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Induction of estrogen-responsive gene transcription in the embryo, larval, juvenile and adult life stages of zebrafish as biomarkers of short-term exposure to endocrine disrupting chemicals

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ABSTRACT

Real-time quantitative RT-PCR was adopted to investigate the transcription of the estrogen-responsive genes in different developmental stages of zebrafish exposed to endocrine disrupting chemicals (EDCs). The lowest observed effect concentrations (LOECs) of 17β-estradiol for inducing vtg1 transcription were 0.25, 0.5, 0.25 and ≤0.1 μg/L for embryo, larvae, juvenile and adult male zebrafish, respectively, while the LOECs of nonylphenol for induction of vtgs transcription were 50 μg/L in embryo and 100 μg/L in larvae and adult stages. The mRNA levels of the two vtgs were low in both the embryo and larvae stages, even at the highest 17β-estradiol or nonylphenol exposure concentrations, while the mRNA levels in liver of adult zebrafish of the two vtgs were 10^2 or even 10^4 times higher than those of the control groups at the corresponding nonylphenol or 17β-estradiol exposure concentrations. Similarity, the ERα and ERβ mRNA levels in juvenile and adult zebrafish livers were also higher. Results suggest that in the early developmental stages of zebrafish might be more sensitive (low LOECs) to the presence of EDCs such as nonylphenol, but juvenile and adult zebrafish have a more effective (high induction levels). The use of zebrafish juveniles, larvae and embryos offers an alternative stage to detect EDCs.

1. Introduction

It has been clearly established that a number of endocrine-disrupting chemicals (EDCs), including natural and synthetic compounds in the environment, are able to disturb normal physiology and endocrinology in wildlife (Mills and Chichester, 2005). In recent years, the potential adverse effects of EDCs in organisms have become a major research topic (Rhind, 2005; Patisaul et al., 2006). The reported adverse effects of EDCs in teleost fish include population decline, inhibition of reproductive function, induction of the yolk precursor protein vitellogenin (VTG) in males and juveniles, and effects on of the development of the gonads (Weber et al., 2003; Song et al., 2008).

It should be realized that one of the main mechanisms of endocrine disruption is interference with the function of the steroid hormone estrogen; thus, the induction of VTG in male fish, which is normally synthesized by the liver in female fish, is widely used as a sensitive biomarker of exposure to estrogenic compounds (Arukwe et al., 1997; Kime et al., 1999). Concentrations of 17β-estradiol (E2) in aquatic systems range from a few nanograms per liter up to hundreds of nanograms per liter (Shore et al., 1993; Desbrow et al., 1998). It is believed that E2 can bind to estrogen receptors and subsequently interact with estrogen responsive elements, resulting in the activation and expression of the target gene. As an estrogen mimicking compound, nonylphenol (NP) exerts numerous direct and indirect effects in fish species through induction of estrogen responsive genes (Larkin et al., 2003) and proteins (Andreassen et al., 2005; Yang et al., 2006). NP has also been shown to have adverse impacts on sexual differentiation and reproduction in fish (Hill and Janz, 2003; Seki et al., 2003). A number of *in vitro* and *in vivo* techniques have been developed for the analysis of biomarkers sensitive to estrogen-like chemicals in vertebrates, and in fish in particular (Arukwe et al., 2001; Shrader et al., 2003; Barucca et al., 2006; Bogers et al., 2006). One of these methods, the induction of gene expression, had already been used to investigate the endocrine disruption effect in some aquatic species such as zebrafish, medaka, and fathead minnows. However, the established experimental systems have typically focused on adult fish; in particular, the effects of EDCs on the different developmental stages of any fish species have so far received limited attention.

Different developmental stages, including the embryo, larva, juvenile and adult stages of zebrafish have been studied in toxicity investigation (Brion et al., 2004). Recently, zebrafish has been used as a predominant test model for the assessment of EDCs (Kime et al., 1999). A three-month study by Song et al. (2008) suggested that 17a-ethynylestradiol (EE2) could adversely affect the fecundity, sex differentiation, gamete development, and other reproductive functions of both male and female zebrafish. Voelker et al. (2007) demonstrated that gene expression analysis could be used as a

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toxicant-sensitive endpoint in zebrafish embryos and larvae. Moreover, of the several distinct subtypes of estrogen-responsive genes identified in zebrafish, the gene most sensitive to the short-term exposure to low concentrations of EDCs, especially in different developmental stages, also remains to be identified. Thus, it is necessary to develop an assay using different developmental stages of zebrafish, such as embryos, larvae, juvenile and adult fish, as a whole animal in vitro screening system for the detection of EDCs.

This study thus has the following two aims: to identify the gene(s) most sensitive to short-term exposure to low concentrations of E2 and NP in different key developmental stages, and to determine which stage of the zebrafish life cycle is the most sensitive to exposure to EDCs. A real-time quantitative RT-PCR method was adopted to investigate the expression of the estrogen responsive genes, vtg1, vtg2, ERα and ERβ in embryo, larva, juvenile and the hepatic tissue of adult male zebrafish for a short period of time exposure.

2. Material and methods

2.1. Exposure experiments and sample collection

Estradiol (E2; CAS 50-28-2, ≥98%) and nonylphenol (NP; CAS 84852-15-3, technical grade) were purchased from Sigma-Aldrich (USA) and used as-received. Stock solutions of E2 and NP were prepared by dissolving in ethanol (100%) at concentrations of 1 g/L and 10 g/L, respectively. The solutions were stored in the dark at 5 °C.

Zebrafish (*Danio rerio*) were kindly provided by the Institute of Hydrobiology of the Chinese Academy of Science. Healthy 5-month old adult female and male fish were selected and acclimatized separately in glass tanks at ambient temperature (26 ± 1 °C) for at least 10 days with a 14-h light /10-h dark cycles. The fish were fed twice a day with *brine shrimp*. Fifteen male and ten female fish were maintained together. Embryos were collected and staged using standard procedures as outlined in Westerfield (1993).

Fertilized eggs were harvested and hatched for embryo and larvae respectively; and juveniles were raised to 17 days post fertilization (dpf) according to the references of Trant et al. (2001) and Kazeto et al. (2004). Then, zebrafish were exposed for 3 or 7 days to relevant concentrations of E2 (0.1, 0.25, 0.5 and 1 μg/L) and NP (10, 25, 50 and 100 μg/L) containing 0.1% ethanol (v/v) as either embryos (from fertilization to 3 dpf), larvae (from 4 to 11 dpf), juvenile (from 17 to 24 dpf) or 5-month old adult male fish for 7 days. Control embryos, larvae, juvenile and adult zebrafish were raised in water with 0.1% ethanol but not dosed with E2 or NP. All the zebrafish were exposed to the above solutions under static conditions for 3 (embryo stage) or 7 days (larvae, juvenile and adult stages) at ambient temperature (26 ± 1 °C), and the rearing water and exposure solutions were completely changed (100%) every day. During the exposure, the larval fish were fed with paramecium, and juvenile and adult fish were fed with brine shrimp twice a day. There were no statistically significant differences in hatching and death rate during embryo and larval stages both in E2 and NP exposure, and no fish died during the juvenile and adult stage exposure correspondingly.

About 15 eggs and larvae, and 10 juveniles were exposed to 100 mL of each of the above solutions, and 10 adult male fish were exposed to 3 L of each of the solutions in glass vessels. Each treatment concentration was replicated in four identical vessels. At least eight larvae and juvenile fish were collected directly as one sample; the livers of seven or eight male adult fish were minced as one sample, resulting in a minimum of four pooled samples for analysis per treatment. Samples were kept on dry ice during preparation and then stored at −80 °C until analysis.

2.2. Isolation of RNA and reverse transcription

The method of isolation of the total RNA and reverse transcription were performed with an established method described as Jin et al. (2009). Briefly, Zebrafish and liver tissues were homogenized in 0.5 mL TRIzol reagent (Invitrogen, USA) with a homogenizer (Polytron, Switzerland). The ratio of absorbance at 260 nm to that at 280 nm as well as the banding patterns on a 1% agarose formaldehyde gel were used to verify the quality of the RNA in each sample. Reverse transcription (RT) was carried out using an M-MLV reverse transcriptase kit (Takara Biochemicals, China). First-strand complementary DNA (cDNA) synthesis was performed by priming with 500 ng RNA and 1 × M-MLV buffer in 10 μL reaction mixture containing 5 U M-MLV reverse transcriptase, 1 U of RNase inhibitor, 0.5 mM each of dNTP mixture and 50 pM oligo-dT. Finally, a portion of 0.5 μL RT products was used directly for the real-time polymerase chain reaction (PCR).

2.3. Gene expression analysis

Oligonucleotide primer pairs of vtg1, vtg2, ERα and ERβ and housekeeping genes (β-actin) used in the present study were as the same as in previous report (Jin et al., 2008b; Liu et al., 2009). β-actin transcript was used to standardize the results by eliminating variations in mRNA and cDNA quantity and quality because it was found that its expression did not change following any of the treatments. Each mRNA level was expressed as its ratio to β-actin mRNA (Chen et al., 2003).

The SYBR reaction mixture consisted of modified polymerase (including SYBR green, optimized PCR buffer, $MgCl_2$, and dNTP mix), ROX reference Dye, and RNase-free water (Takara Biochemicals, China). The real-time quantitative PCR amplification and quantification were performed in a real-time PCR system (ABI-7300, USA) as described in our previous report (Jin et al., 2008b).

2.4. Data analysis

The experimental data were checked for normality and homogeneity of variance using the Kolmogorov–Smirnov one-sample test and Levene's test, respectively. When necessary, the data were transformed for normalization and to reduce heterogeneity of variance. Intergroup differences were assessed by analysis of variance (ANOVA), followed by Fisher's post hoc test, using the StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when p was less than 0.05 or 0.01.

3. Results

3.1. Induction of estrogenic-responsive gene expression in embryo stage exposure

Fig. 1 shows the mRNA levels of vtg1, vtg2, ERα and ERβ of zebrafish exposed to various concentrations of E2 and NP for 3 days post fertilization. The lowest concentrations of E2 and NP to significantly induce vtg1 transcripts were 0.25 and 50 μg/L, and at these concentrations, the induced mRNA levels were about 3 times and twice that of the control (Fig. 1A, E). When the exposure concentration increased, the induction also increased, but not proportionally. For example, when the concentration increased to 1 μg/L E2 or 100 μg/L NP, the induction levels of vtg1 were only 3.8 or 2.3 times higher than that of the control. As for vtg2, the significant induction concentration was 50 μg/L NP (Fig. 1F). However, exposure to the currently tested E2 concentrations for 3 days at the embryo stage did not induce significant increase in expression of vtg2 even in the highest E2 treatment group (1 μg/L)(Fig. 1B). In addition, in comparison with vtg1 and vtg2, the kinetics of ERα mRNA expression was similar to those of vtg1 induction when exposed to E2. The lowest concentration of E2 to significantly induce ERα transcripts was also 0.25 μg/L, and the induced mRNA level was 1.5-fold higher than that of the control (Fig. 1C). No significant effect was observed on the ERα expression at the given NP exposure concentrations. Moreover, no significant effect was observed on the ERβ expression at the given E2 and NP exposure concentrations (Fig. 1D, H).