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Binding of Xenoestrogens and Phytoestrogens to Estrogen Receptor β of Japanese Quail (*Coturnix japonica*)

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In order to examine the binding affinity of various estrogenic compounds to estrogen receptor β (ERβ), the cDNA encoding the hinge domain, the ligand-binding domain, and the C-terminal domain of quail ERβ was constructed and transfected into competent *Escherichia coli.* The binding assay performed using the supernatant of the cell lysates showed that bacterially-expressed ERβ has one single class of binding site for estradiol-17β with a dissociation constant of $4.90 \pm 0.16 \times 10^{-9}$ M. The competition studies indicated that the relative binding affinities for the synthetic estrogens, diethylstilbestrol and ethinyl estradiol, are very high, while those for the xenoestrogens, bisphenol A and nonylphenol, are very low. Coumestrol, known as one of phytoestrogens, can compete with estradiol-17β with higher binding affinity for ERβ than ERα.

Key words : endocrine-disrupting chemicals, estrogen receptor β, Japanese quail, phytoestrogen, xenoestrogen

Introduction

A variety of synthetic chemicals found in the environment have the capacity to alter the endocrine function and development in humans, wild animals, and domestic animals. These chemicals are called endocrine-disrupting chemicals (McLachlan, 2001). Much attention has been focused on estrogenic activity of endocrine-disrupting chemicals, which trigger their effects through the binding to estrogen receptor (ER) (Fielden *et al.,* 1997 ; Hiroi *et al.,* 1999 ; Loomis and Thomas, 2000).

In avian species like as in mammals, subtypes of ER, ERα and ERβ, have been identified, and the expression of ERβ mRNA was detected in liver, kidney, ovary, and oviduct of Japanese quail (Foidart *et al.,* 1999) and in gonads of chicken embryo (Sakimura *et al.,* 2002). We previously reported that bacterially-expressed quail ERα showed strong affinity to synthetic estrogens but xenoestrogens can merely compete with estradiol-17β (E_2) (Hanafy *et al.,* 2004). While E_2 bind to both ER subtypes with a comparable affinity, some estrogenic compounds show a different affinity for ERα and ERβ (Kuiper *et al.,* 1997 ; 1998 ; Bowers *et al.,* 2000).

The purpose of the present study was to examine the binding affinity of estrogenic

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chemicals to quail ERβ. A competitive binding study was undertaken using bacterially-expressed ERβ fusion protein. Estrogenic compounds we chose in this study were diethylstilbestrol and ethinyl estradiol for synthetic estrogens, which have been known to bind to ER as strong as natural estrogens in other species (Blair *et al.,* 2000 ; Tollefsen *et al.,* 2002), and two xenoestrogens, nonylphenol and bisphenol A, which are the most probable candidates for endocrine-disrupting chemicals (Hiroi *et al.,* 1999 ; Matthews and Zacharewski, 2000). We also examined the binding affinity of genistein and coumestrol, both are known as the phytoestrogens which are estrogenic compounds naturally present in plants in milligram order and a matter to current concern because of their beneficial effects for health (Jefferson *et al.,* 2002 ; Wuttke *et al.,* 2003 ; Greim, 2004).

Materials and Methods

Animal and Tissue Preparation

Female Japanese quail, 15–30 weeks of age (Tokai-Yuki, Toyohashi, Japan), were maintained individually under a photoperiod of 14L : 10D with the light on at 0500, and were provided with water and a commercial diet (Tokai-Kigyo, Toyohashi, Japan) *ad libitum.* The liver was removed after cervical dislocation and was flash-frozen in liquid nitrogen. All experimental procedures for the use and care of animals in the present study were approved by the animal care committee of the faculty of agriculture, Shizuoka university.

Chemicals

[2,4,6,7–^{3}H] E_2 (specific activity, 118.0 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). Unlabeled E_2, progesterone, testosterone, bovine serum albumin, diethylstilbestrol (4,4′-(1,2-diethyl-1,2-ethene-diyl)-bisphenol), ethinyl estradiol (17β-ethynyl-1,3,5(10)-estratriene-3,17β-diol), and genistein (4′,5,7-trihydroxyisoflavone) were obtained from Sigma (St. Louis, MO, USA). Bisphenol A (4,4′-isopropylidenediphenol) was obtained from ICN Biomedical (Aurora, OH, USA) and *p*-nonylphenol (a mixture of isomers of monoalkyl phenols) was obtained from Kanto Chemical (Tokyo, Japan). Coumestrol (3,9-dihydroxy-6H-benzofuro [3,2-c]-[1] benzopyran-6-one) was obtained from Helix Research (Springfield, OR, USA).

All other chemicals were of the highest quality available from commercial sources.

Generation of ERβ Expression Construct

Total RNA was extracted from a piece of liver with a commercial kit, ISOGEN (Nippon Gene, Tokyo, Japan), according to manufacturer′s instructions. The first-strand cDNA was synthesized from 2 μg of total RNA using the SuperScript First-Strand Synthesis System for an RT-PCR (Gibco BRL, Rockville, MD, USA) with oligo (dT)-primed reverse transcription. The entire cDNA products were amplified by polymerase chain reaction (30 cycles of which each cycle consisted of denaturation at 94℃ for 1 min, annealing at 61℃ for 1 min, and extension at 72℃ for 1 min) with 0.2 μM primers (forward : 5′-AAAAGAATTCCCGAATCCTGCGCCGCCATCG-TAAT-3′ and reverse : 5′-AAAAGTCGACTCAGACCTGGAAATGTGAA-3′) designed based on the reported cDNA sequence of quail ERβ (GenBank Database

accession number, AF045149 ; Lakaye *et al.,* 1998). An amplicon that included the hinge domain (D domain), the ligand-binding domain (E domain), and the C-terminal domain (F domain) was digested with *EcoRI* and *SalI* and ligated into the glutathione-S-transferase (GST) fusion protein expression vector, pGEX-6P-3 (Amersham Pharmacia Biotech, Piscateway, NJ, USA) treated with the same restriction enzymes. The resulting construct, qERβ-def, was transformed into competent *Escherichia coli* (*E. coli*), strain DH5α (Takara, Tokyo, Japan), and the ampicillin-resistant clone containing qERβ-def was selected after analysis of the nucleotide sequence was performed.

Expression of qERβ-def Fusion Protein

The *E. coli* containing qERβ-def were cultured overnight at 37℃ with constant shaking in 2 ml of LB medium supplemented with 100μg/m*l* ampicillin. The culture medium was diluted 25 times with LB medium and was grown to become an optical density of approximately 1.0 at 600 nm. To express GST-qERβ-def fusion protein in the cell, isopropylthio-β-D-galactoside at a final concentration of 0.1 mM was added to the medium, and the *E. coli* was incubated for 3 h at 28℃ with constant shaking. After the culture, the cell were collected by centrifugation at 10,000$\times$g for 1 min, and were suspended in buffer (pH 7.5) containing 50 mM HEPES, 3 mM EDTA, 5 mM DTT, 50 mM NaCl and 10% glycerol. The cells were lysed by sonication on ice 10 times each for 15 sec, separated by 10-sec intervals. Tween 20 was added to a final concentration of 0.1%, and the cell debris was incubated for 20 min at 4℃. Insoluble materials were removed by centrifugation at 10,000$\times$g for 10 min at 4℃, and clear supernatant was stored at -80℃ until use. The protein concentration of the cell lysates was determined by the method of Lowry *et al.* (1951).

Binding Assay

The binding assay was performed as previously described (Hanafy *et al.,* 2004). The equilibrium dissociation constant (Kd) was calculated as the slope of the line plotted by the method of Scatchard (1949). For the competition study, the specific binding was plotted as the percentage of $[^3H]E_2$ binding versus log of the molar concentration of test chemicals, and the relative binding affinity (RBA) was obtained by comparing the concentration of test chemicals causing 50% inhibition of $[^3H]E_2$ binding to that of E_2. All assays were replicated at least three times, and the RBA of the test chemicals are the means of the replicates.

Results and Discussion

The ERβ has been cloned in quail as the second subtype of ER (Foidart *et al.,* 1999). In order to investigate E_2 binding to ERβ, the specific binding of E_2 to the supernatant of the competent *E. coli* was analyzed by the Scatchard plot and the presence of a single class of saturable binding site was detected with Kd of $4.90\pm0.16\times10^{-9}$ M (Fig. 1). Although this value was approximately 4-folds lower than that of GST- ERα-def for E_2, which was 1.74×10^{-10} M in our previous study (Hanafy *et al.,* 2004), the Kd was comparable to the range of reported values of various species (Tollefsen *et al.,* 2002 ; Fitzpatrick *et al.,* 1999). A recent report by Matthews *et al.,* (2000) using GST-ERα-def fusion protein from five different species (human, mouse,

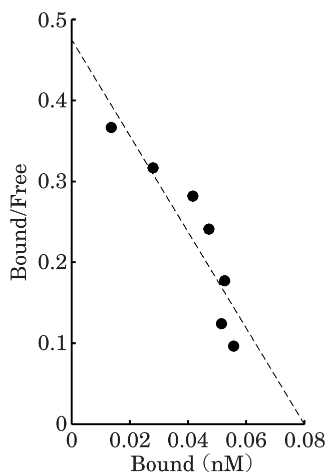

Fig. 1. Representative Scatchard plot of specific binding of estradiol-17β to GST-qERβ-def fusion protein.

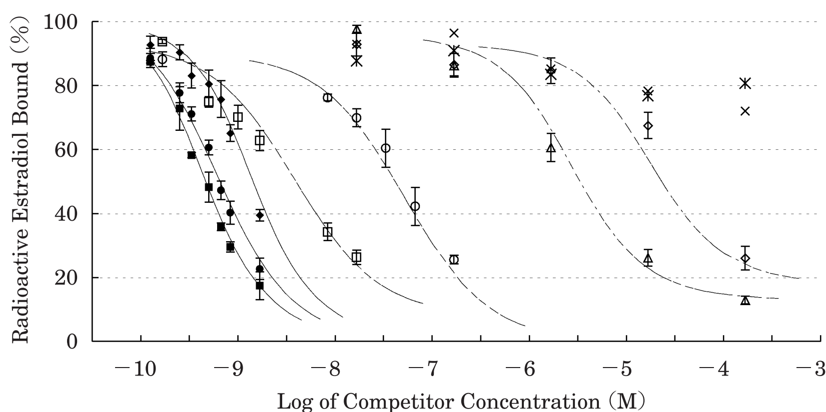

Fig. 2. Ligand-binding specificities of the GST-qERβ-def fusion protein. Concentrations of competitors, estradiol-17β (●), diethylstilbestrol (■), ethinyl estradiol (◆), coumestrol (□), genistein (○), nonylphenol (◇), bisphenol A (△), progesterone (×), and testostereone (∗), were plotted against the percentage of [^{3}H] estradiol-17β binding to the GST-qERβ-def fusion protein. Each point represents the mean±SEM of three observations.

chicken, green anole and rainbow trout) demonstrated that there exists a considerable species difference in the binding affinities to E_2 with Kd values ranging from 3 to 9×10^{-10} M.

Competitive binding assays showed that diethylstilbestrol and ethinyl estradiol bound to GST-qERβ-def fusion protein with the affinity similar to that of E_2 (Fig. 2). None of the other steroids tested (progesterone, testosterone) caused competitive displacement of E_2 binding at concentrations up to 100 nM. As summarized in Table 1, the RBAs of diethylstilbestrol and ethinyl estradiol were 114.5 and 36.5, respectively.

Table 1. Comparison of relative binding affinity of quail estrogen receptor α and β to synthetic estrogens, phytoestrogens, and xenoestrogens

	Estrogen Receptor α[1)]	Estrogen Receptor β	Ratio
Estradiol-17β	100	100	1
Diethylstilbestrol	143.8	114.5	0.796
Ethinyl estradiol	117.1	36.5	0.312
Coumestrol	1.52	16.7	10.99
Genistein	0.25	1.4	5.60
Nonylphenol	0.017	0.001	0.059
Bisphenol A	0.0035	0.011	3.14

[1)] Data adapted from Hanafy *et al.* (2004).

While diethylstilbestrol showed a higher binding affinity than E_2 for ERβ as well as for ERα, ethinyl estradiol had a lower binding affinity for ERβ. The deduced amino acid sequence of quail ERβ exhibits a higher homology with mammalian ERβ than avian ERα (Foidart *et al.,* 1999). In fact, overall identity of their amino acid residues was 80% between quail ERβ and rat ERβ. On the other hand, it was low (50.9%) between quail ERα and quail ERβ (Ichikawa *et al.,* 2003 b). The differences in the affinities of these synthetic estrogens to ER subtypes may be due to the differences in amino acid sequences of ligand binding domain (Matthews and Zacharewski, 2000).

A considerable amount of interest has been generated for the possibility of the involvement of ERβ for estrogenic effects of the endocrine-disrupting chemicals, because many of the candidates of endocrine-disrupting chemicals interact only weakly, if at all, with ERα. As shown in Fig. 2 and Table 1, nonylphenol and bisphenol A exhibited very low binding affinities to GST-qERβ-def fusion protein with RBAs of 0.001 and 0.011, respectively. These values were extremely low when compared with the RBAs of 4.3 for nonylphenol and 6.67 for bisphenol A (Maekawa *et al.,* 2003), which were calculated from the concentrations causing 50% inhibition of E_2 binding to that of DES. The difference might be due to the assay method or the construct of fusion protein : Maekawa *et al.* (2003) employed the competitive enzyme immunoassay to measure the E_2 binding and their fusion protein was limited only the ligand-binding domain of qER. Hiroi *et al.* (1999) reported that bisphenol A exhibits only an agonistic action for ERβ whereas it has dual action as an agonist and antagonist of estrogens for ERα. Although the effects of nonylphenol on estrogen-inducible mRNA expression in the liver have been confirmed in chicken (Sakimura *et al.,* 2001 ; 2002) and quail (Ichikawa *et al.,* 2003 a), our results suggest that these chemicals do not compete the E_2 binding sites of ERα and ERβ, and alternative mechanisms of action of xenoestrogens besides via ER should be considered.

In contrast to the weak binding of the xenoestrogens to ER, coumestrol and genistein bound to GST-qERβ-def fusion protein with high affinity, and the RBAs for coumestrol and genistein were 16.7 and 1.4, respectively (Table 1). These findings are consistent with data showing that some phytoestrogens bind human ERβ with higher

affinity than ERα (Kuiper *et al.,* 1997 ; 1998). In addition, a recent report revealed that the phytoestrogens preferentially stimulated some human gene expression via ERβ (Stossi *et al.,* 2004). The adverse effects of phytoestrogens on reproduction and development have been documented in quail (Leopold *et al.,* 1976). It is therefore important to investigate the effects of exposure to these chemicals to birds, as their contents in ration is estimated far larger than that of synthetic estrogens and xenoestrogens.

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