERα (*A*) and ERβ (*B*). B/B_0, sample bound/maximum binding. The representative dose-dependent binding curves show the IC_{50} value closest to the mean IC_{50} from at least five independent assays. The IC_{50} values showed a between-experiment coefficient of variation of 5–12%.

Table 1. Receptor-binding characteristics of BPA and bisphenol AF for ERα, ERβ, and ERRγ.

Compound	IC_{50} (nM)		
	ERα	ERβ	ERRγ
17β-estradiol	0.88 ± 0.04	2.17 ± 0.12	NB
4-OHT	2.88 ± 0.15	3.17 ± 0.24	10.3 ± 0.8
BPA	1,030 ± 70	900 ± 70	9.70 ± 0.59
Bisphenol AF	53.4 ± 3.1	18.9 ± 0.84	358 ± 3.1
HPTE	59.1 ± 1.5	18.1 ± 1.9	36.4 ± 4.4

Abbreviations: HPTE, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; NB, not bound (no significant receptor binding at 10 μM, 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 μg reporter gene (pGL3/3xERE) and 3 μg of ERα or ERβ 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 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. The assay for ERRγ was carried out as previously reported (Okada et al. 2008).

To measure the antagonistic activity of bisphenol AF for ERβ, we examined four concentrations (0.01, 0.1, 1.0, and 10 μM) of bisphenol AF for a serial concentration of 17β-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 17β-estradiol, which normally elicit full activation of ERβ.

Results

Strong binding activity of bisphenol AF to ERβ 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 ERα (IC_{50} = 1,030 nM) based on its ability to inhibit [^{3}H]17β-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 ERβ (IC_{50} = 900 nM; Figure 2B, Table 1), indicating comparable interactions of BPA with ERα and ERβ despite the subtle structural differences between these ERs. In contrast, bisphenol AF was 20 times more potent than BPA as a ligand for ERα (IC_{50} = 53.4 nM; Figure 2A, Table 1) and was approximately 48 times more potent for ERβ (IC_{50} = 18.9 nM; Figure 2B, Table 1). This high binding activity for ERβ suggests that the binding pocket of ERβ possesses specific structural elements that interact much more favorably with the CF_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 ERα and ERβ (Table 1), but approximately 10 times more potent than bisphenol AF for ERRγ.

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 ERα, ERβ, and ERRγ) to estimate receptor selectivity ratios for BPA and bisphenol AF (Table 2). The results indicate that BPA is exclusively selective for ERRγ, being 90–100 times more active for ERRγ than for ERα or ERβ. In contrast, bisphenol AF receptor binding is much more selective for ERα and ERβ than for ERRγ (6.70 times more selective for ERα than for ERRγ and 18.94 times more selective for ERβ than for ERRγ; Table 2). Bisphenol AF binding is also about three times more potent for ERβ than for ERα.

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 ERα or ERβ expression plasmid and an estrogen-response element (ERE)-luciferase reporter plasmid. Bisphenol AF fully activated ERα (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 ERα activation versus ERα binding to determine receptor activation potency [expressed as EC_{50} (nM)/IC_{50} (nM)], we found a clear discrepancy between 17β-estradiol and bisphenol AF. As shown in Table 3, we estimated the receptor activation potency for 17β-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 ERα, ERβ, and ERRγ.

Compound	Receptor-binding selectivity			
	ERα vs. ERβ	ERα vs. ERRγ	ERβ vs. ERRγ	Preferred receptor(s)
17β-estradiol	2.47 ERα	(ERα)[a]	(ERβ)[a]	ERα
4-OHT	1.10 ERα	3.58 ERα	3.25 ERβ	ERα ~ ERβ
BPA	1.14 ERβ	106.18 ERRγ	92.78 ERRγ	ERRγ
Bisphenol AF	2.83 ERβ	6.70 ERα	18.94 ERβ	ERβ
HPTE	3.27 ERβ	1.63 ERRγ	2.01 ERβ	ERβ

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 ERα" means that 17β-estradiol binds to ERα 2.47 times more strongly than to ERβ.
[a]Because of inactivity of 17β-estradiol in ERRγ, 17β-estradiol is active exclusively in ERα and ERβ.

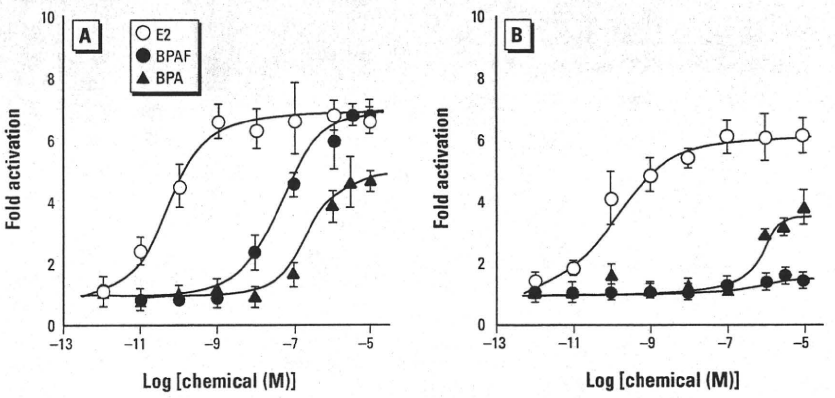

Figure 3. Luciferase-reporter gene assays of bisphenol AF (BPAF), BPA, and 17β-estradiol (E2) for ERα and ERβ using reporter gene (pGL3/3xERE) and either ERα or ERβ expression plasmid (pcDNA3/ERα or pcDNA3/ERβ) in HeLa cells. Concentration-dependent responses of 17β-estradiol, bisphenol AF, and BPA in the luciferase-reporter gene assay for ERα (*A*) and ERβ (*B*). For ERα, bisphenol AF displays full activation in a concentration-dependent manner, whereas for ERβ it displays extremely weak activity. 17β-Estradiol exhibits very strong activity, with approximately 4.5 times more activity induced at 10^{-14} to 10^{-5} M than at baseline.

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

Compound	EC_{50} (nM)/IC_{50} (nM)	
	ERα	ERβ
17β-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 (17β-estradiol = 1.0).

of 17β-estradiol (Table 3). This means that the concentration of 17β-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 17β-estradiol when measured in HeLa cells.

BPA was an extremely weak activator of both ERα (EC_{50} = 317 nM) and ERβ (EC_{50} = 693 nM) based on the luciferase reporter gene assay. The receptor activation potencies of BPA for ERα (0.308) and ERβ (0.770) were 3.6 and 18.8 times greater than the receptor activation potencies of 17β-estradiol for ERα and ERβ, respectively (Table 3). These suggests that, compared with 17β-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 ERβ (3.5 times greater activity relative to baseline in response to BPA vs. an increase to 6 times the baseline level in response to 17β-estradiol). This difference in efficacy indicates that BPA does not have the same ability as 17β-estradiol to induce activation conformation when measured in HeLa cells on this promoter.

Antagonist activity of bisphenol AF on ERβ. For ERβ, bisphenol AF was almost completely inactive, with very little increase in activity even at 10 μM, the highest concentration tested (Figure 3B). Based on the strong receptor-binding activity of bisphenol AF for ERβ (IC_{50} = 18.9 nM; Table 1), we expected that bisphenol AF would also have a high receptor activation potency for ERβ. This unexpected inactivity in the reporter gene assay suggests that bisphenol AF binding disrupts the ERβ-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 17β-estradiol. When we examined 17β-estradiol, an endogenous agonist ligand of ERβ, in the presence of 0.01, 0.1, 1.0, and 10 μM bisphenol AF, its activity (EC_{50} = 0.075 nM) was gradually weakened. As shown in Figure 4A, the dose-dependent curves of 17β-estradiol shifted to the right with increasing concentrations of bisphenol AF, indicating that bisphenol AF effectively inhibits the interaction between 17β-estradiol and ERβ. When the results of Figure 4A were analyzed using a Schild plot, pA_2, 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 17β-estradiol/ERβ was further evidenced by assays in which we added serial concentrations of bisphenol AF (10^{-12} to 10^{-5} M) to a solution of 17β-estradiol maintained at a constant concentration. When 1×10^{-8} M 17β-estradiol was treated with bisphenol AF, the activity of 17β-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 1×10^{-7} M 17β-estradiol. These results demonstrate that bisphenol AF can antagonize the activity of 17β-estradiol on the ERβ receptor.

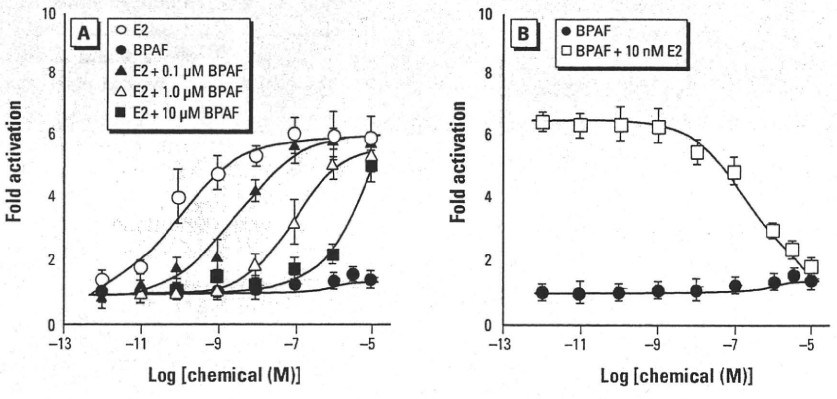

Figure 4. Effects of bisphenol AF (BPAF) on the agonist activity of 17β-estradiol (E2) in the luciferase-reporter gene assays for ERβ. (*A*) Concentration-dependent luciferase-reporter activities of 17β-estradiol by fold activation in the presence and absence of bisphenol AF (0.1, 1, or 10 μM); these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

Discussion

Structural characteristics of bisphenols and ERs/ERRγ receptors. The differences in receptor selectivity between bisphenol AF and BPA are due to the $CH_3 \leftrightarrow CF_3$ substitution on the bisphenol backbone structure. Bisphenol AF is a hexafluoro derivative of BPA with the $CH_3 \rightarrow CF_3$ substitution on the backbone structure of 2,2-disubstituted propane CH_3–C–CH_3. BPA binds strongly to ERRγ, but bisphenol AF binds to ERRγ only weakly; we therefore judged that the binding pocket of ERRγ-LBD possesses structural elements unfavorable for interaction with the trifluoro groups. The molecular size of CF_3 is almost the same as that of CH_3, and thus there would be no structural repulsion or steric hindrance between these groups. However, because the CF_3 group is very electron rich, the structural elements standing face to face with CF_3 must also be electron rich, resulting in their electrostatic repulsion.

In our previous study (Matsushima et al. 2007, 2008), we found that the ERRγ binding sites for BPA CH_3 groups were Phe435 and Met306. Because the aromatic phenyl and S–CH_3 groups of Phe435 and Met306 are electron rich, conditions would be unfavorable for binding of bisphenol AF's electron-rich CF_3 groups. Corresponding receptor residues in ERα are Leu525 and Leu384, respectively. Apparently, there would be no electrostatic repulsion between the bisphenol AF's CF_3 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 ERα.

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

Among the amino acid residues lining the binding pockets of ERα and ERβ, two residues differ significantly: Leu384 in α-helix 5 (H5) of ERα is replaced by Met336 in ERβ, and Met421 in loop 6–7 of ERα is replaced by Ile373 in ERβ. These two residues are most probably responsible for the discriminative affinity and reverse functional activity of bisphenol AF for ERα and ERβ. Furthermore, because bisphenol AF is an ERβ antagonist, the binding of bisphenol AF to the ERβ ligand-binding pocket must damage the ERβ-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 ERα and ERβ 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 ERα and a potent estrogen antagonist for ERβ. ERα and ERβ are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues. ERα is expressed primarily in the uterus, liver, kidneys, and heart (Couse and Korach 1999), whereas ERβ 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).] 17β-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 ERα and ERβ 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 ERα and an antagonist for ERβ. 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 ERα agonist and an ERβ 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 ERα with IC_{50} = 59.1 nM and of ERβ with IC_{50} = 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 ERα but a strong antagonist for ERβ. However, bisphenol AF and HPTE differ in their receptor preference for ERRγ. HPTE was approximately 10 times more potent than bisphenol AF for ERRγ binding, although both chemicals were most strongly bound to ERβ (Tables 1, 2). As an antagonist for ERβ, 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 ERβ antagonists.

Chemical structures of bisphenol AF and HPTE differ, with one of two CF_3 groups of bisphenol AF replaced by CCl_3 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 ERα and ERβ via their interactions with the ligand-binding pockets of each ER, namely, Leu384 in H5 of ERα ↔ Met336 in ERβ, and Met421 in loop 6–7 of ERα ↔ Ile373 in ERβ.

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 uterotropic 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 ERα or ERβ, or both.

Conclusions

BPA binds strongly to ERRγ but very weakly to ERα and ERβ. In contrast, bisphenol AF binds very weakly to ERRγ but strongly to ERα and ERβ. These differences in receptor selectivity reflect subtle but distinct structural differences resulting from the CH_3 ↔ CF_3 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 ERα and ERβ. Given that bisphenol AF and BPA function as endocrine disruptors, these chemicals would work differently via differentNRs.

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Distinction of the binding modes for human nuclear receptor ERRγ between bisphenol A and 4-hydroxytamoxifen

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Bisphenol A (BPA) strongly binds to human estrogen-related receptor γ (ERRγ). BPA is an oestrogenic endocrine disruptor that influences various physiological functions at very low doses. BPA functions as an inverse-type antagonist of ERRγ to retain its high basal constitutive activity by inhibiting the deactivating inverse agonist activity of 4-hydroxytamoxifen (4-OHT). We recently demonstrated that ERRγ receptor residues Glu275 and Arg316 function as the intrinsic binding site of BPA's phenol-hydroxyl group. We also determined the chief importance of phenol-hydroxyl↔Arg316 hydrogen bonding and the corroborative role of phenol-hydroxyl↔Glu275 hydrogen bonding. However, there appeared to be a distinct difference between the receptor binding modes of BPA and 4-OHT. In the present study, using tritium-labelled or non-labelled BPA and 4-OHT, we evaluated in detail the receptor binding capabilities of wild-type ERRγ and its mutants with amino acid alterations at positions 275 and 316. Both compounds exhibited a strong binding ability to wild-type ERRγ due to the hydrogen bonding to Glu275 and Arg316. However, 4-OHT revealed significantly reduced occupancy for both wild-type and mutant receptors. The data obtained suggest that 4-OHT barely binds to ERRγ due to the strong ability of Glu275 and Arg316 to recruit phenol compounds.

Keywords: bisphenol A/estrogen-related receptor γ/4-hydroxytamoxifen/receptor binding mode/receptor binding assay.

Abbreviations: BPA, bisphenol A; DCC, dextran-coated charcoal; DES, diethylstilbestrol; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ERR, estrogen-related receptor; ERRE, ERR-response element; ERRγ, estrogen-related receptor γ; LBD, ligand-binding domain; LBP, ligand-binding pocket; NR, nuclear receptor; 4-OHT, 4-hydroxytamoxifen.

Bisphenol A [BPA; 2,2-*bis*(4-hydroxyphenyl)propane], has recently been found to bind strongly to estrogen-related receptor γ (ERRγ) with high constitutive basal activity (*1*). ERRγ is one of the 48 human nuclear receptors (NRs) (*2*, *3*), while BPA has long been recognized as an estrogenic chemical able to interact with human estrogen receptor (ER) (*4–6*). Various 'low-dose effects' of BPA have recently been reported *in vivo* for many organ tissues and systems in mice and rats (*7–10*). However, since the discovery of ERRγ, it became an immediate and important requirement to evaluate whether the previously reported effects of BPA at low doses are mediated through ERRγ and its specific target gene(s) (*11*).

BPA has the chemical structure of $HO–C_6H_4–C(CH_3)_2–C_6H_4–OH$ with two phenol groups (Fig. 1). We have recently reported the crystallization and structural analysis of the BPA/ERRγ-ligand binding domain (LBD) complex (*12*). 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. The crystal structure of the complex suggests several essential interactions between the BPA and ERRγ-LBD molecules. For instance, BPA's phenol-hydroxyl group is tethered by hydrogen bonds to the Glu275 and Arg316 residues in the ERRγ-LBD (Fig. 2). These hydrogen bonds were also observed in the ERRγ–LBD complex with 4-α-cumylphenol (*13*). Since 4-α-cumylphenol is a compound that lacks one of BPA's phenol-hydroxyl groups, the results clearly indicated that the hydrogen bonds of the phenol-hydroxyl group to the Glu275 and Arg316 residues are crucial for binding of the phenol compounds to ERRγ. Indeed, by examining the ERRγ–LBD analogues through site-directed mutagenesis, we demonstrated these residues as the intrinsic binding site of BPA's phenol-hydroxyl group (*14*).

Similar hydrogen bonding was also found in the complex between 4-hydroxytamoxifen (4-OHT, Fig. 1) and ERRγ–LBD (*15*). 4-OHT deactivates ERRγ in, for example, the luciferase reporter gene assay (*1*, *16*, *17*). BPA inhibits such inverse agonist activity of 4-OHT. BPA reverses the deactivation to the originally high basal activation state in a dose-dependent manner, and thus acts as an inverse antagonist of ERRγ (*1*). Although the phenol-hydroxyl group of 4-OHT shares the same site for its binding to ERRγ, the difference in receptor binding modes between BPA and 4-OHT remains to be clarified.

In the previous study we elucidated the chief importance of phenol-hydroxyl↔Arg316 hydrogen bonding

and the corroborative role of phenol-hydroxyl ↔Glu275 hydrogen bonding (*14*). This result strongly suggested that the formation of these double hydrogen bonds is critical for phenol compounds to bind to ERRγ. In the present study, to shed light on the structural significance of these double hydrogen bonds, we carried out a detailed comparison between BPA and 4-OHT for binding to ERRγ–LBD in a site-directed point mutagenesis series. We here report that human NR ERRγ binds highly specifically to BPA, but barely binds to the inverse agonist 4-OHT.

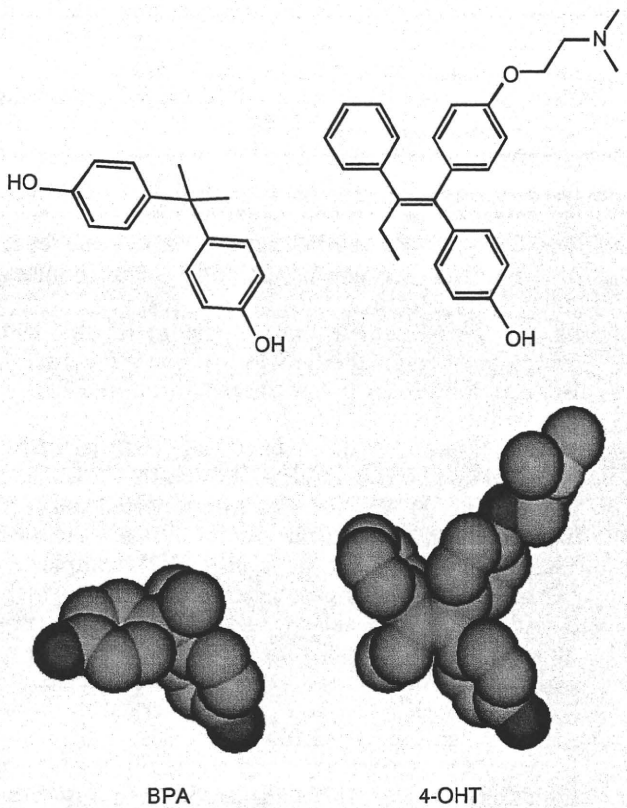

Fig. 1 Chemical structures of BPA and 4-OHT and their space-filling structures in the ligand-binding pocket of the ERRγ. The space-filling structure of BPA originated from the X-ray crystal structure [Protein Data Bank with accession code 2E2R (*12*)], and that of 4-OHT was also from the deposited structure [2GPU, (*30*)].

Experimental procedures

Chemicals

BPA was purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo). 4-Hydroxytamoxifen (4-OHT) was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). [^{3}H]BPA (5 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA) and [^{3}H]4-OHT (80 Ci/mmol) from American Radiolabelled Chemicals Inc. (St Louis, MO, USA).

Plasmid construction and site-directed mutagenesis

A cDNA fragment encoding wild-type ERRγ–LBD (residues 222–458) was generated by polymerase chain reaction (PCR) with specific primers using the human kidney cDNA library (Clontech Laboratories; Mountain View, CA, USA) and cloned into the vector pGEX-6p-1 (Amersham Biosciences, Piscataway, NJ, USA) at the *Eco*RI and *Xho*I sites. The resulting plasmids were designated as pGEX–ERRγ–LBD.

ERRγ mutants were generated using *PfuTurbo*® DNA Polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions using pGEX–ERRγ–LBD as a template. The mutations were introduced by PCR mutagenesis in a two-step reaction essentially as reported (*14*, *18*). Each mutant LBD was amplified and cloned into the vector pGEX-6p-1 at the *Eco*RI and *Xho*I sites. All PCR products were verified for their accuracy in the sequences.

ERRγ–LBD protein expression

Two glutathione *S*-transferase (GST)-fused receptor proteins—the wild-type and mutant GST–ERRγ–LBD—were expressed in *Escherichia coli* BL21 as described previously (*1*). The receptor protein was purified using an affinity column of glutathione–sepharose 4B (GE Healthcare BioSciences Co., Piscataway, NJ, USA). After incubation for 1 h at 4°C, the column was washed three times with PBS containing 0.2% (v/v) Triton X-100 and once with sonication buffer. Fusion protein was eluted with 1 M Tris–HCl (pH 8.0) containing 20 mM reduced glutathione, which was removed by gel filtration on a column of Sephadex G-10 (15 × 100 mm, GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 8.0). The purity was confirmed by SDS–PAGE using 12.5% polyacrylamide

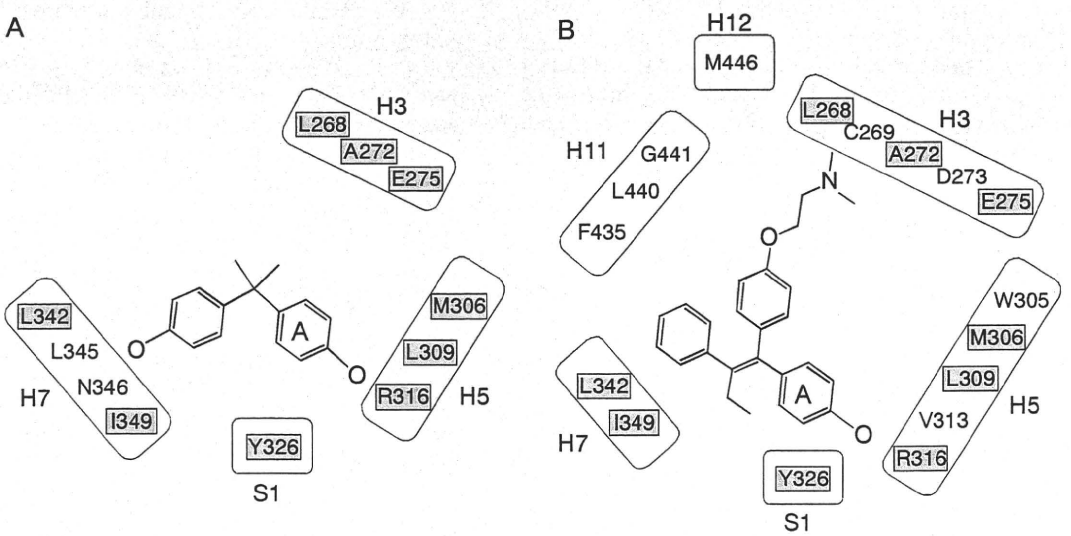

Fig. 2 Structural environments of BPA and 4-OHT in the ligand-binding pocket of the ERRγ. The proximity of each amino acid residue (within the distance 4 Å) to BPA (A) and to 4-OHT (B) is shown in the boxes depicting the α-helices. The amino acids in a grey backboard are the residues shared by both BPA and 4-OHT.

gel. The protein concentrations were determined by the Bradford method (*19*).

Radio-ligand receptor binding assays

Saturation binding. A saturation binding assay was conducted (*20*) using [^{3}H]4-OHT or [^{3}H]BPA. The reaction mixture was incubated at 4°C for 2 h with the receptor proteins—GST-fused wild-type ERRγ–LBD or its mutants—in 100 µl of binding buffer [10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM CHAPS and 2 mg/ml γ-globulin]. The assay was performed with or without the addition of unlabelled BPA or 4-OHT (final concentration of 1×10^{-5} M) to quantify the specific and nonspecific binding. After incubation with 100 µl of 1% dextran-coated charcoal (DCC) (Sigma) (*21*) in PBS (pH 7.4) for 10 min at 4°C, free radioligand was removed by the direct vacuum filtration method using a 96-well filtration plate (Millipore, Bedford, MA, USA) for the B/F separation. The specific binding of [^{3}H]BPA or [^{3}H]4-OHT was calculated by subtracting the non-specific binding from the total binding, and the results were examined by Scatchard plot analysis (*22*). The assay was carried out at least in triplicate.

Competitive binding. Competitive binding assays were performed in the presence of GST-fused wild-type ERRγ–LBD or its mutants at the most appropriate concentration of each. Reaction mixtures were incubated with either [^{3}H]BPA or [^{3}H]4-OHT (5 nM in final) at 4°C for 2 h, and free radioligand was removed by the method described above after incubation with 100 µl of 1% DCC in PBS (pH 7.4) for 10 min at 4°C. To estimate the binding affinity, the IC_{50} values (the concentrations for the half-maximal inhibition) were calculated from the dose-response curves evaluated by the nonlinear analysis program ALLFIT (*23*). Each assay was performed in duplicate and repeated at least three times.

Results

Critical structural elements to detain 4-OHT and BPA to ERRγ

For the receptor binding assays, the ERRγ–LBD (residues 222–458) was expressed in *E. coli* as a protein fused with glutathione *S*-transferase (GST). Mutations were introduced by the PCR mutagenesis method for the original nucleotide triplet codons GAG of Glu275 and CGG of Arg316 (*24*). To evaluate the ligand-binding ability of mutant receptors, a saturation binding assay was first performed using GST–ERRγ–LBD and [^{3}H]4-OHT. In this assay for the mutant receptors, especially when no specific binding was measurable under the same experimental conditions for the wild-type ERRγ receptor, the assay was carried out repeatedly for the specified times using varied concentrations of the receptor GST–ERRγ–LBD or radio-ligand [^{3}H]4-OHT.

In the previous study using [^{3}H]BPA, it was found that Glu275 and Arg316 are necessary for holding BPA and 4-OHT in ERRγ, but with different degrees of involvement in the hydrogen bonding (*14*). This result was further evidenced in the present study using [^{3}H]4-OHT and a series of mutant receptors, in which site-directed mutations in the LBD of ERRγ were carried out for positions 275 and 316. When Glu275 and Arg316 were simultaneously mutated to Ala, the resulting (*Ala*, *Ala*)-ERRγ mutant receptor exhibited no specific binding of [^{3}H]4-OHT, as reported for [^{3}H]BPA (*14*). As the X-ray crystal structure has suggested (*12*, *15*), the results clearly indicated that Glu275 and Arg316 are crucial for the binding of 4-OHT and BPA, whose phenol-hydroxyl groups are indeed engaged in hydrogen bonding with the side chain carboxyl (Glu) and guanidino groups (Arg).

Differential ability of hydrogen bonds evidenced by [^{3}H]4-OHT

When the Glu275→Ala substitution was achieved, the resulting mutant receptor (275*Ala*)-ERRγ was found to exhibit considerably decreased binding potency for 4-OHT. [^{3}H]4-OHT showed significantly diminished binding ability, with dissociation constants of 28.8 nM (35% of the binding affinity for the wild-type ERRγ) (Table I). [^{3}H]BPA's dissociation constant was 17.8 nM (32% of the binding affinity for the wild-type ERRγ). The Arg316→Ala substitution resulted in a further reduction of activity. The dissociation constants were 210 nM (only 4.8% of the binding affinity for the wild-type ERRγ) for [^{3}H]4-OHT [171 nM (3.3%) for [^{3}H]BPA] (Table I).

The present results clearly indicate that the hydrogen bonds between the phenol-hydroxyl group of 4-OHT or BPA and the Glu275 and Arg316 residues are necessary for capturing these chemicals in the binding pocket of ERRγ–LBD. However, it is also clear that the hydrogen bond between 4-OHT/BPA and the Arg316 is much more important than that between 4-OHT/BPA and the Glu275.

Receptor binding results were also obtained by a competitive binding assay using [^{3}H]4-OHT as a tracer (Table II). BPA and 4-OHT elicited almost the same results for the wild-type ERRγ, with IC_{50} values of 13.8 and 10.3 nM, respectively. For (275*Ala*)-ERRγ, the IC_{50} values were 365 nM (3.6% of the binding affinity for the wild-type ERRγ) and 356 nM (3.9%), respectively. However, for (316*Ala*)-ERRγ with the Arg→Ala mutation, the competitive binding assay could not be carried out because of the extremely small specific binding in the saturation-binding assay. These results clearly indicate that the hydrogen bonding to the Arg316 residue is much more important for capturing BPA and 4-OHT than that to the Glu275 residue.

Table I. Receptor binding potency of ERRγ and its mutants by tritium-labelled BPA and 4-OHT.

Amino acid residues of ERRγ receptors[a]		Dissociation constant (K_D, nM)	
Position 275	Position 316	[^{3}H]BPA	[^{3}H]4-OHT
Glu (Wild-type)	Arg	5.70 ± 0.88	10.1 ± 0.29
Ala	Arg	17.8 ± 2.74	28.8 ± 3.28
Asp	Arg	22.0 ± 2.86	42.0 ± 6.73
Gln	Arg	23.4 ± 3.34	29.4 ± 3.74
Leu	Arg	NSB[b]	NSB
Glu	*Ala*	171 ± 39.5	210 ± 38.3
Glu	*Lys*	22.5 ± 4.26	29.4 ± 3.79
Glu	*Leu*	NSB	NSB
Ala	*Ala*	NSB	NSB
Arg	*Glu*	59.7 ± 6.79	77.3 ± 9.38
Ala	*Glu*	NSB	NSB
Arg	*Ala*	54.3 ± 6.82	74.8 ± 6.15

[a]Specifically mutated residues are designated in italics.
[b]NSB means 'no specific binding' in the saturation binding assay.

Table II. Receptor binding potency of BPA and 4-OHT in the competitive binding assay for ERRγ and its mutants by tritium-labelled 4-hydroxytamoxifen [^{3}H] 4-OHT.

Amino acid residues of ERRγ receptors[a]		Receptor binding potency IC_{50} (nM)	
Position 275	Position 316	BPA	4-OHT
Glu (Wild-type)	Arg	13.8 ± 2.55	10.3 ± 0.07
Ala	Arg	365 ± 16.1	356 ± 58.9
Asp	Arg	179 ± 24.1	184 ± 27.1
Gln	Arg	256 ± 21.2	222 ± 26.7
Leu	Arg	*Not carried out*[b]	
Glu	*Ala*	*Impossible to carry out*[c]	
Glu	*Lys*	228 ± 24.4	293 ± 36.7
Glu	*Leu*	*Not carried out*[b]	
Ala	*Ala*	*Not carried out*[b]	
Arg	*Glu*	*Impossible to carry out*[c]	
Ala	*Glu*	*Not carried out*[b]	
Arg	*Ala*	*Impossible to carry out*[c]	

[a]Specifically mutated residues are designated in italics.
[b]Because of 'no specific binding' in the saturation binding assay, the competitive binding assay was not carried out.
[c]Because of the 'extremely small specific binding' in the saturation binding assay, the competitive binding assay could not be carried out.

Glu275 and Arg316 as hydrogen-bonding anchors to tether the ligands

When Glu275 and Arg316 were each replaced by Leu instead of Ala, the resulting (275*Leu*)-ERRγ and (316*Leu*)-ERRγ mutant receptors were completely inactive, with no specific binding (Table I). Thus, it was impossible to carry out their competitive binding assays (Table II). When Glu275 was replaced by glutamine (Gln), the resulting (275*Gln*)-ERRγ mutant receptor exhibited a sufficient specific binding (~70% of the total binding) for [^{3}H]BPA, but just barely sufficient specific binding (~35% of the total binding) for [^{3}H]4-OHT. The K_D values were 23.4 nM (~25% of the binding affinity for the wild-type ERRγ) for [^{3}H]BPA and 29.4 nM (34%) for [^{3}H]4-OHT (Table I). The IC_{50} values of BPA and 4-OHT were 179 nM (5.1% of the binding affinity for the wild type) and 184 nM (6.2%), respectively (Table II). These results are rather worse than those obtained for (275*Ala*)-ERRγ. It is therefore clear that Gln does not compensate for Glu, indicating that the carboxyl (COOH) group, but not the carboxyl amide ($CONH_2$) group, is crucial to capturing BPA and 4-OHT.

For (275*Asp*)-ERRγ with the Glu275→Asp substitution, [^{3}H]4-OHT exhibited just barely sufficient specific binding (~30% of the total binding) (data not shown). It was found that (275*Asp*)-ERRγ affords almost the same results obtained for (275*Ala*)-ERRγ and (275*Gln*)-ERRγ (Tables I and II). In particular, BPA and 4-OHT are significantly weak for binding to this mutant receptor (4-8% of the binding affinity for the wild-type ERRγ) (Table II). All these results indicate that the γ-carboxyl group of Glu275 is crucially important to binding BPA and 4-OHT.

The importance of Arg316 was also demonstrated by another mutation, in which the basic Arg residue was replaced by lysine (Lys) with the amino group. Prepared (316*Lys*)-ERRγ was found to be considerably weak for binding [^{3}H]4-OHT (K_D = 29.4 nM) and [^{3}H]BPA (22.5 nM) (Table I), since these activities are only ~25% that of the parent wild-type receptor ERRγ. In the competitive binding assay using (316*Lys*)-ERRγ and [^{3}H]4-OHT, BPA and 4-OHT were significantly weak for binding to this mutant receptor (~5% of the binding affinity for the wild-type ERRγ) (Table II). Furthermore, the inactivity of (316*Leu*)-ERRγ and the extremely weak activity of (316*Ala*)-ERRγ (Tables I and II) definitely reveal the importance of the basic Arg residue for receptor activation. All these results indicate that Arg316 is a very important structural element for the binding of BPA and 4-OHT to the binding pocket of ERRγ–LBD by hydrogen bonding.

Collectively, it is now clear that Glu275 and Arg316 are necessary for holding BPA and 4-OHT in ERRγ, but with different degrees of involvement in the hydrogen bonding. The results clearly indicate the major importance of phenol-hydroxyl↔Arg316 hydrogen bonding and the supportive role of the phenol-hydroxyl↔Glu275 hydrogen bonding. The difference in their significance might be attributable to the importance and/or necessity of receipt of the phenol-hydroxyl group, even by using an assisting group to facilitate the receptor function. These results are coincident with those evidenced by [^{3}H]BPA, as reported previously (*14*).

(Glu275, Arg316)-binding site arranged ideally to arrest the phenol-hydroxyl groups

In our previous study (*14*), hypothesizing that no amino acids other than 316Arg and 275Glu would play such an intrinsic role in capturing the phenol-hydroxyl group, we prepared a (*Arg*, *Glu*)-ERRγ double mutant receptor, in which Arg and Glu were placed simply in opposite order. Due to the significance of the receipt of the phenol-hydroxyl group, for example, of BPA and 4-OHT, we expected that the Arg and Glu residues would be transferable. In fact, [^{3}H]BPA was found to bind to this (*Arg*, *Glu*)-ERRγ double mutant receptor (*14*). However, its binding potency was almost 10 times weaker than that to the wild-type receptor (Table I). In the present study, almost the same result was obtained for [^{3}H]4-OHT, as shown in Table I. [^{3}H]4-OHT bound to (*Arg*, *Glu*)-ERRγ with a dissociation constant of 77.3 nM, showing only one-seventh the strength of the bond to the wild-type receptor (Table I).

The specific binding of [^{3}H]BPA was ~65%. In contrast, [^{3}H]4-OHT exhibited only a very small specific binding (<20%) in relation to the total binding. Thus, since [^{3}H]4-OHT could only barely bind to (*Arg*, *Glu*)-ERRγ, it was impossible to carry out the competitive binding assay (Table II).

As to the (*Arg*, *Glu*)-ERRγ receptor, we made further Ala substitutions for 275*Arg* and 316*Glu*, respectively. The resulting (*Ala*, *Glu*)-ERRγ mutant receptor with the 275*Arg*→Ala substitution was found to completely lack the binding capability for [^{3}H]BPA and [^{3}H]4-OHT (Table I). In contrast, the Arg-containing (*Arg*, *Ala*)-ERRγ mutant receptor was still active

for [^{3}H]BPA and [^{3}H]4-OHT (Table I). In the saturation binding assay for (*Arg, Ala*)-ERRγ, these tritium-labelled compounds exhibited similar results to those obtained for (*Arg, Glu*)-ERRγ—the dissociation constant K_D values of 54.3 nM for [^{3}H]BPA and 74.8 nM for [^{3}H]4-OHT. However, for this (*Arg, Ala*)-ERRγ receptor, [^{3}H]4-OHT also exhibited only a very small specific binding (<20%) of the total binding. Due to the very small amount of specific binding, we could carry out only the Scatchard plot analysis. It was impossible to carry out the competitive binding assay for the (*Arg, Glu*)-ERRγ receptor by using [^{3}H]4-OHT as a tracer (Table II).

The fact that the Arg-containing (*Arg, Glu*)-ERRγ and (*Arg, Ala*)-ERRγ mutant receptors were still active, but (*Ala, Glu*)-ERRγ with no Arg was completely inactive, led us to conclude that phenol-hydroxyl↔275*Arg* hydrogen bonding plays a primary role, while the role of phenol-hydroxyl↔316*Glu* hydrogen bonding is only supportive. Furthermore, our finding that (*Arg, Glu*)-ERRγ and (*Arg, Ala*)-ERRγ are almost equipotent (Table I) indicates that the supportive role of the phenol-hydroxyl↔316*Glu* hydrogen bond is actually almost insignificant. This together with the result that these mutant receptors have considerably lower affinity to BPA and 4-OHT is apparently due to the mismatched proximity to the phenol-hydroxyl group of BPA and that of 4-OHT. This assumption was proved recently by the X-ray crystal structure analysis of the BPA/(*Arg, Glu*)–ERRγ complex (under submission). Although Glu275 and Arg316 in ERRγ are interchangeable for keeping up the interaction with BPA and 4-OHT, ERRγ appears to afford simultaneously an ideal space and the capability of arresting the phenol-hydroxyl groups by arranging the Glu and Arg residues at positions 275 and 316, respectively.

Discrepancy in potency to replace [^{3}H]4-OHT and [^{3}H]BPA

When the competitive receptor binding assay was carried out for BPA using [^{3}H]4-OHT as a tracer, the wild-type ERRγ afforded a slightly weakened result as compared with that from the assay using [^{3}H]BPA. In the competitive receptor binding assay using [^{3}H]BPA as a tracer, BPA showed an IC_{50} value of 9.70 nM (*14*). In the assay using [^{3}H]4-OHT, however, the IC_{50} value of BPA was 13.8 nM, ~40% larger than that when using [^{3}H]BPA (Table II). These results clearly indicate that BPA has certain difficulty in displacing [^{3}H]4-OHT, probably due to the increased binding attachment points with [^{3}H]4-OHT as compared with [^{3}H]BPA.

On the other hand, in the assays using [^{3}H]4-OHT as a tracer for the mutant receptors that afforded the specific binding, (275*Ala*)-, (275*Asp*)-, (275*Gln*)-, (316*Lys*)- and (*Arg, Glu*)-ERRγ, the IC_{50} values of the compounds were one order of magnitude larger (3–10 times larger) than those obtained from the assays using [^{3}H]BPA as a tracer. For example, for the (275*Ala*)-ERRγ mutant receptor, BPA exhibited an IC_{50} value of 35.7 nM with [^{3}H]BPA (*14*), while this value was 10 times larger, 365 nM, with [^{3}H]4-OHT (Fig. 3, Table II). It should be noted that the competitive binding assay measures the ability of the compound to replace the radiolabelled tracer in the ligand-binding pocket of the receptor. Thus, the results simply imply that, for BPA, to replace [^{3}H]4-OHT is more difficult than to replace [^{3}H]BPA.

Large difference in receptor occupancy between [^{3}H]4-OHT and [^{3}H]BPA

The results described above suggest that 4-OHT binds more solidly to ERRγ than BPA. On the contrary, 4-OHT must have more difficulty fastening to the binding site because of the increased number of attachment points. This difference between [^{3}H]4-OHT and [^{3}H]BPA was clearly shown by the difference in their occupied receptor populations.

The maximal receptor density (B_{max}) was estimated by Scatchard plot analysis for each mutant receptor in which specific binding was observed (Fig. 4). It is clear that the B_{max} values of [^{3}H]4-OHT are significantly

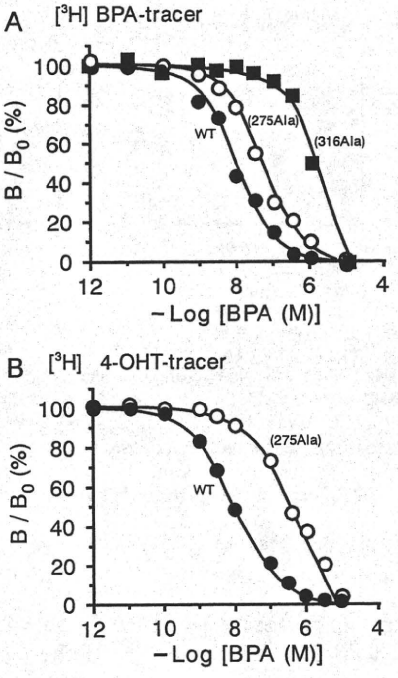

Fig. 3 Concentration-dependent curves of BPA for ERRγ–LBD and its site-directed mutant derivatives by using tritium-labelled BPA (A) and 4-OHT (B). The receptor competitive binding assays were carried out to measure the ability to displace [^{3}H]BPA (A) and [^{3}H]4-OHT (B). Used receptors are wild-type ERRγ (filled circle), (275*Ala*)-ERRγ with the Glu275→Ala substitution (open circle), and (316*Ala*)-ERRγ with the Arg316→Ala substitution (filled square). The graphs show representative dose-dependent binding curves, which give the IC_{50} value closest to the mean IC_{50} from at least five independent assays for BPA. The IC_{50} values showed a between-experiment coefficient of variation of 3–10%. All the receptors used are the ligand-binding domain (LBD) of the human ERRγ and its mutant receptors. The IC_{50} values of BPA in the [^{3}H]BPA binding assay (A) were 9.70 ± 0.59 nM for wild-type ERRγ, 35.7 ± 5.45 nM for (275*Ala*)-ERRγ and 990 ± 78.4 nM for (316*Ala*)-ERRγ (*14*). Those in the [^{3}H]4-OHT binding assay (B) were 10.3 ± 0.07 nM for wild-type ERRγ and 365 ± 16.1 nM for (275*Ala*)-ERRγ, as shown in Table II. The assay for (316*Ala*)-ERRγ was not feasible due to the extremely small amounts of specific binding observed for [^{3}H]4-OHT and the very weak binding potency of 4-OHT (IC_{50} = 818 nM) (*14*).

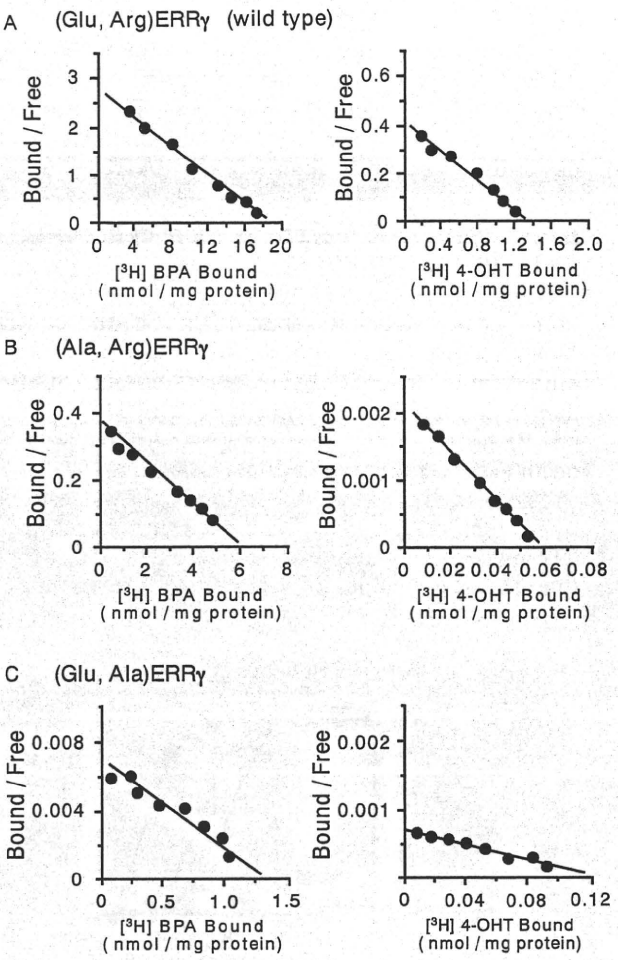

Fig. 4 Scatchard plot analyses showing a single binding mode with a binding affinity constant (K_d) and receptor density (B_{max}). Analyses were carried out from the radioligand receptor saturation binding curves of [^{3}H]BPA (left-side figures) and [^{3}H]4-OHT (right-side figures) for the human ERRγ ligand-binding domain (LBD) and its site-directed mutant derivatives. Those include the wild-type ERRγ (A), (275*Ala*)-ERRγ with the Glu275→Ala substitution (B), and (316*Ala*)-ERRγ with the Arg316→Ala substitution (C). Note that the B_{max} value (nmol/mg protein on the abscissa) of [^{3}H]4-OHT is much smaller than that of [^{3}H]BPA in each receptor assay.

smaller than those of [^{3}H]BPA (Table III). As to the parent wild-type ERRγ receptor, the [^{3}H]BPA's B_{max} value, 18.4 nmol/mg protein, is very compatible with the 18.8 nmol/mg protein that was estimated for the mature ERRγ receptor protein based on its molecular weight (53 500). However, the [^{3}H]4-OHT's B_{max} value, 1.26 nmol/mg protein, was only 1/16th of the estimated value (Fig. 4, Table III). Obviously, these results imply that the feasibility of [^{3}H]4-OHT binding to ERRγ is relatively small, as is also true for other mutant receptors.

Discussion

Structural requirements of ERRγ to bind BPA and 4-OHT

Among the total 48 human NRs, 26 NRs have Arg at the position corresponding to ERRγ's 316 (*14*). In contrast to this Arg316, Glu275 is conserved among only five NRs: ERα, ERβ, ERRα, ERRβ and ERRγ.

Table III. Receptor density of ERRγ and its mutants by tritium-labelled BPA and 4-OHT.

Amino acid residues of ERRγ receptors[a]		Receptor density (B_{max}, nmol/mg)	
Position 275	Position 316	[^{3}H]BPA	[^{3}H]4-OHT
Glu (Wild-type)	Arg	18.4 ± 0.78	1.26 ± 0.055
Ala	Arg	6.72 ± 0.62	0.059 ± 0.0030
Asp	Arg	12.4 ± 0.46	0.28 ± 0.024
Gln	Arg	7.81 ± 0.47	0.35 ± 0.041
Leu	Arg	NSB[b]	NSB
Glu	*Ala*	1.34 ± 0.16	0.12 ± 0.016
Glu	*Lys*	9.98 ± 0.76	0.18 ± 0.049
Glu	*Leu*	NSB	NSB
Ala	*Ala*	NSB	NSB
Arg	*Glu*	3.66 ± 0.29	0.095 ± 0.043
Ala	*Glu*	NSB	NSB
Arg	*Ala*	3.56 ± 0.38	0.30 ± 0.070

[a]Specifically mutated residues are designated in italics.
[b]NSB means 'no specific binding' in the saturation binding assay, and thus the B_{max} value was not obtained.

Since all these Glu275-containing NRs contain Arg316, it is very reasonable to assume that these (Glu275, Arg316)-containing NRs are able to bind to the phenol compounds. We have recently demonstrated that a number of 4-alkylphenols bind to ERRγ considerably more strongly (*11*), and that their phenol-hydroxyl group is arrested by the Glu275 and Arg316 residues, as shown by the X-ray structural analysis of the phenol/ERRγ complexes (unpublished data). Although the natural hormone of ERα and ERβ is 17β-estradiol, ERRα, ERRβ and ERRγ are all orphan receptors whose endogenous ligand is not identified (*25–28*). It is highly likely that if the ERRs have any endogenous ligands, they must be phenol compounds.

When the Glu275→Ala substitution was accomplished, the resulting mutant receptor (275*Ala*)-ERRγ was found to exhibit just about sufficient specific binding (~35% of the total binding) for [^{3}H]4-OHT, in contrast to the clearly sufficient specific binding (~65% of the total binding) for [^{3}H]BPA. For (316*Ala*)-ERRγ with the Arg316→Ala substitution, [^{3}H]4-OHT exhibited only barely sufficient specific binding (~20% of the total binding), which was insufficient to perform the competitive binding assay. [^{3}H]BPA exhibited a sufficient specific binding (~50% of the total binding) for this (316*Ala*)-ERRγ. It is clear that the same structural changes in ERRγ differently affect the binding ability of 4-OHT and BPA, which is suggestive of their different receptor binding modes. BPA and 4-OHT share only a phenol group (Fig. 1), which has been demonstrated to occupy the same (Glu275, Arg316)-binding site in the ERRγ receptor (*12*, *15*, *29*, *30*). Thus, their different receptor binding modes are definitely due to structures other than the phenol group.

For the ERRγ receptor, 4-OHT acts as an inverse agonist (*1*, *16*), the binding of which dissociates the α-helix 12 (H12) region from the LBD body (*12*, *15*, *29*). This discussion results in a deactivation

of the receptor. 4-OHT puts aside the H12 from an activation conformation to an inactivation conformation, as evidenced by the X-ray crystallography of the 4-OHT–ERRγ complex (*15*). This process appears to be a highly energy-consuming option. In contrast, H12 of non-liganded ERRγ–LBD is folded into the activation conformation (*12*, *29*), and BPA is able to bind to this conformation without causing any structural changes in the receptor, as demonstrated by the X-ray crystallography of the BPA–ERRγ complex (*12*). Thus, there is indeed a distinct difference in binding modes between 4-OHT and BPA for ERRγ.

BPA suppressed the inverse agonist activity of 4-OHT, being a specific inhibitor against the inverse agonist 4-OHT. It should be noted that BPA's ability to antagonize 4-OHT is approximately one-order lower than its binding potency to ERRγ, as reported previously by Okada *et al.* (*11*). This discrepancy is probably due to the BPA's difficulty in displacing 4-OHT (see above in the Results section), originating from the distinction in binding modes between BPA and 4-OHT for ERRγ.

Different potentials of BPA and 4-OHT in binding to ERRγ

The difference in binding modes would induce novel differences in the binding and dissociation energies. For instance, with the wild-type ERRγ, [^{3}H]4-OHT exhibited an ~1.8-fold larger K_D value than that of [^{3}H]BPA (Table I). Also, for a series of mutant receptors, [^{3}H]4-OHT and [^{3}H]BPA showed similar differences (Table I). [^{3}H]BPA did bind to (316*Ala*)-ERRγ, (*Arg*, *Glu*)-ERRγ and (*Arg*, *Ala*)-ERRγ, but [^{3}H]4-OHT could not bind to any of them (Table I).

The difference between [^{3}H]4-OHT and [^{3}H]BPA is further revealed in their maximal receptor density B_{max} values. [^{3}H]BPA afforded well-matched B_{max} values for the wild-type ERRγ; *i.e.* 18.4 nmol/mg protein as the observed value and 18.8 nmol/mg protein as the calculated value (Table III). However, the B_{max} value of [^{3}H]4-OHT was diminished significantly, with only 1.26 nmol/mg protein (~7% compared with that of [^{3}H]BPA) (Table III). This result must be due to 4-OHT repositioning H12 from an activation conformation to an inactivation conformation, making it difficult for [^{3}H]4-OHT to bind to ERRγ. BPA binds directly to ERRγ–LBD folded in the activation conformation without any conformational changes in the receptor.

As to the mutant receptors such as (275*Ala*)-, (275*Gln*)-, (275*Asp*)- and (316*Lys*)-ERRγ, [^{3}H]BPA gave fairly large B_{max} values: 6.72–12.4 nmol/mg protein (Table III). All of these contain Arg or Lys at position 316. However, the B_{max} values of [^{3}H]4-OHT for these mutant receptors were drastically reduced, showing only 0.9–4.5% values as compared with that of [^{3}H]BPA. Although it is not clear whether or not LBD in these mutant receptors is folded in the same activation conformation, at least 4-OHT is able to reposition H12 into an inactivation conformation. It is therefore obvious that 4-OHT barely binds to ERRγ receptors.

Another important deduction from the present results is the crucial role of the Arg316 residue to bind BPA and 4-OHT. To the mutant receptor lacking Arg316 due to an Arg→Ala substitution, namely, (316*Ala*)-ERRγ, [^{3}H]BPA and [^{3}H]4-OHT bound only weakly. For the ERRγ mutant receptors containing Arg at position 275, namely (*Arg*, *Glu*)-ERRγ and (*Arg*, *Ala*)-ERRγ, [^{3}H]BPA and [^{3}H]4-OHT could bind, but with fairly small B_{max} values. By contrast, to the mutant ERRγ receptors that are entirely lacking Arg at positions either 275 or 316, [^{3}H]4-OHT and [^{3}H]BPA did not reveal any specific binding. All of these results clearly indicate that the Arg316 residue plays a crucial role in the binding of BPA and 4-OHT to ERRγ. Thus, this Arg316 residue would play a central role in capturing phenolic chemicals.

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Conflict of interest

None declared.

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Functional Role of the C-terminal Helix 12 Peptide in the Receptor Activation Mechanism of Estrogen-related Receptor γ (ERRγ)

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We have demonstrated that estrogen-related receptor γ (ERRγ) binds strongly bisphenol A (BPA) with high basal constitutive activity. X-ray crystal structural analysis of the BPA/ERRγ complex indicated that BPA sits in the pocked with 'active α-helix (H12) peptide' in an activation. 4-hydroxytamoxifen (4-OHT) is an inverse agonist ligand of ERRγ, it has no interaction with H12-peptide. In this study, we identified that H12-peptide is crucially important for the receptor activation and the selection of ligands.

Keywords: bisphenol A, estrogen-related receptor γ, helix 12-peptide, ligand binding, receptor activation

Introduction

Estrogen-related receptor γ (ERRγ) is an orphan receptor that belongs to the 48 human nuclear receptors. ERRγ is widely expressed in humans, especially strongly in the placenta and fetal brain. ERRγ reveals a high constitutive activity, although its endogenous ligand and physiological roles has not been clarified yet. Almost all of the nuclear receptors have a unique ligand binding domain (LBD), in which the ligand binding pocket is constructed with 12 α-helices (H1-H12) peptide and 2 β-strands. These helices play extremely important role to select a specific ligand for the receptor activation. Recently, we have demonstrated that bisphenol A (BPA), an estrogenic endocrine disruptor, binds very strongly to ERRγ with high constitutive activity [1]. We have also successfully achieved in crystallization and structural analysis of the BPA/ERRγ-LBD complex [2]. It was found that BPA sits in the pocked with 'active α-helix (helix 12) peptide' in an activation conformation in accord with no-ligand bind of ERRγ. However, when 4-hydroxytamoxifen (4-OHT) -- an inverse agonist ligand of ERRγ -- binds to receptor, 4-OHT has no interaction with helix 12-peptide [3]. It may be that BPA/4-OHT binds ERRγ with different mechanism by the H12-peptide.

In the present study, in order to identify the structural elements important for ligand binding and constitutive receptor activation, we prepared a series of mutant ERRγ receptor, in which ERRγ's helix 12-peptide was truncated every three amino acid residues (Fig. 1).

```
                          420         430         440         450        458
ERRγ-wild type:   ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEMLEAKV*
ERRγ-Δ(458):      ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEMLEAK*
ERRγ-Δ(457-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEMLEA*
ERRγ-Δ(456-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEMLE*
ERRγ-Δ(455-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEML*
ERRγ-Δ(454-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEM*
ERRγ-Δ(453-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLE*
ERRγ-Δ(450-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPMHKL*
ERRγ-Δ(447-458): ---TLPLLRQTSTKAVQHFYNIKLEGKVPM*
ERRγ-Δ(444-458): ---TLPLLRQTSTKAVQHFYNIKLEGK*
ERRγ-Δ(441-458): ---TLPLLRQTSTKAVQHFYNIKL*
ERRγ-Δ(438-458): ---TLPLLRQTSTKAVQHFYN*
ERRγ-Δ(435-458): ---TLPLLRQTSTKAVQH*
ERRγ-Δ(441-458): ---TLPLLRQTSTKA*
```

Fig.1. Helix 12-peptide mutant receptors of ERRγ design ("___": H11 peptide part; "___": H12 peptide part)

Results and Discussion

The mutant receptors were prepared by the two-step PCR method using wild type ERRγ as template, and cloned into the expression vector pGEX-6p-1 or pcDNA3.1. For the receptor binding assays, the wild-type and mutant LBD of ERRγ were expressed in *E. coli* as a protein fused with glutathione *S*-transferase (GST) protein. Firstly, we used [^{3}H]BPA as a tracer for the saturation binding assay. The Scatchard plot analysis was carried out for all the mutant receptor to estimate both the dissociation constant (K_d) and the receptor protein density (B_{max}). We found that the truncated one/two amino acid residues ERRγ from C-terminal mutant receptors (ERRγ-Δ(458) and ERRγ-Δ(457-458)) were strongly bind to [^{3}H]BPA the same as wile-type ERRγ. However, the truncated three amino acid residues ERRγ from C-terminal mutant receptor (ERRγ-Δ(456-458)) was remarkable reduced the binding ability to [^{3}H]BPA. All of the mutant receptors with helix 12-peptide truncated completely absent the binding ability to [^{3}H]BPA. Furthermore, using the [^{3}H]4-OHT as a tracer, all of the mutant receptors showed the specific binding with 50~100% binding affinity of wild-type ERRγ.

For examine the constitutive receptor activation of the mutant receptors, luciferase reporter gene assay method was used. All mutant receptors and estrogen-related receptor response element (ERRE) transiently expressed in the HeLa cells and luciferase activity was measured after 24 hr for the cells treated with 1% BSA/PBS. We found that the mutant receptors of the C-terminal three amino acid residues truncated every one amino acid residue were kept the constitutive receptor activation as wild-type. The other mutant receptors with helix 12-peptide truncated partly or completely lost the constitutive receptor activity in the reporter gene assay.

The binding assay and reporter gene assay data indicated that helix 12-peptide of ERRγ is crucially important for the constitutive activation and the selection of ligands.

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The Agonist/Antagonist Differential-docking Screening (AADS) Method for Exploration of the Estrogen Receptor-binding Chemicals

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We have established a novel method to screen endocrine-disrupting chemicals by in silico *docking calculations, which utilized the both agonist-bound- and antagonist-bond-ligand binding domains as templates. This agonist/antagonist differential-docking screening (AADS) method predicted, for example, 4-(1-adamantyl) phenol as an agonist of the human estrogen receptor α. The AADS method is an approach that appears to foresee both the binding potency and the agonist or antagonist activity of chemicals for the target nuclear receptors.*

Keywords: agonist/antagonist differential-docking screening (AADS), docking calculation, nuclear receptor

Introduction

Nuclear receptors (NRs), which play a central role as transcription factors in various biological processes, consist of five domains: N-terminal domain, DNA-binding domain, hinge domain, ligand-binding domain (LBD), and C-terminal domain. In general, the LBD has a pocket to capture a chemical, which has agonist or antagonist activity [1]. Because the pocket, which was named ligand binding pocket (LBP), exists on the LBD, the LBD is considered to be the major binding site for the pharmacological agents and the endocrine disrupting chemicals (EDCs).

Currently, numerous industrial chemicals are suspected to be EDCs. If we could predict that the chemicals would bind to the LBP, it would definitely be advantageous for the design of agonists or antagonists of particular NR. It would also be very helpful to predict which chemicals would cause serious disruptions in the endocrine system. In this study, to establish a novel method that screens a huge number of chemicals to explore EDCs, the docking calculation procedure was newly developed by using one of NRs, the human estrogen receptor α (ERα). The ERα-LBD changes its conformation, depending upon the ligand docks as either agonist or antagonist. Such differences of the conformation were observed in 3D structures of ERα-LBD/ligand complexes, which determined by X-ray crystallography [2,3]. Therefore, we used two agonist-bound (PDB: 1ERE and 3ERD) and two another antagonist-bound (1ERR and 3ERT) ERα-LBD's crystal structures as templates for the docking calculation, and compared binding energies elucidated from

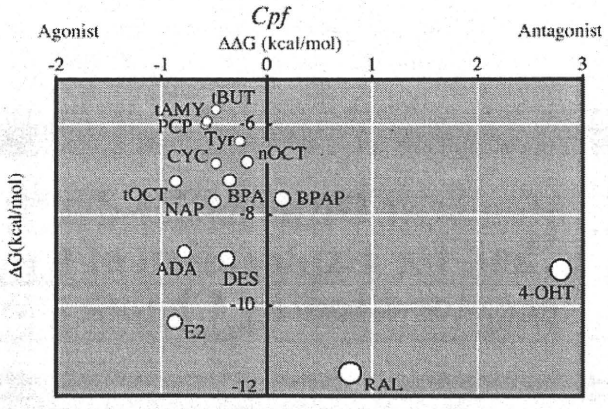

Fig. 1. Plotting analysis of the ADDS method.
ΔG versus ΔΔG plotting analysis. The size of each plot corresponds to the relative molecular size of the test chemical. Abbreviations of chemicals are denoted in parentheses: pentachlorophenol (PCP), 4-tert-butylphenol (tBUT), 4-tert-amylphenol (tAMY), 4-cyclohexylphenol (CYC), 4-n-octylphenol (nOCT), 4-tert-octylphenol (tOCT), bisphenol A (BPA), 4-(2-naphthyl)phenol (NAP), 4-(1-adamantyl)phenol (ADA), bisphenol AP (BPAP), 17β-estradiol (E2), dietylstilbestrol (DES), raloxifene (RAL), and 4-hydroxytamoxifen (4-OHT).

the docking calculations by using each template. This calculation method was named as the agonist/antagonist differential-docking screening (AADS) [4].

Results and Discussion

Docking calculations were carried out by the program Autodock 3.0 (The Scripps Research Institute) and tested 16 chemicals. The average binding energies (ΔG) of the chemicals were obtained from the results of the docking calculations using four LBDs. The agonist/antagonist factor (ΔΔG=Cpf: conformation preference factor) was calculated as the difference between ΔG from the docking calculations used by agonist-bound receptors and antagonist-bound receptors.

The calculated free energies of binding (ΔG) of the known ER-ligands, E2, DES, RAL and 4-OHT, ranged from –8.98 to –11.47 kcal/mol. Among test chemicals, there was only one putative antagonist (Fig. 1). Because of its small minus ΔΔG value, it is possible that BPAP has weak antagonist-like activity. On the other hand, we identified 4-(1-adamantyl) phenol (ADA) as a relatively strong agonist for hERα. ADA attained a binding energy (–8.84 kcal/mol) greater than that of the other test chemicals and it had a plus ΔΔG value. The AADS method successfully predicted that ADA is almost as active as DES (ΔG = –8.98 kcal/mol).

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