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Aquatic Toxicology 77 (2006) 348–358

AQUATIC TOXICOLOGY

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Induction of hepatic choriogenin mRNA expression in male marine medaka: A highly sensitive biomarker for environmental estrogens

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Received 12 April 2005; received in revised form 9 January 2006; accepted 9 January 2006

Abstract

Teleost choriogenins, precursors of the inner layer subunits of egg envelope, have been recently introduced as sensitive biomarkers for exposure to estrogenic compounds. In this study, two full-length cDNAs—*ojChgH* and *ojChgL* which encode the choriogenin H and L forms, respectively, were cloned from the marine medaka, *Oryzias javanicus*. The deduced protein sequences of *ojChgH* and *ojChgL* are highly similar to the corresponding homologues in the freshwater medaka (*O. latipes*) with identities of 77.2 and 87.6%, respectively. Phylogenetic analysis indicated that ojChgH and ojChgL are members of two different classes of liver-specific ZP-domain containing proteins (ZPB and ZPC, respectively). Computer analysis of ca. 2 kb of the 5′-flanking sequences of *ojChgH* and *ojChgL* revealed that both genes contain a number of putative estrogen response elements (EREs) and/or half-site EREs. In vivo mRNA expression patterns of the genes were examined by quantitative real-time RT-PCR. *ojChgH* is expressed exclusively in the liver while *ojChgL* is co-expressed in the liver (major) and ovary (minor). Exposure of fish to waterborne 17β-estradiol (E2) at environmentally relevant concentrations (1, 5, 10 and 100 ng/L) resulted in dose-dependent induction of both genes in the liver, with higher sensitivity and magnitude of induction in males than in females. In the male liver, induction of *ojChgH* is more sensitive to E2 than that of *ojChgL* and two other estrogen-responsive genes, estrogen receptor α (*ojERα*) and vitellogenin (*ojVTG*). The lowest-observed-effect concentration (LOEC) of E2 on induction of hepatic *ojChgH* mRNA is 1 ng/L. In the ovary, expression of *ojChgL* is non-responsive to E2 treatment. In conclusion, the present study suggested that induction of hepatic *ojChgH* mRNA in male fish may be a highly sensitive biomarker for exposure to environmental estrogens.

Keywords: Choriogenin; Gene expression; Promoter; Real-time PCR; *Oryzias javanicus*

1. Introduction

Developing oocytes of vertebrates are surrounded by a thick acellular coat generally termed the egg envelope. This envelope functions to prevent polyspermy and also protect the egg and the developing embryo from mechanical injury. The nomenclature used to describe the egg envelope has been quite ambiguous and has included terms such as zona pellucida (ZP), zona radiata (ZR), eggshell, chorion and vitelline envelope (reviewed by Arukwe and Goksøyr, 2003).

In teleosts, the egg envelope is composed of a thin outer layer and a thicker inner layer (Hyllner et al., 1994). The inner layer comprises at least two groups of glycoprotein subunits, designated ZI1,2 and ZI3 (Hamazaki et al., 1989); the ZI1,2 group consists of three subunits of molecular masses from 74 to 76 kDa (Murata et al., 1993) while ZI3 is a single protein of ca. 49 kDa (Murata et al., 1995). In the Japanese medaka (*Oryzias latipes*), the respective precursors of ZI1,2 and ZI3 are named choriogenin (Chg) H (which is represented by two different isoforms—Chg H and Chg Hminor; Murata et al., 1997; Sugiyama et al., 1998) and Chg L (Murata et al., 1995). Both of these precursor proteins contain a ZP domain (characterized by aligned cysteine residues) that is consistently found in the egg envelope glycoproteins of different vertebrates. The ZP-domain containing genes are classified into five groups; *ZPA*, *ZPB*, *ZPC*, *ZPAX* and *ZPD* (Harris et al., 1994; Lindsay et al., 2001). In human, *ZPA*, *ZPB* and *ZPC* are also called *ZP2*, *ZP1* and *ZP3*, respectively. Phylogenetic analyses indicated that *ZPA*,

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doi:10.1016/j.aquatox.2006.01.003

ZPAX and *ZPB* are more closely related than *ZPC* (Kanamori et al., 2003). *Chg H* and *Chg Hminor* belong to the *ZPB* family while *Chg L* belongs to the *ZPC* family.

Fish Chgs are normally synthesized in the liver of spawning female fish during oogenesis before the onset of vitellogenesis. Estrogen-dependent activation of the Chg genes in *O. latipes* may be brought about by the binding of the 17β-estradiol (E2)-estrogen receptor (ER) complex to the estrogen responsive elements (EREs) in the 5′-flanking regions of the Chg genes (Ueno et al., 2004). Secreted Chgs are transported via the blood stream to the ovary and taken up by maturing oocytes to form the rigid eggshell inner layer. In male medaka, the Chg genes are transcribed at almost undetectable levels under normal conditions (Murata et al., 1997; Lee et al., 2002). However, when the males are exposed to estrogens, hepatic synthesis of Chgs is strongly induced. In addition to the liver-specific *Chgs*, seven other oocyte-specific ZP genes have been identified in medaka (Kanamori, 2000), although the function of these genes in egg envelope formation is still undefined.

The induced synthesis of Chgs and vitellogenin (VTG) in male fish has been proposed as sensitive biomarkers for estrogenic chemicals (Rotchell and Ostrander, 2003). Though Chg is used to a lesser extent than VTG in routine toxicological risk assessment for estrogenicity, several recent studies demonstrated that expression of *Chgs* (as well as some other closely related ZP genes) are more sensitive to E2 than that of *VTG*. Using ELISA, Celius and Walther (1998) showed that in Atlantic salmon hepatocyte cultures, ZR when compared with VTG was secreted at lower E2 doses and in shorter exposure time. By quantitative real-time PCR, Celius et al. (2000) showed that induction of ZR mRNA in the rainbow trout liver was more responsive than that of VTG mRNA to low doses of E2 (0.01, 0.1 and 1.0 mg/kg BW). More recently, Fujita et al. (2004) reported that the plasma levels of Chgs in masu salmon were induced to a greater extent than that of VTG after a low dose of E2 injection (0.01 mg/kg BW). Despite the potential of fish Chgs as highly sensitive estrogen biomarkers, relatively little is known about their expression patterns and regulation. Nevertheless, induction of hepatic Chg H and Chg L mRNAs in male medaka has been shown to exhibit a dose-dependent response to 17β-ethinylestradiol (EE2) whereby EE2 induction of *Chg L* was more sensitive than that of *Chg H* (Lee et al., 2002). More recently, fusion *Chg-L* promoter-green fluorescent protein (GFP) gene constructs were used to establish estrogen-responsive transgenic medaka fish lines for in vivo monitoring of estrogenic chemicals in freshwater environments (Ueno et al., 2004).

As a first step in the development of an in vivo assay system for monitoring estrogenic compounds in the marine environment, we report here the isolation and characterization of the full-length cDNAs of choriogenin H (*ojChgH*) and choriogenin L (*ojChgL*), and ca. 2 kb of their 5′-flanking promoter sequences from the marine medaka, *Oryzias javanicus*. Quantitative real-time RT-PCR assays were developed to examine the dose-response expression patterns of the ojChgH and ojChgL genes in different tissues of fish exposed to environmentally relevant concentrations of E2. For comparison, the dose-response patterns of the estrogen receptor α (*ojERα*) and *ojVTG* genes were also investigated.

2. Materials and methods

2.1. Fish and treatment

Sexually mature *O. javanicus* (Singapore strain; ∼12-week-old) were maintained in seawater at 25 °C with a 12 h light/12 h dark regime at a mean density of 1 g fish/L. Male and female fish ($n = 5$) were mixed at an approximate ratio of 1:1 and were fed three times daily with commercial flake food (Aquatox Food, Aquatic Eco-Systems Inc., USA) and allowed to acclimatize for 7 days prior to 17β-estradiol (E2) exposure. E2 (Sigma) was dissolved in methanol (20 μg/mL stock solution) and added to various tank waters to yield final concentrations of 1, 5, 10 and 100 ng/L. For the control group, fish were reared in seawater treated with the same concentration of methanol without E2. On alternate days, 50% of the tank water was renewed to maintain a constant concentration of active E2. One week after treatment, fish were sacrificed and the livers and gonads were dissected out, snap-frozen in liquid nitrogen and stored at −80 °C until used. To examine the pattern of Chg mRNA expression in different organs, brain, eye, gut, heart, spleen, kidney and muscle tissues were also dissected out from control fish for analysis.

2.2. RNA isolation and reverse transcription

Organ samples were disrupted in Trizol reagent (Invitrogen) using a Brinkman Polytron homogenizer and total RNA was extracted according to the manufacturer's instructions. Contaminating DNA was removed using RQ1 RNase-free DNase (Promega). First-strand cDNA was synthesized using 1 μg total RNA, 1.25 μL of dNTP (10 mM), 2.4 μL of random hexamer (50 ng/μL), 1 μL of RNaseOUT (40 U; Invitrogen) and 1 μL of M-MLVRT (H-) (200 U/μL; Promega) in a total volume of 25 μL in 1× M-MLVRT reaction buffer at 42 °C for 50 min. The reaction was terminated by incubating at 70 °C for 15 min.

2.3. Cloning of ojChgH and ojChgL cDNAs by degenerate RT-PCR

To clone the *O. javanicus* ojChgH and ojChgL cDNAs by RT-PCR, degenerate primers directed at conserved regions were identified by multiple alignment of different fish egg envelope genes. For the cloning of *ojChgH*, gene sequences from Japanese medaka (AF500195), sheepshead minnow (AY598615), winter flounder (U03674), rainbow trout (AF231707) and common carp (Z72494) were aligned and resulted in the design of primers, fChgH-F (5′-ATGGCAAGGCACTGGAGTATHAC-3′; forward) and fChgH-R (5′-ATCAAAATRTCCCAYTGNGG-3′; reverse). For the cloning of *ojChgL*, gene sequences from Japanese medaka (AF500194), gilthead seabream (X93306), Atlantic snailfish (AY547503), sheepshead minnows (AY598616) and rainbow trout (AF231708) were aligned and resulted in the design of primers, fChgL-F (5′-GCTCACCTGG-AARTAYCC-3′; forward) and fChgL-R (5′-AGCACYTGAG-