Development of Standardized *in Vitro* Assay System for Estrogen Receptors and Species Specificity of Binding Ability of 4-Nonylphenol and *p*-Octylphenol

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The *in vitro* binding assay seems to be a useful first screening method for endocrine disrupting chemicals. The various methods have been developed and applied to the testing of chemicals. Although these assays should be applied to estrogen receptors (ER) of not only humans but also wildlife, a standardized system is yet to be established. Furthermore, a method for *Xenopus* ER is not yet developed. We previously expressed the ligand-binding domain (LBD) of quail ERα and ERβ as a fusion protein with glutathione S-transferase, and developed a competitive enzyme immunoassay for detecting the capacity of chemicals to bind ERs. It seems that this system is a powerful tool, since it needs no special equipment. In this report, we first produced ER-LBD protein of human, *Xenopus* and medaka as well as quail. Then, we established a competitive enzyme immunoassay for these ERs as a standardized method, and compared the species specificity of the ability of 4-nonylphenol and *p*-octylphenol to bind ERs. Although a significant difference was not detected among ERβ of human, quail and medaka, 4-nonylphenol and *p*-octylphenol exhibited the higher affinity for the medaka ERα than human ERα. These results indicate the species specificity of the capacity of chemicals to bind ERs.

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

INTRODUCTION

Endocrine disrupting chemicals (EDCs) also called endocrine disruptors (EDs) seem to be substances affecting reproductive functions through the estrogen-estrogen receptor (ER) signaling pathway.[1,2] Although the mechanisms of disruption are not fully understood, screening methods are definitely needed. Indeed, *in vivo* approaches including a one-generation study and uterotrophic assay, and *in vitro* techniques including a receptor binding assay and reporter gene assay 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. For initial screening *in vitro*, the receptor binding assay is often utilized. Using the fluorescence polalization method, the capacity of chemicals to bind human ERα and ERβ was evaluated. The Japanese medaka (*Oryzias latipes*) ERα and ERβ were tested using a radio-competitive assay (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html). We previously established the competitive enzyme immunoassay (EIA) for analysis of the capacity to bind quail ERα and ERβ, and then tested the chemicals selected by MoE.[5]

Recently, it has been proposed that the effects of EDCs are considered not only in humans but also in wildlife, such as birds, frogs and fish, and indeed adverse effects on human and wildlife were reported. However, a standardized method is not yet established. Moreover, a method for use with frog ER

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has not been developed. The Organization for Economic Cooperation and Development (OECD) and MoE have started the development of various test methods for humans, fish, reptiles, amphibians, and birds (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html, OECD; http://www.oecd.org/home/).[6] For this purpose, a cheap and easy screening method was required. However, the competitive assay system using radiolabeled estrogen needs special equipment and regulation for handling. Although a kit for the fluorescence polarization method for human ERα and ERβ is available, this system also needs special equipment. The yeast two-hybrid system is a superior method in terms of cost and handling.[7,8] However, since a cofactor is needed for each species, it is not suitable for an evaluation of the binding to many species of ERs.

The competitive EIA for detecting the ability of EDCs to bind ERs has several advantages. First, it needs no special equipment and no special techniques. Second, it is less expensive than other methods. Therefore, once the recombinant ERs are ready, the assay system can be established quickly for ERs in all species.

In this report, we first established a competitive EIA system for *Xenopus laevis* ER. Furthermore, we developed similar assay systems for ERs in human and medaka as well as quail. Using these standardized assay systems, we evaluated the species specificity of the binding of 4-nonylphenol and *p*-octylphenol, which are known as EDCs, to various ERs.

MATERIALS AND METHODS

Chemicals —— 4-Nonylphenol and *p*-octylphenol were supplied by Dr. Kawashima at JAPAN NUS Co., Ltd. (Tokyo, Japan), where large amounts of chemicals of reagent grade have been stocked for various tests at the request of MoE. These chemicals were dissolved in dimethyl sulfoxide (DMSO). All other chemicals are of reagent grade.

Plasmid Construction —— A DNA fragment containing the ligand-binding domain (LBD) of *Xenopus* ER (721–1758 bp and 241–586 aa in open reading frame) was subcloned into the *Eco*RI-*Sal*I sites in pGEX-4T-1, coding glutathione S-transferase (GST) (Amersham Biosciences Corp., U.S.A.). Finally, the recombinant plasmid was introduced into *Escherichia coli* (*E. coli*), BL21 (DE3) (Novagen, EMD Biosciences Inc., Germany). The construction

of pGEX-4T-1-ERα- and ERβ-LBD for human and quail was described previously.[5,9] pGEX-4T-1-ERα- and ERβ-LBDs for medaka were a gift from Dr. Nakai at Chemicals Evaluation and Research Institute, Japan. Thus, pGEX-4T-1 was used for all constructs for the expression of ER-LBDs.

Expression and Purification of GST-ER Fusion Protein —— BL21 harboring pGEX-4T-1-ERα- or ERβ-LBD was cultured in LB medium at 30°C. At $OD_{600} = 0.4$, isopropyl-1-thio-β-D-galacto pyranoside (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]. 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 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-ER-LBDs, 0.42 ml of GSH-Sepharose 4B (Amersham Biosciences Corp., U.S.A.) 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 principle of *in vitro* binding assay for ERs is based on the enzyme-linked competitive immunoassay. This assay was performed using the kit, Ligand Screening System-ERα or ERβ, (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The only change was the replacement of the human ER in the kit with GST-ERα-LBD or ERβ-LBD of human, quail, *Xenopus* and medaka. 17β-Estradiol (E2) and diethylstilbestrol (DES) were diluted with DMSO at a concentration of $8/3 \times 10^{-4}$–$8/3 \times 10^{-8}$ M, and further diluted with the dilution buffer attached in the kit at a final concentration of $8/3 \times 10^{-6}$–$8/3 \times 10^{-10}$ M (final DMSO concentration, 1%). The test chemicals were diluted in the same way at a final concentration of $8/3 \times 10^{-4}$–$8/3 \times 10^{-8}$ M.

The assay was done according to the manufacturer's instructions. The protocol consists of three steps as follows. Step 1: receptor-ligand binding; 20 μl (0.45–12.6 pmol; see RESULTS in detail) of GST-ER in B-1 buffer including 20 mM GSH described above, 30 μl of various amounts of test

chemicals or standard DES in the dilution buffer attached in the kit, and 30 μl of E2 ($8/3 \times 4$ nM) in the dilution buffer attached in the kit were mixed and incubated for 1 hr on ice. Step 2: antigen-antibody reaction; after the incubation, 50 μl out of 80 μl of the mixture was transferred to an E2-coated plate, and 50 μl of E2-horse radish peroxidase (HRP) solution was added. The mixture was incubated for 1 hr on ice. Step 3: enzyme reaction; the plate was washed and the enzyme reaction was done at 37°C for 20 min. The absorbance at 450 nm was measured with a 1420 ARVO Multilabel Counter (Wallac, Gaithersburg, U.S.A.).

The % inhibition of binding was calculated as follows: $(A_{\text{DMSO control}}-A_{\text{test sample}})/(A_{\text{DMSO control}}-A_{\text{DES at 112 nM}}) \times 100$. 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 the IC_{50} of the test sample.

RESULTS

Comparison of Amino Acid Sequences of ER-LBDs in Various Species

In the nuclear hormone receptors, DNA binding domains (DBDs) were found in the N-terminal half, and LBD were located in the C-terminal half. The nucleotide and amino acid sequences of ER-DBDs were well conserved among human, quail, *Xenopus*, and medaka. The similarity of amino acid sequences is more than 95%. On the other hand, the similarity of amino acid sequences of ER-LBDs was slightly lower among the four species. As shown in Fig. 1, both ERα and ERβ of medaka revealed less similarity than the other species, suggesting that the characteristics of ligand binding might be different. Only one form of *Xenopus* ER was identified. Although the amino acid sequences of *Xenopus* ER show 99% similarity to DBDs of ERα and ERβ in human and quail, these show 85 and 87% similarity to LBDs of ERα in human and quail, respectively (Fig. 1). On the other hand, it shows only 59 and 60% similarity to LBDs of ERβ in human and quail, respectively. Therefore, *Xenopus* ER seems to be ERα.

Expression and Purification of GST-ER Fusion Protein

For the development of an *in vitro* binding assay system for *Xenopus* ERα, we first expressed the LBD of *Xenopus* ERα as a GST fusion protein in *E.*
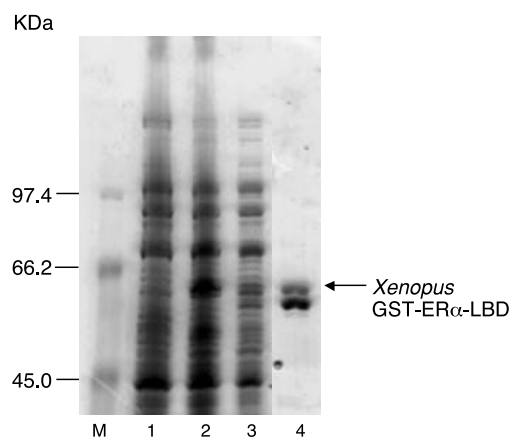

Fig. 1. Similarity of Amino Acids in the Ligand Binding Domain of ERα and ERβ in Various Species

(A); ERα, (B); ERβ. The left panels show a schematic representation of DBD and LBD in ERs. The number of amino acids is shown. The right panels show the % similarity of amino acids in LBD in various species.

Fig. 2. Expression and Purification of *Xenopus* GST-ERα-LBD

The *Xenopus* GST-ERα-LBD fusion protein was expressed in *E. coli* BL21 (DE3), and then purified using GSH-Sepharose. The samples were loaded on 8% SDS-polyacrylamide gel, and stained with coomassie brilliant blue. Lane M: size marker; Lanes 1: whole protein solubilized from untreated cells; Lanes 2: whole protein solubilized from IPTG-treated cells; Lanes 3: soluble fraction from IPTG-treated cells; Lanes 4: purified GST-ERα-LBD from soluble fraction. The arrow shows a band for *Xenopus* GST-ERα-LBD. The band below the GST-ERα-LBD is a nonspecific band.

coli. With the addition of IPTG, GST-ERα-LBD was induced and recovered partly in the soluble fraction (Fig. 2). This soluble fraction was applied to a GSH-Sepharose column and eluted with GSH. The purity
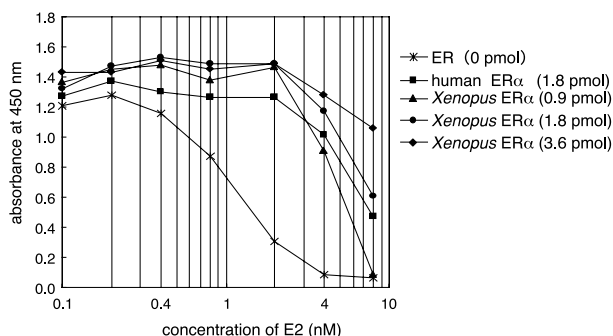
Fig. 3. Measurement of the Amount of Free Ligand in the Presence of *Xenopus* GST-ERα-LBD

For determination of the most suitable amount of GST-ERα-LBD used, the free ligand test was performed using various amounts of GST-ERα-LBD. As a control, human ERα was used.

and amount of the resultant purified GST-*Xenopus* ERα-LBD (MW = 63.8 kDa) was checked by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Although the purified GST-*Xenopus* ERα-LBD was identified by the position of induced proteins by IPTG and the molecular mass and also the purification fold, the nonspecific protein was also co-purified. This nonspecific protein was always recovered with the purification of GST-ERs for other species, and found at the same position in SDS-PAGE when GSH-Sepharose column was used for purification. It seems that this protein does not have any effect on the competitive enzyme immunoassay.[5] GST-ER-LBDs for other species were expressed and purified in the same way.
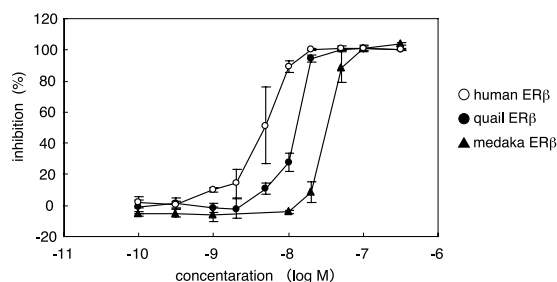
Development of Competitive Enzyme Immunoassay for *Xenopus* ERα and ERs in Other Species

For the development of a competitive EIA for *Xenopus* ERα, the optimum amount of GST-ERα-LBD used should be determined. Using various amounts of E2 and *Xenopus* GST-ERα-LBD, the amount of free ligand which does not make a complex with GST-ERα-LBD was determined (Fig. 3). Since the final concentration of E2 used in the kit is 4 nM, it is required that up to 4 nM of E2 be trapped with GST-ERα-LBD, whereas the E2 above 4 nM exists free. As shown in Fig. 3, when used 1.8 pmol/well, 2 nM of E2 was completely trapped, while small amounts of E2 exists free in 4 nM of E2. In this regard, the optimum amount of *Xenopus* ERα used was determined as 1.8 pmol/well. In the same way, the optimum amounts of ERs in other species were also determined. The amounts determined were
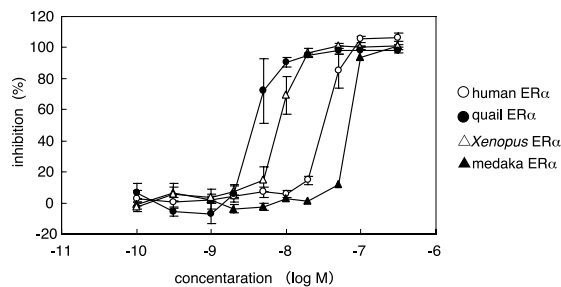
Fig. 4. Standard Curves of DES Obtained by Competitive Enzyme Immunoassay

The inhibition curves against DES for ERα (A) and ERβ (B) are shown. Values are the mean and standard deviation (*n* = 3–4).

0.45, 3.6, 12.6, 3.6, 3.6 and 7.2 pmol/well for human ERα and ERβ, quail ERα and ERβ and medaka ERα and ERβ, respectively.

Next, we developed the competitive EIA using these amounts of recombinant ERs. The standard curve for *Xenopus* ERα was made as percent inhibition when that by 112 nM DES was 100%. Those for other ERs were made in the same way. In Fig. 4, the standard curves of ERα in four different species and of ERβ in three different species are shown. IC_{50}s of DES for various ERs are listed in Table 1. The quail ERα and medaka ERα showed the highest and lowest affinities, 3.8×10^{-9} M and 6.5×10^{-8} M, respectively. Thus, IC_{50} values among the four species and two types of ER revealed differences of one order of magnitude.

Comparison of Binding Capacity of 4-Nonylphenol and *p*-Octylphenol for ERs in Four Species

In the previous report, 4-nonylphenol and *p*-octylphenol revealed relatively strong binding to both quail ERα and ERβ.[5] Using this competitive enzyme immunoassay system, we next tested the capacity of 4-nonylphenol and *p*-octylphenol to bind 7 kinds of ERs. The results are shown in Figs. 5 and

Table 1. The Relative Binding Affinity of 4-Nonylphenol and *p*-Octylphenol for Various ERα and ERβ Determined by Competitive Enzyme Immunoassay

(A) ERα

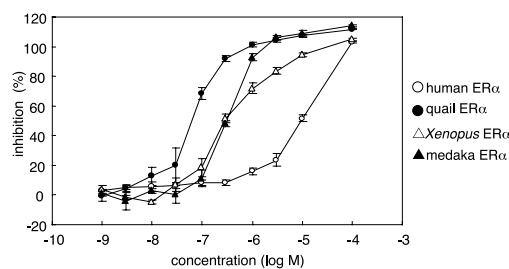|	DES	
	IC$_{50}$ (M)	RBA (%)
human	$3.2 \times 10^{-8} \pm 3.0 \times 10^{-9}$	100
quail	$3.8 \times 10^{-9} \pm 1.0 \times 10^{-9}$	100
Xenopus	$7.8 \times 10^{-9} \pm 9.2 \times 10^{-10}$	100
medaka	$6.5 \times 10^{-8} \pm 1.5 \times 10^{-9}$	100
	NP	
	IC$_{50}$ (M)	RBA (%)
human	$9.2 \times 10^{-6} \pm 1.1 \times 10^{-6}$	0.35 ± 0.047
quail	$6.0 \times 10^{-8} \pm 9.7 \times 10^{-9}$	6.4 ± 1.9
Xenopus	$2.9 \times 10^{-7} \pm 3.6 \times 10^{-8}$	2.8 ± 0.43
medaka	$3.1 \times 10^{-7} \pm 1.2 \times 10^{-8}$	21 ± 0.98
	OP	
	IC$_{50}$ (M)	RBA (%)
human	$1.4 \times 10^{-5} \pm 1.0 \times 10^{-6}$	0.23 ± 0.030
quail	$1.3 \times 10^{-7} \pm 2.3 \times 10^{-8}$	3.3 ± 1.2
Xenopus	$1.3 \times 10^{-6} \pm 1.5 \times 10^{-7}$	0.63 ± 0.091
medaka	$1.9 \times 10^{-7} \pm 6.3 \times 10^{-8}$	34 ± 2.4

(B) ERβ

	DES	
	IC$_{50}$ (M)	RBA (%)
human	$5.3 \times 10^{-9} \pm 1.8 \times 10^{-9}$	100
quail	$1.3 \times 10^{-8} \pm 4.2 \times 10^{-10}$	100
medaka	$3.2 \times 10^{-8} \pm 2.3 \times 10^{-9}$	100
	NP	
	IC$_{50}$ (M)	RBA (%)
human	$5.7 \times 10^{-7} \pm 1.3 \times 10^{-8}$	0.83 ± 0.33
quail	$5.1 \times 10^{-7} \pm 3.5 \times 10^{-8}$	2.5 ± 0.19
medaka	$7.3 \times 10^{-7} \pm 7.2 \times 10^{-8}$	4.5 ± 0.79
	OP	
	IC$_{50}$ (M)	RBA (%)
human	$4.9 \times 10^{-7} \pm 2.7 \times 10^{-8}$	0.98 ± 0.41
quail	$9.9 \times 10^{-7} \pm 2.3 \times 10^{-7}$	1.3 ± 0.32
medaka	$1.4 \times 10^{-6} \pm 1.3 \times 10^{-7}$	2.3 ± 0.093

RBA (%) = (DES IC$_{50}$)/(test sample IC$_{50}$) × 100.

(A) 4-Nonylphenol-ERα

(B) 4-Nonylphenol-ERβ

Fig. 5. Inhibition Curves of 4-Nonylphenol for Various ERα and ERβ Obtained by Competitive Enzyme Immunoassay
Values are the mean and standard deviation (n = 3–4).

(A) *p*-Octylphenol-ERα

(B) *p*-Octylphenol-ERβ

Fig. 6. Inhibition Curves of *p*-Octylphenol for Various ERα and ERβ Obtained by Competitive Enzyme Immunoassay
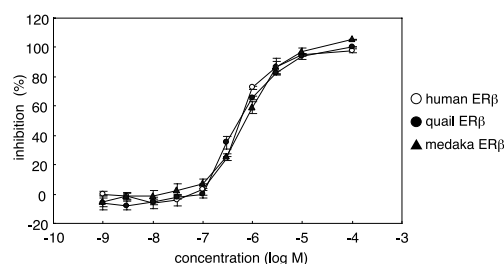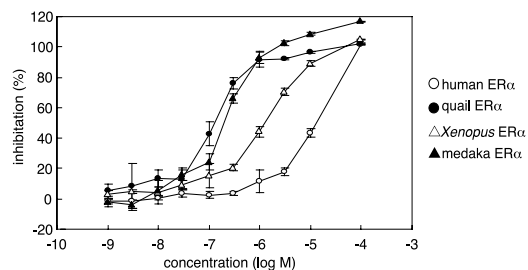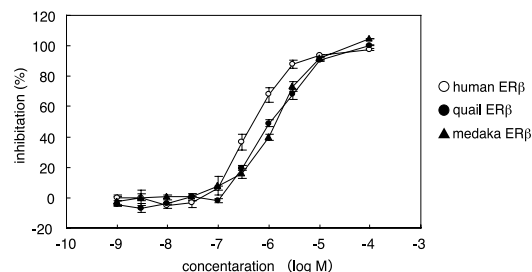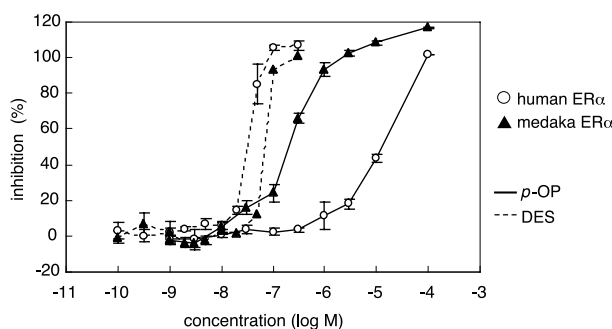Values are the mean and standard deviation (n = 3–4).

6. Both 4-nonylphenol and *p*-octylphenol bound to ERβ of human, quail and medaka with similar affinity. On the other hand, in the case of ERα, different binding patterns were obtained. In Fig. 5A, 4-nonylphenol bound to quail ERα with higher affinity, whereas it bound to human ERα with lower affinity. Almost the same pattern was obtained in the binding of *p*-octylphenol to ERα (Fig. 6A).

The results are summarized by showing the IC$_{50}$ and RBA (relative binding affinity) (%) in Table 1. The RBA (%) values were lower than 7% in all cases,

except for medaka ERα which showed 21 and 34% for 4-nonylphenol and *p*-octylphenol, respectively. Thus, 4-nonylphenol and *p*-octylphenol seem to have a strong ability to bind medaka ERα. Fig. 7 shows the inhibition curves of DES and *p*-octylphenol for ERα in human and medaka. These data were from Figs. 4 and 6. Although the affinity of DES for ERα in human and medaka was almost the same, that of *p*-octylphenol was not, indicating that *p*-octylphenol bound to medaka ERα with higher affinity than human ERα. On the other hand, in the case of quail ERα, the inhibition curve of *p*-octylphenol revealed a similar pattern to medaka ERα. However, the patterns of DES were different between quail and medaka ERα, resulting in the marked difference in RBA (%) (Fig. 8, Table 1). These results indicate that there is species specificity in the ability of not only 4-nonylphenol and *p*-octylphenol but also DES to bind ERs.

DISCUSSION

As a first screening method for the evaluation of EDCs, *in vitro* binding analyses are very useful, and many methods have been developed. These include the yeast two-hybrid assay, fluorescence polarization method, radio-competitive assay and competitive EIA. Although the capacity to bind human ERα and ERβ is well characterized, it is pointed out that the effects of EDCs on not only humans but also wildlife should be clarified.

We have previously established a competitive EIA for the detection of EDCs bound to quail ERα and ERβ. Using this system, we evaluated 20 test chemicals selected by MoE.[5] The competitive EIA has several advantages described above. Since no *in vitro* binding assay has been established for the frog, we first developed a competitive EIA for *Xenopus* ERα. Then, we established similar systems for human and medaka ERα and ERβ. The only difference among these systems is GST-ER-LBD. Therefore, once the GST-ER-LBD of other species is available, the development of a species specific EIA for ERs would seem to be very easy.

Next, we characterized the species specificities of the binding of 4-nonylphenol and *p*-octylphenol to various ERs, since there is no report of a comparison of the binding affinity of 4-nonylphenol and *p*-octylphenol for various ERs using the same protocol. No significant differences in the ability of 4-nonylphenol and *p*-octylphenol to bind ERβ in hu-

Fig. 7. Inhibition Curves of *p*-Octylphenol for Human and Medaka ERα Obtained by Competitive Enzyme Immunoassay

The solid and dotted lines show inhibition curves of *p*-octylphenol and DES, respectively. Values are the mean and standard deviation ($n = 3$–4).
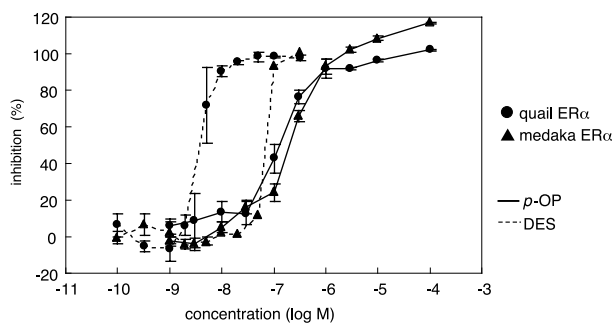
Fig. 8. Inhibition Curves of *p*-Octylphenol for Quail and Medaka ERα Obtained by Competitive Enzyme Immunoassay

The solid and dotted lines show inhibition curves of *p*-octylphenol and DES, respectively. Values are the mean and standard deviation ($n = 3$–4).

man, quail and medaka were observed. However, in the case of medaka ERα, the RBA (%) was a large value compared with that of ERα in human, quail and *Xenopus*. These results are partly due to the sequence difference with medaka ERα. However, it should be noted that RBA (%) is a relative binding affinity compared with the IC_{50} for DES. IC_{50}s obtained from inhibition curves for 4-nonylphenol and *p*-octylphenol revealed higher values for human ERα in comparison with those for quail, *Xenopus* and medaka (Table 1 and Figs. 5 and 6).

It is also pointed out that IC_{50}s obtained from standard curves for DES to ERα and ERβ in various species showed slight differences in binding abilities. These data strongly suggest that the binding affinities of E2, DES, 4-nonylphenol and *p*-octylphenol for ER, especially ERα, might differ in a species-specific manner. The reports presented

previously are all based on the relative binding affinity for E2 or DES. Therefore, for further analyses of the binding of chemicals to ERs, a comparison of the affinity constant of the chemical itself as well as E2 and DES for the various ERs is required.

REFERENCES

1) Crews, D., Willingham, E. and Skipper, J. K. (2000) Endocrine disruptors: present issues, future directions. *Q. Rev. Biol.*, **75**, 243–260.
2) Hutchinson, T. H., Brown, R., Brugger, K. E., Campbell, P. M., Holt, M., Lange, R., McCahon, P., Tattersfield, L. J. and van Egmond, R. (2000) Ecological risk assessment of endocrine disruptors. *Environ. Health Perspect.*, **108**, 1007–1014.
3) Kavlock, R. J. (1999) Overview of endocrine disruptor research activity in the United States. *Chemosphere*, **39**, 1227–1236.
4) Iguchi, T., Sumi, M. and Tanabe, S. (2002) Endocrine disruptor issues in Japan. *Congenit. Anom.* (Kyoto), **42**, 106–119.
5) Maekawa, S., Nishizuka, M., Heitaku, S., Kunimoto, M., Nishikawa, J., Ichikawa, K., Shimada, K. and Imagawa, M. (2004) Development of a competitive enzyme immunoassay for detection of capacity of chemicals to bind quail estrogen receptor α and β. *J. Health Sci.*, **50**, 25–32.
6) Baker, V. A. (2001) Endocrine disrupters-testing strategies to assess human hazard. *Toxicol. In Vitro*, **15**, 413–419.
7) Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M. and Nishihara, T. (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmcol.*, **154**, 76–83.
8) Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, H. (2000) Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci.*, **46**, 282–298.
9) Kanayama, T., Mamiya, S., Nishihara, T. and Nishikawa, J. (2003) Basis of a high-throughput method for nuclear receptor ligands. *J. Biochem.*, **133**, 791–797.