In vivo and *in vitro* screening of endocrine disrupting chemicals with estrogenic activity in Japanese quail

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ABSTRACT

This review paper describes (1) establishment of an *in vitro* method for screening endocrine disrupting chemicals with estrogenic activity and (2) study on effects of nonylphenol (NP) and octylphenol (OP) on mRNA expression of vitellogenin (VTG) II and very low density lipoprotein (apoVLDL) II in the liver of quail embryos. For *in vitro* screening, the cDNA of the ligand binding domain of quail estrogen receptor (ER) α or quail β was ligated into the gluthathione S-transferase expression vector and expressed in *E. coli*. The fusion protein was used for competitive enzyme immunoassay. Among 20 substances examined, 9 and 5 showed binding to ER α and β, respectively. NP and OP showed the highest binding to ER α, and bisphenol-A to ER β, with binding affinities relative to that of diethylstilbesterol (10^{-9}M order) of 6% and 16.2%, respectively. For the *in vivo* study, quail fertile eggs were treated with a single injection of either NP or OP (10 and 100 nmole/egg, in 20 μl) on day 13 of incubation. VTGII and apoVLDLII mRNA levels on day 15 of incubation were determined by RT-PCR. A weak but distinct expression of VTG II and ApoVLDLII mRNA was observed after NP and OP. NP and OP could be endocrine disruptors in birds.

Keywords: endocrine disrupters, estrogen receptor, competitive enzyme immunoassay

INTRODUCTION

Varieties of man-made chemicals were released in the environment and a lot of them cause adversive effects on endocrine functions in animals. These chemicals are noted as endocrine disrupters. Many decades ago, several studies demonstrated the effects of insecticides on reproductive functions in birds. For example, DDT and Kepone were demonstrated to possess estrogenic activity (Eroschenko and Wilson, 1975; Palmitter and Mulvihill, 1978; Eroschenko, 1981) Although these chemicals were banned from use,

many other substances such as alkylphenols, bisphenol compounds, and phthalate compounds have been claimed to be endocrine disrupters in different species of animals. However, the information in birds has been limited.

Alkylphenols, such as nonylphenol (NP) and octylphenol (OP) are used in industrial detergents. They have recently been included in a growing list of environmental chemicals with detrimental effects on endocrine, reproductive, and immune systems in humans, wildlife, and fish. Recent evidence from *in vitro* studies has raised the concern that NPs may be capable of disrupting endocrine systems in fish (Warhurst, 1995). NPs have been shown to be weakly estrogenic as indicated by elevated vitellogenin (VTG) II production in cultured rainbow trout hepatocytes (Jobling and Sumpter, 1993) and in the liver of chicken embryos (Sakimura *et al*. 2001).

Recently, the Organization for Economic Cooperation and Development (OECD) expert group is attempting to establish the test guidelines to assess the environmental chemicals whether or not they cause endocrine disrupting impacts in birds. The Ministry of Environment in Japan (MoE) also support this movement with providing considerable amount of research funds to a Japanese group. Firstly, how can we select the EDC out of so many environmental substances. In order to answer this question, they suggested to establish *in vitro* screening method to evaluate the binding capacity of chemicals to estrogen receptor, if the chemicals possess estrogenic activity. Consequently, only some of chemicals are focused next for *in vivo* assessment correctly. In this review, establishment of competitive enzyme immunoassay and the relative binding capacity are described for testing substances with estrogenic activity. For this assay, since the cDNA of quail estrogen receptor is necessary, the cDNA is cloned.

Although the competitive enzyme immunoassay is extremely useful to

demonstrate the binding capacity of some chemicals specifically to bind to receptor, because the binding is the first step for biological action, it does not reflect a whole biological status. The receptor in question must be in the living system and truly mediate action of substance in the target organs at least. In this regard, studies of the tissue distribution of mRNA expression of the receptors are meritorius.

Next, how can we connect the binding data to biological system? Potency of the selected EDC must be assessed in *in vivo* studies. This is still the debating subject among investigators of the OECD expert as well as elsewhere. In this review, as one of molecular approaches to assess the EDC, some of our results are described in relation to expression of yolk protein precursors such as VTGII and very low density lipoprotein (apoVLDL) II.

***In vitro* screening of EDC with estrogenic activity :**

(1) Competitive enzyme immunoassay

The receptor binding assay is advantageous for the initial *in vitro* screening. As *in vitro* binding assay, several methods have been established. These are the yeast two-hybrid assay, the fluorescence polalization method, a competitive enzyme immunoassay (EIA) and a radio-competitive assay. The yeast two-hybrid assay is useful but needs the coactivator as well as the hormone receptor. The radio-competitive binding assay is sensitive and specific but it requires special facility for managing the radio-chemicals. Accordingly, the rapid and relatively easy-handling competitive enzyme immunoassay is recommended. For this assay, although quail ER α and β are essential, a commercial kit for human estrogen receptor is available and Maekawa *et al.* (2004) developed the assay system for the quail estrogen receptor as described herein.

cDNA cloning of quail ER α

Although both types of α and β of chicken ER cDNA have been identified (Krust *et al*., 1986), only the β form of ER cDNA was cloned in quail (Foidart *et al*., 1999). Recently, Ichikawa *et al*. (2003) sequenced the ER α cDNA as illustrated in Fig. 1. The quail ER cDNA contains the sequence of open-reading frame of 1770 bp and encodes the 589 predicted amino acid residues. The encoded protein has several unique characteristics common to other steroid receptors. They consist of 6 regions (A-F) in which the C region contains 9 cysteines and forms the unique Zinc finger motif responsible for DNA binding as reported previously in the chicken (Krust *et al*., 1986). A-F regions consist of 37, 136, 83, 39, 251 and 43 amino acid residues, respectively (Fig. 1). Comparison of the quail ER α amino acid residues to those of human, mouse and frog are shown in Fig. 2. As shown in Table 1, overall identity of their amino acid residues was 98.8 % in the proteins of quail and chicken ER α. Two regions of high homology were found in C (100% amino acid identity) and E regions (99.6 %). In contrast, overall identity of the amino acid residues was low (50.9 %) between quail ER α and ER β.

Expression and Purification of GST-ER Fusion Protein

For the development of *in vitro* binding assay systems, the LBD of quail ER α and β were expressed as a GST fusion protein in *E. coli* (Fig. 3). IPTG addition in the medium induces GST-ERs efficiently and recovered mainly in the soluble fraction (Fig. 3). This soluble fraction was applied to a GSH-Sepharose column and eluted with GSH.

Figure 4 shows the purity and amount of the resultant purified GST-ER α (MW = 65 KDa) and GST-ER β (MW = 60 KDa) after SDS-PAGE.

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

The optimum amount of ER used was determined as 5.4 pmol/well and 1.8 pmol/well for quail ER α and β, respectively. The standard curve was made taking percent inhibition with 112 nM DES as 100 % (Fig. 5). The IC_{50} of DES for quail ER α and ER β was 6.0 x 10^{-9} M and 1.3 x 10^{-8} M, respectively. These values are comparable to the results in the assay for the human ERs with 1.0 x 10^{-8} M and 5.0 x 10^{-9} M, respectively. The Ministry of Environment in Japan (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. Table 2 shows the results for 20 chemicals. Among those chemicals tested, NP, OP and bisphenol A exhibited relatively strong binding to both quail ER α and β although in each case the IC_{50} was 10^{-7} M and the RBA was less than 10 %. Figure 5 shows the inhibition curves of NP, OP, bisphenol A, dichlorophenol and pentachlorophenol for quail ER α and ER β, respectively. Accordingly, it was conclucted tentatively that seventeen test substances may be excluded from a list of EDC. Three other substances may not be potent enough to cause biological response after binding in some organs. Although the binding activity was weak in 3 other substances, there is no deny that these chemicals cannot induce transcription of the target genes in some organs, causing estrogenic responses. Therefore, it is necessary to test possible EDC impact in some *in vivo* studies.

It is worth noting the relatively high binding of bisphenol A to quail receptor in comparison to other substances. This indicates that bisphenol A could be an EDC in quail. Our previous data using Northern blot analysis show marked expression of ER α mRNA in the liver, ovary (white follicle),and oviduct, whereas little expression of ER β mRNA (Ichikawa et al., 2003b, Fig. 6). However, expression of ER β mRNA was detected by RT-PCR analysis in cerebrum, cerebellum, hypothalamus, gizzard, heart, liver and gonads (Fig. 7). These tissues can be a possible target if the transcript is translated into a protein form. At present, where and what kind of biological responses

are induced by bisphenol A remains to be investigated. Foidart *et al*. (1999) reported that ER β mRNA is expressed in the different regions in the brain of the quail and may be involved in sexual behavior.

Effect of endocrine disrupters on mRNA expression of VTG II and apoVLDL II in the liver of quail embryos

It is probably true that tier 1 screens for the EDC impact should be very inexpensive, quick and easy to perform and the endpoint should be more sensitive rather than specific. In terms of expense, molecular approaches to assess the EDC impact may not be the bet choice. However, sophisticatted assessment with this approach is sensitive and quick. In addition, the resultant molecular data may contribute to a better understanding of the mechanisms underlying in EDC effects.

We have conducted to assess the effects of estrogen, and NP and OP on hepatic expression of VTGII and apoVLDLII mRNA in the quail embryo because some previous literatures described clear effects of estrogen on the mRNA expression in the chick embryo. Elbrecht *et al*. (1984) demonstrated that VTGII and apoVLDL mRNA expression in the liver of chicken embryos was induced by estrogen treatment. Northern blot analysis revealed that apoVLDLII was induced at day 9 of incubation but VTGII mRNA was not found until day 11. In this study Estradiol 17 β (1.25 mg) in 50 ul of propylene glycol was placed just beneath the air sac membrane 48 hours before the embryos were killed. In consistent with these data, other studies showed estrogen-induced mRNA expression of other yolk proteins in the liver of the chicken embryo (van het Schip *et al*. 1987; Evans et al. 1988; Sakimura *et al*. 2001).

We conducted our study in the quail to assess estrogenic activity of NP and OP in embryos by determining mRNA levels of liver VTG II and apoVLDL II. The fertile eggs

were treated with a single injection of either NP, OP or ethynyl estradiol (EE) at doses of 10 and 100 nmole/egg in 20 μl on day 13 of incubation. In the control group the eggs were treated with the vehicle (corn oil, 20 μl/egg). On day 15 of incubation the liver was collected and mRNA levels were determined by RT-PCR assay. No expression of VTG II mRNA was detected in the control group, whereas a marked induction of VTG II mRNA was revealed in the EE treatment (Fig. 8). A weak but distinct expression of VTG II mRNA was evident in the NP and OP treatment groups. ApoVLDLII transcripts were detected in the control group and induced markedly by the injection of EE with higher expression in females (Fig. 9). NP also induced considerable expression in females, whereas no transcripts were detected in males. OP also induced the transcript in females but in males OP at 10 nmole was effective. This study indicates that NP and OP possess estrogenic activity in terms of liver VTG II and apoVLDLII mRNA expression in the quail embryo. Our study clearly showed the sexual dimorphism in apoVLDLII mRNA expression in the quail embryonic liver. The higher response of apoVLDLII mRNA expression to NP was also evident in females. Estrogen receptor binding might reflect this higher response of apoVLDLII mRNA expression in females.

Although Sakimura *et al*. (2001) using the chicken embryos demonstrated a slight induction of VTGII mRNA by repetitive injections of NP on days 13 and 15 of incubation, the present study clearly showed a definite response of VTGII mRNA to a single injection of NP at equivalent dose as used previously in females. This result suggests that the Japanese quail is more sensitive to endocrine disrupters like NP and OP in terms of mRNA expression of the liver yolk proteins when compared to chicken. Although the estrogen receptor binding affinity of NP and OP was not shown in this study, these compounds may have provoked the estrogenic activity by binding to estrogen receptor as reported in the different species (Routledge and Sumpter, 1997; Matthews *et al*., 2000). Together with *in vitro* binding study, it is likely that both NP and O P can be endocrine

disruptors in birds.

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Table 1. Comparison of quail ER α amino acid residues to those of chicken, human, mouse and frog and those of ER β of quail and chicken. Identity is shown in %.

Adopted from Ichikawa *et al*. (*J. Poult. Sci*., 40:121-129. 2003)

Gene	ORF	DBD	LBD
chicken ER α	98.8	100	99.6
human ER α	78.4	100	93.6
mouse ER α	77.3	100	93.6
frog ER α	76.3	98.6	85.3
quail ER β	50.9	81.7	58.4
chicken ER β	50.9	81.7	57.8

Table 2. The relative binding affinity of test chemicals to quail ER α and ER β 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^{-6}		0.030	9.0×10^{-6}		0.014
5	Bis(2-ethylhexyl) adipate		$>10^{-4}$			$>10^{-4}$	
6	Triphenyltin chloride	1.0×10^{-6}		0.060	1.0×10^{-6}		0.130
7	Bis(2-ethylhexyl) phthalate	3.0×10^{-6}		0.200		2.0×10^{-6}	
8	Dicyclohexyl phthalate	1.0×10^{-6}		0.005		9.0×10^{-6}	
9	Di-n-butyl phthalate		$>10^{-4}$			$>10^{-4}$	
10	Tributyltin(IV) chloride	2.0×10^{-8}		0.030		2.0×10^{-8}	
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	7.4×10^{-8}		16.2
14	2,4-Dichlorophenol	1.0×10^{-6}		0.047		2.8×10^{-6}	
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		$>10^{-4}$			$>10^{-4}$	

Adopted from Maekawa, S. *et al*. (*J. Health Science*. 50:25-32. 2004.)

Figure legends

Fig. 1 Nucleotide and deduced amino acid sequences of quail ER α. The numbers

On the nucleotides refer to the position of the nucleotides and on the left to that of the amino acids. The initiation and stop codon for the reading frame was underlined. The sequence was divided into six regions (A-F) based on sequence homology between quail ER α and chicken ER α. The three most highly conserved regions (A, amino acids 1-37, C, 180-262, E, 302-552) are boxed. Conserved cysteines (*) are indicated. (Redrawn by permission from Ichikawa, K., Yamamoto, I., Tsukada, A., Saito, N. and Shimada, K. 2003. cloning and mRNA expression of estrogen receptor α in Japanese quail. *J. Poult. Sci*., 40:121-129.)

Fig. 2. Alignment of ER α proteins among quail, chicken, human, mouse and frog. The amino acids alignment of ER α was divided into six regions (A-F) as described in Fig. 1. Numbers in each side represent the position of the amino acid residues in each sequence. Identical amino acids in 3-4 sequences are shadowed and in all 5 sequences are darkened . (Redrawn by permission from Ichikawa, K., Yamamoto, I., Tsukada, A., Saito, N. and Shimada, K. 2003. Cloning and mRNA expression of estrogen receptor α in Japanese quail. *J. Poult. Sci*., 40:121-129.)

Fig. 3. Production of recombinant proteins (estrogen receptors α and β) in *Escherichia coli.*

Fig. 4. Expression and purification of quail GST-ERαLBD and ERβLBD 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: cytosol fraction from IPTG-treated cells; Lanes 4&9: purified GST-ERs from cytosol fraction; Lanes 5&10: purified GST-ERs from cytosol fraction (40-fold more than in Lanes 4 &9). (Redrawn by permission from Maekawa, S. Nishizuka, M. Heitaku, S. Kunimoto, M. Nishikawa, .J. Ichikawa, K. Shimada, K. and Imagawa, M. 2003. Development of a competitive enzyme immunoassay for detection of capacity of chemicals to bind quail estrogen receptor α and β. *J. Health Science*. Vol. 50: 25-32)

Fig. 5. Inhibition curves of DES, NP, OP, BisA, DCP and PCP for quail estrogen receptor α and β determined by competitive enzyme immunoassay. Values are the mean and standard deviation (n=4). (Redrawn by permission from Maekawa, S. Nishizuka, M. Heitaku, S. Kunimoto, M. Nishikawa, .J. Ichikawa, K. Shimada, K. and Imagawa, M. 2003. Development of a competitive enzyme immunoassay for detection of capacity of chemicals to bind quail estrogen receptor α and β. *J. Health Science*. Vol. 50: 25-32)

Fig. 6. Northern blot analysis of ERα and ER β in quail tissues. Autoradiograph exposure was 1 day. Cbr, cerebrum; CBl, cerebellum; hyp, hypothalamus; Giz, gizzard; Hrt, heart; Spl, spleen; Liv, liver; Kid, kidney; Adr, adrenal; Wf, white follicle; Mag, magnum; She, shell gland; Int, intestine, Tes, testis. (Redrawn by permission from Ichikawa, K., Yamamoto, I., Tsukada, A., Saito, N. and Shimada, K. 2003. cloning and mRNA expression of estrogen receptor α in Japanese quail. *J. Poult. Sci*., 40:121-129.)

Fig. 7 . RT-PCR analyses of ER α and ER β in quail tissues. Abbreviation: same as in Fig. 6. M, molecular marker (Hinc II-Eco RI digested DNA). (Redrawn by permission from Ichikawa, K., Yamamoto, I., Tsukada, A., Saito, N. and Shimada, K. 2003. Cloning and mRNA expression of estrogen receptor α in Japanese quail. *J. Poult. Sci*., 40:121-129.)

Fig. 8. VTG II mRNA expression in liver of quail embryo. mRNA expressions were expressed as relative density of RT-PCR products compared toβ actin.

(Means ± SEM, N = 3 – 6.) (Redrawn by permission from Ichikawa, K., Ha, Y., Tsukada, H., Saito, N. and Shimada, K. 2003. Effect of endocrine disrupters on mRNA expression of vitellogenin (VTG) II and very low density lipoprotein (apoVLDL) II in the liver of quail embryos. *J. Poult. Sci*., 40: 45-52.)

Fig. 9. ApoVLDLII mRNA expression in liver of quail embryo. mRNA expressions

were expressed as relative density of RT-PCR products compared to β actin.

(Means ± SEM, N = 3 – 6.) (Redrawn by permission from Ichikawa, K., Ha, Y., Tsukada, H., Saito, N. and Shimada, K. 2003. Effect of endocrine disrupters on mRNA expression of vitellogenin (VTG) II and very low density lipoprotein (apoVLDL) II in the liver of quail embryos. *J. Poult. Sci*., 40: 45-52.)