EFFECTS OF 17α-ETHINYLESTRADIOL AND NONYLPHENOL ON THE INDUCTION OF VITELLOGENIN GENE EXPRESSION IN MEDAKA, *Oryzias latipes*

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Introduction

Endocrine disruptors are hormone mimics that modify hormonal action in humans and wildlife. They bind to the estrogen receptor or androgen receptor, mimic natural hormones and influence vital endocrine functions[1,2]. Recently, environmentally induced alterations of endocrine function in fishes are well being studied and sex reversal or intersexuality are known to be induced following exposure of egg or juvenile fish to environmental estrogens during the early part of a fishes life[3-7].

Vitellogenin which is the precursor of yolk protein in oviparous animal is known to be regulated by estrogen and induced by the interaction between estrogen receptor and the chemicals through the transcriptional activation[8,9]. Vitellogenin synthesis and secretion is known to be normally restricted to adult female. However, its production can be induced in males and immature females by exogenous estrogenic chemicals[10, 11]. So, the measurement of this protein in the plasma of male or juvenile fish provides a ready means of testing for the exposure of environmental estrogens[12,13]

Despite the widespread use of medaka *Oryzias latipes* for various toxicological research field, not many studies have been carried out for screening and assessment for the endocrine disruption. However, researches on endocrine disruption, especially the estrogenic effects of endocrine disruptors using medaka are increasing recently because experiments with medaka are relatively rapid and sensitive *in vivo* assay for assessing estrogenicity[14-16]. Here, we performed screening the effects of estrogen-like chemicals 17 α -ethinylestradiol and nonylphenol on the vitellogenin gene expression of male medaka, using reverse transcription (RT)-PCR method. The RT-PCR method has become increasingly popular for analysis of gene transcripts, primarily because it is highly sensitive and rapid. Thus, by using this method, we examined the possibility of medaka vitellogenin mRNA expression as a biomarker for the screening of environmental estrogenic chemicals.

Materials and Methods

Experimental animals and exposure

The orange-red variety of the medaka were fed on artemia daily and kept in 100L aquaria at 25℃ on 18hr light/6hr dark condition. Sexually matured male and spawning female with mean weight of 250mg were exposed to 17α-ethinylestradiol at nominal concentrations of 1, 5, 10, 20, 50, 100 and 200 μg/L for 144 hours. Nonylphenol and bisphenol A were treated with concentrations of 5, 50, 100, 200 and 500 μg/L for 144 hours, respectively. Ethanol was used as a vehicle for the chemicals.

Preparation of total RNA

Total RNA was carefully extracted from the livers of treated males and spawning females. For tissue disruption and preparation of total RNA, QIA-Shredder and RNeasy mini-kits(*QIAgene Co.*) were used according to the manufacture's instructions. Purified RNA samples were diluted at 1 μg/μℓ for RT-PCR or store at -80℃until further use.

RT-PCR, cloning and sequencing

Reverse transcription PCR was performed using RNA template, AMV reverse transcriptase, RNase inhibitor, dNTPs and oligo dT primers in 20 μℓ volume at 42℃ for 30min, and PCR reaction was then performed for 30 cycles at 94℃for 30sec, 60℃for 30sec and 72℃for 1min in 50 μℓ total mixture volume. For the sequence analysis of vitellogenin(VTG) cDNA, amplification of cDNA specific for the VTG gene of spawning female medaka was performed using degenerated primers based on the published VTG sequences of rainbow trout[17], resulting in 1200bp. The slices of 1200bp amplified cDNA fregments was cut out from the agarose gel and was cloned into pGEM-T easy vector(*Promega co*) and then the cDNA clone was applied to the automatic DNA sequencer. Based on the partial VTG cDNA analysis, VTG primers used for RT-PCR analysis were selected. The housekeeping protein β-actin of medaka was used as internal standard .

Results and Discussion

17α-ethinylestradiol(EE2) is synthetic estrogen used as oral contraceptives and nonylphenol is widely used in detergents, plasticizer, paints and cleaning agents. Both EE2 and nonylphenol have been shown to interact with estrogen receptor and to induce human breast cancer cell growth[18]. In this study, the estrogenic effect of EE2 and NP on the vitellogenin gene expression was performed *in vivo*. The 1200bp long cDNA was amplified with RT-PCR from the spawning female medaka and cloned into pGEM-T easy vector for seqence analysis. Comparison of the cDNA sequence and deduced amino acids sequence of this cloned cDNA expressed protein with the published sequences of the vitellogenin proteins using NCBI blast search programm revealed significant similarity with *Fundulus heteroclitus*(mummichog) and *Oreochromis aureus* (tilapia) vitellogenin. The identity of the deduced amino acids sequence of this fragment with vitellogenin proteins of *Fundulus heteroclitus*, *Oreochromis aureus* and *Oncorhynchus mykiss*(rainbow trout) was 62%,

54% and 49%, respectively. From these results, 1200bp cDNA fragment were identified as a part of vitellogenin, so we selected a pair of primers from this region resulting 750bp fragment for assessing the estrogenic effects of estrogen-like chemicals.

Medaka males were exposed to EE2 ranging from 1 to 200 μg/L for 144hrs to measure the vitellogenin induction level. 50 μg/L of EE2 was sufficient to induce the vitellogenin mRNA expression and 750bp fragments of the vitellogenin were detected in the treated group in concentrations of 20～200 μg/L EE2 (Fig 1). Vitellogenin gene induction caused by EE2 reached saturation at a concentration of 100 μg/L in this experiment. The 540bp β-actin cDNA fragments were approximately expressed equally in all treated and control group. These results showed that the induction of vitellogenin mRNA caused by synthetic estrogen EE2 was dose-dependent and no differences concerning expression of β-actin could be observed in parallel in all test fish.

There is an evidence that the estrogenic activity of nonylphenol is mediated by binding to the estrogen receptor. However, the binding potency of nonylphenol to the estrogen receptor was found to be very weak. In this study, although the estrogenic activity of nonylphenol is shown to be weak relative to EE2, vitellogenin fragment(750bp) appeared at 100 μg/L of nonylphenol in agarose gel electrophoresis. The amplified cDNA band was observed with a faint at this concentration, but 200 μg/L of nonylphenol could induce the vitellogenin mRNA expression sufficiently (Fig 2). Michelle A *et al.* reported that 86% of males developed testis-ova among medaka exposed to 100 μg/L of nonylphenol from 1 day posthatch to 3 months[19].

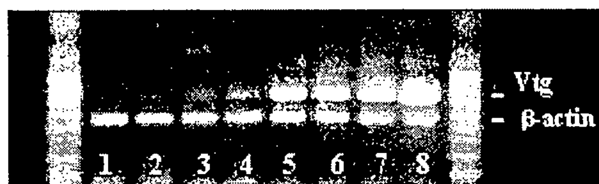

Fig 1. Dose-response of EE2 on the vitellogenin gene expression. RT-PCR was performed for 30 cycles. In treated groups, 50 μg/L of EE2 was sufficient to induce the Vtg expression(750bp band). β-actin primers for a 540bp fragment were used for control expression.
1: control, 2: 5 μg/L EE2, 3: 10 μg/L EE2, 4: 20 μg/L EE2, 5: 50 μg/L EE2, 6: 100 μg/L EE2, 7: 200 μg/L EE2, 8: Spawning female

Fig 2. Dose-response of nonylphenol on the vitellogenin gene expression. RT-PCR was performed for 30 cycles. In treated groups, Vtg cDNA fragments(750bp) started to be seen at 100 μg/L of nonylphenol(NP). β-actin primers for a 540bp fragment were used for control expression.
1: control, 2: 5 μg/L NP, 3: 50 μg/L NP, 4: 100 μg/L NP, 5: 200 μg/L NP, 6: 500 μg/L NP, 7: Spawning female

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