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Cloning, expression and functional characterization on vitellogenesis of estrogen receptors in *Scatophagus argus*

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ABSTRACT

Estrogen receptors (Er) play a critical role in vitellogenesis. Three *ers* (*erα*, *erβ1* and *erβ2*) and *vitellogenins* (*vtg-A*, *vtg-B* and *vtg-C*) subtypes were isolated in various fish species, while the contribution of each Er to the regulation of *vtgs* expression was not analyzed in detail. Here, *erα*, *erβ1* and *erβ2* were cloned and all were found to be expressed in female liver in *Scatophagus argus*. During proteic vitellogenesis stage, *erα* was simultaneously up-regulated, while *erβ1* and *erβ2* were not, with three *vtgs* in female liver. The effects of 17β-estradiol (E_2) alone or combined with Er antagonists on *ers*, *vtgs* mRNA expressions and Vtg protein content in incubated male liver were examined by real-time PCR and enzyme-linked immunosorbent assay (ELISA), respectively. The expressions of *erα*, *erβ1*, *vtgs* mRNA and Vtg protein increased significantly after 24 h incubation with E_2 (0.1, 1 and 10 μM), while Er nonselective antagonist ICI 182 780 (0.01, 0.1 and 1 μM) significantly attenuated the up-regulation effects of E_2 on *ers*, *vtgs* mRNA and Vtg protein in a dose-dependent manner. Erα selective antagonist Methyl-piperidinopyrazole (MPP) (0.01, 0.1 and 1 μM) significantly attenuated the up-regulation effects of E_2 on *erα*, *vtg-B*, *vtg-C* mRNA and Vtg protein, while promoted the expression of *erβ1* and *vtg-A*. Erβ selective antagonist Cyclofenil (0.01, 0.1 and 1 μM) attenuated the up-regulation effects of E_2 on *erβ1*, *erβ2*, *vtg-A*, *vtg-C* mRNA and Vtg protein while promoted the expression of *erα* and *vtg-B*. Our results suggest that the regulation of Ers on different *vtgs* was divergent in *S. argus*.

1. Introduction

In vertebrates, estrogens, primarily 17β-estradiol (E_2), play critical regulatory roles in many physiological processes including reproduction, growth and development (Korach, 1994; Hewitt and Korach, 2002). The actions of estrogens are mediated by estrogen receptors (Ers), Erα and Erβ. A third form of Er is being characterized in a growing number of fish species. This Er results from the fish specific duplication and the three *ers* were named as *erα*, *erβ1* and *erβ2* (previously *erγ*), respectively. Ers belong to the nuclear receptor (NR) superfamily, which includes receptors for sex steroids, thyroid hormone, retinoids and orphan receptors with no ligands or unidentified ligands (Cheng et al., 2015). After binding to their ligand (estrogens), Ers begin to dimerize and bind to estrogen responsive elements (ERE) in the promoter regions of estrogen target genes, modulating transcription through interactions with other transcription factors and components of the transcription initiation complex (Hall et al., 2002; Hewitt and Korach, 2002).

Vitellogenin (Vtg) is a yolk precursor protein, mainly synthesized in the female liver of oviparous vertebrates upon E_2 stimulation, and is secreted into the blood, from where it is taken up by growing oocytes via receptor-mediated endocytosis and enzymatically processed by cathepsin D into the major yolk proteins (Utarabhand and Bunlipatanon, 1996; Polzonetti-Magni et al., 2004; Yilmaz et al., 2015). The processes of synthesis, secretion, and uptake of Vtg are important for reproductive development in fish. To date, three types of *vtg* genes have been identified in several fish species: (1) one of the major *vtgs* (type A) has been shown to have homology to common killifish (*Fundulus heteroclitus*) *vtg-I* or haddock (*Melanogrammus aeglefinus*) *vtg-A*; (2) the other major

Abbreviations: Er, estrogen receptors; NR, nuclear receptors; Vtg, vitellogenin; E_2, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; ERE, estrogen responsive elements; MPP, methyl-piperidinopyrazole.

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vtg (type B) is homologous to *F. heteroclitus vtg-II* or *M. aeglefinus vtg-B*; and (3) a minor *vtg* that lacks the phosvitin (Pv) domain (*vtg-C*) is most homologous to zebrafish *vtg-3* (Wang et al., 2000; Sawaguchi et al., 2006). Although Ers are co-expressed in both genders, higher Vtg protein expression was only induced in mature female by relative higher estrogen levels. In male and immature female, the Vtg protein level is low, but exogenous estrogens or estrogen analogues can induce Vtg accumulation (Loomis and Thomas, 2000). There are many possible pathways of estrogen induced Vtg accumulation (Roman-Blas et al., 2009). The best understood way is the estrogen-Er complexes combine with the ERE in the promoter region of the *vtg* genes and activate the expression of *vtgs* mRNA (Mader et al., 1993; Menuet et al., 2004). However, which subtype of Ers is critical for Vtg synthesis is contrary among fish species. Several studies provide support for Erα mediated vitellogenesis in fish (Sabo-Attwood et al., 2004; Filby and Tyler, 2005; Davis et al., 2008, 2010; Marlatt et al., 2008; Unal et al., 2014), some others suggest Erβ carries out that role (Soverchia et al., 2005; Leaños-Castañeda and Van Der Kraak, 2007), while some studies suggest both are important for vitellogenesis (Nelson and Habibi, 2010). These conflicting results highlight the need for specific examination of individual teleost Ers function in hepatic vitellogenesis.

The development of compounds with specific binding capabilities for Erα and Erβ has provided valuable tools for elucidating the roles of Er subtypes in mammals (Arias-Loza et al., 2008; Morissette et al., 2008). ICI 182 780 is pure estrogen receptors antagonist, functioning to inhibit all activity of estrogens in mammals and teleosts (Hermenegildo and Cano, 2000; Notch and Mayer, 2011). Methyl-piperidinopyrazole (MPP) is a pyrazole compound that strongly antagonizes Erα, with a 200-fold greater binding affinity for Erα than for Erβ in mammals (Sun et al., 2002). In cell-based assays for transcriptional activity, Cyclofenil is proved to be weak partial antagonist on Erα and full antagonist on Erβ (Muthyala et al., 2003). Recently, a number of studies have examined the effects of mammalian Ers agonists and antagonists on hepatic vitellogenesis, including ICI 182 780, MPP and Cyclofenil (Leaños-Castañeda and Van Der Kraak, 2007; Davis et al., 2008, 2010; Nelson and Habibi, 2010). However, results from these studies was not consistent across species. In goldfish and tilapia, the Erα antagonist MPP was able to block induction of vitellogenin (Davis et al., 2010; Nelson and Habibi, 2010), while other studies in rainbow trout showed that MPP was unable to block vitellogenin production (Leaños-Castañeda and Van Der Kraak, 2007). These discrepancies suggested that Ers might contribute differently to vitellogenin production in fish.

Scatophagus argus is a euryhaline subtropical fish that is widely distributed in Indian-Pacific waters. The *S. argus* is a species of Scatophagidae and has high economic value in aquaculture (Li et al., 2015). At present, juvenile *S. argus* has to be obtained from the wild and only limited harvests are available. Although there is increasing demand for *S. argus* seedlings, female ovaries cannot fully mature for breeding in artificial environments (Zhang et al., 2013; Li et al., 2015). Therefore, greater understanding of molecular mechanisms of ovarian development and vitellogenesis is required. Herein, three *ers* (*erα, erβ1* and *erβ2*) and *vtgs* (*vtg-A*, *vtg-B* and *vtg-C*) partial cDNA sequences from *S. argus* were cloned. Tissue distribution and ontogeny expression profile of these *ers* and *vtgs* were examined. To better understand the role of specific *ers* in hepatic vitellogenesis in *S. argus*, the male liver was incubated *in vitro* with E_2 alone, or combined with ICI 182 780, MPP and Cyclofenil, respectively. The expression patterns of *ers* and *vtgs* mRNA, and Vtg protein contents after the *in vitro* incubation were examined. Additionally, the contribution of each Er to the regulation of different *vtgs* was investigated.

2. Materials and methods

2.1. Experimental fish and chemical reagents

S. argus were purchased from Dongfeng Market (Zhangjiang, Guangdong, China). 17β-Estradiol (E_2) (491187, 98% purity), ICI 182 780 (1286650, 98% purity), MPP (M7068, 97% purity) and Cyclofenil (C3490, 98% purity) were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). E_2 was dissolved in alcohol. ICI 182 780, MPP and Cyclofenil were dissolved in dimethylsulfoxide (DMSO). The Vtg ELISA Kit was purchased from CUSABIO (CSB-E14116Fh, Wuhan, Hubei, China).

2.2. cDNA cloning and sequence analysis

After alignment of three *ers* cDNA sequences from *Epinephelus coioides, Oreochromis niloticus* and *Acanthopagrus schlegelii* (as deposited in GenBank), the highly conserved regions were identified and primers for 5′ partial and 3′ partial *ers* cDNA of *S. argus* were designed. These two pieces of cDNA were spliced together according to the overlap area to form the target cDNA sequence of the *ers*. Three *er* cDNA sequences were also available from our transcriptome sequencing of the *S. argus* (unpublished data). We designed primers based on the predicted Open Reading Frame (ORF) to confirm the cDNA sequence. The partial cDNA sequences of the *S. argus vtgs* were also cloned in this way. All primers used in this study were listed in Table 1. The cDNAs of *S. argus* were used as template. The PCR program was as follows: initial denaturation at 94 °C for 3 min; 37 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min, followed by a final extension of 10 min at 72 °C in a PCR Thermal Cycler (BIO-RAD-C1000, USA). The PCR products were separated by electrophoresis on 1.5% agarose gel, and the gel was stained with ethidium bromide (EB). The specific fragments were extracted and cloned into pMD19-T vector (Takara, Japan). Positive clones were sequenced by ABI 377 sequencer.

The cDNA sequences of the three *er* subtypes (*erα, erβ 1* and *erβ 2*) were spliced by DNASTAR (http://www.dnastar.com/). ORFs and amino acid (aa) sequence were predicted by ORFfinder software from the NCBI website (https://www.ncbi.nlm.nih.gov/). Nucleotide and aa sequence homology alignments were performed using BLAST software from the NCBI website. Multiple alignments of three Er aa sequences were performed using ClustalX (http://www.clustal.org/). The neighbor-joining method was used to construct the phylogenetic tree by MEGA 5.0 (http://www.megasoftware.net/).

2.3. Tissue distribution of ers and vtgs in S. argus

Female (n = 6) and male (n = 6) adult fish were anesthetized with 100 mg/L tricaine methane sulfonate (MS 222, Sigma, Saint Louis, MO) and dissected. Intestine, gill, heart, spleen, kidney, testis, ovary, brain, muscle, pituitary and liver tissues were collected, frozen with liquid nitrogen and stored at -80 °C prior to RNA extraction. Total RNA (2.0 μg) was isolated from various tissues of female and male adult fish respectively. The quality and concentration of the RNA were assessed by NanoDrop 2000 (Thermo Scientific, USA). Total RNA was treated with RNase-free DNase I (Thermo Scientific Corp, Waltham, MA, USA). M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) was used for cDNA synthesis with oligo-dT (12–18) primers. Tissue distribution was performed by Reverse transcription PCR (RT-PCR). *β-actin* was used as an internal control. The *ers*, *vtgs* and *β-actin* primers for tissue distribution were listed in Table 1. The amplification regime consisted of 35 cycles of 15 s at 95 °C, 55 °C for 15 s, and 72 °C for