Gene 552 (2014) 8–17

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Gene expression profiling of key genes in hypothalamus–pituitary–gonad axis of rare minnow *Gobiocypris rarus* in response to EE2

Fang Qin, Xueqin Wang, Shaozhen Liu, Yao Zheng, Meng Li, Yingying Zhang, Zaizhao Wang *

College of Animal Science and Technology, Northwest A&F University, Shaanxi Key Laboratory of Molecular Biology for Agriculture, Yangling, Shaanxi 712100, China

ARTICLE INFO

Article history:
Received 16 December 2013
Received in revised form 28 August 2014
Accepted 3 September 2014
Available online 4 September 2014

Keywords:
17α-Ethinylestradiol
Gobiocypris rarus
Gonadotropin
Gonadotropin receptor
Estrogen receptor

ABSTRACT

The 17α-ethinylestradiol (EE2), which could induce estrogenic effects, is found in different aquatic systems. The current study aimed to assess in vivo effects of short-term EE2 exposure on the transcriptional activity of genes in the brain and gonad tissues in order to characterize the mode of action of EE2 on the hypothalamus–pituitary–gonad axis in rare minnow (*Gobiocypris rarus*). The full length cDNAs of *fshβ*, *lhβ*, *fshr* and *lhr* were first characterized in *G. rarus*. The homology and phylogenetic analyses of the amino acid sequences revealed that these four genes share high identity in cyprinid fish. The tissue distribution analysis by qRT-PCR showed that *fshβ* and *lhβ* were mainly expressed in the brain and *fshr* and *lhr* were mainly expressed in gonads. Adult *G. rarus* was exposed to EE2 at 1, 5, 25 and 125 ng/L for 3 and 6 days and the expression of brain *cyp19a1b*, *fshβ* and *lhβ*, estrogen receptors (*esr1*, *esr2a*, and *esr2b*) and gonadal *fshr*, *lhr* and *cyp19a1a* were assessed. *Cyp19a1b* was significantly up-regulated in the brains of female exposed to EE2 at 1–125 ng/L for 6 days. The brain *lhβ*, but not *fshβ* was strongly suppressed in most EE2 exposure groups of both sexes. The brain *esr2b* was inhibited in both sexes exposed to EE2 at all of the four concentrations for 6 days. *Esr2a* was up-regulated in the females by 6-day EE2 treatment at 1 and 25 ng/L. The high responsiveness of brain *lhβ* and *esr2*s to EE2 and their significant correlation in both sexes suggested that the transcriptional activity of Esr2s could play key roles in modulation of *lhβ* expression via direct action on gonadotropic cells in response to EE2. In gonads, *fshr* was strongly inhibited by EE2 in males, while *lhr* was significantly stimulated by EE2 in females. *Cyp19a1a* was inhibited by EE2 in both sexes. The positive correlations of gene expressions of both *fshr* and *lhr* with *cyp19a1a* in testes suggest that the suppression of 17α-estradiol (E2) synthesis in testis by exogenous estrogen could mediate via both Fsh/Fshr and Lh/Lhr signaling in male *G. rarus*.

1. Introduction

Endocrine disrupting chemicals (EDCs) refer to natural organic compounds or synthetic chemicals that are discharged into the environment and cause disorder in the endocrine system of human and wildlife (Brucker-Davis et al., 2001). Specifically, EDCs interfere with the hypothalamus–pituitary–gonad (HPG) axis and influence sexual development and function (Rasier et al., 2006). Evidences suggest that EDCs in the environment are linked to disturbing the normal endogenous hormone pathway, interrupting reproductive and sexual development in wildlife species (Segner et al., 2003). The mechanisms of EDC influences are complicated and participate in all the three hierarchies of HPG axis. Previous studies support that EDCs could be involved in the reproductive regulations of neuroendocrine system (Diamanti-Kandarakis et al., 2009; Dickerson and Gore, 2007; Qin et al., 2013; Vosges et al., 2010). At upper levels of the hierarchy of the reproductive axis, complex networks of neuroendocrine system act coordinately to regulate the reproductive functions.

Two gonadotropins (Gths), a follicle-stimulating hormone (Fsh), and a luteinizing hormone (Lh), have been proven to be key pituitary hormones in reproduction of fish (Yaron et al., 2003). They are heterodimers, which consist of a common α-subunit and the corresponding hormone-specific β-subunit Fshβ or Lhβ (Pierce and Parsons, 1981). Fsh and Lh are secreted by the pituitary and act through binding to their specific receptors (Fshr and Lhr) in the gonads of vertebrates, inducing steroidogenesis and gametogenesis (Dufau, 1998; Nagahama et al., 1995; Simoni et al., 1997). The main functions of gonadotropins were attributed to gonadal maturation, proliferation of germ and their somatic cells, or the stimulation of steroidogenesis (Swanson et al., 2003; Urbatzka et al., 2010; Weltzien et al., 2004). It is evidenced that Fsh and Lh in teleosts, as in mammals, have different functions and

Abbreviations: EE2, 17α-ethinylestradiol; E2, 17β-estradiol; BPA, bisphenol A; E, efficiency; EDCs, endocrine disrupting chemicals; Er, estrogen receptor; ERE, estrogen responsive elements; Fsh, follicle-stimulating hormone; Gth, gonadotropin; HPG, hypothalamus–pituitary–gonad; KO, knockout; Lgr, leucine-rich repeat-containing G protein-coupled receptors; Lh, luteinizing hormone; ORF, open-reading frame; R, Pearson's correlation coefficient; C_q, quantification cycle; qRT-PCR, quantitative real-time PCR; TM, transmembrane.

* Corresponding author at: College of Animal Science and Technology, Northwest A&F University, 22 Xinong Road, Yangling, Shaanxi 712100, China.
E-mail address: zzwang@nwsuaf.edu.cn (Z. Wang).

http://dx.doi.org/10.1016/j.gene.2014.09.006
0378-1119/

expression patterns at different stages of the reproductive cycle (Jeng et al., 2007; Prat et al., 1996; Swanson et al., 1991; Yaron et al., 2001). Fsh is involved in the control of puberty and gametogenesis, whereas Lh mainly regulates final gonadal maturation and spawning (Prat et al., 1996; Schulz and Miura, 2002). Sexual steroids are able to change the secretion of gonadotropins through feedback mechanisms on the pituitary and therefore, an interference of chemicals mimicking steroid hormones with the HPG axis can be expected (Urbatzka et al., 2006). Endogenous estrogens are critical for directing initial ovarian differentiation in fish. Cytochrome P450 aromatase is a key enzyme in the hormonal pathway catalyzing the conversion of sex steroids such as androgens to estrogens. *Cyp19a1a* and *cyp19a1b*, the two *cyp19a1* subtypes in teleost species, encode gonad and brain type aromatase respectively (Guiguen et al., 2010). Estrogen and EDCs may exert their biological effects by interacting with the estrogen receptors (ERs), including *esr1*, *esr2a* and *esr2b*, in two distinct estrogenic transduction pathways (Edwards, 2005).

17α-Ethinylestradiol (EE2), a synthetic estrogen used in the production of contraceptive pills and hormone replacement therapies (Bjerselius et al., 2001; Lange et al., 2001), was observed in aquatic systems and has been shown to induce estrogenic effects in fish. The maximum concentrations of EE2 ranged from undetectable level to 35.6 ng/L in rivers of China (Zhou et al., 2014), while in different effluents of Beijing, China, the mean concentration of EE2 reached 250 ng/L (Zhou et al., 2012). EE2 affects body growth, sexual differentiation, maturation, and reproductive functions (Andersen et al., 2003; Van den Belt et al., 2003; Watts et al., 2001) even at low levels of exposure. EE2 could affect the telencephalic proteome and might alter normal central nervous system function at the environmentally relevant concentration (Martyniuk et al., 2010).

Rare minnow (*Gobiocypris rarus*), a Chinese freshwater cyprinid, is an appropriate test animal for EDCs. In the present study, adult *G. rarus* of both sexes were exposed to 1, 5, 25 and 125 ng/L EE2 for 3 and 6 days, and the expression profiles of *cyp19a1b*, *esr1*, *esr2a*, *esr2b*, *fshβ*, *lhβ* and gonadal *cyp19a1a*, gonadotropin receptors were analyzed using quantitative real-time PCR (qRT-PCR). The aim of this study was to assess in vivo effects of short-term EE2 exposure on the transcriptional activity of genes in the brain and gonad tissues which are involved in the regulation of steroidogenesis.

2. Materials and methods

2.1. Ethics statement

This study has been carried out in accordance with the regulations on experimental animals of Management Methods of Laboratory Animals in Shaanxi Province, China (orders of the People's Government of Shanxi Province, No. 150, 2011). During the whole experiment, fish were humanely treated. Before being sacrificed, all fish were anesthetized using tricaine methanesulphonate (MS-222, 500 mg L^{-1}), and every effort was made to minimize suffering. All experimental procedures were approved by the Animal Ethics Committee of Northwest A&F University.

2.2. Fish rearing conditions and EE2 exposure

G. rarus was obtained from fertilized eggs by artificial fertilization in our lab. The fish larvae were fed *Artemia* nauplii twice daily. After 30 days post-fertilization (dpf), fish were fed chironomid larvae twice a day. They were raised in re-circulating aquaculture system with dechlorinated tap water at 25 ± 2 °C and the photoperiod was 14 h:10 h light/dark. To analyze gene expression patterns, one sample set was derived from seven different tissues, including the brain, eye, gill, intestine, liver, muscle and gonad, of eight-month normal adult fish (eight males and eight females). Three-month old adult fish (0.42 ± 0.08 g in weight, 3.39 ± 0.28 cm in length) were exposed to EE2 (Sigma Chemicals Inc., St. Louis, MO, USA) or the solvent control (0.001% dimethyl sulfoxide, DMSO, v/v) for 3 and 6 days with the maximum loading of 1.0 g fish/L. The nominal EE2 concentrations were 1, 5, 25 and 125 ng/L. Half of the exposure solution was renewed every day. After 3 and 6 days exposure to EE2, the brain and gonad tissues were dissected and then frozen using liquid nitrogen and stored individually at −80 °C until ready for use. All exposure experiments were conducted concomitantly in triplicate in separate glass tanks (five fish per tanks per time point). Samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.3. RNA extraction and reverse transcription (RT)

Tissue samples were homogenized in TRIzol (Invitrogen) and total RNAs were isolated as previously described (Wu et al., 2012). The concentration and purity of isolated RNA were assessed using the spectrophotometric method with a nanodrop spectrophotometer (Thermo Electron Corporation, USA). The RNA integrity was also checked by analyzing 28S ribosomal RNA (rRNA) and 18S rRNA ratios with 1% agarose gel eletrophoresis. Total RNAs were further treated with RNase-free DNase I (Promega, USA) to remove genomic DNA contamination. The cDNAs were synthesized from 3 μg total RNA with M-MLV reverse transcriptase (Invitrogen) and oligo$(dT)_{18}$ primer in a 20 μL final reaction volume.

2.4. cDNA cloning and sequence analysis

The same approach, described in our previous studies (Liu et al., 2012; Qin et al., 2013; Wang et al., 2011), was employed to obtain and analyze the full length cDNA sequences of *G. rarus fshβ*, *lhβ*, *fshr* and *lhr* genes. *Fshβ* and *lhβ* were cloned from the brain tissues, while *fshr* and *lhr* were cloned from the gonad tissues. The cDNA fragments of these genes were obtained respectively by PCR using primers designed from the consensus sequences of cyprinid species closely related to *G. rarus* (Table S1).

Potential signal peptide cleavage site was predicted using freely available SignalP 3.01 software (http://www.cbs.dtu.dk/services/SignalP). Potential N-linked glycosylation sites in the deduced amino acid sequences were identified using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). Membrane topology and putative membrane-spanning regions were determined by hidden Markov Model analysis with HMMTOP v.2.0 software (http://www.enzim.hu/hmmtop).

2.5. qRT-PCR

The qRT-PCR was performed using SYBR Green ExTaq II kit (TaKaRa) and CFX96 real-time PCR system (Bio-Rad). The PCR reaction was carried out in a final 25 μL volume, with SYBR Green Premix Ex Taq™, 0.4 μM of each forward and reverse primer, and 2.5 μL RT reaction solution. Each sample was analyzed in triplicate using the following protocol: 95 °C/30 s; 40 cycles of 95 °C/5 s and 60 °C/30 s. To ensure the specificity of each amplicon, a melt curve analysis was performed at the end of each PCR run and the PCR product was finally checked on a 2.0% agarose gel. CFX Manager software was used to analyze the density of SYBR Green I and to determine the quantification cycle (C_q) value. The qRT-PCR efficiency (E) of each PCR reaction was calculated on the slope of a standard curve generated by a 10-fold diluted cDNA sample series with five dilution points measured in triplicate. E was determined by the equation $E = 10^{(-1/\text{slope})}$ (Rasmussen, 2001).

2.6. Screening of reference genes

To obtain a reliable reference gene for gene expression analysis in the brain tissues of the EE2-treated fish, four reference genes including *β-actin* (DQ539421), elongation factor 1 alpha (*ef1α*) (HM017974), glycldehyde-3-phosphate dehydrogenas (*gapdh*) (HM017973), and