Effect of Estradiol and Nonylphenol on mRNA Levels of Vitellogenin II in the Liver of Chicken Embryos

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Nonylphenol is one of the endocrine disrupting agents with estrogenic activity in some vertebrates. The present study was conducted to assess estrogenic activity of p-nonylphenol (NP) in chicken embryos by determining mRNA levels of liver vitellogenin II (VTG II). Fertile chicken eggs were incubated at 37.5°C using standard conditions. In the group 1, the eggs were treated with a single injection of either NP at doses of 50, 10, 1, 0.5, 0.2, 0.1, 0.01 and 0.001 mg/egg or estradiol-17β (E2) at doses of 1.0, 0.1 and 0.01 mg/egg in 50 µl on day 16 of incubation. In the group 2, the eggs were treated with double injections of either NP or E2 on days 13 and 16 of incubation. In the control group the eggs were treated with the vehicle (propanediol (PD), 50 µl/egg). On day 18 of incubation the liver was collected and total RNA was extracted. VTG II mRNA levels were determined by reverse transcription-polymerase chain reaction (RT-PCR) assay. All the embryos were alive in the group of E2, PD or 0.01 and 0.001 mg/egg of NP. Only 17% (3/18) of embryos were alive in the group of NP at 10 mg/egg. No VTG II mRNA was detected in the control group, whereas distinct VTG II mRNA was revealed in the E2 treatment group where there was higher expression in the group 2 than in the group 1. Weak but distinct VTG II mRNA was detected in the NP treatment group. This study indicates that NP may have estrogenic activity in terms of liver VTG II mRNA assessed by RT-PCR assay in the chicken embryo.

Key words: nonylphenol, estradiol, vitellogenin II, chicken embryo, RT-PCR

Introduction

Estrogens are essential for the normal development and function of the female reproductive system of vertebrates. However, excessive amounts of these compounds can have adverse effects on the function of reproductive organs in both sexes. All of the physiologically important estrogens are 18-carbon steroids, e.g. estradiol-17β (E2), estrone and estriol, that are synthesized in the ovary in response to pituitary hormones. A balance between synthesis, binding to serum proteins, metabolism to inactive com-
pounds, and clearance keeps the effective concentration of these naturally occurring estrogens within a normal range during reproductive cycles. In addition to these naturally occurring steroidal estrogens, an increasing number of nonsteroidal compounds have been shown to have estrogenic activity.

Alkylphenols, such as nonylphenol are used in industrial detergents, in the form of alkylphenol ethoxylates. Alkylphenol ethoxylates (such as nonylphenol ethoxylate) break down slowly in the environment or in sewage treatment works, with important breakdown products being short chain alkylphenol ethoxylates (e.g. nonylphenol mono-ethoxylate, nonylphenol di-ethoxylate), alkylphenols (e.g. nonylphenol) and alkylphenoxy carboxylic acids (e.g. nonylphenoxy mono-carboxylic acid, nonylphenoxy di-carboxylic acid). Alkylphenols such as nonylphenol persist in sediments; the alkylphenoxy carboxylic acids tend to persist in the water column (Warhurst, 1995). These alkylphenols 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 nonylphenols may be capable of disrupting endocrine systems in fish (Warhurst, 1995). Nonylphenols have been shown to be weakly estrogenic as indicated by elevated vitellogenin (VTG) production in cultured rainbow trout hepatocytes (Jobling and Sumpter, 1993). Similar activity has also been demonstrated in estrogen-responsive human breast cancer cell cultures (White et al., 1994). However, the significance of these findings relative to whole animal responses is not clear at this time. Therefore, this study was conducted to reveal the estrogenic effects of nonylphenol on the VTG activity of the chicken embryo in vivo. VTG II was used in this experiment because the levels of mRNAs encoding VTG I, VTG II, and VTG III in the liver of egg-laying hen are approximately 11,000, 30,000, and 3,000 molecules per cell, respectively (Evans et al., 1988). Embryonic chicken liver was chosen in this study because it is a useful system to study the acquisition of responsiveness to the steroid hormone. VTG II mRNA is inducible by E2 from day 15 of incubation using Northern hybridization in embryonic chicken liver (Elbrecht et al., 1984).

The present study aimed first to reveal a detection of the estrogenic effects of nonylphenol using the whole chicken embryos.

Materials and Methods

Embryos and Treatments

Sex-linked, feather colored-cross eggs (Rhode Island Red ♂ × Barred Plymouth Rock ♀) were obtained from Okazaki Station, National Livestock Breeding Center (Okazaki, Aichi, Japan). They were incubated at 37.5°C in a commercial incubator using standard conditions. In the group 1, the eggs were treated with a single injection of either p-nonylphenol (NP, Kanto Chemical, Tokyo, Japan) at doses of 50, 10, 1, 0.5, 0.2, 0.1, 0.01 and 0.001 mg or estradiol-17β (E2, Sigma, St. Louis, MO, USA) at doses of 1.0, 0.1 and 0.01 mg in 50 μl of 100% 1,2-propanediol (PD, Kanto Chemical, Tokyo, Japan) into the air sac of the eggs on day 16 of incubation (the number of injected eggs was shown in Table 1). Eggshell holes were sealed and incubation was continued as
described previously (Abinawanto et al., 1996). In the group 2, the eggs were treated with double injections of either NP or E2 on day 13 and 16 of incubation in the same way (injection volume and concentration of the compounds) as group 1. The control group received 50µl of 100% PD. On day 18 of incubation, the embryos were weighed and the liver was collected individually and total RNA was extracted from the liver of 1 embryo/sex/group.

**RNA extraction**

Liver tissues from individual embryos were homogenized in 1 ml Trizol™ Reagent (GIBCO BRL). After incubation of the homogenized samples at least 5 min at room temperature, the samples were centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected and 0.3 ml of chloroform was added per 1 ml of Trizol Reagent. The samples were mixed vigorously and then centrifuged at 12,000 g for 10 min at 4°C. The upper colorless, aqueous phase were collected, and mixed with 0.5 ml of isopropanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 70% ethanol. The pellet was air dried and dissolved in 150 µl of diethyl pyrocarbonate (DEPC)-treated water. The RNA quantity was determined by spectrophotometry at 260 nm. Samples were stored at −70°C until analysis.

**Reverse transcription**

Five µg of total RNA was reverse transcribed in 20 µl reverse transcription buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3) containing 10 mM dithiothreitol (DTT), 0.5 mM dNTPs, 0.5 µg oligo (dT) 12–18, and 200 units SuperScript™ II Reverse Transcriptase (GIBCO BRL). Reverse transcription was carried out at 42°C for 50 min, and samples were then incubated at 70°C for 15 min to inactivate the reverse transcriptase.

**PCR amplification**

Two µl of each cDNA product from reverse transcription reaction was used for PCR amplification. For VTG II, PCR primers were: 5’-CTT GTA AAC TGC ATC GTT CA-3’ and 5’-TCA TGT ACT GCA TTC CTC AT-3’. These primers correspond to nucleotide positions 18919–18938 and 20102–20121, respectively, of chicken vitellogenin II gene (van het Schip et al., 1987). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sequences of the upstream and downstream primers were: 5’-GTG GAG AGA TGA CAG AGG TG-3’ (608–627) and 5’-AAC AAG CTT GAC GAA ATG GT-3’ (937–956), respectively (Dugaiczyk et al., 1983). PCR reactions were carried out in 25 µl PCR buffer (20 mM Tris HCl, 100 mM KCl, 2 mM MgCl₂, pH 8.0) contains 0.2 mM dNTPs, 0.5 µM primers and 0.3125 unit Ex Taq DNA polymerase (Takara, Japan). After the initial denaturation for 2 min at 94°C, the amplification profile consisted of 10 sec of denaturation at 97°C, 30 sec of annealing at 55°C, and 1 min of extension at 72°C for 25 cycles. Amplification was completed with an additional extension at 72°C 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 × TAE buffer, and bands were visualized by ethidium bromide staining. The bands of
the electrophoretic gels were analyzed using Scion Image for Windows (Scion Corporation).

**Southern hybridization**

The electrophoresed agarose gel was prepared as described above and cDNA was transferred to Hybond-N+ nylon membrane (Amersham Inc., UK) in 10×SSC buffer (166.5 mM sodium citrate and 166.5 mM NaCl) by capillary transfer. The membrane was probed with a cloned chicken VTG II specific cDNA labeled with [32P]dCTP with a specific activity of 3000 Ci/mmol by a Rediprime II random prime labeling system (Amersham Pharmacia Biotech, UK). After prehybridization for 2 hours, hybridization was carried out at 42°C for 18 hours in a solution consisting of 50% formamide, 5×Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA), 0.1% SDS, and 5×SSPE (900 mM NaCl, 5 mM EDTA, 50 mM Na2HPO4 12H2O), 100 μg/ml salmon sperm DNA, and denatured cDNA probe. The membranes was washed in 1×SSC containing 0.1% SDS at 65°C for 20 min and was washed again in 0.1×SSC containing 0.1% SDS at 65°C for 20 min. The membrane was then exposed to x-ray film with intensifying screens for 30 min.

**Statistical analyses**

The data of embryonic body weight were analyzed using the F test for homogeneity of variance followed by the Student's t test or the Aspin & Welch t test (Gad and Weil, 2000). Statistical analyses were made between vehicle control (PD) group and treated groups (NP or E2) at two-tailed 5% levels of significance.

**Results**

**Embryo survival and Body weight**

The results of survival of embryos and body weights were summarized in Table 1. Survival of embryos injected with vehicle control solution (100% PD) and all E2 groups was 100%, while for those receiving NP it varied from 0 to 100%. Survival of 0.001, 0.01, 0.1, 0.2, 0.5, 1.0, 10 and 50 mg dose group (group 1: single injection group) of NP was 100%, 100%, 71%, 33%, 33%, 13%, 11% and 0%, respectively. In the same way, survival of group 2 (double injection group) embryos was 100%, 100%, 100%, 67%, 67%, 0%, 22% and 0%, respectively. Survival was significantly affected above 0.2 mg dose of NP.

Average body weights of control embryos given PD alone were 25.21 g (group 1) and 25.51 g (group 2). Treatment of E2 and 0.01 mg/egg or lower doses of NP did not affect body weights. NP slightly suppressed the body weights of embryos at 0.1 mg or higher doses.

**Results of RT-PCR and Southern hybridization**

At the RT-PCR analysis, no VTG II mRNA expression was detected in the control group, whereas distinct VTG II mRNA was revealed in the E2 treatment embryos and one of the 10 mg/egg NP treated embryo (Group 2 male) at 279-bp (Fig. 1). Control GAPDH products were detected at 348-bp on the gel. In the semi-quantitative evaluation of VTG II expression levels, VTG II products were not different between male and female embryos (Fig. 2). In comparison with injection frequency, VTG II
Table 1. Number of treated embryos, survival and weight determinations after injection of NP or E2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/egg)</th>
<th>Group 1: single injection on day 16</th>
<th>Group 2: double injection on days 13 and 16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number injected</td>
<td>Survival (%)</td>
<td>Body weight (g)</td>
</tr>
<tr>
<td>Vehicle (PD)</td>
<td>—</td>
<td>7</td>
<td>7 (100)</td>
</tr>
<tr>
<td>E2</td>
<td>0.01</td>
<td>3</td>
<td>3 (100)</td>
</tr>
<tr>
<td>E2</td>
<td>0.1</td>
<td>8</td>
<td>8 (100)</td>
</tr>
<tr>
<td>E2</td>
<td>1.0</td>
<td>8</td>
<td>8 (100)</td>
</tr>
<tr>
<td>NP</td>
<td>0.001</td>
<td>3</td>
<td>3 (100)</td>
</tr>
<tr>
<td>NP</td>
<td>0.01</td>
<td>8</td>
<td>8 (100)</td>
</tr>
<tr>
<td>NP</td>
<td>0.1</td>
<td>7</td>
<td>5 (71)</td>
</tr>
<tr>
<td>NP</td>
<td>0.2</td>
<td>3</td>
<td>1 (33)</td>
</tr>
<tr>
<td>NP</td>
<td>0.5</td>
<td>3</td>
<td>1 (33)</td>
</tr>
<tr>
<td>NP</td>
<td>1.0</td>
<td>8</td>
<td>1 (13)</td>
</tr>
<tr>
<td>NP</td>
<td>10</td>
<td>9</td>
<td>1 (11)</td>
</tr>
<tr>
<td>NP</td>
<td>50</td>
<td>5</td>
<td>0 (0)</td>
</tr>
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</table>

Group 1 eggs were treated with a single injection of each solution on day 16 of incubation.

Group 2 eggs were treated with double injections on day 13 and 16 of incubation in the same way (injection volume and concentration of the compounds) as group 1.

*: Statistically significant compared with injection of vehicle solution. ND: Body weight could not be determined.

Expression levels after double injections of E2 were 20 to 70% higher than that of single injection. VTG II expression level of 10 mg NP treated embryo was around 20% of 0.1 mg E2 treated embryo. Neither sex synthesized VTG II mRNA without artificial estrogen or NP stimulation. Fig. 2 shows that there was no sexual difference in VTG II mRNA levels by estradiol stimulation and VTG II mRNA level was enhanced by double injections of estradiol.

In the autoradiogram of Southern blotting, the hybridization patterns corresponded to the VTG II mRNA bands of ethidium bromide staining (Fig. 3).

Discussion

Estradiol is a hormone that influences the development and maintenance of female sex characteristics, and the maturation and function of accessory sex organs. In the male chicken embryo, the injection of estradiol significantly induced P450arom mRNA expression but suppressed Anti-Müllerian hormone (AMH) mRNA from day 6 of incubation (Shimada et al., 1999, Nishikimi et al., 2000). This suggests that the sexual difference of susceptibility for estrogen was seen in chicken embryo from day 6 of incubation, but in this study, there was no sexual difference in VTG II mRNA levels by estrogen stimulation. The reason of this phenomenon is unknown, but these data
Fig. 1. Chicken VTG II gene expression in liver of embryos. The liver mRNA from PD (vehicle control), E2, and NP injected embryos were analyzed by RT-PCR. Gels were stained by ethidium bromide.

Fig. 2. Relative density of the bands compared with GAPDH products. Ethidium bromide stained gels showed in Fig. 1 were analyzed using Scion Image for Windows. The levels were expressed as percentages compared with GAPDH levels.
suggest that male embryos seem to retain the ability of VTG II mRNA synthesis. The enhancement of estrogenic effects by double stimulation was also shown in chicken oviduct cells assayed by induction of ovalbumin and conalbumin synthesis (Moen and Palmiter, 1980). This report suggests that this phenomenon occurred by mRNA accumulation after primary stimulation of estrogen.

Alkylphenols including nonylphenol were first found to be estrogenic (estrogen-mimicking) about 60 years ago (Dodds and Lawson, 1938). 4-propylphenol caused vaginal cornification in the ovariectomized rats, as occurs during a normal estrous cycle, thus mimicking the activity of estradiol. Cornification also occurred with 4-tert-pentylphenol, but not with 2-n-pentylphenol, indicating the importance of the two groups being para-on the ring. More recent research has highlighted the implications of these effects. The growth of cultured human breast cancer cells was affected by 4-nonylphenol at concentrations as low as 1 μM (220 μg L⁻¹), and the proliferation was induced by 10 μM 4-nonylphenol similar to that produced by 30 pM estradiol, a concentration about 300,000 times lower (Soto et al., 1991). Further work showed that 4-nonylphenol could also induce expression of the progesterone receptor in human breast cancer cell (as estradiol does), and caused cell proliferation in ovariectomized rats (Soto et al., 1991). Estrogenic effects have also been shown on rainbow trout hepatocytes, chicken embryo fibroblasts and a mouse estrogen receptor (White et al., 1994). All of them showed estrogenic responses to alkylphenolic compounds and the most potent alkylphenolic compound was found to be octylphenol, which was active in many assays at 0.1 μM, and was able to stimulate responses similar to those produced by estradiol at concentrations only 1000 times higher than those of estradiol. Nonylphenol was 10- to 20-fold less potent than octylphenol. In multigeneration reproduction study in rats, epididymal sperm density and testicular spermatid count were reduced in F₂ male rats over 3 generations of 4-nonylphenol administration.
This study suggests that RT-PCR method using the whole chicken embryos is valid to detect weak estrogenic potencies and that may be applicable to reveal the estrogenic activity of other chemicals.

References


