Development of a Competitive Enzyme Immunoassay for Detection of Capacity of Chemicals to Bind Quail Estrogen Receptor α and β

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In vitro binding assays are useful in the initial screening of endocrine disrupting chemicals. Such assays should be applied to the estrogen receptors (ER) of not only humans but also wildlife. As a system for birds is yet to established, we expressed the ligand binding domain (LBD) of quail ERα and ERβ as a fusion protein with glutathione S-transferase and using these proteins, developed two systems (a competitive enzyme immunoassay and a fluorescence polarization assay) for assaying the capacity to bind ERs *in vitro*. Moreover, 20 test chemicals selected by Ministry of the Environment of Japan were evaluated in terms of binding ability. Both systems worked well, the competitive enzyme immunoassay proving especially powerful, since it needs no special equipment. This system is applicable to other species including fish, amphibians and reptiles when information on the LBD of ER is available.

Key words —— endocrine disrupting chemicals, estrogen receptor, enzyme immunoassay, quail, endocrine disruptor, *in vitro* binding assay

INTRODUCTION

Endocrine disrupting chemicals (EDCs) also called endocrine disruptors (EDs) seem to affect reproductive functions through the estrogen-estrogen receptor (ER) signaling pathway.[1,2] Although the mechanisms behind the disruption are not fully understood, the development of screening methods is definitely needed. Indeed, one-generation study and uterotrophic assay for use *in vivo*, and the receptor binding assay and reporter gene assay for use *in vitro* have been developed.[1–4] The Ministry of the Environment of Japan (MoE) released a document entitled "Strategic Programs on Environmental Endocrine Disrupters '98 (SPEED'98)" in 1998, and selected 65 substances as high-priority chemicals to be tested. The tests for some chemicals have been completed.[5]

For the initial screening *in vitro*, the receptor binding assay is often utilized. The yeast two-hybrid assay is particularly useful, since it is easy and relatively cheap to perform, and also no special equipment is needed.[6,7] This method is based on the interaction between the ligand binding domain (LBD) in the hormone receptor and the coactivator in the ligand-dependent manner.[6] Recently, it was proposed that the effect of EDCs should be considered not only in humans but also in wildlife, and indeed adverse effects on humans and wildlife were reported. However, information on coactivators is restricted to specific species, such as humans, rats and mice.[1,2] Therefore, the yeast two-hybrid assay does not seem to be a suitable method for use *in vitro* among various species.

The Organization for Economic Cooperation and Development (OECD) and MoE have started to develop various test methods for humans, fish, reptiles, amphibians, and birds.[5,8,9] As *in vitro* binding assay, several methods have been established, and

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some for human ERs are commercially available. These include the fluorescence polalization method[10] (TAKARA BIO Inc., Otsu, Shiga, Japan), a competitive enzyme immunoassay (EIA) (TOYOBO Co. Ltd., Osaka, Japan) and a radio-competitive assay. For the testing birds, Japanese quail was used in a vitellogenin assay, and a sex-reversal assay.[5] We have previously reported the cloning of quail ERα.[11] The cloning of ERβ was also reported.[12] However, an *in vitro* binding assay for birds is yet to be established.

We report here the establishment of a competitive EIA for analysis of the capacity to bind quail ERα and ERβ, and the subsequent testing of chemicals selected by MoE. We also established a fluorescence polalization method for quail ERα and ERβ, and compared the usefulness of these two approaches.

MATERIALS AND METHODS

Chemicals —— The test chemicals (12 and 8 chemicals selected by MoE in 2000 and 2001, respectively)[5] were supplied by Dr. Kawashima at JAPAN NUS Co. Ltd. (Japan), where large amounts of chemicals of reagent grade have been stocked for the various tests at the request of MoE. All test chemicals were dissolved in dimethyl sulfoxide (DMSO). All other chemicals are of reagent grade.

Plasmid Construction —— A DNA fragment containing LBD of quail ERα or ERβ was amplified by polymerase chain reaction (PCR) using KOD-Plus (TOYOBO Co. Ltd.), and the products were recovered. The 5′-end of these fragments was phosphorylated by T4 polynucleotide kinase and subcloned into the *Eco*RV site of pBluescript KS+. After confirmation of the nucleotide sequences using an automated DNA sequencer DSQ1000 (Shimadzu Corp., Kyoto, Japan), the *Eco*RI-*Sal*I fragment of ERα-LBD or ERβ-LBD was cloned into the same restriction sites in pGEX-4T-1, which codes glutathione S-transferase (GST) (Amersham Pharmacia Biotech). Finally, the resultant recombinant plasmid was transformed into *Escherichia coli* (*E. coli*), BL21 (DE3) (Novagen, EMD Biosciences Inc., Germany).

Expression and Purification of GST-ER Fusion Protein —— BL21 (DE3) harboring pGEX-4T-1-quail ERα or ERβ was cultured in Luria-Bertani (LB) medium at 30°C. At an OD_{600} of 0.4, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM. After incubation for 2 hr, the cells were harvested, suspended in 14.4 ml of B-0.1 [20 mM Tris (pH 7.5), 10% glycerol, 0.1 M KCl, 5 mM $MgCl_2$, and 1 mM DTT]. Then, 1.6 ml of 1 mg/ml lysozyme in B-1 [20 mM Tris (pH 7.5), 10% glycerol, 1 M KCl, 5 mM $MgCl_2$, and 1 mM dithiothreital (DTT)] was added and the cells were disrupted by sonication. This suspension was mixed with 16 ml of B-1, and further stirred gently for 0.5 hr at 4°C. By centrifugation at 12000 rpm for 15 min at 4°C, the soluble fraction including GST-ERs was obtained.

For the purification of GST-ERs, 0.42 ml of glutathione (GSH)-Sepharose 4B (Amersham Pharmacia Biotech) was added to 32 ml of the soluble fraction, and stirred gently for 0.5 hr at 4°C, and then the mixture was packed into the column. After washing thoroughly, the bound GST-ERs were eluted with 1 ml of 20 mM GSH in B-1.

Competitive Enzyme Immunoassay —— The competitive enzyme immunoassay was performed using the kit, Ligand Screening System-ERα or ERβ (TOYOBO Co. Ltd.), according to the manufacturer's instructions. However, the human ERα or ERβ in the kit was replaced with quail GST-ERα or GST-ERβ. 17β-Estradiol (E2) and diethylstilbestrol (DES) were diluted with DMSO at a concentration of $8/3 \times 10^{-4}$ M–$8/3 \times 10^{-8}$ M, and further diluted with the dilution buffer in the kit at a final concentration of $8/3 \times 10^{-6}$ M–$8/3 \times 10^{-10}$ M (The final DMSO concentration is 1%). The test chemicals were diluted in the same way at a final concentrations of $8/3 \times 10^{-4}$ M–$8/3 \times 10^{-8}$ M.

Next, 20 μl of quail GST-ER, 30 μl of test chemical or standard DES, and 30 μl of E2 were mixed and incubated for 1 hr on ice. After the incubation, 50 μl of the mixture was transferred to an anti-E2 antibody-coated plate, and 50 μl of E2-horse radish peroxidase (HRP) solution was added. After incubation for 1 hr on ice, the plate was washed and the enzyme reaction was run. The absorbance at 450 nm was measured with a 1420 ARVO Multilabel Counter (Wallac, Gaithersburg, U.S.A.).

The percent inhibition of binding was calculated as follows: $(A_{\text{DMSO control}} - A_{\text{test sample}})/(A_{\text{DMSO control}} - A_{\text{DES at 112 nM}}) \times 100$. The IC_{50} was obtained from the concentration giving 50% inhibition when the inhibition by 112 nM DES is 100%. Relative binding affinity (RBA) was obtained by dividing the IC_{50} of DES by that of the IC_{50} at test sample. When percent inhibition was less than 50%, 20% relative effective concentration (REC20) was also calculated.

Fluorescence Polarization Method —— For the

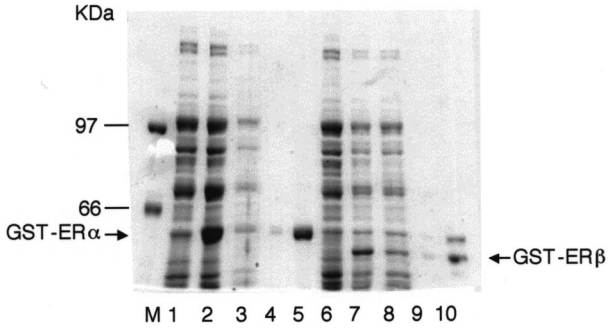

Fig. 1. Expression and Purification of Quail GST-ERα and ERβ

The quail GST-ERα and GST-ERβ fusion proteins were expressed in *E. coli* BL21 (DE3), and then purified using GSH-Sepharose. The samples were loaded on SDS-polyacrylamide gel, and stained with coomassie brilliant blue. Lane M: size marker; Lanes 1–5: GST-ERα; Lanes 6–10: GST-ERβ; Lanes 1&6: whole protein solubilized from untreated cells; Lanes 2&7: whole protein solubilized from IPTG-treated cells; Lanes 3&8: soluble fraction from IPTG-treated cells; Lanes 4&9: purified GST-ERs from soluble fraction; Lanes 5&10: purified GST-ERs from soluble fraction (40-fold more than in Lanes 4 &9).

fluorescence polarization method of assaying binding to ERs,[10] an FP Screen-for-Competitors Kit, ER-α or ERβ, high sensitivity (TAKARA BIO Inc.), was used. The human ERα or ERβ in the kit was replaced with quail GST-ERα or GST-ERβ. The assay was performed according to the manufacturer's instructions. In brief, the test chemicals were diluted with DMSO at a concentration of 10^{-2} M–10^{-6} M, and 1 μl of each chemical was added to 99 μl of Fluomone ES1 and quail GST-ER complex. After incubation for 1 hr at room temperature, the fluorescence polarization was measured with a Full-Range Beacon 2000 (TAKARA BIO Inc.).

RESULTS

Expression and Purification of GST-ER Fusion Protein

For the development of *in vitro* binding assay systems, we first expressed the LBD of quail ERα and ERβ as a GST fusion protein in *E. coli*. With the addition of IPTG, GST-ERs were induced and recovered mainly in the soluble fraction (Fig. 1). This soluble fraction was applied to a GSH-Sepharose column and eluted with GSH. The purity and amount of the resultant purified GST-ERα (MW = 65 kDa) and GST-ERβ (MW = 60 kDa) were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).

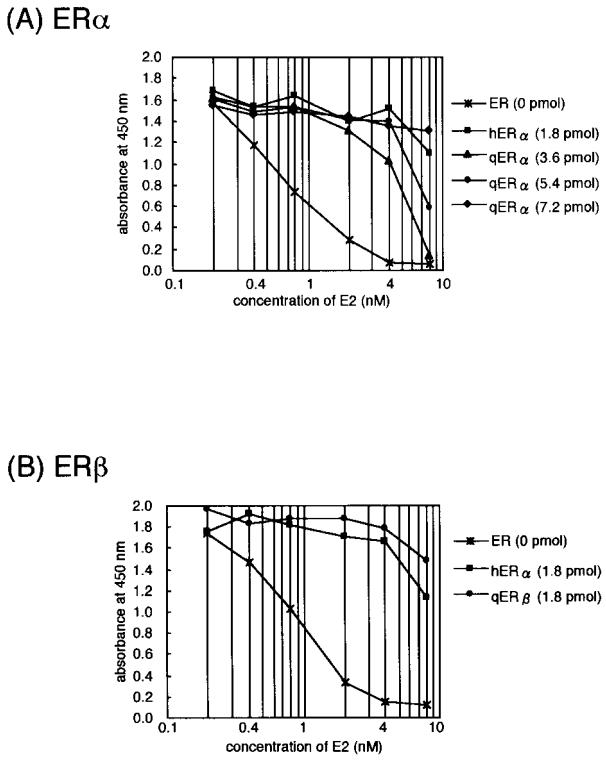

Fig. 2. Measurement of the Amount of Free Ligand in the Presence of Quail ERs

For determination of the most suitable amount of ERs to use, the free ligand test was performed using various amounts of quail ERs. As a control, human ERα was used. (A): quail ERα; (B): quail ERβ.

Development of a Competitive Enzyme Immunoassay for the Capacity to Bind Quail ERs

For the development of a competitive EIA for quail ERs, the optimum amount of ERs used should be determined. Using various amounts of E2 and quail ERs, the amount of free ligand which does not make a complex with ERs was determined (Fig. 2). Since the concentration of E2 used in the kit is 4 nM, it is required that up to 4 nM be trapped by ER. At above 4 nM, E2 exists freely. In this regard, the optimum amount of ER used was determined as 5.4 pmol/well and 1.8 pmol/well for quail ERα and ERβ, respectively.

Next, we developed a competitive EIA using these amounts of recombinant ERs. The standard curve was made taking percent inhibition with 112 nM DES as 100% (Fig. 3). The IC_{50} of DES for quail ERα and ERβ was 6.0×10^{-9} M and 1.3×10^{-8} M, respectively. When using the human ERs in the kit, those values were 1.0×10^{-8} M and 5.0×10^{-9} M, respectively. Thus, IC_{50} values were similar among the two species and two types of ER.

The competitive EIA established for quail ERs

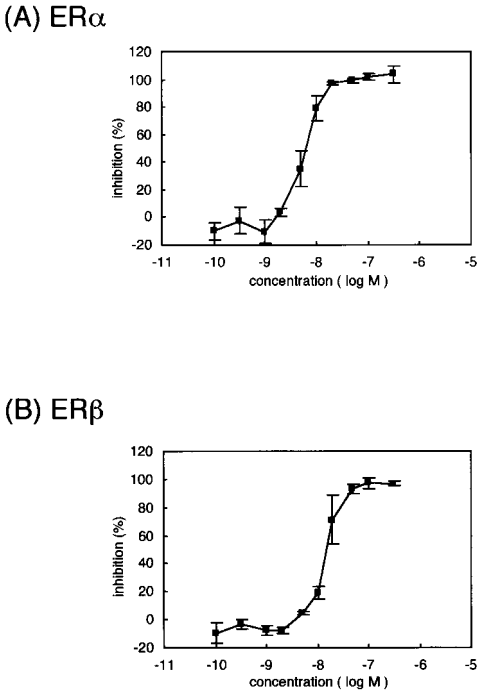

Fig. 3. Standard Curve of DES Obtained by Competitive Enzyme Immunoassay

The inhibition curves against DES for quail ERα (A) and ERβ (B) are shown. Values are the mean and standard deviation ($n = 4$).

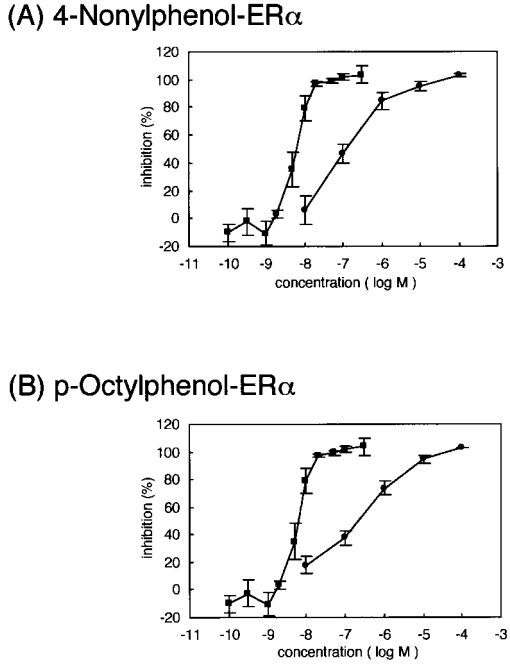

Fig. 4. Inhibition Curves of 4-Nonylphenol and *p*-Octylphenol for Quail ERα Determined by Competitive Enzyme Immunoassay

The squares show the inhibition curves of DES. The circles show the inhibition curves of 4-nonylphenol (A) and *p*-octylphenol (B). Values are the mean and standard deviation ($n = 4$).

was next applied to the test chemicals. MoE listed 65 chemicals suspected of having endocrine-disrupting effects in SPEED'98. Of these, 12 and 8 chemicals were selected in 2000 and 2001, respectively, as high-priority chemicals to be tested. Therefore, we next tested the capacity of these 20 chemicals to bind quail ERs using the competitive EIA developed above. Figures 4 and 5 show the inhibition curves of 4-nonylphenol and *p*-octylphenol for quail ERα and ERβ, respectively. The results for 20 chemicals were summarized by showing the IC_{50} or REC20 in Table 1. Among those chemicals tested, 4-nonylphenol, p-octylphenol and bisphenol A exhibited relatively strong binding to both quail ERα and ERβ, although in each case the IC_{50} was 10^{-7} M and the RBA was less than 10%.

The competitive EIA is based on the antigen-antibody reaction. If the test chemical inhibits this reaction, a false positive result might be obtained. Therefore, it is necessary to check the inhibitory effect of the test chemical on the antigen-antibody interaction. Figures 6–8 show the inhibitory effects on the immunoreaction as well as the results for the competitive EIA for ERα and ERβ. Bisphenol A (Fig. 6) had the capacity to bind quail ERα and ERβ, although an inhibitory effect on the immunoreaction

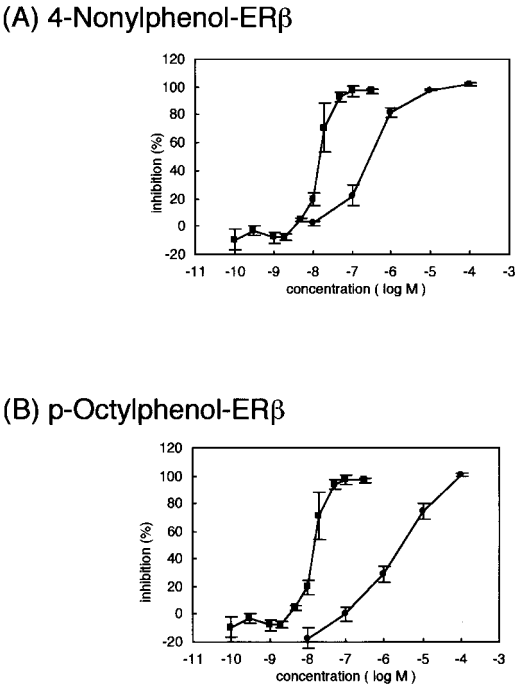

Fig. 5. Inhibition Curves of 4-Nonylphenol and *p*-Octylphenol for Quail ERβ Determined by Competitive Enzyme Immunoassay

The squares show the standard curves of DES. The circles show the inhibition curves of 4-nonylphenol (A) and *p*-octylphenol (B). Values are the mean and standard deviation ($n = 4$).

Table 1. The Relative Binding Affinity of Test Chemicals to Quail ERα and ERβ Evaluated by Competitive Enzyme Immunoassay

		qERα			qERβ		
	Chemicals	IC_{50} (M)	REC20 (M)	RBA (%)	IC_{50} (M)	REC20 (M)	RBA (%)
	DES	6.0×10^{-9}		100	1.3×10^{-8}		100
1	Benzophenone		$>10^{-4}$			$>10^{-4}$	
2	Octachlorostyrene		$>10^{-4}$			4.0×10^{-5}	
3	Diethyl phthalate		$>10^{-4}$			$>10^{-4}$	
4	Benzyl-n-butyl phthalate	2.0×10^{-5}		0.030	9.0×10^{-5}		0.014
5	Bis(2-ethylhexyl) adipate		$>10^{-4}$			$>10^{-4}$	
6	Triphenyltin chloride	1.0×10^{-5}		0.060	1.0×10^{-5}		0.130
7	Bis(2-ethylhexyl) phthalate	3.0×10^{-6}		0.200		2.0×10^{-5}	
8	Dicyclohexyl phthalate	1.0×10^{-4}		0.006		9.0×10^{-6}	
9	Di-n-butyl phthalate		$>10^{-4}$			$>10^{-4}$	
10	Tributyltin(IV) chloride	2.0×10^{-5}		0.030		2.0×10^{-5}	
11	4-Nonylphenol	1.0×10^{-7}		6.00	3.0×10^{-7}		4.30
12	p-Octylphenol	1.0×10^{-7}		6.00	3.0×10^{-6}		0.430
	DES	4.7×10^{-9}		100	1.2×10^{-8}		100
13	Bisphenol A	3.0×10^{-7}		1.57	1.8×10^{-7}		6.67
14	2,4-Dichlorophenol	1.0×10^{-5}		0.047		2.8×10^{-5}	
15	4-Nitrotoluene		$>10^{-4}$			$>10^{-4}$	
16	Di-n-pentyl phthalate		$>10^{-4}$			$>10^{-4}$	
17	Di-n-propyl phthalate		$>10^{-4}$			$>10^{-4}$	
18	Pentachlorophenol		5.0×10^{-5}			1.9×10^{-5}	
19	Amitrole		$>10^{-4}$			$>10^{-4}$	
20	Di-n-hexyl phthalate		$>10^{-4}$			$>10^{-4}$	

IC_{50} (M): the concentration giving 50% inhibition when the inhibition by 112 nM DES is 100%. REC20 (M): 20% relative effective concentration when percent inhibition was less than 50%. RBA (%): relative binding affinity was obtained by dividing the IC_{50} of DES by that of the IC_{50} at test sample. Since the experiments of Nos. 1–12 and Nos. 13–20 were performed separately, IC_{50} for DES were shown in each experiment.

was also obtained at a higher concentration.

On the other hand, the percent inhibition by pentachlorophenol was similar to the inhibitory effect on the immunoreaction, indicating that this chemical had no capacity to bind ERs at the concentration tested (Fig. 7). In the case of 2, 4-dichlorophenol, a slight binding capacity was obtained only for ERα (Fig. 8). Both triphenyltin and tributyltin exhibited the weak binding, but had the same inhibitory effect on the immunoreaction (data not shown). Thus, it is necessary to check whether test chemicals having the capacity to bind ERα or ERβ exhibit an inhibitory effect on the immunoreaction.

Development of the Fluorescence Polarization Method for Assaying the Capacity to Bind Quail ERs

A kit for the fluorescence polarization method for human ERα and ERβ is available. Therefore, by replacing human ERs with quail ERs, we established a fluorescence polarization method for assaying the capacity to bind quail ERs. First, we determined the optimum (saturating) concentration of quail ERs to be 100 nM ERα and 50 nM ERβ with Fluomone ES1. Under these conditions, we established the fluorescence polarization method, and obtained a standard curve and the inhibition curve of 4-nonylphenol and *p*-octylphenol (Figs. 9 and 10, respectively). Moreover, 12 chemicals selected in 2000 by MoE were tested. The results on binding capacity were basically the same as those obtained by competitive EIA (data not shown).

DISCUSSION

As a method for screening EDCs, *in vitro* binding analysis is a quite useful. So far, the capacity of EDCs to bind human ERα and ERβ has been well characterized. Recently, it was pointed out that the effects of EDCs on not only humans but also wildlife should be clarified. For this purpose, a cheap

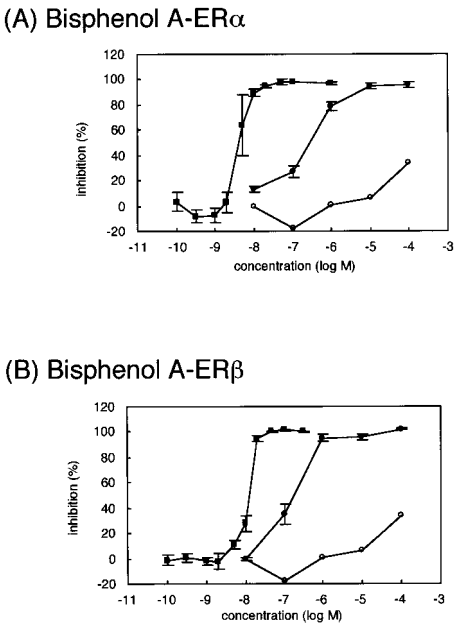

Fig. 6. Inhibition Curves of Bisphenol A for Quail ERα and ERβ Determined by Competitive Enzyme Immunoassay and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of bisphenol A for ERα (A) and ERβ (B). The open circles show the inhibitory effect of bisphenol A on the antigen-antibody reaction. Values are the mean and standard deviation ($n = 4$).

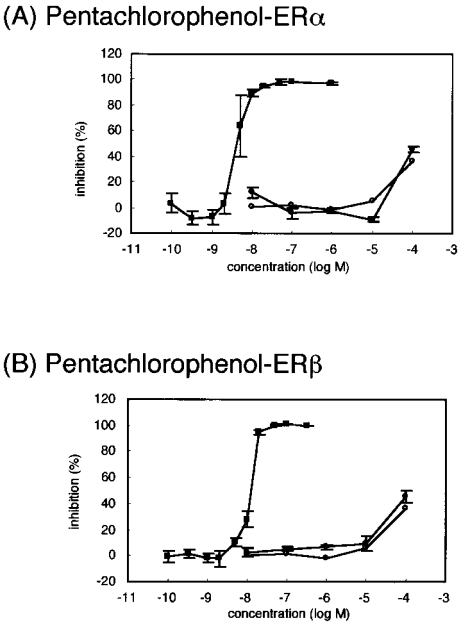

Fig. 7. Inhibition Curves of Pentachlorophenol for Quail ERα and ERβ Determined by Competitive Enzyme Immunoassay and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of pentachlorophenol for ERα (A) and ERβ (B). The open circles show the inhibitory effect of pentachlorophenol on antigen-antibody reaction. Values showed the mean and standard deviation ($n = 4$).

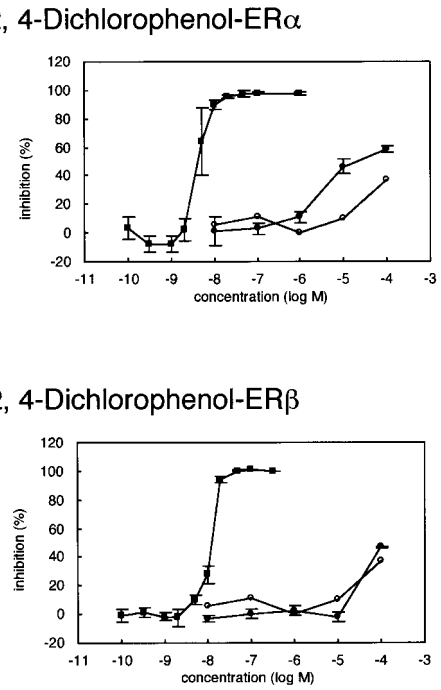

Fig. 8. Inhibition Curves of 2, 4-Dichlorophenol for Quail ERα and ERβ Determined by Competitive Enzyme Immunoassay and the Inhibitory Effect on the Antigen-Antibody Reaction

The squares show the standard curves of DES. The closed circles show the inhibition curves of 2, 4-dichlorophenol for ERα (A) and ERβ (B). The open circles show the inhibitory effect of 2, 4-dichlorophenol on the antigen-antibody reaction. Values are the mean and standard deviation ($n = 4$).

and easy screening method was required. However, competitive assays using radiolabeled estrogen need special equipment and handling. Although a kit for fluorescence polarization is available for human ERα and ERβ, this system also needs special equipment. The yeast two-hybrid system is superior in terms of cost and handling. However, since the cofactor of each species is necessary, it has disadvantages for the evaluation of the binding capacity for many species of ERs.

Competitive EIAs for detecting the ability of EDCs to bind ERs have several advantages. First, it requires neither special equipment nor special techniques. Second, it costs less. Therefore, once the recombinant ERs are ready, the assay system can be established quickly for ERs in all species. Although quail is used as a test bird for the evaluation of EDCs, an *in vitro* binding assay system for birds is yet to be established. In this report, we have established a competitive EIA for the detection of EDCs bound to quail ERα and ERβ. Using this system, we evaluated the 20 test chemicals selected by MoE.

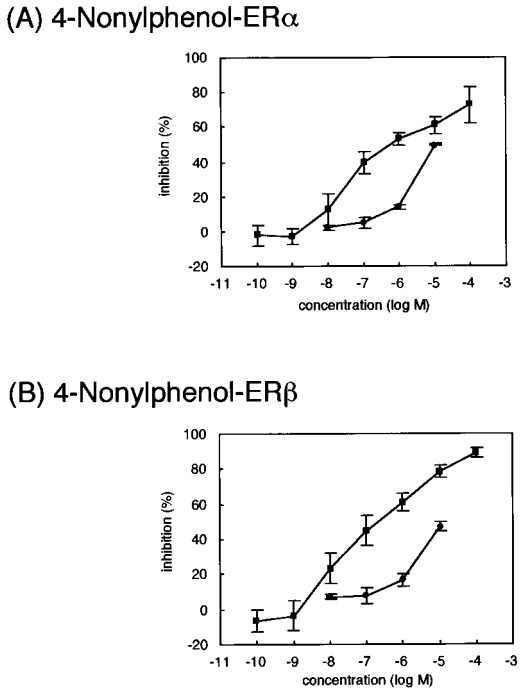

Fig. 9. Standard Curve of E2 and Inhibition Curve of 4-Nonylphenol Obtained by the Fluorescence Polalization Method

The squares show the standard curve of E2. The circles show the inhibition curves of 4-nonylphenol for ERα (A) and ERβ (B). Values are the mean and standard deviation (n = 3).

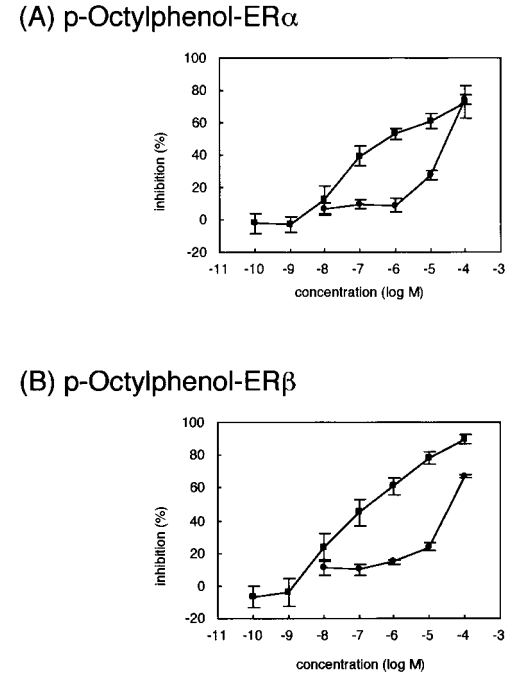

Fig.10. Standard Curve of E2 and Inhibition Curve of p-Octylphenol Obtained by the Fluorescence Polalization Method

The squares show the standard curve of E2. The circles show the inhibition curves of p-octylphenol for ERα (A) and ERβ (B). Values are the mean and standard deviation (n = 3).

The *in vitro* binding method for birds presented in this paper seems to be well developed, since the relative affinity concentrations of 20 test chemicals in this assay system are well interrelated with those obtained by the fluorescence polarization method for human ER,[5] the yeast two-hybrid assay using rat ER and rat cofactor,[6,7] and the radio-competitive assay for fish.[5] Moreover, the fold differences of the binding concentration between E2 and test chemicals including 4-nonylphenol, *p*-octylphenol and bisphenol A are basically the same when compared with those obtained by *in vivo* studies, which is 3-day uterotrophic assay in rats.[13]

However, the species specificity was also observed. Unfortunately, it is hard to discuss with this specificity in detail, because all *in vitro* binding assays estabilished previously have shown the relative binding activity to E2 or DES, and the binding affinity of E2 and DES itself to ER are not characterized yet. Therefore, for characterization of the species specificity, the affinity constant itself of E2, DES and other test chemicals including 4-nonylphenol, *p*-octylphenol and bisphenol A are definitely needed. We are now establishing an assay system for use in frogs as well as humans and medaka, and also the affinity constant of several chemicals having estrogenic activity for evaluating species-specific effects of EDCs.

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