ESTROGEN-RELARTED RECEPTOR γ (ERRγ) AS A SPECIFIC RECEPTOR FOR ENDOCRINE DISRUPTOR BISPHENOL A

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Abstract
Estrogen-related receptor γ (ERRγ) is a member of 48 human nuclear receptors. We have recently found that bisphenol A (BPA) strongly binds to ERRγ, but not to estrogen receptor (ER). We suggested that BPA's low-dose estrogenic activities acknowledged as an endocrine disruption might be caused by the crosstalk between the ER and ERRγ receptors. To analyze such a crosstalk, the discovery of ERRγ-specific agonist and antagonist is crucial. Thus, in this study we carried out the analyses of structure-function relationships of BPA and ERRγ. X-ray crystallography of the BPA-ERRγ complex revealed the important attachments that complete the binding of BPA to ERRγ. Such structural requirements were confirmed by means of experimentations to evaluate the activities for both the ERRγ receptors with site-directed mutagenesis and the BPA derivatives lacking some of structural elements. We concluded that ERRγ possesses a ligand binding pocket just for BPA and there are some other compounds feasible to bind to ERRγ more potently than BPA. The present result is particularly important because ERRγ is expressed in a tissue-restricted manner, for example very strongly in the mammalian fetal brain and also in the placenta, at sites that could have important outcomes for the newborn.

Introduction
Bisphenol A (BPA) is used mainly in the production of polycarbonate plastics and epoxy resins. Its worldwide manufacture is approximate 3.2 million metric tons per year at this point. BPA has a symmetrical chemical structure of HO-C_6H_4-$C(CH_3)_2$-C_6H_4-OH, and had been acknowledged as an estrogenic chemical able to interact with human estrogen receptors (ERs). Various so-called "low-dose effects" of BPA have extensively been reported *in vivo* for the central nervous system and reproductive system in mice and rats. However, its binding to ER and hormonal activity is extremely weak, 1,000–10,000 times lower than for natural hormones, and the intrinsic significance of low dose effects has been intangible[1-3]. This discrepancy on low dose effects promoted us to enquire whether BPA may interact with NRs other than ER.

We could identify recently that BPA strongly binds to human estrogen-related receptor γ (ERRγ).[4] The ERRγ belongs to a subfamily of orphan NRs closely related to ERs, ERα and ERβ.[5] There are three ERR family members, ERRα, ERRβ, and ERRγ, and all of ERRs have considerably high amino acid sequence similarity to ERs. ERRs have a high constitutive activity with no ligand, and their intrinsic ligands are still unknown to date. The selective ER modulator, 4-hydroxytaomxifen (4-OHT), has been identified as an inverse agonist of ERRγ, deactivating the receptor by decreasing such constitutive activity.[6] We have shown that BPA reverses this 4-OHT's inverse agonist activity in a dose-dependent manner, exhibiting a distinct inverse antagonist activity. BPA completely preserves the high constitutive activity of ERRγ, since BPA fits and stays at a ligand-binding pocket of ERRγ as evidenced by X-ray structural analysis of the BPA-ERRγ complex.[7]

In the present study, in order to clarify the important attachments between the BPA and ERRγ, we conducted tests to evaluate the activities to complete their binding. Those include the experimentations such as site-directed mutagenesis for the ERRγ receptor and the receptor binding assay for a series of BPA derivatives lacking some of structural elements. We concluded that ERRγ possesses a ligand binding pocket just for BPA and there are some other compounds feasible to bind to ERRγ more potently than BPA.

Materials and Methods
The ligand binding domain (LBD) of ERRγ was amplified from a human kidney cDNA library by PCR using gene-specific primers and cloned into a plasmid pGEX6P-1. The glutathion *S*-transferase (GST)-fused ERRγ-LBD was expressed in *E. coli* BL21 and purified on an affinity column of Glutathione-Sepharose 4B to obtain GST-ERRγ-LBD for radio-ligand binding assays.

Saturation binding assay using [^{3}H]BPA was conducted essentially as reported.[8] Free radio-ligand was removed by centrifugation or filtration after incubation with 1% dextran-coated charcoal. For competitive binding assay, BPA and other chemicals were dissolved in 0.3% DMSO, and examined for their ability to inhibit the binding of [^{3}H]BPA to GST-ERRγ-LBD. The mutated ERRγ expression plasmids were produced by a polymerase chain reaction method. Luciferase reporter gene assay was performed by using HeLa cells as reported.[4]

Results and Discussion

In order to evaluate the capability of chemicals and hormones to bind to ERRγ, we first tested the specific binding of their triturated derivatives by the Scatchard plot analysis to estimate the dissociation constant. The assay revealed that BPA binds to ERRγ very strongly with an K_D value of 5.50 nM, slightly more potent than 4-OHT (10.0 nM).[4] The natural estrogen 17β-estradiol (E2) failed to show any specific binding to ERRγ even at its concentration of 10 μM. The competitive binding assay using [^{3}H]BPA as a tracer was carried out to evaluate the ability of compounds to displace BPA, and the results were compatible to those from the saturation binding assay.

The competitive binding assay was carried out also to analyze the structural requirements of BPA analogues. When one of phenyl-hydroxy groups of BPA HO-C_6H_4-$CH(CH_3)_2$-C_6H_4-OH was eliminated, the resulting 4-α-cumylphenol HO-C_6H_4-$C(CH_3)_2$-C_6H_4 was found to bind still very strongly to ERRγ. This implied that the hydrogen bonding between this hydroxy group and ERRγ-Asn346 is not so strong. Indeed, Asn346→Ala mutant ERRγ was able to bind BPA considerably strongly.

All of the structural elements of BPA should get a checkup, and the receptor residues likewise. It was found that multiple interaction keeps BPA as 'space filler' without the conformation change of activated ERRγ. The receptor residues necessary to keep BPA in a binding pocket include Glu275, Met306, Leu309, Arg316, Tyr 326 and Phe435. In particular, Tyr326 must be the most important residue to hold BPA in the pocket. Whilst, it should be noted that bisphenol E, HO-C_6H_4-$CH(CH_3)$-C_6H_4-OH, turned out as the most potent BPA analogue. It elicited an inverse antagonist activity against 4-OHT's inverse agonist activity in the luciferase reporter gene assay.

It is now crucial to expand the risk assessment of endocrine disruptor candidate compounds to all 48 nuclear receptors, and more importantly to evaluate whether BPA's previously reported low-dose effects are mediated through ERRγ and its specific target genes.

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