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Expression of two zona pellucida genes is regulated by 17α-ethinylestradiol in adult rare minnow *Gobiocypris rarus*

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ABSTRACT

Zona pellucida (ZP) proteins are glycoproteins synthesized in liver, ovary or in both tissues in fish. In the present study, we aimed to determine the responsiveness of *ZP2* and *ZP3* to 17α-ethinylestradiol (EE2) in adult rare minnow *Gobiocypris rarus*. The full length of *ZP3* cDNA was firstly characterized and its tissue distribution revealed that *ZP3* mRNA was predominantly expressed in ovary of *G. rarus*. The gene expression profiles of *ZP2*, *ZP3* and vitellogenin (*VTG*) were analyzed in gonad and liver of adult *G. rarus* exposed to EE2 at 1, 5, 25, and 125 ng/L for 3 and 6 days. The results show that *ZP2* is more sensitive than *ZP3* in gonads of both genders, and *VTG* in liver is extremely sensitive to EE2 in male fish. However, at lower concentrations (1 and 5 ng/L), the *ZP2* in testes shows higher responsiveness to EE2 compared with *VTG* in rare minnow. The 5′ flanking regions of *ZP2* and *ZP3* were isolated and the comparison of transcription factors in the regions of *ZP2* and *ZP3* suggested that the disparity for the responsiveness of *ZP2* and *ZP3* to EE2 could partly be a result of differential *cis*-elements such as oocyte-specific protein (Osp1) binding sites or/and sex-determining region Y (SRY) binding site.

1. Introduction

Global concern about the occurrence of endocrine disrupting chemicals (EDCs) in the water environment and their possible disruptive effects on aquatic plants and animals were increasingly serious. EDCs have been defined as exogenous agents that interfere with the production, release, transport, metabolism, binding action, or elimination of the natural hormones in the body responsible for maintenance of homeostasis and the regulation of developmental processes (Kavlock et al., 1996). Some natural and synthetic estrogens such as estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) are identified as the most potent EDCs. Some non-steroidal chemicals are less potent EDCs such as alkylphenols and bisphenol-A (BPA) that are widely encountered at significant concentration levels (Pojana et al., 2007). Abundant evidence from field and laboratory studies provided that exposure to these chemicals can lead to abnormal modulation or disruption of physical development, reproduction and sexual behavior in aquatic wildlife, especially in fish (Scholz and Gutzeit, 2000; Kang et al., 2002; Sária et al., 2011).

All vertebrate eggs are surrounded by an acellular envelope whose nomenclature is not consistent and the different names are used for different animal groups such as zona pellucida (ZP) in mammals, vitelline envelope in amphibians, perivitelline envelope in reptiles and birds, and chorion in fish. These extracellular matrices are involved in species-specific sperm-egg binding to undergo the acrosome reaction during fertilization and protect the embryo during early development in mammals (Wassarman, 2008), while there is generally lack of an acrosome in teleost sperm and fertilized through the micropyle (Monné et al., 2006). The major protein constituents of the envelope are glycoproteins and consist of two to four major proteins (Lefièvre et al., 2004; Wassarman, 2008). As these proteins are the main constituents of the zona pellucida, they have been named the zona pellucida proteins. In a recent study, *ZP*s were classified by phylogenetic analysis under six subfamilies: *zp1*, *zpa*/*zp2.4*, *zpax*, *zpb*/*zp4*, *zpc*/*zp3*, and *zpd* (Goudet et al., 2008). Unfortunately, the nomenclature used to describe *ZP*s is confusing, especially in fish. For simplicity we refer to these proteins as the zona pellucida proteins (ZPs) hereafter.

ZP proteins contain a single homologous region known as the ZP domain which has many conservative cysteine residues (Yonezawa and Nakano, 2003). Mammal ZPs consist of a signal peptide sequence, a ZP domain whose function is to support protein-protein interactions during the ZP assembly, a consensus furin cleavage site for secretion, and a C-terminal transmembrane domain (Wassarman, 2008), while the ZPs of the teleost have no obvious transmembrane domain (Wang and Gong, 1999).

In mammals, almost all the *ZP*s are expressed in the ovary (Wassarman et al., 2004), while in teleost, the ZPs were synthesized

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in liver, ovary, or these two tissues (Celius and Walther, 1998; Kanamori et al., 2003; Fujita et al., 2008). The ZPs synthesized in liver was regulated by estrogen in medaka (Lee et al., 2002b; Ueno et al., 2004) and winter flounder (Lyons et al., 1993), while the ZPs synthesized in ovary can either be under control or independent of estrogen (Celius et al., 2000; Liu et al., 2006). The mechanism of *ZP*s regulation was not very clear. In mammals, regulation of ovarian expression of the *ZP*s has been shown to be mediated by some binding factors including E-box (Hu et al., 2012), ZAP-1 (Millar et al., 1993), OSP-1 (Wassarman, 2008) and ERE (Hamada et al., 1989) in *ZP* promoter region. In fish, many *cis*-elements including ERE, C/EBP (Ueno et al., 2004), pou5f1, OSP1 (Liu et al., 2006), CAAT box (Mold et al., 2009), Sp1 and E-box (Kanamori et al., 2003) were considered to be indispensable for the regulation of *ZP*s.

Vitellogenins (*VTG*) are egg-yolk precursor proteins crucial for reproductive success in oviparous animals and the number of *VTG* gene subtypes of oviparous species is different (Wahli et al., 1979; Blumenthal et al., 1984; Trichet et al., 2000). In teleost, *VTG*s are specifically expressed in the liver. However, *VTG*s are also expressed in the gonads of E2 treated zebrafish (Wang et al., 2005). The induction of vitellogenin (*VTG*) by environmental estrogens in teleost has been proposed as a sensitive biomarker of estrogens (Rotchell and Ostrander, 2003; Jin et al., 2008; Kanungo et al., 2012). Although *ZP*s was used to a less extent than *VTG* in toxicological assessment for estrogenicity, several studies indicated that *ZP*s, which are also induced by estrogens, may be alternative biomarkers for environmental estrogens and are more sensitive to estrogen than that of *VTG* in low dose of estrogen (Celius et al., 1999; Fujita et al., 2004; Yu et al., 2006). Chen et al. (2008) considered that *ZP2* could be used as a highly sensitive biomarker for monitoring estrogenic chemicals in the marine environment. Moreover, different ZP gene has different response capability for estrogenic chemicals (Mommsen and Korsgaard, 2008).

Gobiocypris rarus, a small Chinese fresh water cyprinid fish, is parochial to the upstream of Yangze River, Sichuan Province, China. Owing to their small size, short life cycle and sensitivity to aquatic pollutants, they were considered to be an appropriate animal model for aquatic toxicology research (Zha et al., 2007; Zhang et al., 2008). Many previous studies have shown that this small cyprinid fish was sensitive to estrogenic chemicals (Wang et al., 2010, 2012; Qin et al., 2013).

The aim of this study was to determine the responsiveness of *ZP2* and *ZP3* to EE2 in adult *G. rarus*. In the previous study, *ZP2*s were isolated in *G. rarus* and gene expression of *ZP2*s upon EE2 exposure indicated that *ZP2*s are insensitive to EE2 induction in the juveniles (Wu et al., 2012). In the present study, we firstly cloned *ZP3*, a member of *ZP* subfamilies and analyzed its tissue distribution. In the short-term EE2 exposure, gene expression profiling of *ZP2* and *ZP3* was analyzed in the adult fish upon EE2 exposure and *VTG* served as a positive control for the estrogen induction. Our data will provide foundation for screening of sensitive molecular biomarker for early warning to monitor the environmental estrogenic chemicals in fresh water environment.

2. Materials and methods

2.1. Animals and rearing conditions

Fertilized eggs of *G. rarus* were obtained from female fish spawned in our laboratory. After hatching, the larvae were fed *Artemia* sp. nauplii (<24 h after hatching) twice a day. After 30 days post fertilization (dpf), fish were fed chironomid larvae once a day. The fish were raised in 125 L glass tanks with dechlorinated tap water at 25 ± 2 °C. The photoperiod was 14 h:10 h light/dark.

2.2. Estrogen treatment

Adult *G. rarus* at 105 dpf (0.42 ± 0.08 g in mass, 3.39 ± 0.28 cm in length) was exposed to 17α-ethinylestradiol (EE2) or solvent control dimethylsulfoxide (DMSO; 0.001%, v/v) in 30 L glass tanks for 3 and 6 days. All the EE2 exposure and control groups contained twenty fish. The nominal concentrations of EE2 were 1, 5, 25 and 125 ng/L and it was dissolved in DMSO (Katsiadaki et al., 2010; Wu et al., 2012). All exposure experiments were repeated 3 times in separate aquarium at the same time. Half of exposure solution was renewed every day.

2.3. RNA isolation and reverse transcription (RT)

Tissue samples of gonad, brain, liver, muscle, eye, intestine, and gill of *G. rarus* for cDNA cloning and quantitative RT-PCR were synthesized from adult fish using Trizol reagent (Invitrogen) and further treated with RNase-free DNase I (Promega, USA) and stored at −80 °C until RNA extraction. The yield and quality of RNA samples was ascertained by the spectrophotometric method with an A_{260}/A_{280} ratio from 1.8 to 2.0 and 1% agarose gels electrophoresis based on the integrity of 18S and 28S rRNA bands. cDNAs were synthesized from 3 μg of Dnase-treated total RNA using oligo $(dT)_{20}$ primer and M-MLV reverse transcriptase (Invitrogen) in 20 μL final volume according to the manufacturer's instructions.

2.4. Cloning of the full-length cDNA of ZP3

Primers (Table S1) for cDNA amplification of *ZP3* sequence were designed based on *ZP3* sequences of different fish species available in the GenBank database. The full length of *G. rarus ZP3* cDNA sequence was generated in three consecutive steps using reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) strategies. The purified DNA fragments were sub-cloned into the pMD18-T vector using TA cloning kit (TaKaRa). The confirmed recombinant plasmids were sequenced by GenScript Corporation (Nanjing, China) using BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA) and ABI 3730 automated DNA sequencer (BigDye Terminator Chemistry). In order to ensure the accuracy of the sequencing results, the nucleotide sequences were determined for both sense and antisense strands.

To obtain the 5′- and 3′-ends of *ZP3* cDNA, a pair of transcript-specific primers (Table S1) was designed for *ZP3* transcripts to replicate sequence regions of their core fragment which were sequenced above. 5′- and 3′-RACE was performed with the RACE cDNA Amplification Kit (Invitrogen) using ovarian total RNA following manufacturer's protocols. Nested 5′- and 3′-RACE products were sub-cloned and sequenced as described above.

2.5. Computer-aided sequence analysis

The cDNA of *ZP3* were assembled by alignment of the internal core fragments, 5′- and 3′-RACE fragments using SeqMan program of Lasergene software. To ensure that the cDNA sequence was the right fragment, a support from the Blast program at the web server of National Center of Biotechnology Information (NCBI) also received. The cDNA sequence was also submitted to BLASTX analysis for tentative functional identification at NCBI and a phylogenetic tree was established using multifarious of vertebrate ZPs by Neighbour-Joining algorithms method of Mega 4.0 with bootstrap of 1000 replicates (Tamura et al., 2007). Bioinformatics analysis of predicting signal peptides and transmembrane domain were carried out through online programs such as SignalP Server (Nielsen et al., 1997) and TMHMM Server (Krogh et al., 2001).

2.6. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed by CFX96 real-time PCR detection systems thermocycler (Bio-Rad) and SYBR Premix ExTaq II kit (TaKaRa). The qRT-PCR reactions were carried