Suppressive Effect of p-Nonylphenol on Male-Specific mRNA Expression in the Embryonic Gonad of Chickens

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Effects of estradiol-17β (E2) and p-nonylphenol (NP) on the mRNA expression of sex determination-related genes were examined in the embryonic gonad of chickens. Fertilized eggs were treated with either E2 (0.1 and 1.0 mg/egg) or NP (0.001, 0.01, 0.1 and 0.2 mg/egg) twice on days 13 and 16 of incubation. The mRNA expressions of anti-Müllerian hormone (AMH), SRY-related HMG box 9 (SOX9), cytochrome P450 aromatase (P450arom) and steroidogenic factor 1 (SF-1) in the embryonic gonads were determined by the reverse transcription-polymerase chain reaction (RT-PCR) on day 18 of incubation. AMH, SOX9 and P450arom, but not SF-1, showed sexually dimorphic expression in the control; AMH and SOX9 were male-specific while P450arom was female-specific. E2 had no significant effects on these expressions in either sex. In contrast, NP reduced the expressions of AMH and SOX9 only in the males but had no effects on the expressions of P450arom and SF-1. These results suggest that NP has endocrine disrupting effects on the mRNA expression of sex determination-related genes in the gonads of chicken embryos.

Key words: p-nonylphenol, AMH, SOX9, P450arom, SF-1

Introduction

p-Nonylphenol (NP), which is industrially used as a detergent constituent, is one of the environmental chemicals with estrogenic activity in some vertebrates (Warhurst, 1995). For example, NP has been shown to be weakly estrogenic as indicated by elevated vitellogenin 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). In chicken embryos, NP was shown to have weak estrogenic activity by assessment of liver vitellogenin II mRNA using a reverse transcription-polymerase chain reaction (RT-PCR) assay (Sakimura et al., 2001).
In birds, it is supposed that estrogenic environmental chemicals such as NP can affect their sex differentiation, because the differentiation of the gonad is sensitive to estrogens or antiestrogens. It has been known that estrogen treatment in bird embryos before the differentiation of the gonad causes feminization of the male gonad at hatching although they become male-phenotypic at sexual maturity, while tamoxifen, an antiestrogen, increased the phenotypic males (Coco et al., 1992). Furthermore, in ovo administration of a non-steroidal aromatase inhibitor caused female-to-male sex-reversal in chicken embryos with testes capable of producing the sperm (Elbrecht and Smith, 1992; Abinawanto et al., 1998).

Some molecular aspects of sex determination and differentiation in birds have been revealed in recent years (Shimada, in press). SRY gene, found as sex determining region Y in mammals, or its equivalent has not been found in birds (Griffiths, 1991; Coriat et al., 1993). It has been shown, however, that the expression of the cytochrome P450-aromatase (P450arom), the key enzyme converting androgen to estrogen, is shown in female gonads only and is one of the essential steps of ovarian differentiation. Anti-Müllerian hormone (AMH) causes regression of the Müllerian ducts in males during embryonic development (Vigier et al., 1987) and represses the P450arom expression (Vigier et al., 1989). In chickens, SOX9, a transcription factor belonging to SRY-related high mobility group box, is expressed in male gonads only and activates AMH in humans, but it may not activate AMH in chickens (Oreal et al., 1998; Smith et al., 1999a). The expression of steroidogenic factor 1 (SF-1), a transcription factor that may be involved in the expression of the AMH gene in humans, is higher in the ovary than in the testis in chickens (Smith et al., 1999b) although the relationship between AMH and SF-1 is unknown.

The objective of the present study is to examine the effects of E2 and NP on the mRNA expression of sex determination-related genes in chicken embryos. E2 or NP was injected to chicken eggs twice on days 13 and 16 of incubation according to an experimental protocol in our previous study where E2 had induced the vitellogenin II mRNA expression more effectively than by single injection on day 16 (Sakimura et al., 2001). The mRNA expressions of AMH, SOX9, P450arom and SF-1 in the embryonic gonad on day 18 of incubation were determined as the amount of their RT-PCR products.

**Materials and Methods**

*Chicken embryos and treatments with E2 and NP*

Fertilized chicken eggs with sex-linked feather color (Rhode Island Red $\sigma^\times$ Barred Plymouth Rock $\varphi$) were obtained from Okazaki Station, National Livestock Breeding Center (Okazaki, Aichi, Japan) and incubated at 37.5°C under standard conditions. The eggs were treated with either E2 (Sigma, St. Louis, MO, USA) or NP (Kanto Chemical, Tokyo, Japan) twice on days 13 and 16 of incubation. E2 (0.1 and 1.0 mg/egg/injection) and NP (0.001, 0.01, 0.1 and 0.2 mg/egg/injection) dissolved in 50 µl of 1,2-propanediol (PD, Kanto Chemical, Tokyo, Japan) were injected into the air sac of the eggs as described previously (Sakimura et al., 2001). The eggs in the control
group received 50 μl of PD. Embryos were removed from the eggs on day 18 of incubation and their genetic sex were examined by a white spot on the head of males. The gonads of the embryos were collected individually and stored at −80°C until the extraction of their total RNA. The right gonad of the female embryos could not be collected because of its regression.

**RNA extraction and reverse transcription**

Total RNA of the gonad was extracted from each of the embryos using RNeasy™ Mini Kit (Quiagen, Hilden, Germany). The extracted RNA (1 μg) was reverse transcribed in a reverse transcription mixture (20 μl) as described previously (Sakimura et al., 2001).

**PCR amplification**

The reverse-transcribed product (1 μl) was amplified by PCR in a reaction mixture (25 μl) as described previously (Sakimura et al., 2001). Sequences and nucleotide positions of the primers used, which were designed from respective chicken genes, were as follows. The primers for AMH (Carré-Eusèbe et al., 1996) were 5′-CTG CAG CGC TTC CTT ATC CT-3′ (553–572) and 5′-GCA GTG GCA AAC CGT GAG TG-3′ (959–978). The primers for SOX9 (accession number AB012236) were 5′-GAG GAA GTC GGT GAA GAA CG-3′ (650–669) and 5′-ATG GCC ACG TCT CGT AAA TC-3′ (927–946). The primers for P450arom (McPhaul et al., 1988) were 5′-CAG CCA GTT GTG GAC TTA AT-3′ (1195–1214) and 5′-CCT CTT CCT TTC ATT GTC TG-3′ (1477–1496). The primers for SF-1 (Kudo and Sutou, 1997) were 5′-TGA AGT ACC TGG AGA ACC AC-3′ (1290–1309) and 5′-TCA AGT CCG CTT GGC GTG CA-3′ (1515–1534). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Dugaiczyk et al., 1983) were 5′-GTG GAG AGA TGA CAG AGG TG-3′ (608–627) and 5′-AAG CAG AAG CTT GAC GAA ATG GT-3′ (937–956). The cycle number of the reaction was 30 for SOX9, and was 25 for AMH, P450 arom and SF-1. Primers for GAPDH were served as an internal standard using same cycle number as each primer pair. The amount of the PCR product was semi-quantitatively determined from the density of the gel band using the Scion Image for Windows (Scion Corporation, Maryland, USA). Relative density of each gene was compared with GAPDH products.

**Statistical analyses**

Because of the limited number of embryos in each of the NP and E2 groups due to the complicated experimental method, the data were combined to the NP treatment group and the E2 treatment group for the statistical analysis. Statistical significance of the differences between the control and the NP or E2 treatment groups were examined by the Student’s t test or the Aspin & Welch t test (Gad, 2001). This analysis was not applied when the number of embryos expressing the mRNA is less than two in the control, E2 treatment and/or NP treatment groups.

**Results**

**Embryotoxicity of E2 and NP**

Embryonic body weight is shown in Table 1. E2 did not affect the embryonic
Table 1. Number of examined embryos and mean body weight after injection of E2 or NP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/egg)</th>
<th>Number of embryos</th>
<th>Mean body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>Male: 3, Female: 4</td>
<td>25.53</td>
</tr>
<tr>
<td>E2</td>
<td>0.1</td>
<td>Male: 3, Female: 2</td>
<td>26.04</td>
</tr>
<tr>
<td>E2</td>
<td>1.0</td>
<td>Male: 2, Female: 1</td>
<td>27.88*</td>
</tr>
<tr>
<td>NP</td>
<td>0.001</td>
<td>Male: 1, Female: 1</td>
<td>25.80</td>
</tr>
<tr>
<td>NP</td>
<td>0.01</td>
<td>Male: 1, Female: 1</td>
<td>26.15</td>
</tr>
<tr>
<td>NP</td>
<td>0.1</td>
<td>Male: 1, Female: 1</td>
<td>23.36</td>
</tr>
<tr>
<td>NP</td>
<td>0.2</td>
<td>Male: 1, Female: 1</td>
<td>20.30**</td>
</tr>
</tbody>
</table>

The eggs were treated with either NP or E2 on days 13 and 16 of incubation. The body weight of males and females were combined for statistical analysis.

*: Statistically significant compared with the control value (p < 0.05, p < 0.01).

body weight. NP caused significant decrease in the embryonic body weight at 0.2 mg/egg. Embryonic death was not observed except for NP at 0.2 mg/egg (one of three embryos dead). Thus, only NP at the highest dose caused embryotoxicity.

**AMH mRNA expression**

AMH mRNA showed male-specific, sexually dimorphic expression (Fig. 1). The AMH expression in the control group was nearly ten-fold higher in the males than in the females. Effects of the E2 on the AMH expression were not observed in either sex. On the other hand, NP showed a tendency to reduce the AMH expression in the males, and there was a significant difference in the male left gonad between the control and the NP treatment groups, although there was no significant difference in the right gonad.

![Fig. 1. AMH mRNA expression in the gonads of chicken embryo. mRNA expressions are expressed as relative density of RT-PCR products compared with GAPDH. *: Statistically significant difference between the control and the treatment groups (p < 0.05).](image)
probably due to somewhat larger variance. Effects of NP on the AMH expression, however, were not observed in the females.

**SOX9 mRNA expression**

Similar to AMH, SOX9 mRNA showed male-specific, sexually dimorphic expression (Fig. 2). In the control group, SOX9 was clearly expressed in the males but was barely expressed in the females. E2 showed a tendency to reduce the SOX9 expression at 1.0 mg/egg, but there was no significant difference between the control and the E2 treatment groups in either sex. As in the AMH expression, NP had a tendency to reduce the SOX9 expression in the males. There was a significant difference in the male right gonad between the control and the NP treatment groups. Effects of NP on the SOX9 expression were not observed in the females as in the AMH expression.

**Fig. 2.** SOX9 mRNA expression in the gonads of chicken embryo. mRNA expressions are expressed as relative density of RT-PCR products compared with GAPDH. *: Statistically significant difference between the control and the treatment groups (p < 0.05).

**Fig. 3.** P450arom mRNA expression in the gonads of chicken embryo. mRNA expressions are expressed as relative density of RT-PCR products compared with GAPDH.
Fig. 4. SF-1 mRNA expression in the gonads of chicken embryo.

mRNA expressions are expressed as relative density of RT-PCR products compared with GAPDH.

**P450arom mRNA expression**

P450arom mRNA also showed sexually dimorphic expression, but was exclusively expressed in the females (Fig. 3). Significant effects of the E2 on the P450arom expression were not observed in either sex as in the AMH and SOX9 expressions. Similarly, but unlike in the AMH and SOX9 expressions, NP had no effects on the P450 arom expression in either sex.

**SF-1 mRNA expression**

SF-1 mRNA showed no sexually dimorphic expression, being expressed at almost the same level in the males and females (Fig. 4). E2 showed a tendency to reduce the SF-1 expression, but there was no significant difference between the control and the E2 treatment groups in either sex. NP had no significant effects on SF-1 expression in either sex as in the P450arom expression.

**Discussion**

The present results demonstrated that NP reduced the AMH and SOX9 mRNA expressions which were specific for the male gonad, but had no effects on the P450arom and SF-1 mRNA expressions. It is unlikely that these effects were due to the embryotoxicity of NP because any obvious symptoms for embryotoxicity was not observed except for the highest dose. It is considered that these effects of NP are close but not identical with E2 effects because E2 had no significant effects on these mRNA expressions and reduction of mRNAs by NP are consistent with the expression profiles of these mRNAs during female sex differentiation that occur after the onset of estrogen production in chicken embryos. In the gonad of female chicken embryos, estrogens are produced after day 3.5 of incubation (Woods and Erton, 1978) and about 3.0 days later, the down-regulation of AMH and SOX9 mRNA expressions begins (Smith et al., 1999a). It has also been indicated that the E2 injection into chicken eggs reduced the AMH mRNA expression and enhanced aromatase mRNA expression in the embryonic
The mechanisms of the suppressive effects of NP on the expression of AMH and SOX9 mRNA may involve the interaction with the estrogen receptor (ER) because NP binds to the rat ER (Odum et al., 2001; Mueller et al., 1978) and human ER (Bolger et al., 1998). The cause of the asymmetric reduction of the SOX9 mRNA expression in the right gonad of the males by NP is not known but may be involved in the asymmetric effects development of female also be induced by exogenous estrogens (Etches and Kagami, 1997). Unlike NP, E2 did not affect the expression of AMH, SOX9 and P450arom mRNA in spite of its high affinity to the ER. This may suggest that the effects of NP do not involve or differently involve the interaction with the ER.

No significant effects of E2 were observed in the AMH mRNA expression in the gonad of chicken embryos in the present study. It has been reported, however, that E2 reduced the AMH mRNA expression and increased the P450arom mRNA expression in the chicken embryonic gonad when E2 was injected on day 5 of incubation and the mRNA expressions were examined on day 14 (Nakabayashi et al., 1998). The suppression of these mRNA expressions was also confirmed in the embryos on day 6–8 of incubation when E2 was injected on day 3 (Nishikimi et al., 2000). This discrepancy may be explained by the critical period of susceptibility of the developing gonad to estrogens. The day of E2 injection or the day of examination in the present study might be too late to cause significant effects on these mRNA expressions even though they are suited to observe the effects of E2 on vitellogenin II mRNA expression in the embryonic liver (Sakimura et al., 2001).

Because E2 induced vitellogenin II mRNA expression in the embryonic liver at the doses as low as 0.1 mg/egg by the same experimental protocol as in the present study (Sakimura et al., 2001), it is considered that the effects of E2 on the embryonic gonad were regulated in a distinct manner. The effects of exogenous E2 on the embryonic gonad may be weak at the later incubation days due to the maturation of the endocrine system and/or the metabolic system, while the embryonic liver becomes more responsive like the adult liver. In this context, it is considered that the effects of NP are not regulated by the endocrine and/or the metabolic system unlike E2 because the extent of the effects on the mRNA expressions is different between E2 and NP.

As for toxicologic assessment of NP, the effective dose is in the range of 0.01 to 0.2 mg/egg for the suppressive effects on the AMH and SOX9 mRNA expressions in the chicken embryonic gonad in the present study. On the other hand, NP induced vitellogenin II mRNA expression in the embryonic liver at an embryotoxic dose of 10 mg/egg by the same experimental protocol (Sakimura et al., 2001). One of the novel findings in the present study is that the AMH and SOX9 mRNA expressions in the embryonic gonad are about 50 fold more sensitive than the vitellogenin II mRNA expression in the embryonic liver. This means that the selection of these parameters is critical for the assessment of endocrine disrupting activity of NP.

The sexually dimorphic mRNA expression profiles observed in the present study is essentially consistent with those reported so far. However, the SF-1 mRNA expression was not sexually dimorphic and tended to be reduced by E2 in the present study. This
is inconsistent with the observation that the SF-1 mRNA expression was up-regulated and was higher in the female gonad than in the male gonad of chicken embryos after day 7 of incubation (Smith et al., 1999b). This discrepancy cannot be explained at present but may indicate that the expression of SF-1 is not critical for the sex differentiation in chicken embryos. The finding that the sexually dimorphic expression of chicken SF-1 begins after the AMH expression in male embryos (Smith et al., 1999a) and the P450 arom expression in female embryos (Nishikimi et al., 2000), supports this notion.

In conclusion, it is considered that NP suppresses the male-specific mRNA expression of sex determination-related genes in the gonad of chicken embryos by its endocrine disrupting activity, which is close but not identical with the estrogenic activity. Furthermore, the AMH and SOX9 mRNA expressions in the embryonic gonad are much more sensitive than the vitellogenin II mRNA expression in the embryonic liver.

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