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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).

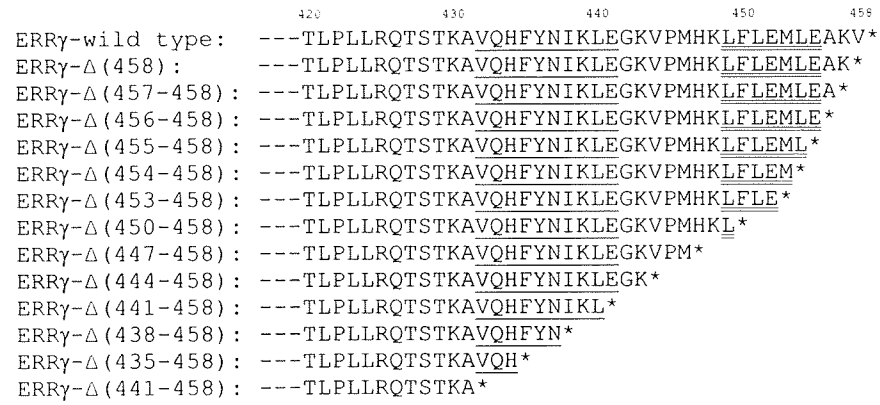

Fig.1. Helix 12-peptide mutant receptors of ERRγ design ("___": H11 peptide part; "___": H12 peptide part)

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.

References

1. Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., and Shimohigashi, Y. (2006) *Toxicol Lett.* **167**, 95-105.J.
2. Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M., and Shimohigashi, Y. (2007) *J. Biochem.* **142**, 517-524.
3. Greschik, H., Flaig, R., Renaud, J.P., and Moras, D. (2004) *J. Biol. Chem.* **279,** 33639-33646.

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The authors declare they have no competing financial interests.

Short running head: Bisphenol AF as ERα-Agonist but ERβ-Antagonist

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Key words: bisphenol A, bisphenol AF, endocrine disruptor, estrogen receptors, receptor antagonist, receptor binding

Abbreviations:

AR	androgen receptor
BSA	bovine serum albumin
DDT	dichlorodiphenyltrichloroethane
ER	estrogen receptor
ERRγ	estrogen-related receptor γ
GST	glutathione *S*-transferase
LBD	ligand binding domain
NR	nuclear receptors
NTP	National Toxicology Program
4-OHT	4-hydroxytamoxifen

Outline of section headers:

Abstract

BACKGROUND: Bisphenol AF has been acknowledged to be a 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 intended to determine the relative preference of bisphenol AF for the human nuclear estrogenic receptors ERα and ERβ and the bisphenol A-specific receptor ERRγ. Also, we aimed 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} = 18.9 nM) than 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: The present 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.

Introduction

Bisphenol AF (also referred to as hexafluoro-bisphenol A) is a homolog of bisphenol A (Figure 1). Bisphenol AF has a symmetrical chemical structure of HO-C_6H_4-$C(CF_3)_2$-C_6H_4-OH, designated as 1,1,1,3,3,3-hexafluoro-2,2-bis(4-hydroxyphenyl)propane by the IUPAC nomenclature rule. 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.

(Figure 1)

In 2008, the US National Institute of Environmental Health Sciences nominated bisphenol AF for comprehensive toxicological characterization based on the lack of adequate toxicity data (National Toxicology Program 2008a). In this nomination report, the National Toxicology Program (NTP) noted concern regarding potential exposure of the general population to bisphenol AF. Structural dissimilarities between bisphenol AF and bisphenol A are determined by the presence of a trifluoromethyl or methyl group, respectively. The potential toxicity of bisphenol AF is of concern in part because the trifluoromethyl CF_3 group is much more electronegative (an potentially reactive) than the methyl CH_3 group of bisphenol A.

Various "low-dose effects" of bisphenol A have recently been reported *in vivo* for reproductive organ tissues in mice and rats. For example, in utero exposures to very low-dose levels of bisphenol A 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 bisphenol A have effects on the central nervous system as well (vom Saal and Welshons 2005; Welshons et al. 2003, 2006). All of these low-dose effects of bisphenol A 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 proceeding report by NTP on the potential for bisphenol A 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 (National Toxicology Program 2008b).

Bisphenol A exhibits extremely weak binding activity for ER and AR. Based on the idea that bisphenol A 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 bisphenol A target receptor (Takayanagi et al. 2006). Bisphenol A binds to ERRγ very strongly (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 bisphenol A to ERRγ was further demonstrated by direct X-ray crystallographic analysis of this complex (Matsushima et al. 2007, 2008). Moreover, using real-time PCR, 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 bisphenol A suggested that it is essential for endocrine chemicals to be examined for interactions with all 48 human NRs. We previously reported that bisphenol AF binds to ERα more strongly than ?, and that its receptor selectivity is seven times higher for ERα than ERRγ (Okada et al. 2008). There are two subtypes of estrogen receptors, ERα and ERβ with distinctly different physiological distributions and functions. Since 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 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.

Materials and Methods

Test compounds. 17β-Estradiol, CAS no. 50-28-2, 98.9%, Research Biochemicals International, Natick, MA. Bisphenol A, CAS no. 80-05-7, purity 99%, Tokyo Kasei Kogyo Co. LTD, Tokyo, Japan. Bisphenol AF, CAS no. 1478-61-1, purity 99%, Tokyo Kasei Kogyo Co. LTD, Tokyo, Japan. 4-Hydroxytamoxyphen (4-OHT), CAS no. 68047-06-3, purity 98%, Sigma-Aldrich Inc., St Louis, MO.

Preparation of GST-fused nuclear receptor LBD protein. cDNA clones of ERα and ERβ were purchased from OriGene human cDNA clone (OriGene Technologies, Inc., Rockville, MD). Glutathione *S*-transferase (GST)-fused receptor ligand binding domain (LBD) expressed in *E. coli* BL21α, namely, 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) followed by gel filtration on a column of Sephadex G-10 (15 x 10 mm, GE Healthcare BioSciences).

Radio-ligand binding assays for saturation binding —Saturation binding assays for ERα and ERβ were conducted essentially as reported (Nakai et al. 1999) by 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 of binding buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 10% glycerol; pH 7.4). Non-specific binding was determined in a parallel set of incubations that included 10 μM of non-radiolabeled 17β-estradiol. After incubation for 2 hr at 20°C, free radioligand was removed by incubation with 0.4% dextran-coated charcoal (DCC) (Sigma) in phosphate buffer saline (pH 7.4) for 10 min on ice and centrifugation for 10 min at 15,000 rpm.

Saturation binding assay for ERRγ was carried out as reported (Okada et al. 2008) by using [^{3}H]bisphenol A (5.05 TBq/mmol; Moravek Biochemicals, Brea, CA). Specific

binding of tritium-labeled ligand was calculated by subtracting the non-specific binding from the total binding. Receptor proteins that were expressed and purified were evaluated in a saturation binding assay to estimate their dissociation constant 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.

Radio-ligand binding assays for competitive binding. Bisphenol AF, bisphenol A, 17β-estradiol, and 4-OHT were dissolved in 0.3% *N,N*-dimethylsulfoxide (DMSO) in 1% bovine serum albumin (BSA; a blocker of non-specific adsorption to the reaction vessels). 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 radio-ligand was removed with 1% dextran-coated charcoal by filtration. Radioactivity was determined on a liquid scintillation counter (TopCount NXT; Perkin Elmer Life Sciences Japan, Tokyo). IC_{50} values (concentrations resulting in half maximal inhibition of 17β-estradiol binding), were calculated from dose-response curves obtained using the non-linear analysis program ALLFIT (De Lean et al. 1978). Each assay was performed in duplicate and repeated at least five times. For reconfirmation, the binding assay for ERRγ was also performed using [^{3}H]bisphenol A (5 nM in final) and GST-ERRγ-LBD (26 ng).

Luciferase reporter gene assay. HeLa cells were maintained in Eagle's Minimum Essential Medium (MEM) (Nissui, Tokyo) in the presence of 10% (v/v) fetal bovine serum at 37°C. For luciferase assays, HeLa cells were seeded at 5×10^5 cells / 6 cm dish for 24 h and then transfected with 4 μg of 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 h after transfection, cells were harvested and plated into 96 well plates at 5×10^4 cells per well. The cells were then treated with varying doses of chemicals

diluted with 1% BSA/PBS (v/v). After 24 h, luciferase activity was measured with the appropriate reagent using a Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WI). Light emissions were measured using a Wallace 1420 ARVOsx mutilabel 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).

In order to measure the antagonistic activity of bisphenol AF for ERβ, four different concentrations (0.01, 0.1, 1.0, and 10 μM) of bisphenol AF were examined for a serial concentration of 17β-estradiol (10^{-12} – 10^{-5} M in the final solution). Also, a serial concentration of bisphenol AF (10^{-12} – 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. Receptor protein preparations suitable for the competitive receptor-binding assay were selected 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 radio-ligand receptor-binding assay. Since all the nuclear receptors are secreted protein preparations, observed B_{max} values were comparable to those calculated from their molecular weight.

Bisphenol A was very weak ligand for ERα (IC_{50} = 1,030 nM) based on its ability to inhibit [^{3}H]17β-estradiol binding, as previously reported by us (Okada et al. 2008) (Figure 2A, Table 1). In the present study, we confirmed that bisphenol A is also a very weak ligand for ERβ (IC_{50} = 900 nM, Figure 2B, Table 1), indicating comparable interactions of bisphenol A with ERα and ERβ despite the subtle structural differences

between these ERs. In contrast, bisphenol AF was 20 times more potent than bisphenol A as a ligand for ERα (IC_{50} = 53.4 nM, Figure 2A, Table1), and was approximately 48 times more potent for ERβ (IC_{50} = 18.9 nM, Figure 2B, Table1). 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 the CH_3 groups of bisphenol A.

(Figure 2) (Table 1)

Receptor-binding selectivity of bisphenol AF and bisphenol A. 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 bisphenol A and bisphenol AF (Table 2). The results indicate that bisphenol A 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 ERRγ (6.70 times more selective for ERα than ERRγ and 18.94 times more selective for ERβ than ERRγ (Table 2). Bisphenol AF binding is also about three times more potent for ERβ than ERα.

(Table 2)

Differential effects of bisphenol AF in the reporter gene assay. We next examined reporter gene activity following bisphenol AF exposure in HeLa cells transiently co-transfected with an ERα or ERβ expression plasmid and an ERE-luciferase reporter plasmid. Bisphenol AF fully activated ERα (increasing activity to approximately 7 times the baseline level) in a dose-dependent manner at concentrations in the range of 10^{-10} – 10^{-5} M (Figure 3A). The half-maximal effective concentration (EC_{50}) of bisphenol AF was 58.7 nM.

(Figure 3)

When potencies for ERα activation versus ERα binding were compared to determine receptor activation potency [expressed as EC_{50} (nM) / IC_{50} (nM)], there was a clear discrepancy between 17β-estradiol and bisphenol AF. As shown in Table 3, the

receptor activation potency for 17β-estradiol was estimated to be 0.085 (EC_{50} 0.075 nM/ IC_{50} 0.88 nM based on values from Figure 3A and Table 1, respectively). In contrast, the receptor activation potency of bisphenol AF [58.7 nM (EC_{50}) / 53.4 nM (IC_{50}) = 1.099], was approximately 13 times greater than that 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, while 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.

(Table 3)

Bisphenol A 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 bisphenol A for ERα (0.308) and ERβ (0.772) 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 bisphenol A 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, bisphenol A exhibited a reduced ability to bring about full activation of ERβ (3.5 times greater activity relative to baseline in response to bisphenol A versus an increase to 6 times the baseline level in response to 17β-estradiol). This difference in efficacy indicates that bisphenol A 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 the 10 μM concentration (Figure 3B). Based on the results of 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 ERβ. This unexpected inactivity in the reporter gene assay

suggests that bisphenol AF binding disrupts the ERβ-LBD (ligand binding domain) 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 17β-estradiol, an endogenous agonist ligand of ERβ, was examined in the presence of 0.01, 0.1, 1.0, and 10 μM concentrations of 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, the 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.

(Figure 4)

The antagonist activity of bisphenol AF for 17β-estradiol/ERβ was further evidenced by assays in which a serial concentration of bisphenol AF ($10^{-12} - 10^{-5}$ M) was added to a solution of 17β-estradiol maintained at a constant concentration. When a 1×10^{-8} M concentration of 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 $10^{-10} - 10^{-5}$ M (Figure 4B). A similar result was obtained for a 1×10^{-7} M concentration of 17β-estradiol. These results demonstrate that bisphenol AF can antagonize the activity of 17β-estradiol on the ERβ receptor.

Discussion

Structural characteristics of bisphenols and ERs/ERRγ receptors. It should be noted that the differences in receptor selectivity between bisphenol AF and bisphenol A are due to the $CH_3 \leftrightarrow CF_3$ substitution on the bisphenol backbone structure. Bisphenol AF is a hexafluoro derivative of bisphenol A with the $CH_3 \rightarrow CF_3$ substitution on the backbone structure of 2,2-disubsutituted propane CH_3-C-CH_3. Bisphenol A binds strongly to ERRγ, but bisphenol AF binds to ERRγ only weakly; and it was therefore judged that the binding pocket of ERRγ-LBD possesses structural elements unfavorable for interaction with the trifluoro CF_3 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, since 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, we found that the ERRγ binding sites for bisphenol A CH_3 groups were Phe435 and Met306 (Matsushima et al. 2007, 2008). Since the aromatic phenyl and S-CH_3 groups of Phe435 and Met306 are electron-rich, conditions would be unfavourable for binding of bisphenal 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, bisphenol AF was found to be a strong ligand for both ERα and ERβ receptors, although it shows a three 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 ligand binding domains of ERα and ERβ share a high sequence identity (59%) and similar three-dimensional structures. No obvious differences between ERα and ERβ were

observed in the estrogen-responsive element 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 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, as 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 will be analyzed in the light of the crystal structures, and such studies are in progress in our laboratory.

Bisphenol AF as a candidate of potential endocrine disruptor. It should be noted that 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 (Couse and Korach 1999), liver (Couse and Korach, 1999), kidneys (Couse and Korach 1999), and heart (Couse and Korach 1999), while ERβ expressed primarily in the ovaries (Couse and Korach 1999), prostate (Couse and Korach 1999), lungs (Kuiper et al. 1997), gastrointestinal tract and bladder (Nilsson et al. 2001). Co-expression of both receptors occurs in the mammary glands (Pettersson and Gustafsson 2001), epodidymis (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 http://www.nursa.org/datasets.cfm?doi=10.1621/datasets.02001 (accessed 30 March 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 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 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), a bisphenolic metabolite of methoxychlor, 1,1,1-trichloro-2,2-bis(4-methoxyphenol)ethane. HPTE was found to behave as an ERα agonist and an ERβ antagonist with estrogen-responsive promoters in HeLa cells (Gaido et al. 1999). We confirmed these results also 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 others (Gaido et al. 1999; 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 ten times more potent than bisphenol AF for ERRγ binding, although both chemicals were most strongly bound to ERβ (Tables 1, 2). As antagonists for ERβ, bisphenol AF (pA_2 = 7.87) was found to be somewhat stronger than HPTE, the pA_2 of which was reported to be 7.52 (Gaido et al. 1999). It should be noted, however, that 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 and the other by H in HPTE (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, 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, since 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). It should be noted, however, that HPTE is approximately 100 times more active at ER than 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 to both.

Conclusion

Bisphenol A 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 $CH_3 \leftrightarrow CF_3$ 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 nuclear receptors 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 nuclear receptors. Collectively, it is now required to examine the degree and ways in which bisphenol AF may influence the physiological roles of ERα and ERβ. Given that bisphenol AF and bisphenol A function as endocrine disruptors, these chemicals would work out differently via different nuclear receptors.

REFERENCES

Alm H, Tiemann U, Torner H. 1996. Influence of organochlorine pesticides on development of mouse embryos in vitro. Reprod Toxicol 10(4):321–326.

Arts J, Kuiper GGJM, Janssen JMMF, Gustafsson J-Å, Löwik CWGM, Pols HAP, et al. 1997. Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138(11): 5067–5070.

Brandenberger AW, Tee MK, Lee JY, Chao V, Jaffe RB. 1997. Tissue distribution of estrogen receptors alpha (ER-α) and beta (ER-β) mRNA in the midgestational human fetus. J Clin Endocrinol Metab. 82(10): 3509–3512.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758.

Couse JF, Korach KS. 1999. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20(3): 358–417.

Cummings AM. 1997. Methoxychlor as a model for environmental estrogens. Crit Rev Toxicol 27(4):367–379.

DeLean A, Munson PJ, Rodbard D. 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am J Physiol 235(2):E97-E102.

Gaido KW, Leonard LS, Maness SC, Hall JM, McDonnell DP, Saville B, et al. 1999. Differential interaction of the methoxychlor metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors α and β. Endocrinology 140(12):5746-5753.

Gupta C. 2000. Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc Soc Exp Biol Med 224(2):61-68.