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Structural Characteristics of *Drosophila* Estrogen-related Receptor Ligand Binding Domain to Capture the Peptide and Non-peptide Ligands

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In order to establish the binding assay system for Drosophila *estrogen-related receptor (dERR), we examined a few triturated ligands for expressed dERR-ligand binding domain (LBD). None of those compounds that were reported as binders for mammalian ERR revealed binding activity. When constructed a 3D model of the dERR-LBD, its ligand-binding pocket exhibited a very limited space, suggesting a rather tiny size of ligand for receptor activation.*

Keywords: computational analysis, estrogen receptor, estrogen-related receptor, ligand binding domain.

Introduction

A series of estrogen-related receptors (hERRα, β, γ) have recently been found in human as components to modulate the ordinary estrogen receptors (hERα, β), and they appeared to be a novel target of endocrine disruptors. No ER-like receptors have been found in the genome of the fruit fly *Drosophila melanogaster*, but instead an ERR-like nuclear receptor was revealed. When the amino acid sequences were compared, this termed as the *Drosophila* estrogen-related receptor (dERR) was found to resemble most closely the hERRs at both the DNA binding domain (DBD) and the ligand-binding domain (LBD) [1]. Thus, this receptor likely belongs to the steroid hormone subfamily NR3 of nuclear receptor.

In the present study, we carried out the cDNA cloning of full-length dERR, expression of dERR-LBD, and the receptor-binding assay. In addition, we carried out a computational analysis of dERR-LBD to understand the result of the receptor binding assay.

Results and Discussion

We first amplified dERR cDNA clone by PCR using dERR-gene-specific primers (ERR-*Eco*RI:5'CCGGAATTCATGTCCGACGGCGTCAGCAT3', ERR-*Sal*I: 5'CGAGTCGACTCACCTGGCCTGGCCAGCGGCTCGA3') for five different

cDNA libraries derived from *Drosophila* embryo, larvae-pupae, adult head, ovary, and adult testis. Eventually, from the testis cDNA library, we obtained a full-length of dERR cDNA clone, which is comprised of 1,455 bp oligonucleotides with a mature protein of 482 amino acid residues.

The amino acid sequence of clarified dERR was aligned together with hERRs and hERs for analysis of domains. A cDNA fragment (residues 734-1455, n=241) corresponding to the LBD was subcloned into pGEX-6p-1 vector for its expression in *E. coli* BL-21 cells as a GST-fusion protein. For screening of the ligands of dERR, we used purified GST-dERR-LBD fusion protein and newly developed the radio-ligand binding assay system. In this binding assay, we utilized polyethylene glycol for receptor protein precipitation followed by rapid filtration. While natural ligand is not known yet, the ER agonist diethylstilbestrol (DES) and the selective ER modulator 4-hydroxytamoxifen (4-OHT) have been identified as hERRγ antagonists [2-4]. Thus, we used triturated analogues of these compounds as tracers. However, no obvious specific binding was observed, suggesting that the structure of dERR binding site is different from that of hERRγ.

Homology modeling using four different ERR-LBD structural data as templates was carried out to construct a 3D model structure of dERR. The binding site analysis revealed that dERR-ligand binding pocket (LBP) has a binding pocket rather smaller than mouse ERRγ-LBD-LBP (data not shown). When compared the structure of mERRγ-LBD containing DES or 4-OHT, it was found that dERR-LBD places the residual indole group of Trp-459 in the LBP. Apparently, this makes DES and 4-OHT to be unable to bind to this small pocket of dERR (Fig. 1).

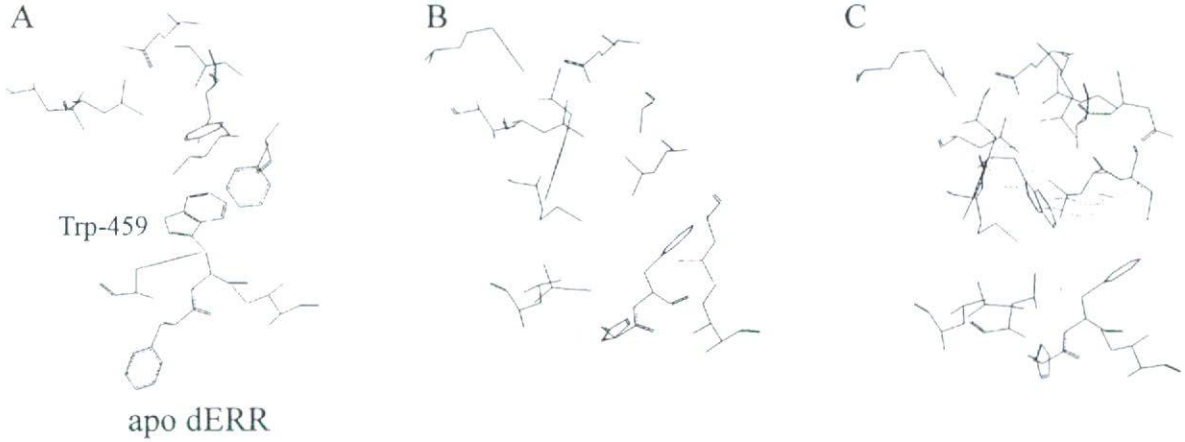

Figure 1. Comparison of ligand binding pockets of dERR (A: by homology modeling), mERRγ with DES (B: 1S9P), and mERRγ with 4-OHT (C: 1S9Q). Ligand molecules of DES (B) and 4-OHT (C) were depicted in gray scale.

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Highly Sensitive Monoclonal Antibodies to Distinguish Conformational Changes Induced by Endocrine Disrupting Chemicals in the Estrogen Receptor

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Estrogen receptor (ER) changes its conformation upon ligand binding. Mouse monoclonal antibodies (mAbs) were raised against an ER C-terminal 17-mer peptide involved in such a conformation change, and a set of mAbs were demonstrated to detect efficiently the receptor conformation change. The results indicate that these mAbs can be used as a sensitive molecular tool to evaluate the potential risk of environmental xenoestro-genic chemicals.

Keywords: conformation change, conformation-sensing, endocrine disruptors, estrogen receptor, monoclonal antibody.

Introduction

Rapid and effective evaluation of the biological effects of suspected environmental chemicals has become increasingly important to shed light on the critical issue of endocrine disruption in humans and animal wildlife. Xenoestrogenic effects caused by environmental chemicals cause most threat to mammalian hormonal activities, and the estrogen receptor (ER) is the major target of endocrine disrupting chemicals (EDCs). The ER and other nuclear receptors are known to change their conformation after binding ligands, as evidenced by X-ray crystallographic analyses. The resulting relocation of the C-terminal α-helix numbered 12 (H12) within the ligand-binding domain of the receptors is the most prominent and common feature of ligand-induced conformation change, which leads to an ultimate hormonal action. We have previously demonstrated that a rabbit antiserum raised against human ERα-H12 peptide was able to detect this conformation change in ER [1]. Here we present the preparation of a number of mouse monoclonal antibodies (mAbs) that detect such changes even more efficiently.

Results and Discussion

A 17-mer peptide corresponding to the ERα H12 moiety was synthesized and immunized into the footpads of Balb/c mice. Lymph cells were isolated ten days

after immunization and fused with mouse myeloma cells to obtain hybridomas. Among hundreds of antibody-secreting hybridomas tested, a number exhibited affinities towards both the peptide and ERα in the early rounds of screening by competitive ELISA. From among these, antibodies were selected that could detect the conformation change induced by 17β-estradiol (E_2), an agonist of ER. This selection assay (*conformation sensing assay*) was carried out using the competitive ELISA method to detect and differentiate the different affinities toward ligand-bound and -free ERs. Several mAbs showed conformation-sensing capabilities similar to those of the previous polyclonal antibodies. Eventually, a few were found to possess higher efficiency in sensing, because they required smaller amounts of the ER as the competitor and of H12 peptide as the immobilized antigen.

The conformation-sensing mAbs were further applied to assay several tens of known chemicals. The effective concentrations of chemicals to elicit a half maximal immunological response from the mAb, the EC_{50} value, correlated well with, but were lower than those of the polyclonal antibody (Fig. 1). These results clearly indicate that the mAbs were > 10 times more sensitive. The fact that their EC_{50} values are almost compatible with those obtained in the competitive receptor binding assays suggests that the conformation change detected by our mAbs reflects appropriately the ligand-binding.

For mAbs that displayed conformation-sensing capabilities, the epitopes were analyzed using sequence analogs of antigen peptides that were additionally synthesized. In a conformation sensing assay using different ligands, one set of mAbs was sensitive to the conformation change induced only by E_2, while another mAb displayed a differential sensitivity toward an antagonist-induced change as well. The difference in the ligand sensitivity among these sets of mAbs is relevant to the difference in the location of their respective epitopes within the H12 moiety, indicating that the mAbs are sufficiently specific to distinguish the different conformation changes induced by agonists and antagonists. In conclusion, the use of these mAbs should allow a highly effective and accurate evaluation of the latent risk of EDCs.

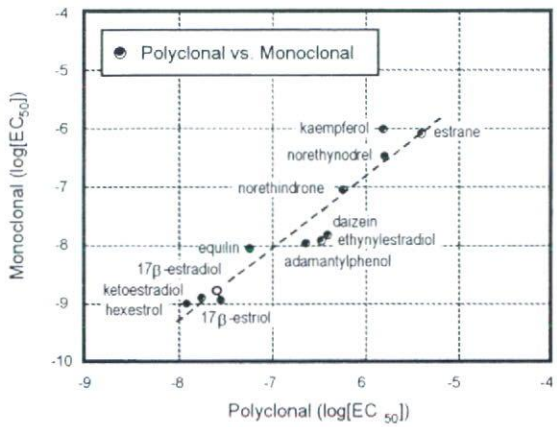

Figure 1. Correlation of the conformation sensing assays using polyclonal and monoclonal antibodies against ERα. Chemicals were assayed and their effective concentrations to elicit half-maximal immunological responses were plotted against each other. Correlation coefficient was 0.96, and regression coefficient was 0.63.

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Ligand-inducing Conformation Changes in the Estrogen Receptor C-Terminal Tail Moiety and Their Sensing by Polyclonal Antibodies

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Ligand binding to the estrogen receptor induces a conformation change. By using a polyclonal antibody to sense such a change, we have established the assay method to assess simultaneously the binding ability and hormonal activity of endocrine disruptors. In order to improve the immunoreactivity, we prepared four varieties of antibodies in this study. It was revealed that the ability of antibody to sense the conformation change is related to the structural characteristics of each antigen peptide.

Keywords: antigen peptide, conformation change, endocrine disruptors, estrogen receptor, polyclonal antibody.

Introduction

The estrogen receptor (ER) is a member of the nuclear receptor family which functions as a transactivation factor. The conformations of the ligand-bound (holo-ER) and ligand-free (apo-ER) forms of the ER are intrinsically different from each other [1]. This is due mainly to the change in positioning of the amphiphilic α-helix numbered as 12 (H12) present in the receptor C-terminal portion. This ligand-induced conformation change of the receptor is essential to bind the coactivator protein [2]. Antibodies provide a feasible tool to differentiate between these conformations, provided they could recognize specifically and selectively either the apo or holo conformation. In fact, an anti-ER H12 antibody was already found to discriminate apo-ER and holo-ER. In addition, this antibody was found to quantify the amount of ligand-bound and ligand-free ERs [3]. When compared with H12 *per se*, ER possesses a C-terminal tail including H12 which is about four times longer. It is highly likely that this tail moiety would also be involved in some conformation change. Thus, the purpose of the present study is to test the structural availability of this tail region as an antigen.

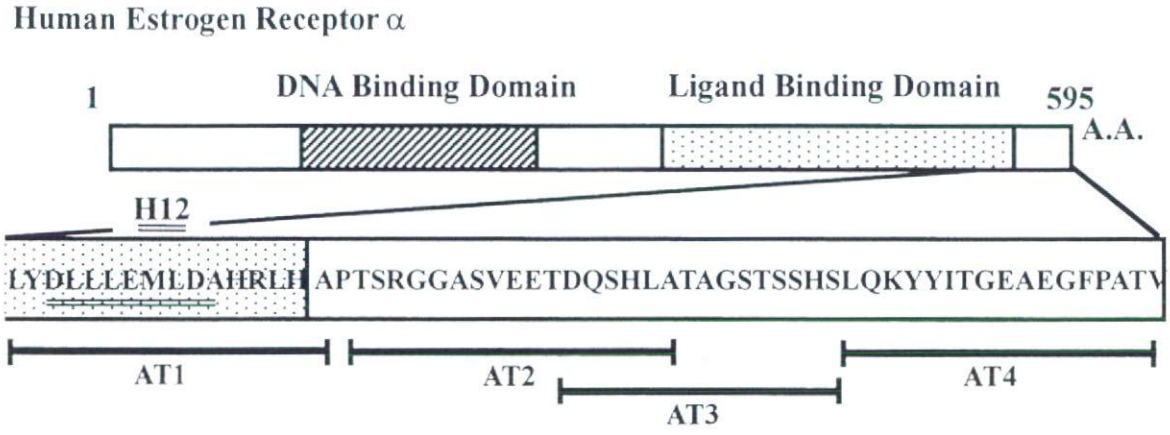

Figure 1. Design of antigen peptides for preparation of conformation-sensing antibody.

Results and Discussion

The C-terminal tail of ER was segmented into four peptide fragments (Fig. 1). The fragment AT1 contains H12. These peptides were synthesized by the Fmoc-based solid phase method. To conjugate to a carrier protein Keyhole Limpet Hemocyanin (KLH), Cys was incorporated into these peptides at the N-terminus. Peptides were liberated from the resin by treatment with Reagent K and purified by gel filtration (Sephadex G-25, 1.8 x 72 cm) followed by preparative reversed-phase high performance liquid chromatography (RP-HPLC) (Lichrospher RP-18 (e), 25 x 250 mm, 5 μm). The mass spectra of peptides were measured to verify their purity on a mass spectrometry Voyager™ DE-PRO with the method of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF).

The peptides conjugated to KLH were injected into a rabbit, respectively. About three months later from the first immunization, blood was collected. The serum was purified successively by KLH immunoprecipitation, affinity chromatography with antigen-linked agarose gel, and then with a protein A-linked agarose gel. The specificity of antibody was analyzed by the enzyme-linked immunosorbent assay (ELISA) method. Competitive ELISA was employed to evaluate the ability of antibody to bind to apo-ER and/or holo-ER. The production of antibody was checked by preparative ELISA using ER and the antigen peptide. In this titer checking it was found that the serum contains enough amount of antibodies sensitive to both the receptor and peptide. As shown in Fig. 2, the serum obtained from the immunization by AT4 peptide interacted with the antigen peptide and the receptor ER almost equally well. Similar results were obtained for AT2 and AT3 peptides.

When the relative immunoactivity of the antibody was estimated under the certain concentrations of ER (10^{-12} - 10^{-6} M), a dose-dependent reduction was observed against the antigen peptide (10^{-12} - 10^{-11} mol) coated on the plate (Fig. 3). This assay was carried out under the presence of 10^{-5} M 17β-estradiol (E2), and the results were depicted in the same figure. As shown in Fig. 3A, a dose-dependent curve became much more gentle, making a certain deviation between the curves. This deviation (about 30%) corresponds to the sensing based on the conformation change. Thus, it is concluded that anti-AT1 exhibits a high ability to distinguish between holo-ER and apo-ER (Fig. 3). A similar result was obtained for anti-AT3 antibody, although the extent of deviation is much smaller than for anti-AT1 antibody. For anti-AT4

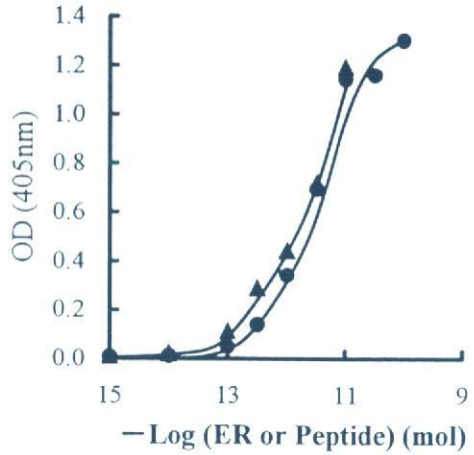

Figure 2. Indirect ELISA using anti-AT4 antibody for titer checking against recognition to antigen peptide (●) and ER (▲). The antibody used is the preparation purified by affinity chromatography with a protein A-linked agarose gel.

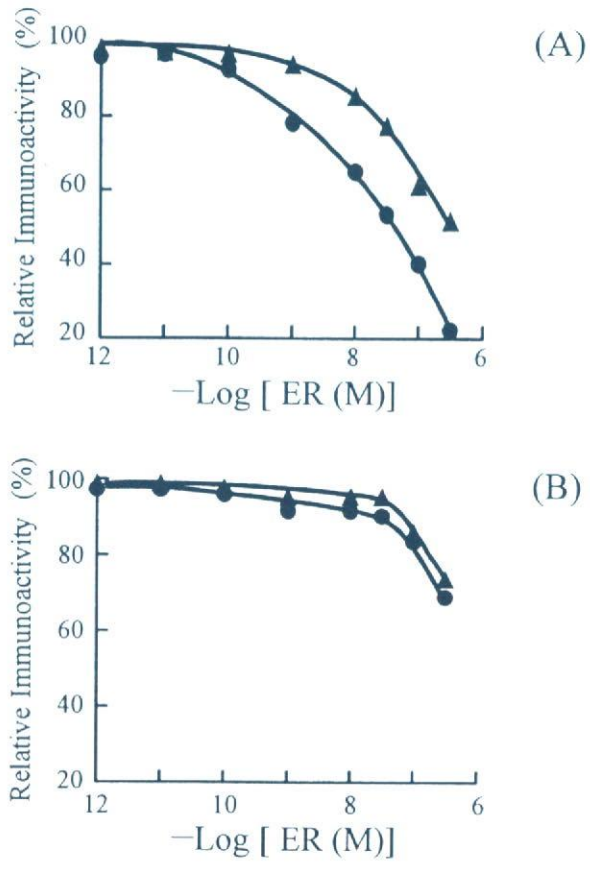

Figure 3. Immunoresponse of anti-AT1 (A) and anti-AT4 (B) antibodies against apo-ER (●) and holo-ER (▲). The antibodies used are the preparation purified by affinity chromatography with a protein A-linked agarose gel. Holo-ER was prepared by treatment with 10 μM 17β-estradiol.

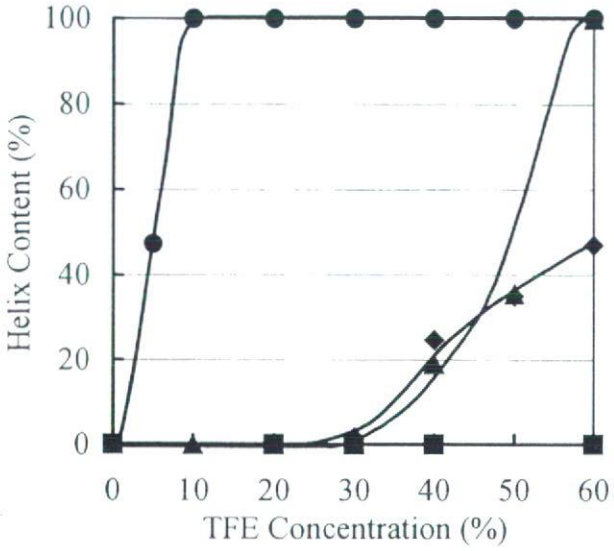

Figure 4. The helical characteristics of each peptide; AT1(●), AT2(■), AT3(▲),and AT4(◆)

antibody, almost no deviation was observed (Fig. 3B). A similar result was shown by anti-AT2 antibody (data not shown).

The CD spectra were masured on a J-725 Spectropolarimeter (Jasco), and the % contents of the secondary structures were calculated by SSE-338W protein secondary structure analysis program (Jasco). It was found that AT1 peptide is easy to adopt an α-helix structure. AT1 exhibited an extremely high content of α-helix (100% even in 10% TFE). AT3 peptide was found to adopt also a 100% α-helix structure, but only in 60% TFE. Almost no helical content was observed for AT2 peptide and a very low for AT4 peptide. These results indicated that the ability of antibody to sense the conformation change is well-related to the structural characteristics of each peptide fragment to adopt an α-helical conformation.

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Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity

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Abstract

Bisphenol A (BPA) has been acknowledged as an estrogenic chemical able to interact with human estrogen receptors (ER). Many lines of evidence reveal that BPA has an impact as an endocrine disruptor even at low doses. However, its binding to ER and hormonal activity is extremely weak, making the intrinsic significance of low dose effects obscure. We thus supposed that BPA might interact with nuclear receptor(s) other than ER. Here we show that BPA strongly binds to human estrogen-related receptor γ (ERRγ), an orphan receptor and one of 48 human nuclear receptors. In a binding assay using [^{3}H]4-hydroxytamoxifen (4-OHT) as a tracer, BPA exhibited a definite dose-dependent receptor binding curve with the IC_{50} value of 13.1 nM. 4-Nonylphenol and diethylstilbestrol were considerably weaker (5–50-fold less than BPA). When examined in the reporter gene assay for ERRγ using HeLa cells, BPA completely preserved ERRγ's high constitutive activity. Notably, BPA exhibited a distinct antagonist action to reverse the inverse agonist activity of 4-OHT, retaining high basal activity. ERRγ is expressed in a tissue-restricted manner, for example very strongly in the mammalian brain during development, and in the adult in the brain, lung and other tissues. It will now be important to evaluate whether BPA's hitherto reported low dose effects may be mediated through ERRγ.

Keywords: Bisphenol A; Endocrine disruptor; Estrogen-related receptor γ; Low dose effects; Nuclear receptors; Receptor binding

1. Introduction

Bisphenol A (BPA[1]), 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, with a worldwide production of approximately 3.2 million t per year, is used mainly in the production of polycarbonate plastics and epoxy resins. It has been long 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 recently to act as an antagonist for a human androgen receptor (AR) (Sohoni

Abbreviations: AR, androgen receptor; BPA, bisphenol A; DBD, DNA-binding domain; DCC, dextran-coated charcoal; DES, diethylstilbestrol; E1, estrone; E2, 17β-estradiol; E3, estriol; ER, estrogen receptor; ERE, estrogen response element; ERR, estrogen-related receptor; ERRE, ERR-response element; ERRγ, estrogen-related receptor γ; GR, glucocorticoid receptor; LBD, ligand-binding domain; NR, nuclear receptor; 4-OHT, 4-hydroxytamoxifen; PR, progesterone receptor

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and Sumpter, 1998; Xu et al., 2005). Both ER and AR belong to the group III steroid hormone receptors, a subfamily of 48 human nuclear receptors (NRs) (Nuclear Receptors Nomenclature Committee, 1999; Robinson-Rechavi et al., 2001).

Various so-called "low dose effects" of BPA have recently been reported *in vivo* for reproductive organ tissues and systems in mice and rats. For instance, very low dose levels of BPA were shown to have an increase in size and weight of the fetal mouse prostate (Nagel et al., 1997; Gupta, 2000), and a decrease in daily sperm production and fertility in male mice (Gupta, 2000; vom Saal et al., 1998). All of these low dose effects of BPA have been explained as the output effects of steroid hormone receptors (Welshons et al., 2003). It should be noted, however, that BPA's binding to ER and AR and hormonal activity is extremely weak, 1000–10,000 times lower than for natural hormones, making the intrinsic significance of low dose effects intangible and obscure (National Toxicology Program, 2001; Safe et al., 2002; Gray et al., 2004; vom Saal and Hughes, 2005). This discrepancy on low dose effects prompted us to enquire whether BPA may interact with NRs other than ER and AR.

The estrogen-related receptors (ERRs) are a subfamily of orphan NRs closely related to ERs, ERα and ERβ (Giguère, 2002; Horard and Vanacker, 2003). There are three ERR family members, ERRα, ERRβ and ERRγ, with ERRγ the most recently identified third member (Eudy et al., 1998; Hong et al., 1999). ERRs and ERs show a considerable level of amino acid sequence similarity and identity in both their DNA-binding (DBD) and ligand-binding (LBD) domains. Although 17β-estradiol (E2), a natural ligand of ERs, does not bind to any of the ERR family, ERRs can bind to functional estrogen response elements (EREs) in ER target genes, suggesting a possible overlap between ERR and ER action (Huppunen and Aarnisalo, 2004). ERRs also bind to ERR-response element (ERRE), but as monomers.

ERRs are all orphan receptors, while efforts to discover synthetic compounds that might modify the activities of the ERRs have identified only a few chemicals that suppress ERRs' spontaneous transcriptional activities. For instance, diethylstilbestrol (DES) was found to repress the molecular activities of ERRs (Tremblay et al., 2001; Coward et al., 2001), although DES is considerably weaker in inhibiting ERR activities compared with its action as an ER-activator. 4-Hydoroxytamoxifen (4-OHT) has also been identified as an inverse agonist of ERRγ, deactivating the receptor by decreasing the very high level of spontaneous constitutive activity (Coward et al., 2001). From evidence in a receptor binding assay in which [^{3}H]4-OHT was used as a tritium-labeled receptor tracer (Coward et al., 2001), 4-OHT binds strongly to ERRγ. Collectively, these results reveal that E2, DES and 4-OHT all bind to ERs very strongly, but that their binding abilities to ERRγ vary.

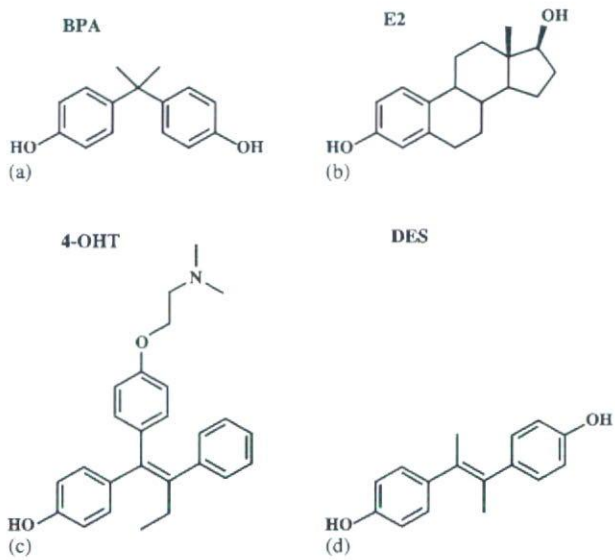

Fig. 1. Chemical structures of (a) bisphenol A (BPA), (b) 17β-estradiol (E2), (c) 4-hydroxytamoxifen (4-OHT), and (d) diethylstilbestrol (DES).

When we compared the chemical structures of these ligands, it became clear that they share only the phenol group usually acknowledged as a key structural element for receptor recognition (Fig. 1). Since BPA in a compact minimum-energy conformation also shares this phenol group, we assumed that BPA is a potent binder to ERRγ. In the present study, we first established the competitive receptor-binding assay using [^{3}H]4-OHT as a tracer. The reported binding assay (Coward et al., 2001) utilized glutathione-coated beads to cargo GST-ERRγ-LBD, namely ERRγ-LBD fused to glutathione *S*-transferase (GST), for B/F separation of the tracer [^{3}H]4-OHT. Instead of expensive glutathione-coated beads, we used 1% dextran-coated charcoal (DCC) to absorb and remove receptor-free [^{3}H]4-OHT. This worked successfully, and we eventually could demonstrate that BPA binds strongly to ERRγ. This initial result has led us further to detailed examination of BPA in the reporter gene assay for ERRγ, and here we report that BPA retains extremely high ERRγ's constitutive activity.

2. Materials and methods

2.1. Test compounds

Bisphenol A (BPA), CAS no. 80-05-7, purity 99%, Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. 4-Hydroxytamoxifen

(4-OHT), CAS no. 68047-06-3, purity 98%, Sigma–Aldrich Inc., St. Louis, MO. Diethylstilbestrol (DES), CAS no. 56-53-1, purity 97%, Wako Pure Chemical Industries Ltd., Osaka, Japan. Nonylphenol, CAS no. 84852-15-3, Technical grade, Sigma–Aldrich. Estrone (E1), CAS no. 53-16-7, 98%, Wako. 17β-estradiol (E2), CAS no. 50-28-2, 98.9%, Research Biochemicals International, Natick, MA. Estriol (E3), CAS no. 50-27-1, 98%, Wako. Dihydrotestosterone (DHT), CAS no. 58-22-0, 97%, Wako. Testrosterone, CAS no. 521-18-6, 95%, Wako. Cortisone, CAS no. 53-06-5, ≥98%, Sigma–Aldrich. Progesterone, CAS no. 57-83-0, ≥99%, Sigma–Aldrich.

2.2. Preparation of receptor protein GST-fused ERRγ-LBD

ERRγ-LBD was amplified from a human kidney cDNA library (Clontech Labolatories, Mountain View, CA, USA) by PCR using gene-specific primers and cloned into pGEX6P-1 (Amersham Biosciences, Piscataway, NJ). GST-fused receptor protein expressed in *E. coli* BL21α was purified on an affinity column of Glutathione-Sepharose 4B to obtain GST-ERRγ-LBD.

2.3. Radio-ligand binding assays for saturation binding

Saturation binding assay was conducted essentially as reported (Nakai et al., 1999) at 4 °C using [^{3}H]4-OHT (3.15 TBq/mmol as methiodide, America Radiolabeled Chemicals Inc., St. Louis, MO) and GST-ERRγ-LBD (1 μg) with or without 4-OHT (10 μM final concentration). Free radio-ligand was removed by centrifugation or filtration after incubation with 1% dextran-coated charcoal (Sigma). Specific binding of [^{3}H]4-OHT was calculated by subtracting the non-specific binding from the total binding.

2.4. Radio-ligand binding assays for competitive binding

BPA and other chemicals were dissolved in 0.3% DMSO in 0.2% γ-globulins (a blocker of non-specific adsorption to the reaction vessels). These chemicals were examined for their ability to inhibit the binding of [^{3}H]4-OHT (5 nM in final) to GST-ERRγ-LBD (26 ng). Reaction mixtures were incubated overnight at 4 °C and free radio-ligand was removed with 1% dextran-coated charcoal by either centrifugation or filtration. The IC_{50} values were calculated from the dose–response curves assessed by the non-linear analysis program ALLFIT (De Lean et al., 1978). Each assay was performed in duplicate and repeated at least five times. For other nuclear receptors, the binding assays were carried out essentially as outlined above by using a combination of specific radio-ligands and receptor preparations: [^{3}H]E2 (5.74 TBq/mmol, Amersham Biosciences, Buckinghamshire, UK) for ERα (prepared by us as GST-fused ERα-LBD protein) and ERβ (Invitrogen, Carlsbad, CA), [^{3}H]DHT (4.48 TBq/mmol, Amersham Biosciences) for AR (Invitrogen), [^{3}H]cortisol (2.90 TBq/mmol, Perkin-Elmer Life and Analytical Sciences, Wellesley, MA) for GR (Invitrogen), and [^{3}H]progesterone (3.48 TBq/mmol, Amersham Biosciences) for PR (Invitrogen).

2.5. Luciferase reporter gene assay

Full-length ERRγ cDNA was amplified by nested PCR using gene-specific primers and cloned into pcDNA3 (Invitrogen) at the *Eco*RI and *Xho*I sites. To generate the ERR response element (ERRE)-luciferase construct, oligonucleotides (5′-CCGGACC*TCAAGGTCA*CGTTCGGACC*TCAAGGTCA*CGTTCGGACC*TCAAGGTCA*GGATCCA-3′), and (5′-GATCTGGATCC*TGACCTTGA*GGTCCGAACG*TGACCTTGA*GAACG*TGACCTTGA*GGTCCGGGTAC-3′) were annealed and ligated into (*Bgl*II, *Kpn*I)-digested pGL3-Luc (Promega, Madison, WI) to generate three copies of ERRE. Both the ERR expression (pcDNA3/ERR, 1 μg) and luciferase reporter (pGL3/3 × ERRE, 3 μg) plasmid were transiently transfected in HeLa cells with 10 μl/ml of Plus Reagent (Invitrogen) and 15 μl/ml of Lipofectamine (Invitrogen), which was exposed to various concentrations of test chemicals to detect agonist/antagonist activity. Luciferase assay reagent (Promega) with the cell culture lysis reagent and the substrate solution was used to measure the luciferase enzyme activity on a microplate reader (Wallac 1420 ARVOsx, Perkin-Elmer, Turku, Finland). Each assay was performed in triplicate and repeated at least five times.

3. Results

3.1. Saturation binding [^{3}H]4-OHT to ERRγ

For the present study, we first attempted to establish a routine radio-labeled receptor binding assay. As mentioned earlier, instead of expensive glutathione-coated beads carrying GST-ERRγ-LBD (Coward et al., 2001), we utilized 1% dextran-coated charcoal (DCC) to absorb and remove receptor-free [^{3}H]4-OHT. We used this B/F separation method successfully for [^{3}H]E2 and the estrogen receptor (Nakai et al., 1999). In the present study, B/F separation of the tracer [^{3}H]4-OHT also worked successfully and effectively with 1% DCC. As shown in Fig. 2a, specific binding of [^{3}H]4-OHT to ERRγ was estimated to be approximately 75%, which is about twice larger than reported value (Coward et al., 2001). It should be noted that non-specific binding of [^{3}H]4-OHT is significantly low as compared with the procedure to use glutathione-coated beads.

A Scatchard plot analysis (Fig. 2b) showed a single binding mode with a binding affinity constant (K_d) of 10.0 nM and a receptor density (B_{max}) of 6.32 nmol/mg. These data are almost compatible with those reported for the glutathione-coated bead assay.

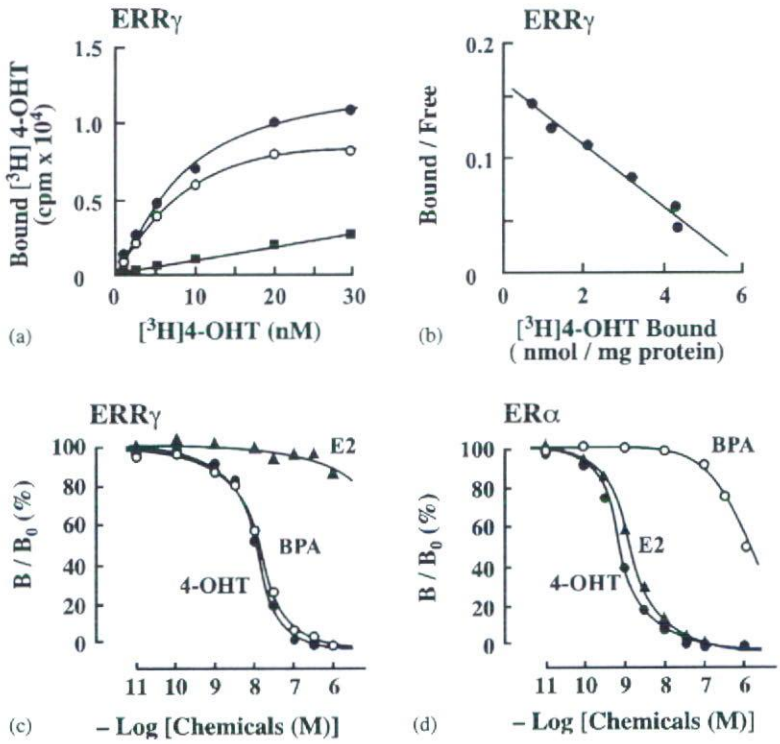

Fig. 2. Radio-ligand receptor binding assays of 4-hydroxytamoxifen (4-OHT), bisphenol A (BPA) and 17β-estradiol (E2) for ERRγ and ERα. (a) Saturation binding curve of [^{3}H]4-OHT for the recombinant human ERRγ. The graph shows total (●), specific (○) and non-specific (■) bindings. Determination of non-specific binding was carried out by excess unlabeled 4-OHT (10 μM). (b) Scatchard plot analysis showing a single binding mode with a binding affinity constant (K_d) of 10.0 nM and receptor density (B_{max}) of 6.32 nmol/mg. (c) Concentration-dependent curves of BPA (○), 4-OHT (●) and E2 (▲) in the receptor competitive binding assay to measure the ability of the compounds to displace [^{3}H]4-OHT in ERRγ, and (d) [^{3}H]E2 in the recombinant human estrogen receptor ERα. 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. IC_{50} values showed a between-experiment coefficient of variation of 5–12%.

3.2. Binding ability of BPA and other chemicals to ERRγ

In the competitive receptor binding assay for ERRγ (10 μg/ml), 4-OHT displaced [^{3}H]4-OHT (2.5 nM in final) in a clear dose-dependent manner (Fig. 2c). The ability of 4-OHT was calculated with an IC_{50} value of 10.3 nM, while DES exhibited IC_{50} = 54.3 nM (Table 1). Surprisingly, in the assay to screen a series of candidate compounds of endocrine disruptors, BPA was found to displace [^{3}H]4-OHT very strongly with an IC_{50} value of 13.1 nM. As shown in Fig. 2c, BPA exhibited a distinct dose-dependent sigmoidal binding curve. BPA and 4-OHT bind to ERRγ equally strongly.

This unexpected result was confirmed repeatedly and substantiated by two independent assay systems. In the first, each competition reaction of a dilution series was carried out in a micro-tube, and the following B/F separation with 1% DCC was performed by centrifugation for all micro-tubes. Alternatively, in a second assay system, dilutions were delivered into the wells of a 96-well polypropylene plate. In this method DCC mixtures were transferred to the 96-well plate with a PVDF membrane filter (0.45 μm pore size) for direct vacuum filtration using a specific system (Millipore). In both

Table 1
The receptor binding affinity of natural hormones and chemicals for the estrogen-related receptor γ, ERRγ

Natural hormones and chemicals	Binding affinity (IC_{50}, nM)
Bisphenol A (BPA)	13.1 ± 2.34
4-Hydroxytamoxifen (4-OHT)	10.3 ± 0.80
Diethylstilbestrol (DES)	54.3 ± 3.14
4-Nonylphenol	194 ± 30.0
Estrone (E1)	549 ± 0.81
17β-Estradiol (E2)	NB[a]
Estriol (E3)	NB
Dihydrotestosterone (DHT)	NB
Testrosterone	NB
Cortisone	NB
Progesterone	NB

[a] NB means "not bound", indicating no significant receptor binding at its 10 μM concentration.

assay systems it was eventually demonstrated that BPA is highly potent binder to ERRγ. Two more individuals further validated these results in a carefully followed protocol.

Another representative endocrine disrupter candidate 4-nonylphenol was also examined for its specific binding to ERRγ. It was moderately active, with IC_{50} = 194 nM (Table 1), about 15 times less potent than BPA. The natural estrogens, E2 and estriol (E3) were by contrast inactive, showing no binding affinity up to a concentration of 10 μM (Fig. 2c). Only estrone (E1) was weakly active (549 nM). All other natural ligands of steroid hormone receptors were inactive (Table 1). These included dihydrotestosterone and testosterone for AR, cortisone for the glucocorticoid receptor (GR), and progesterone for the progesterone receptor (PR). Almost no binding was detected up to a concentration of 10 μM.

3.3. Receptor binding specificity and selectivity of BPA and other chemicals

We next examined BPA for its ability to bind to other group III nuclear receptors, particularly the steroid hormone receptors ERα, ERβ, AR, GR, and PR. Table 2 summarizes the binding affinities of E2, 4-OHT and BPA to illustrate their receptor specificity and selectivity. When compared with BPA and 4-OHT, which are almost equally potent binders to ERRγ, these additional candidates were almost completely inactive for AR, GR, and PR. For ERα and ERβ, 4-OHT is highly potent as reported previously by others, with an IC_{50} value of approximately 1 nM (Kuiper et al., 1998; Fang et al., 2001). In contrast, BPA is very weak for ERα (IC_{50} = 1040 nM) and ERβ (1320 nM). Collectively, BPA is as potent as 4-OHT for ERRγ, but much less potent than 4-OHT for both estrogen receptors ERα and ERβ (Table 2, Fig. 3).

The reason for this discrepancy in receptor selectivity between BPA and 4-OHT probably results from differences in their three-dimensional structures. The lowest-energy conformations of the two compounds differ considerably from each other. Thus, although BPA and 4-OHT are almost equally potent as ERRγ ligands, these data strongly suggest that they occupy different ligand binding subsites of the ERRγ.

3.4. Different effects of BPA and 4-OHT on ERRγ constitutive activity in the reporter gene assay

We next examined the reporter gene activity of BPA in HeLa cells transiently co-transfected with an ERRγ expression plasmid and an ERRE-luciferase reporter plasmid. ERRγ exhibited significantly elevated constitutive activity to yield a reporter enzyme luciferase. Detailed assay results are shown in Fig. 3. Comparing ERRγ with ERα, the constitutive activity levels were considerably different, the basal activity of ERRγ being about 25 times larger than that of ERα (Fig. 3a and b). 4-OHT is definitely an antagonist of ERα as shown Fig. 3a; 10 μM 4-OHT completely suppressed the full agonist activity of 10 nM E2. By contrast, 4-OHT deactivated ERRγ, as reported (Coward et al., 2001), diminishing the basal activity of ERRγ up to 70% at a concentration of 10 μM (Fig. 3b). This suppression effect of 4-OHT on the constitutive activity of ERRγ has been acknowledged as an inverse agonist activity.

BPA, on the other hand, showed no effect on the basal constitutive activity of ERRγ even at its concentration of 10 μM (Fig. 3b), completely preserving ERRγ's high constitutive activity. This BPA action was clearly confirmed in the assay in which 4-OHT exhibited a distinct dose-dependent activity as an inverse agonist (Fig. 3c). It seemed that BPA has no effect on ERRγ, exhibiting neither enhanced agonist activity nor inverse agonist

Table 2
The receptor binding affinities of 17β-estradiol, 4-hydroxytamoxifen, and bisphenol A to various steroid hormone receptors

Steroid hormone receptors (^{3}H-labeled ligand used)	Receptor binding affinities shown by IC_{50} (nM)		
	E2	4-OHT	BPA
ERRγ ([^{3}H]4-OHT)	NB[a]	10.3 ± 0.80	13.1 ± 2.34
ERα ([^{3}H]E2)	0.98 ± 0.15	0.55 ± 0.11	1040 ± 180
ERβ ([^{3}H]E2)	1.27 ± 0.31	0.88 ± 0.14	1320 ± 287
AR ([^{3}H]DHT)	248 ± 52	NB	NB
GR[b] ([^{3}H]cortisol)	465 ± 125	1130 ± 24	ND[c]
PR[b] ([^{3}H]progesterone)	527 ± 128	ND	NB

[a] NB means "not bound", indicating no significant binding up to a concentration of 10 μM.

[b] Standard compounds for these receptors were also evaluated, and those include: dihydroteststerone for AR, 1.76 ± 0.03 nM; dexamthazone for GR, 0.80 ± 0.11 nM; and progesterone for PR, 2.26 ± 0.33 nM. For abbreviations, see the text.

[c] ND means "not determined", indicating that the inhibitory constant IC_{50} cannot be calculated up to a concentration of 10 μM owing to its extremely weak binding activity.

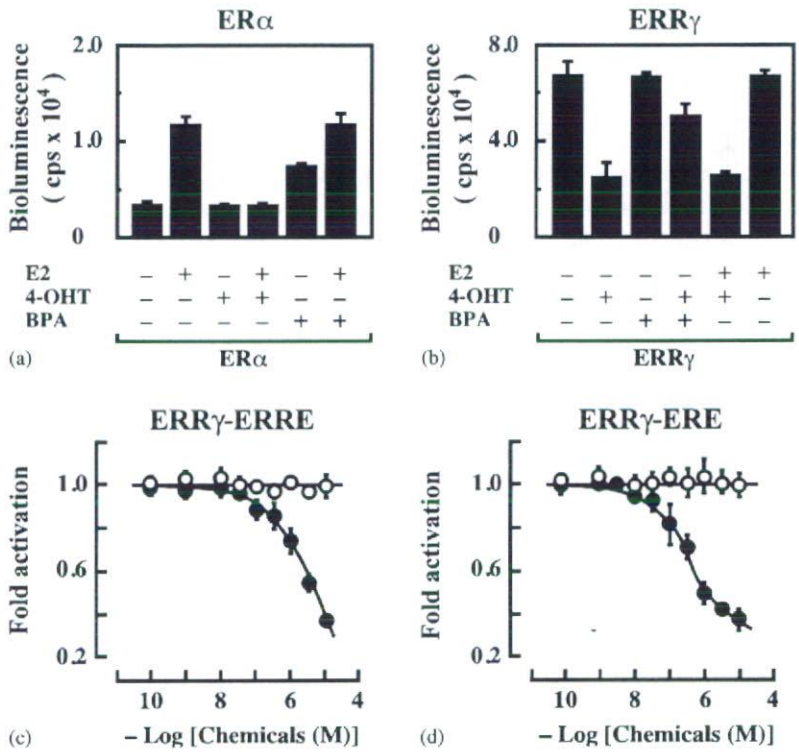

Fig. 3. Luciferase-reporter gene assays of 17β-estradiol (E2), 4-hydroxytamoxifen (4-OHT) and bisphenol A (BPA) for ERα and ERRγ. Comparison of constitutive activities of ERα and ERRγ in the same cell preparations (1.0×10^5 HeLa cells/well) with the luciferase-reporter plasmid (pGL3/3 × ERRE or ERE). (a) For ERα, activities by a single or combination exposure to compounds, for which 10 nM E2, 10 μM 4-OHT and 10 μM BPA are shown. These include the control basal constitutive activity (E2, 4-OHT, BPA; ———), agonist activity of 10 nM E2 (+——) and its inhibitory action on 10 μM 4-OHT (++—). No reaction is elicited by 4-OHT alone (—+—), while very weak agonist activity is elicited by 10 μM BPA (——+). At the saturated condition with 10 nM E2, 10 μM BPA showed no effect (+—+). (b) For ERRγ, the compounds used are 10 μM E2, 10 μM 4-OHT and 10 μM BPA. Control basal constitutive activity (———) of ERRγ is 20–30 times larger than that of ERα (in Fig. 3a). This high constitutive activity is sharply reversed by 10 μM 4-OHT (—+—), while 10 μM BPA (——+) shows no effect on basal constitutive activity. On the other hand, 10 μM BPA reversed almost 80% of the inverse agonist activity of 10 μM 4-OHT (—++). No such suppression activity was shown by 10 μM E2 (++—). E2 itself (+——) exhibited no reaction on ERRγ. (c and d) Concentration-dependent responses of BPA (○) and 4-OHT (●) in the luciferase-reporter gene assay measure the ability of these two compounds to either activate or deactivate the constitutive activity of ERRγ. Reporter plasmids used were pGL3 ERRE for (c) and ERE for (d).

activity, whereas at ERα, it showed extremely weak agonist activity (Fig. 3a).

When an ERE-luciferase reporter plasmid was used in place of a plasmid with ERRE element, BPA exhibited exactly the same activity profile as shown in Fig. 3c, totally retaining ERRγ's high basal activity (Fig. 3d). In contrast, 4-OHT exhibited definitely enhanced inverse agonist activity in this assay (IC_{50} = ca. 0.5 μM, about 20 times enhanced as compared to that in the assay with ERRγ-ERRE) (Fig. 3c and d). However, it is very puzzling why 4-OHT has such a strong binding affinity for ERRγ *in vitro* (10 nM IC_{50}) but has such a low potency in the transient transfection assays (IC_{50} in the range of around micromolar concentration). This problem has been extensively discussed by Coward et al. (2001). The activity change observed by the replacement of receptor response elements strongly suggests that the cell-based assays are not still optimized for ERRγ.

3.5. Inhibitory effect of BPA on the inverse agonist activity of 4-OHT

Since BPA strongly binds to the ERRγ receptor (Fig. 2c), our results from the reporter gene assay suggest that BPA simply occupies the ligand binding site of ERRγ, causing it to retain its basal constitutive activity. We thus assumed that BPA would inhibit the binding of 4-OHT. To examine this possibility, we first examined the inhibitory activity of BPA against the deactivating activity of 4-OHT. As shown in Fig. 4a, 4-OHT's inverse activity was clearly suppressed in the presence of either 1 μM or 10 μM BPA. Thus, 10 μM BPA strongly inhibited the 4-OHT activities, reversing them by about 85% at

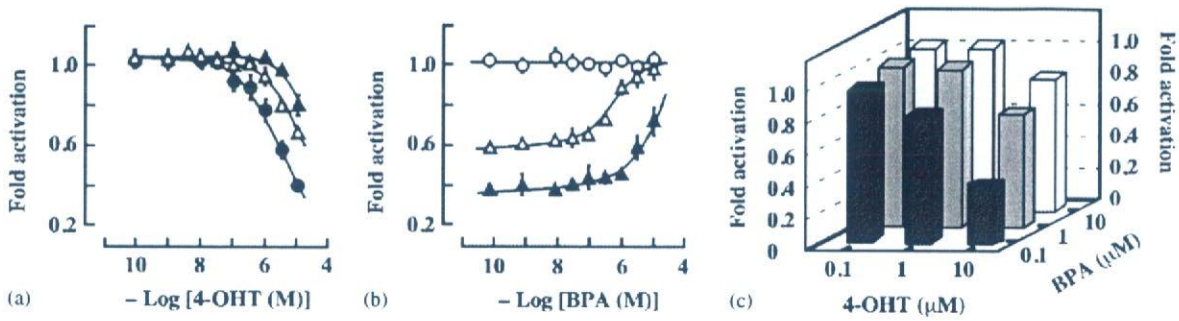

Fig. 4. Effect of bisphenol A (BPA) on the inverse agonist activity of 4-hydroxytamoxifen (4-OHT) in luciferase-reporter gene assays for ERRγ. The basal constitutive activity of ERRγ was evaluated in a cell preparation (1.0×10^5 HeLa cells/well) with the luciferase-reporter plasmid (pGL3/3 × ERRE). (a) Concentration-dependent luciferase-reporter activities of 4-OHT are shown by fold activation in the absence and presence of BPA. 1 μM (△) and 10 μM (▲) BPA clearly reverse the inverse activity of solo 4-OHT (●) against the constitutive activity of ERRγ. (b) Concentration-dependent effect of BPA on the inverse agonist activity of 4-OHT. The inverse agonist activities of 1 μM (△) and 10 μM (▲) 4-OHT were clearly reversed by BPA in a dose-dependent manner. BPA itself (○) sustained high constitutive activity of ERRγ at the concentration range tested. (c) Three-dimensional illustration of the interrelationships between 4-OHT's inverse agonist activity and its suppression activity by BPA in the luciferase-reporter gene assay for ERRγ. The opposing actions of BPA and 4-OHT are dependent upon their concentrations.

each test concentration of 4-OHT. 1 μM BPA was found to reverse by about 65% (Fig. 4a).

As shown in Fig. 4b, BPA completely reversed the inverse activity (about 40% to ERRγ's constitutive activity) of 1 μM 4-OHT in a dose-dependent manner up to a concentration of 10 μM. Against 10 μM 4-OHT, BPA also exhibited a clear ability to reverse its inverse activity. These relationships are shown as a three-dimensional plot, expressing the opposing effectiveness of BPA and 4-OHT on the basal constitutive activity of ERRγ (Fig. 4c): 4-OHT is an inverse agonist, whereas BPA is its antagonist. All these results clearly demonstrate that BPA binds to ERRγ to preserve its native constitutive activity. When the ERE-luciferase reporter plasmid was used instead of the ERRE-luciferase reporter plasmid, similar results were also obtained for a series of reporter gene assays.

4. Discussion

ERRγ, unlike ERα, shows extremely high constitutive activity in the reporter gene assay (Fig. 3b). At present, neither the target gene of ERRγ's constitutive transcriptional activity, nor its natural ligand are known. If ERRγ were to possess an agonistic natural ligand, 4-OHT could act as an antagonist of that natural ligand, and BPA as an antagonist. If the endogenous ligand were to function as a natural inverse agonist, 4-OHT would be interpretable as an analogue of such an agonist. In that case, BPA might also act as an antagonist of such a putative natural inverse agonist ligand. However, as suggested by its very high constitutive activity, if ERRγ were an orphan receptor lacking an identified natural ligand, what then is the significance of BPA's action on this orphan receptor? 4-OHT is simply an inverse agonist that deactivates ERRγ's high transcriptional activity.

The ligand-binding domain (LBD) of ERRγ contains a conserved coactivator-binding surface that interacts with coactivators (Darimont et al., 1998), but only when the bound-agonist form positions the activation function (AF)-2 helix, namely helix 12 of the receptor, to bind the coactivator. The LBD structure of ERRγ has now been solved (Greschik et al., 2002), and as expected from its very high constitutive activity, helix 12 is folded in this active conformation. Most recently, Greschik et al. (2004) reported the three-dimensional structure of the 4-OHT/ERRγ-LBD complex, in which helix 12 is widely separated from the position in the active conformation. This repositioning of helix 12 by 4-OHT deactivates ERRγ because the receptor becomes unable to recruit coactivator proteins at the appropriate position.

It is important that ERRγ-LBD comprises a well-formed pocket (Greschik et al., 2002). Unlike 4-OHT, BPA is apparently allowed to bind to this pocket without changing the positioning of helix 12. Maintaining helix 12 in an active conformation would then result in preservation of high receptor constitutive activity. Thus, BPA acts just as a space filler with no detectable influence on the ERRγ receptor conformation, and preventing 4-OHT from inserting into the pocket. This BPA binding might influence, for example, the rate of turnover or metabolism of ERRγ, perhaps resulting in prolonged transcriptional activities. As described above, BPA would be its antagonist if an endogenous ligand were to act as a natural inverse agonist. Since it is unlikely that ERRγ has an endogenous agonist ligand (Greschik et al., 2002), BPA might act as an "agonist"

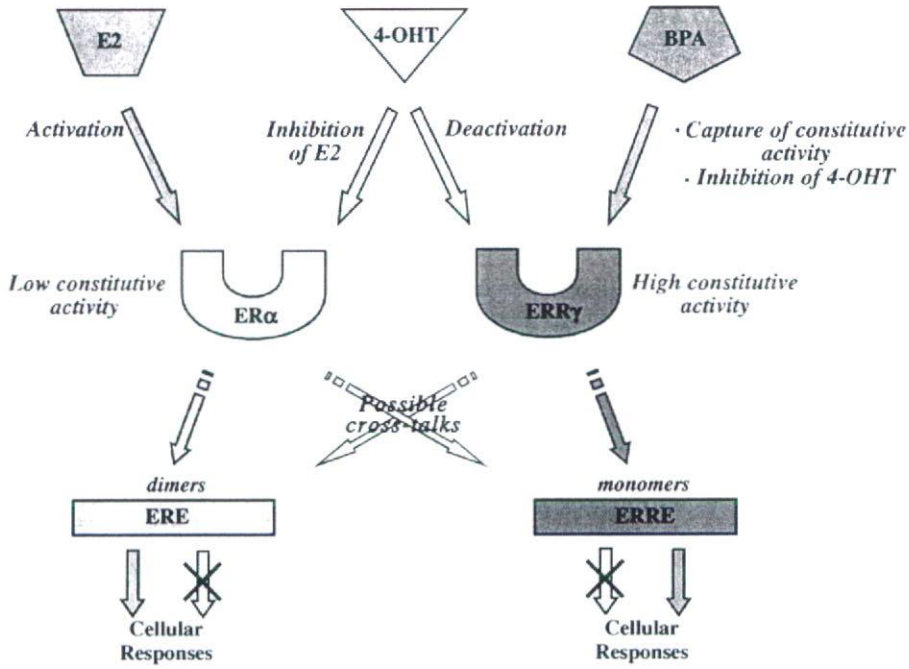

Fig. 5. Schematic flow diagram of cell-specific responses originated by binding of chemicals to nuclear receptors followed by their specific activities against the target genes. ERα's natural ligand E2 and synthetic chemical 4-OHT both bind to ERα, and the resulting ERα dimer binds the ERE-containing target genes to activate it with a coactivator. BPA and 4-OHT both bind to the ERRγ monomer, which in turn binds the ERRE-containing target genes. The ERα dimer may interact with ERRE-containing genes, and the ERRγ monomer would bind to ERE-containing genes as either a homodimer or heterodimer, for example with ERα.

that works to retain or lock the ERRγ's high transcriptional activity, keeping hold of the helix 12 in an active conformation.

Based on these consequences and including the present results, Fig. 5 depicts putative interrelationships between these exogenous chemical ligands and their receptors together with the corresponding target genes. Both E2 and 4-OHT bind to ERα, inducing different interactions with the ERE-containing genes. Both 4-OHT and BPA also bind to ERRγ, eliciting a different set of interactions with the ERRE-containing genes. As a result, 4-OHT may bind to both ERα and ERRγ, although the corresponding 4-OHT-ERRγ and 4-OHT-ERα complexes do not stimulate the transcription of genes. In contrast to this non-selective action of 4-OHT, BPA interacts mainly with ERRγ, and the resulting BPA-ERRγ complex interacts with the ERRE DNA binding element and activates the transcription of genes. This activation is not stimulated more than ERRγ's basal constitutive activity. The BPA-ERRγ complex can also interact with the ERE DNA binding element as a dimer. Given that BPA-ERRγ binds DNA together with E2-ERα via ERE, the resulting heterodimers would interfere or cooperate with each other to regulate the overall estrogenic response in a given cell type. Such potential functional cross-talk between the ERRγ and ERα systems might explain some of BPA's "estrogenic" activities.

Receptor selectivity and specificity of the compounds are both strongly related to the structure of the compounds, especially to their three-dimensional structure. It is clear that the structures of BPA, E2 and 4-OHT share simply the phenol group that is generally known as a key structure for receptor recognition (Fig. 1). When we compared structural features other than the phenol group, it became clear that these compounds possess subtle structural differences in phenol benzene-hydrogen (H) substitutions. In each of the structures, both the *ortho* positions and at least one *meta* position of the phenolic-hydroxy group are unhindered, beyond the minimal substitution of benzene-H atoms. The *para* positions, on the other hand, are blocked or crowded with bulky substitutions that do not share shape, lipophilicity or hydrogen bonding positioning. E2 would be planar, while BPA is not in a planar configuration because of the presence of the sp^3 carbon atom at the *para* position. These features suggest that the receptor fit is likely to arise primarily from the phenolic function, with minimal interference from substitutions at the *ortho* and at least one *meta* position. Thus, substitutes at the *meta* and *para* positions appear to affect the receptor selectivity between

ERα and ERRγ. More detailed structural consideration will need to be made, perhaps with exploration of the 3D-structure of the BPA–ERRγ complex, and such a structural analysis is under way in our laboratory.

The pattern of human ERRγ expression is significant. For example, it expresses during the development and differentiation of the fetal brain at very high levels in a tissue-restricted manner (Hong et al., 1999; Heard et al., 2000; Lorke et al., 2000). Although the function of ERRγ is not yet clarified, various low dose effects of BPA have recently been reported *in vivo* for reproductive organ tissues and systems in mice and rats as mentioned earlier (Nagel et al., 1997; vom Saal et al., 1998; Gupta, 2000; Welshons et al., 2003). In addition, Markey et al. (2001) have shown that *in utero* exposure to low doses of BPA alters the development and tissue organization of the mouse mammary gland. There is also evidence for low dose effects of BPA on sexual differentiation of the brain and behavior in rats (Kubo et al., 2003). Kawai et al. (2003) have recently reported that male mice exposed to BPA during fetal development show a high aggression score at 8 weeks of age. This change in aggressive behavior was seen in male offspring of pregnant females fed BPA at 2 ng/g or 20 ng/g weight, both within the range of current human exposure. Palanza et al. (2002) also noted alterations in maternal behavior in mice exposed to a low dose of BPA during fetal life or in adulthood. Many other low dose effects of BPA have been reported extensively in recent years, including the advanced onset of female puberty (Howdeshell et al., 1999) and insulin resistance (Alonso-Magdalena et al., 2006) (see review article (vom Saal and Hughes, 2005) for others).

By contrast, studies that deny such lowdose effects of BPA have also been repeatedly reported (Ashby et al., 1999; Cagen et al., 1999), and thus the issue of low dose effects has become controversial (Kaiser, 2000). There is also a hypothesis that some estrogenic compounds can be stored in lipid-rich body tissues so that the toxins accumulate over time and eventually reach concentrations that can activate ER. These are to be reviewed also for ERRγ and/or other nuclear receptors, but in case such a bioaccumulation may facilitate greatly the specific interaction of chemicals, for example, of BPA with ERRγ.

It is noteworthy that the IC_{50} value (13.1 nM) of BPA in the receptor binding assay for ERRγ corresponds to about 3.0 ppb, 1/1000 the concentration (3 mg/kg, or 3 ppm) specifically established as the limit for food. In this climate of uncertainty, it seems essential to evaluate what is a physiological significance of BPA's binding to ERRγ and whether ERRγ may be involved in the low dose effects of BPA.

Among a series of steroid hormone receptors including ERs (ERα and ERβ), AR, GR and PR, BPA was found to be highly selective and specific for ERRγ (Table 2). Its receptor selectivity for ERRγ versus ERs is estimated to be approximately 80–100-fold. Although we initially assumed that BPA might interact with nuclear receptor(s) other than ER, BPA's high receptor selectivity together with its high specificity was quite unexpected. In the present study, we succeeded in establishing a receptor binding assay system for ERRγ. However, to attain specific binding against ERRγ was extremely difficult even with [^{3}H]4-OHT, probably because 4-OHT is not a natural ligand of ERRγ. This is also true for ERRα and ERRβ, and in particular no binding of [^{3}H]4-OHT was reported for ERRα (Coward et al., 2001). It should be also noted that BPA elicits a unique combination of reactions in the reporter gene assay, an unchanged high transcriptional activity and suppression of 4-OHT's inverse agonist activity, as we show in the present study.

Notwithstanding the difficulties in our assay, it is now crucial to expand the risk assessment of endocrine disruptor candidate compounds to all nuclear receptors, and more importantly to evaluate whether BPA's previously reported low dose effects are mediated through ERRγ and its specific target genes. Also, what is a physiological significance of BPA's binding to ERRγ is critically important issue. Although we could not provide any evidences for this query in the present study, the binding of BPA to ERRγ has potential and intrinsic importance. To evaluate its physiological relevance may require the examinations to test the abnormality and/or disorder of ERRγ's ordinary but unknown functions by means of sophisticated and well-designed assays. We are going to perform such examination and corroboration due to their fundamental importance on human and animal health.

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