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Effect of Endocrine Disrupters on mRNA Expression of Vitellogenin (VTG) II and Very Low Density Lipoprotein (apoVLDL) II in the Liver of Quail Embryos

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The present study was conducted to assess estrogenic activity of nonylphenol (NP) and octylphenol (OP) in quail embryos by determining mRNA levels of liver vitellogenin (VTG) II and very low density lipoprotein (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 20μl on day 13 of incubation. In the control group the eggs were treated with the vehicle (corn oil, 20μl/egg). On day 15 of incubation the liver was collected and total RNA was extracted. Both mRNA levels were determined by RT-PCR assay and were expressed in relation to β actin mRNA levels. 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. 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. 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, and that apoVLDLII expression in female embryo is more sensitive to estrogenic substances.

Key words : endocrine disrupters, VTG II, apoVLDLII, quail, RT–PCR

Introduction

Among varieties of man-made chemicals in the environment, many interfere with endocrine functions in animals. These chemicals are often referred to as endocrine disrupters. Although the terminology is relatively new, environmental chemicals have long been claimed to cause adverse effects in animals including humans. Many decades ago, several studies demonstrated the effects of insecticides on reproductive functions in

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birds. For example, DDT and Kepone were demonstrated to possess estrogenic activity (Eroschenko and Wilson, 1975 ; Palmitter and Mulvihill, 1978, ; Eroschenko, 1981.) and was subsequently banned from use. However, many other substances such as alkylphenols, bisphenol compounds, and phthalate compounds have been suggested to be endocrine disrupters.

Alkylphenols, such as nonylphenol (NP) and octylphenol (OP) are used in industrial detergents, in the form of alkylphenol ethoxylates. 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). Very low density lipoprotein (apoVLDL) II is also known as an estrogen -induced protein in the chicken (Elbrecht *et al.* 1984). Few studies have been reported in regard to the effect of endocrine disrupters on liver protein production in quail. Japanese quail is a unique species of birds because they produce an enormous amount of egg protein in response to estrogen. Furthermore, recently the Organization for Economic Cooperation and Development (OECD) proposed the standardization of assay system for the evaluation of biological potencies of endocrine disrupters in birds and suggested the Japanese quail as an experimental model. Therefore, this study was conducted to reveal the estrogenic effects of NP and octylphenol (OP) in quail embryos by determining mRNA levels of liver VTG II and apoVLDLII.

Materials and Methods

cDNA cloning of Japanese quail VTG II

Actively laying female Japanese quail were killed by decapitation and the liver was collected, quickly frozen in liquid nitrogen and stored at -80℃. Total RNA was extractd as described below. The total RNA (1μg) was denatured at 65℃ for 10 min with oligo dT primers and reverse transcrived with 200 units of superscript II (Gibco-BRL) in 20μl mixture. Based on the sequence of the chicken VTGII cDNA (van het Schip *et al.,* 1987) primers for quail VTGII were designed. The pairs of olignuleotide primers (sense primer : 5′-GCT ATG AGG GGG ATC ATA CT-3′, antisense : 5′-GAG GSG GTA AGC ATT CTC TG-3′) were synthesized used for the PCR. The reverse-transcribed (RT) product was subjected to 35 cycles of PCR amplification using EX Taq DNA polymaerase (Takara bio inc., Shiga, Japan) in a total volume of 25μl. The cDNAs were denatured at 97℃ for 1 min, followed by 35 cycles of 95℃ for 10 sec, 60℃ for 20 sec, and 72℃ for 1 min, and finally 10-min extension at 72 C. The PCR products were separated on an agarose gel, and two DNA fragments of about 200 bp and 400 bp were subcloned into pGEM-TEASY Vector (Promega). DNA sequencing was performed on plasmids using dye terminator chemistry on an Applied chain-termination method (Sanger *et al.* 1977).

Embryos and Treatments

Sex-linked, fertile eggs were obtained by crossing black female (Z^BW^-) with brown male (Z^bZ^b), which were purchased from Tokai Yuki (Toyohashi, Aichi, Japan). They were incubated at 37.5℃ in a commercial incubator using standard conditions. The eggs were "treated" with a single injection of either NP, OP (Kanto Chemical, Tokyo, Japan) or ethynyl estradiol (EE, Wako Pharmaceutical) at doses of 10 and 100 nmole/egg in 20μl on day 13 of incubation. The solution was injected through a small hole of the shell into the air cell on the shell membrane as described previously (Abinawanto *et al.* 1996). In the control group the eggs were treated with the vehicle in 20 μl of corn oil (Kanto Chemical, Tokyo, Japan). On day 15 of incubation the embryos and the liver were weighed and the liver was collected individually for total RNA extraction.

RNA extraction and PCR assay for VTG II and apoVLDLII mRNA levels

Total RNA was extracted from the liver and was reverse transcribed as described by Sakimura *et al.* (2001). For PCR amplification 2μl of each reverse transcription (RT) reaction mixture was used. For VTG II and apoVLDLII mRNA, the sequences of the upstream and downstream primers used were listed in Table 1. PCR was carried out in 25μl PCR buffer (20 mM Tris HCl, 100 mM KCl, 2 mM $MgCl_2$, pH 8.0) containing 0.2 mM dNTPs, 0.5μM primers and 0.3125 unit of Ex Taq DNA polymerase (Takara bio inc., Shiga, Japan). After the initial denaturation for 2 min at 94℃, the amplification profile consisted of 10 sec of denaturation at 97℃, 30 sec of annealing at 55℃, and 1 min of extension at 72℃ for 25 cycles for VTGII and 30 cycles for apoVLDLII. Amplification was completed with an additional extension at 72 ℃ for 2 min. All PCR reactions were carried out on a Perkin-Elmer 9700 thermocycler. PCR products were run on a 1.5% agarose gel in 1 X TAE buffer and bands were visualized by ethidium bromide staining. The bands of the electrophoretic gels were analyzed using NIH Image software.

Statistical analyses

Data were presented as means + SEM. Statistical significance of the differences between vehicle control (corn oil) group and treated groups (OP, NP or EE) within male or female were examined by student's *t* test or Aspin & welch *t* test (Gad, 2001). This analysis was not applied when the number of embryos expressing the mRNA is less

Table 1. Position and sequence of synthetic oligonucleotide primers used in this study

Gene	Location	Sequence (5′→3′)	Reference and accession number
VTG II	31–50	AGC AGT AGT GCT CAC CCT TG	van het Schip
	394–413	ATA GCC CAC TTG ATC TCT AT	*et al.,* 1987
apo	104–128	GGT ACA ATA CAG GGA ATT GGT GAT A	MacLachlan
VLDL II	397–421	AAC TAG TAG CCT AGT TTT CCG TTC C	*et al.,* 1996
β actin	168–194	ATC GTG GGT CGC CCC AGA CAT CAG GGT	GenBank
	1028–1054	ATC TTG ATT TTC ATT GTG CTA GGT GCC	AF199488

than two in the control and treated groups.

Results

cDNA cloning of Japanese quail VTG II

Fig. 1 showed the nucleotide and deduced amino acid sequences of quail VTGII. It consists of 183 nucleotides for a part of the first exon I, intron, the whole exon II, intron, and a part of the exon III segments encoding 57 amino acids. The amino acids alignment between quail and chicken VTG II was shown in Fig. 2. Out of 57 amino acids, 12 were different between them (78.9% homology).

RNA extraction and PCR assay for VTG II and apoVLDLII mRNA levels

RT-PCR analysis detected no mRNA expression of VTGII in the liver of males and females in the control group, whereas distinct mRNA transcripts were detected in the treatment groups, notably in the EE group based upon the predicted size of the PCR product (183bp) (Fig. 3). For the internal control β actin mRNA expression was examined and the predicted size of the PCR product (887bp) was detected in all samples (Fig. 3). The semi-quantitative data showed that the mRNA levels of the liver VTGII increased markedly after the EE treatment in both groups of males and females when compared to the control and other groups, respectively (Fig. 4). The NP treatment induced a slight increase in the mRNA levels when 100 nmole of NP was injected, but no significant increase was detected with the low dose injection. After the injection of 10 nmole OP, there was no induction of mRNA expression but 100nmole OP induced a slight increase.

The liver mRNA expression of apoVLDLII was detected as 318 bp cDNA product after PCR amplification in most of the samples examined (Fig. 3). β actin mRNA

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        10        20        30        40        50        60        70        80        90
agcagtagtgctcaccottgtaggtaagcttacaaatctcctcttcattcttttttccctggaatttccttgagttcactgacaataatta
 A  V  V  L  T  L  V

       100       110       120       130       140       150       160       170       180
ggcttagactggatataactcctgtggtttttctttttgtaggcagccagaagcttgacattggtaagtacatttctatctacaaactct
                                    G  S  Q  K  L  D  I

       190       200       210       220       230       240       250       260       270
gtgtctatgttatgatgactattcattagaatatgcttatagcttttgtgtaaatggctgctattcccacagacccaggattcagtagt
                                                                  D  P  G  F  S  S

       280       290       300       310       320       330       340       350       360
ggaaagagttacctgtacagctatgaaggttctatgttgaatgggcttcaagaaagaagtctgggcaaagctggtgtgcgcttgagctgc
G  K  S  Y  L  Y  S  Y  E  G  S  M  L  N  G  L  Q  E  R  S  L  G  K  A  G  V  R  L  S  C

       370       380       390
aagatagagatcaggtgggctat
K  I  E  I  R  W  A
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Fig. 1. Partial sequences of nucleotide and deduced amino acid of quail VTG II. Solid letters indicate exon (see in detail in text).

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Quail   1 AVVLTLVGSQKLDIDPGFSSGKSYLYSYEGSMLNGLQERSLGKAGVRLSCKIEIRWA       57
Chicken 1 ALVLTLVGSQKFDIDPGFNSRRSYLYNYEGSMLNGLQDRSLGKAGVRLSSKLEISGL       57
           * ********* ****** *  **** ********** ************ * **
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Fig. 2. The amino acids alignment between quail and chicken VTG II. *indicates amino acids common to chicken and quail.

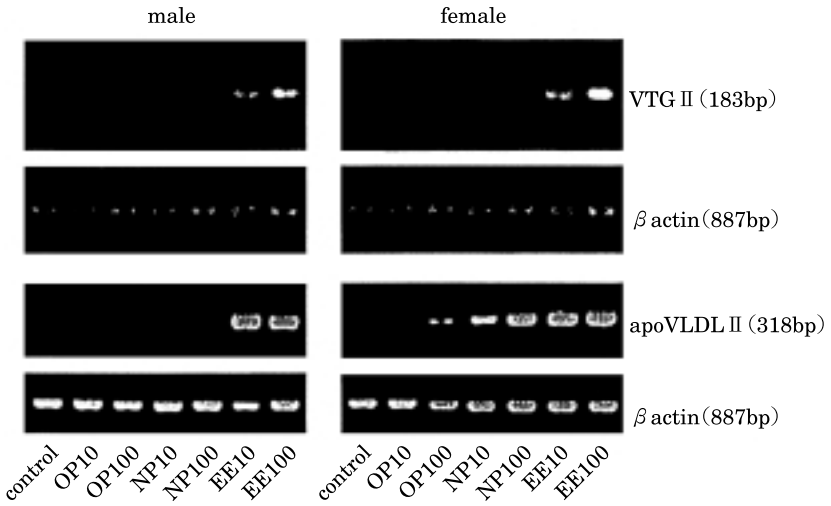

Fig. 3. Detection of mRNA transcripts for quail VTG II and apoVLDL II mRNA. Liver mRNA from corn oil (vehicle control), OP (octylphenol), NP (nonylphenol) and EE (ethynyl estradiol) treated embryos were analyzed by RT-PCR. Gels were stained by ethidium bromide. Upper : VTG II mRNA, lower : apoVLDL II mRNA. β actin mRNA in upper and lower panels corresponds to VTG II and apoVLDL II mRNA, repectively as control.

expression for the internal control was also detected at 887 bp. The semi-quantitative data showed that the levels of apoVLDLII markedly increased in the EE treatment in both males and females. The response was greater in females than in males ($P < 0.05$) (Fig. 4). The low dose of OP induced an increase in apoVLDLII mRNA levels but the higher dose of OP did not induce any expression in males. Either dose of NP did not induce any expression in males. However, in females the levels increased after OP and NP treatments in females.

Discussion

Elbrecht *et al.* (1984) demonstrated that VTGII and apoVLDL mRNA expression in the liver of chicken embryos was induced by estrogen treatment. Estrogen-induced expression of other yolk proteins in the liver of the chicken embryos were also reported (van het Schip *et al.* 1987 ; Evans *et al.* 1988). We also observed in the chicken embryo that VTGII mRNA expression was inducible following estrogen injection at day 13 of incubation and the expression was potentiated with an additional estrogen injection at day 16 of incubation (Sakimura *et al.* 2001). Consistent with these data of previous reports, the present study also demonstrated that both VTGII and apoVLDLII transcripts were induced by estrogen in the liver of quail embryos. Since the apoVLDL mRNA expression responded earlier than VTGII mRNA expression by 2 days, Elbrecht *et al.* (1984) suggest that the two estrogen regulated genes may be independently developmentally regulated. The methods in the present study, however, do not allow

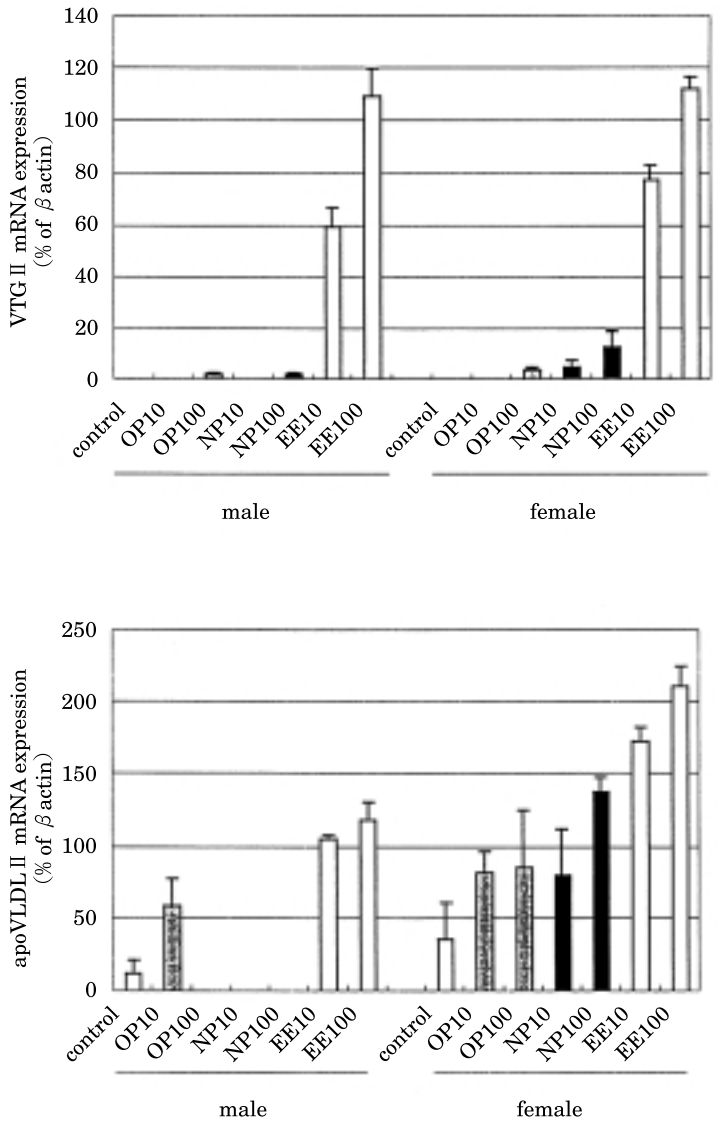

Fig. 4. VTG II and apoVLDL II mRNA expression in liver of quail embryo. mRNA expressions were expressed as relative density of RT-PCR products compared to β actin. (Means+SEM, N=3–6.) Upper : VTG II mRNA, lower : apoVLDL II mRNA.

for the detection of the different degrees of mRNA expression between the two genes due to different cycles of the PCR amplification.

There has been no report previously in regard to the sex differences in the VTGII or apoVLDLII mRNA expression in response to estrogen treatment. The present 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. This higher response of apoVLDLII mRNA expression in females

might be reflected by estrogen receptor binding, however, there was no difference in mRNA expression in estrogen receptor α in the liver between males and females of these embryos (unpublished data).

Although Sakimura *et al.* (2001) using the chicken embryos demonstrated a slight induction of VTGII mRNA by repetitive injections of NP, 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. 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).

In conclusion, this study provides the first evidence that endocrine disrupters act on mRNA expression of major yolk proteins in the quail. Namely, (1) NP induces sexual dimorphic expression of VTGII and apoVLDLII mRNA with higher expression in females and (2) OP induces only apoVLDLII mRNA expression. Hence, mRNA expression of liver yolk protein in quail can be a molecular indicator for assay of endocrine disrupters with estrogenic activity.

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