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Effects of 4-Nonylphenol on the Induction of Plasma Vitellogenin (VTG), Alkaline-Labile Protein Phosphorus (ALPP), Calcium (Ca), Glutamate Pyruvate Transaminase (GPT) and Hepatosomatic Index (HSI) in the Immature Rockfish, *Sebastes schlegeli*

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4-NP가 미성숙 조피볼락, *Sebastes schlegeli*의 혈장 VTG, ALPP, Ca, GPT 및 HSI에 미치는 영향

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요 약

4-nonylphenol (4-NP)이 해산어류인 조피볼락, *Sebastes schlegeli*의 혈장 vitellogenin (VTG), alkaline-labile protein phosphorus (ALPP), calcium (Ca), glutamate pyruvate transaminase (GPT) 및 hepatosomatic index (HSI)에 미치는 영향을 조사하였다. 실험어에 3일간격으로 estradiol-17β (E_2, 5 mg/kg B.W.) 또는 4-NP (0, 10, 50, 100 및 200 mg/kg B.W.)을 복강에 2번 주사한 후, 7일째에 채혈과 적출을 통해 혈장과 간장을 수집해 분석이 실시되었다. 대조 실험어에는 용매로 사용된 70% 에탄올만이 투여되었다. E_2 투여 실험어의 혈장 단백질을 전기 영동상으로 분석한 결과 약 170 kDa의 위치에서 짙은 VTG 밴드가 관찰되었으나, 용매만 투여한 대조 실험어의 혈장에서는 동일 밴드가 관찰되지 않았다. 4-NP 투여한 모든 실험어의 혈장 단백질에서는 E_2 투여 실험어와 동일한 VTG 밴드가 관찰되었다. 혈장 ALPP와 Ca 농도도 4-NP 투여 실험어에서 E_2 투여 실험어와 유사하게 증가하였으며, 이들 농도 변화는 VTG 합성과 더불어 증가하는 경향을 나타냈다. 혈장 전위효소인 GPT와 HSI도 E_2 투여 실험어와 유사하게 4-NP가 투여된 모든 실험어에서 급격히 증가하였다.

이상의 결과로부터 연안생태계 내에서 서식하는 어류가 4-NP과 같은 내분비 장애물질 (Endocrine Disrupting Compounds, EDCs)에 의해 영향을 받는지를 규명하기 위한 생물학적 지표로서 VTG와 더불어 혈장 ALPP와 Ca이 사용가능할 것으로 판단된다. 또한, 조피볼락과 같은 해산어가 EDCs에 노출되어 VTG가 합성될 때 간장 기능의 손상으로 혈장 전위효소인 GPT가 일시적으로 증가하고 간장도 비대해져 HSI가 높아지는 것으로 판단된다.

Key words : 4-nonylphenol, estradiol-17β, vitellogenin, plasma values, biomarker, *Sebastes schlegeli*

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INTRODUCTION

During the recent 10 years, remarkable concern has been emerged over endocrine disrupting compounds (EDCs) which mimic the effects of the steroid hormone and modulate the endocrine functions of human and wildlife (Jobling *et al.*, 1996; Kendall *et al.*, 1998; Tyler *et al.*, 1998). The biodegradation products of surfactants such as nonylphenol (NP) have been regarded as EDCs (Nimrod and Benson, 1996).

Vitellogenin (calcium-binding phosphlipoglycoproteins, VTG), a precursor molecule of egg yolk, is synthesized in the liver of mature female under estrogen (estradiol-17β, E_2) stimulation and is also used as biomarker of EDCs exposure (Christiansen *et al.*, 1998). It has been reported that VTG are induced by the estrogenic activity of 4-NP in the liver of rainbow trout (Smeets *et al.*, 1999). Also, *in vitro* experiments using the hepatocyte of rainbow trout, 4-NP promoted VTG synthesis (Kloas *et al.*, 1999). However, only few studies have been carried out to investigate the effect of EDCs on VTG synthesis in marine fish species. Therefore, in this study, we used the immature rockfish as experimental fish that is an excellent indicator of coastal environment pollution, because this fish is fairly stationary in the coastal rock area.

The present study was undertaken to examine the effect of 4-NP administration on reproductive physiology in the immature rockfish, *Sebastes schlegeli*. The changes of plasma VTG by 4-NP administration were analyzed by electrophoresis. The concentrations of plasma alkaline-labile phosphorus (ALPP) and calcium (Ca) were also analyzed as biomarkers for determining EDCs using the quantivative analysis of inorganic phosphorus (Parsons *et al.*, 1984) and the method of o-cresolphthalein-complexon (Bjornsson and Haux, 1985), respectively. An eventual toxic effect of 4-NP administration was also analyzed using kit of reitman-frankel methods for the concentrations of plasma the glutamate pyruvate transaminase (GPT). This enzyme has been previously been used as an indicator of toxic effects on the liver (Hwang and Kang, 2002). Hepatosomatic index (HSI) was expressed by liver weight × 100/body weight.

MATERIALS AND METHODS

Immature rockfish, *Sebastes schlegeli* weighing about 50 g were obtained from Institute of Fisheries Sciences, Pukyoung National University (Busan, Korea) and kept in indoor tanks with continuously running water with 33‰ salinity at constant temperature of 18°C. They were not fed during experimental periods.

1. 4-NP injection and blood sampling

Fish were intraperitoneally injected with E_2 (5 mg/kg body weight) or 4-NP (4-*t*-nonylphenol hydroxyl, Fluka) (0, 10, 50, 100, and 200 mg/kg body weight) in 70% ethanol twice at 3-day intervals and blood sample were extracted from the fish (n=7 per experiment tanks), as described below, 7 days after the last administration. Control received the vehicle ethanol only. The injected concentrations of E_2 and 4-NP were decided by Hwang and Kang (2002).

Fish were anesthetized with 2-phenoxyethanol and the blood was collected by injecting the tail of fish and by draining the blood into heparinized capillary tubes. Plasma was separated by centrifugation (350 × g, 8 min) and frozen at −20°C until analyzed for VTG, ALPP, Ca and GPT concentrations.

2. Protein determination and electrophoresis

Protein concentrations of the plasma were determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Plasma proteins were analyzed by 5~20% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (1970). The gels were run at 30 mA and stained with 0.25% Coomassie brilliant blue R-250. Standard proteins used for molecular mass determinations were carbonic anhydrase (29 kDa), ovalbumin (45 kDa),

bovine serum albumin (66 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa), and myosin heavy chain (205 kDa).

3. Effect of 4-NP on VTG synthesis

Fish were received twice administration of 4-NP at 3-day intervals and were sampled after 7 days. The dose was the same as described above. Plasma was separated from the blood of fish and analyzed for the main VTG band by SDS-PAGE.

4. Effect of 4-NP on ALPP concentration

ALPP was extracted from 0.01 mL sample according to the method of Wallace and Jared (1968) and determined by colorimetric assay of the acidified phosphomolybdate complex using a commerically available kit (Sigma, 670-A). This kit consists of the necessary reagents including phosphorus reagent and calcium/phosphorus standard.

The assay was conducted according to the kit instruction. Briefly, 1 mL of phosphorus reagent was added to 0.01 mL of calcium/phosphorus for standard and added to 0.01 mL of plasma in 4-NP-injected rockfish and then reacted at room temperature for at least 15 min. The distilled water was also used as the blank. The absorbance of standard and samples were measured by atomic absorption spectrophotometer (DR/4000, HACH) at 340 nm.

5. Effect of 4-NP on Ca concentration

Ca concentration was examined using Ca assay kit (Asan Pharm. Co., Ltd) by the method of o-cresolphthalein-complexon (Bjornsson, 1985). This kit consists of the necessary reagents including standard solution I, II, reaction solution and assay solution.

The assay was conducted according to the kit instruction. Briefly, 0.5 mL of the reaction solution was added standard solution I, II and added to 0.05 mL of plasma in 4-NP-injected rockfish and then mixed at room temperature. The distilled water was used as the blank. The assay solution of 5 mL was added to the standard solutions and samples and then 4 absorbance were measured by atomic absorption spectrophotometer (DR/4000, HACH) at 575 nm.

6. Effect of 4-NP on GPT concentration

GPT was examined using assay kit (Asan Pharm. Co., Ltd) by the method of Reitman-Frankel (Racicot *et al.*, 1975). This kit consists of the necessary reagents including standard solution, reaction solution, assay solution and 4 N NaOH solution.

The assay was conducted according to the kit instruction. Briefly, 1 mL of the reaction solution was added to 0.2 mL of plasma in 4-NP-administrated rockish and then mixed at 37°C for 30 min. After 20 min, 10 mL of 0.4 N NaOH was added and mixed at room temperature for 10 min. The distilled water was used as the blank. The GPT of standard solutions and samples were measured by atomic absorption spectrophotometer (DR/4000, HACH) at 505 nm.

7. Effect of 4-NP on HSI

The body and liver weight of 4-NP-administrated fish were examined and then HSI was expressed as liver weight × 100/body weight.

8. Statistical analysis

Data were analyzed by one-way ANOVA (Fisher PLSD test). Fisher's test was also used to examine the significance of correlation coefficients. Significance was accepted at $P < 0.05$.

RESULTS

1. Effect of 4-NP on VTG synthesis

Plasma sampling was extracted and then analyzed by SDS-PAGE in 7 days after E_2 administration. A newly synthesized VTG band was detected at a molecular weight position of about 170 kDa in E_2-administered fish. However, the control group without E_2 did not produce an equivalent protein (Fig. 1).

The administration of 4-NP to fish also increased the intensity of CBB staining for the VTG band. Intense VTG bands were detected in all the plasma of 4-NP-administered fish as that of E_2-administered fish (Fig. 1).

2. Effect of 4-NP on ALPP concentration

Plasma sampling was extracted and then analyzed by colorimetric assay in 7 days after E_2 administration. Plasma ALPP was suddenly increased in all the group of 4-NP-administered fish than that of the control group with solvent only (Fig. 2). Significant increase was confirmed at the all plasma of 4-NP-administered fish that of E_2-administered fish ($P < 0.05$).

3. Effect of 4-NP on Ca concentration

Plasma sampling was extracted and then analyzed by the method of *o-cresolphthalein*-complexon in 7 days after 4-NP and E_2 administration. Plasma Ca was suddenly increased in all the group of 4-NP-administered fish than that of the control group with solvent only (Fig. 3). Significant increase was confirmed at the all plasma of 4-NP-administered fish as that of E_2-administered fish ($P < 0.05$).

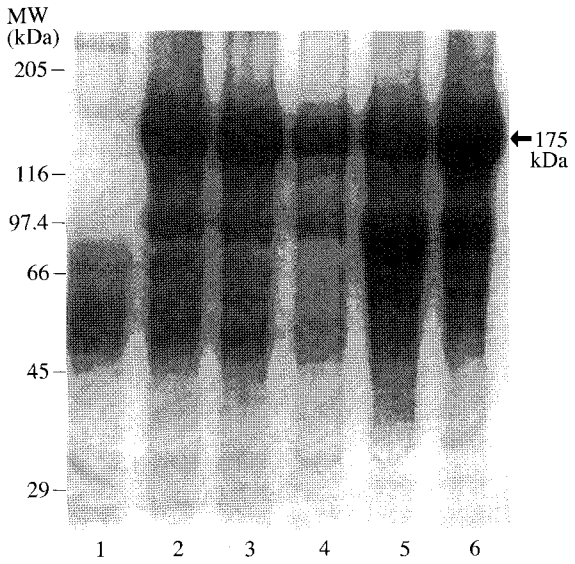

Fig. 1. SDS-PAGE of plasma in 4-NP or E_2-administered immature rockfish, showing the expression of VTG bands (arrowhead). Plasma were analyzed on day 7 after 4-NP (10×2, 50×2, 100×2, 200 mg/kg×2) or E_2 (5 mg/kg×2) administration. Lane 1: control (solvent only); Lane 2: E_2 (5 mg/kg); Lane 3: 10 (mg/kg); Lane 4: 50 (mg/kg); Lane 5: 100 (mg/kg); Lane 6: 200 (mg/kg).

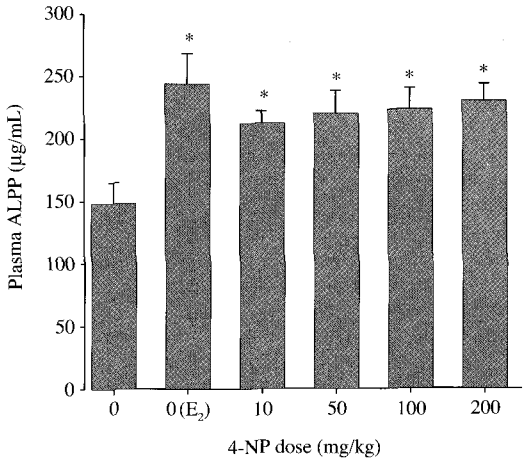

Fig. 2. ALPP changes in the plasma of 4-NP or E_2-administered immature rockfish. Vertical bars represent the SE of the mean for the seven individuals. *$P < 0.05$ for control (solvent only).

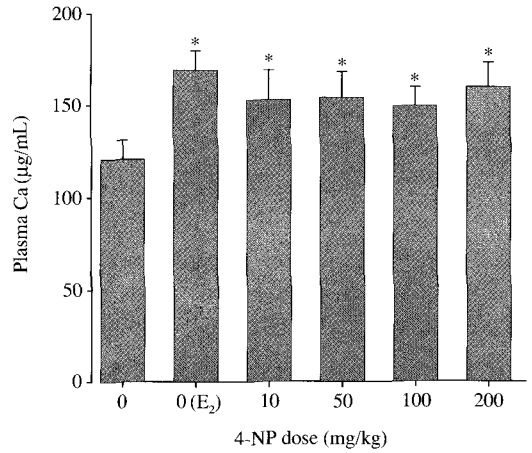

Fig. 3. Ca changes in the plasma of 4-NP or E_2-administered immature rockfish. Vertical bars represent the SE of the mean for the seven individuals. *$P < 0.05$ for control (solvent only).

4. Effect of 4-NP on GPT concentration

GPT was increased with increasing 4-NP dose (Fig. 4). Significant increase was confirmed at 4-NP doses of 10, 50, 100, and 200 (mg/kg), in which the GPT was increased to 24%, 47%, 60% and 67% than that of the control, respectively. GPT was also significantly increased to 27% ($P<0.05$) of the control in E_2-administrated fish (Fig. 4).

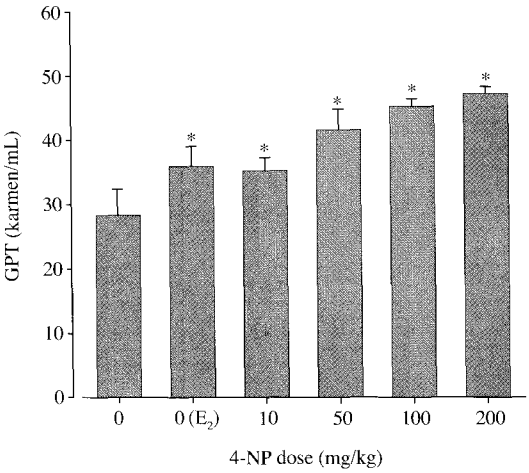

Fig. 4. GPT changes in the plasma of 4-NP or E_2-administered immature rockfish. Vertical bars represent the SE of the mean for the seven individuals. *$P<0.05$ for control (solvent only).

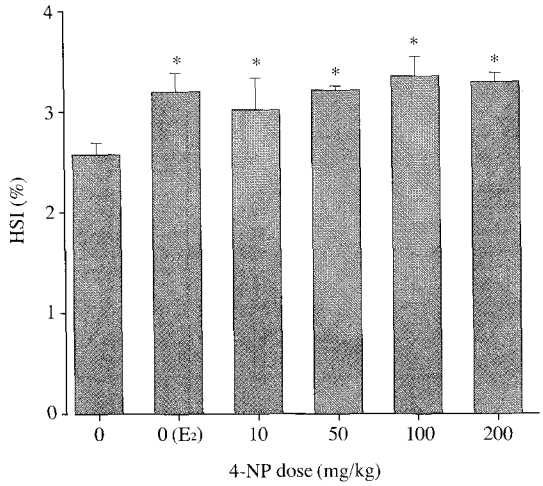

Fig. 5. HSI changes in the plasma of 4-NP or E_2-administered immature rockfish. Vertical bars represent the SE of the mean for the seven individuals. *$P<0.05$ for control (solvent only).

5. Effect of 4-NP on HSI

HSI was measured at 7 days after 4-NP and E_2 administration (Fig. 5). HSI was increased in all the group of 4-NP-administered fish than that of the control group with solvent only. Significant increase was confirmed at 4-NP doses of 10, 50, 100 and 200 (mg/kg), in which the HSI was increased to 15% ($P<0.05$), 20% ($P<0.05$), 24% ($P<0.05$) and 22% ($P<0.05$) of the control, respectively. HSI was also significantly increased to 20% ($P<0.05$) of the control in E_2-administrated fish (Fig. 5).

DISCUSSION

In the present study, immature rockfish were intraperitoneally administrated with 4-NP, because of this method be able to compare the eventual dose-dependent response and avoid large amounts of 4-NP-polluted waste water during experiment periods.

VTG synthesis were observed in the all plasma of 4-NP-administrated immature rockfish in this study. This result is very similar to that of the effects of estrogen (estradiol-17β, E_2) in the male flounders (Emmersen *et al.*, 1979). It is well known that the bulk of synthetic chemicals such as 4-NP with estrogenic activity binding to the E_2 receptor (ER), regulating the activity of E_2 responsive genes and inducing VTG synthesis in the hepatocytes (Villeneuve *et al.*, 2002). Hwang and Kang (2002) also reported that bisphenol-A induced VTG synthesis by BPA-ER binding activity in the hepatocyte of rainbow trout. Therefore, VTG was synthesized by estrogenic activity like 4-NP-ER binding in this study.

Emmersen *et al.* (1979) reported that plasma ALPP concentrations were increased in male flounders with E_2 treatment and this increase is very similar to that of VTG. Also, the increase of VTG synthesis by 4-NP exposure is similar to that of ALPP concentration,

which has been shown to be a very reliable indicator of circulating VTG in fish (Nagler *et al.*, 1987; Kramer *et al.*, 1998) Similarly, the increase of plasma ALPP concentrations by 4-NP adminiatration were similar to that of VTG in this experiment using the juvenil rockfish.

Nagler *et al.* (1987) and Tinsley (1985) reported the concentration of plasma Ca can also indicate circulating vitellogenin. Ca binds to vitellogenin, so that an Ca increase is mainly due to an increase of the protein-bound calcium fraction in the blood (Bjornsson and Haux, 1985). In the present experiment, the concentrations of plasma Ca were increased but the increase of total protein concentrations were not investigated. It is reported that total protein concentrations were decreased with increasing dose of 4-NP, because the effect of 4-NP on the hepatic tissue in the flounder (Christen *et al.*, 1999). Maybe, effect likely to be differed by 4-NP exposure in fish species. Christen *et al.* (1999) reported that mortality was observed with increasing doses of 4-NP treatment in the flounder but not observed in the immature rockfish administrated with the same concentrations in this study.

The concentrations of plasma enzyme GPT has frequently been used to detect an eventual damage to the liver cells. Hwang and Kang (2002) reported VTG induction by exogenous E_2 damage to hepatocyte, and the concentrations of plasma GPT were temporarily increased in the immature rockfish. In this experiment, a significant increase were observed in the concentrations of plasma GPT of the 4-NP-administrated immature rockfish. This result indicate that the effect of 4-NP exposure on the hepatic tissue. The increase of HSI were also observed in immature rockfish treated with E_2 (Hwang *et al.*, 2004). In this experiment, the increase of HSI by 4-NP administration were similar to that of E_2. The liver was primarily hypertrophied during the induction of VTG synthesis by E_2 and 4-NP exposure. In the present study, the concentrations of plasma ALPP and Ca were closely related to the synthesis of VTG by 4-NP administration in the immature rockfish. Also, the plasma GPT concentration and HSI were suddenly increased by 4-NP administration.

In conclusion, these results suggest that the concentrations of plasma ALPP and Ca could be utilized as a biomarkers of EDCs such as 4-NP in coastal ecosystem, because the changes of plasma ALPP and Ca concentration after 4-NP administration were similar to that of VTG. The process of VTG induction by 4-NP administration damage to hepatocyte, and the concentrations of plasma GPT were temporarily increased in the immature rockfish. HSI were also increased by 4-NP administration, because primarily hypertrophic.

CONCLUSION

These results suggest that the changes of plasma ALPP and Ca concentrations could be utilized as a biomarker of EDCs exposure in coastal ecosystem, because the changes of ALPP and Ca concentrations after 4-NP administration were very similar to that of VTG, biomarker of EDCs exposure. The process of VTG induction by EDCs damaged to hepatocyte, and the concentrations of plasma enzyme GPT were temporarily increased. HSI were also increased, because primarily hypertrophic in related to the induction of VTG synthesis by EDCs administration.

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