General and Comparative Endocrinology 159 (2008) 38–45

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Molecular characterization and expression of three GnRH forms mRNA during gonad sex-change process, and effect of GnRHa on GTH subunits mRNA in the protandrous black porgy (*Acanthopagrus schlegeli*)

Kwang Wook An [a], Erik R. Nelson [b], Hamid R. Habibi [b], Cheol Young Choi [a,*]

[a] *Division of Marine Environment & BioScience, Korea Maritime University, #1, Dongsam-dong, Youngdo-gu, Busan 606-791, Republic of Korea*
[b] *Department of Biological Sciences, University of Calgary, Calgary, Alta., Canada T2N 1N4*

ARTICLE INFO

Article history:
Received 15 May 2008
Revised 19 June 2008
Accepted 23 July 2008
Available online 5 August 2008

Keywords:
Black porgy
GnRH forms
GnRHa
GTH subunits
Sex change

ABSTRACT

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in control of reproduction and gonadal maturation in teleost fish. To investigate the action GnRH in black porgy (*Acanthopagrus schlegeli)*, we examined the mRNA expression of GTH subunits (GTHα, FSHβ, and LHβ) in the pituitary as well as plasma estradiol-17β (E_2) level following treatment with a GnRH analog (GnRHa) in immature fish. The expression levels of GTH subunits mRNA and plasma E_2 level were increased after GnRHa injection. We were also able to identify three GnRH forms: salmon GnRH (sGnRH), seabream GnRH (sbGnRH) and chicken GnRH-II (cGnRH-II) by cDNA cloning in the ovary of the black porgy. Black porgy gonadal development is divided into seven stages, involving sex change from male to female (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary). In the present study, we investigated the expression pattern of three GnRH molecular forms in the black porgy gonads at different stages of gonadal development by quantitative polymerase chain reaction (QPCR). The mRNA expressions of sGnRH, sbGnRH and cGnRH-II were found to be higher in mature testis and ovary, compared to gonads at different stages of maturity. The findings support the hypothesis that the three forms of GnRH play important roles in the regulation of hypothalamic–pituitary–gonadal axis, and are likely involved also in gonadal development and sex change in black porgy.

1. Introduction

The regulation of reproduction in teleost fish is a complex process involving the interaction of a number of factors including gonadotropin-releasing hormone (GnRH), gonadotropins, gonadal hormones, and other neurohormones. GnRH is released by the hypothalamus and stimulates the synthesis and release of hypophysial gonadotropin hormones (GTHs): follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The GTHs have two subunits: GTHα, which is common to FSH, LH and thyroid stimulating hormone, and a β subunit which is specific to either FSH or LH. Pituitary LH stimulates the synthesis and secretion of steroid hormones from the gonads, and FSH regulates both vitellogenesis and spermatogenesis, thereby regulating ovarian and testicular function in teleosts (for review see: Ando and Urano, 2005). Thus, gonadal maturation is primarily regulated by the brain–pituitary–gonadal axis, and GnRH plays a central role in the regulation of gonadal maturation and reproduction in fish and other species.

To date, 15 GnRH isoforms have been isolated from vertebrates, comprising a family of highly conserved, decapeptide neurohormones responsible for the control and coordination of reproduction in all vertebrates (Kavanough et al., 2008). Typically, several forms of GnRH are co-expressed in the brain of vertebrates. The majority of vertebrates studied to date express chicken GnRH-II (cGnRH-II), which appears to be largely ubiquitous. Similarly, sea bream, a protandrous hermaphroditic fish, express three forms of GnRH: salmon GnRH (sGnRH), cGnRH-II, and seabream GnRH (sbGnRH) in the brain and gonads (Gothilf et al., 1995; Nabissi et al., 2000).

All GnRH forms identified have a primary gene structure, which is highly conserved. GnRH is encoded as a prepro-hormone, consisting of a "single peptide", directly followed by the decapepdide, and by a "GnRH-associated peptide" (GAP) (Guilgur et al., 2006). Interestingly, extra-hypothalamic GnRH has been reported in the ovaries and testes of various species, including the seabream (Andreu-Vieyra et al., 2005; Nabissi et al., 2000; Soverchia et al., 2007). It has been suggested that GnRH is involved in the regulation of gonadal function as an autocrine or paracrine regulator (Andreu-Vieyra et al., 2005; Leung and Steele, 1992), and direct actions of GnRH on the resumption of oocyte meiosis and effects on steroidogenesis have also been

* Corresponding author. Fax: +82 51 404 3988.
E-mail address: choic@hhu.ac.kr (C.Y. Choi).

0016-6480/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2008.07.012

reported in goldfish (Habibi et al., 1988, 1989). Additionally, gonadal GnRH appears to regulate testicular and ovarian apoptosis in goldfish and gilthead seabream, which might be an important factor in follicular astresia, control of spermatogenesis, and early sex differentiation in fish (Andreu-Vieyra and Habibi, 2000; Andreu-Vieyra et al., 2005; Soverchia et al., 2007). Nabissi et al. (2000) identified GnRH transcripts in the gilthead seabream during the sex-change process, suggesting that GnRHs may be involved in the paracrine/autocrine regulation of seabream sex change from male to female. Despite these recent insights, the molecular mechanism of gonadal sex change in protandrous hermaphroditic fish remains poorly understood.

Black porgy, *Acanthopagrus schlegeli* (Perciformes, Sparidae), are marine protandrous hermaphrodites that are widely distributed and are of particular interest for commercial aquaculture in parts of Asia including Korea. These fish are functional males for their first 2 years of life, but approximately 70% of black porgy change into females during the third spawning season in their natural environment.

The objective of this study was two fold: (1) To test the hypothesis that GnRH regulates induction of pituitary LH and FSH, and the subsequent increase in circulating estradiol-17β (E_2) levels in immature black porgy (1-year-old). (2) To investigate the expression of GnRH transcripts during the sex-change process, with associated changes in pituitary GTHα, LHβ and FSHβ, and circulating E_2 levels.

2. Materials and methods

2.1. Experimental fish

The study was carried out on immature fish (51.0 ± 2.3 g, 1-year-old), mature male (220 ± 14.2 g, 2-year-old), sex changing fish (489.2 ± 11.5 g, 3-year-old) and female black porgy (948.5 ± 51.6 g, 4-year-old). The fish were captured in spawning period (May, water temperature: 20 °C) and sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of mature ova and sperm. All fish were anesthetized in tricaine methane sulfonate (MS-222, Sigma–Aldrich, St. Louis, MO, USA), prior to blood collection. Blood was collected from the caudal vasculature using a 3 ml syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000*g*, 5 min) and stored at −80 °C until RIA analysis. Gonad and pituitary samples from black porgy at each gonad maturity stage (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary and mature ovary) were removed, immediately frozen in liquid nitrogen and stored at −80 °C until the total RNA was extracted for analysis.

2.2. GnRHa treatment

To establish that GnRH was active in black porgy, we first injected immature fish with GnRHa and measured pituitary expression of GTHα, LHβ and FSHβ, and associated changes in circulating E_2. GnRHa (des Gly^{10}-[D-Ala^6] LHRH ethylamide, Sigma) was dissolved and diluted in 0.9% physiological saline. After anesthesia, the fish were given an injection of GnRHa (0.2 μg/g, body weight, BW) at volume of 1 μl/g BW. After injection, pituitary and blood were sampled from three fish at each of the following time periods: 0, 6, 12, 24, and 48 h. Water temperature was maintained 20 ± 1 °C during the injection periods. All fish survived the experimental periods.

2.3. Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from gonad and pituitary of black porgy at the each gonad maturity stages during sex-change process (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary) and pituitary (GnRHa treatment fish), using the Trizol method, according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. About 2.5 μg of total RNA was reverse transcribed in a total volume of 20 μl, using an oligo-$d(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at −20 °C for use in a polymerase chain reaction (PCR) and quantitative PCR (QPCR).

2.4. Identification of sGnRH, sbGnRH, and cGnRH-II cDNA

The primers used for three GnRHs amplification were designed using highly conserved regions of other teleost fish; sGnRH forward primer (5′-GCA GAG TGA CGG TGC AGG TG-3′), sGnRH reverse primer (5′-CTT CCG GTC GAA AGG ACT GG-3′), sbGnRH forward primer (5′-CCA CAG ACT TCA AAC CTC TGG-3′), sbGnRH reverse primer (5′-GTA CGT TCT GTG TCC GTT GT-3′), cGnRH-II forward primer (5′-CTC GGC TGG TTT TGC TGC TC-3′), and cGnRH-II reverse primer (5′-CTC TTC TGG AGC TCT CTT GC-3′). Total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL). PCR amplification was performed using a 2× Taq Premix I (Solgent, Daeseon, Korea) according to the manufacturer's instructions. PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 40 s, and extension at 72 °C for 60 s; followed by 7 min at 72 °C for the final extension. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Sungnam, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Seoul, Korea) and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD, USA). Based on the plasmid DNA, the sGnRH, sbGnRH, and cGnRH-II cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR, total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL). Using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo-$(dT)_{18}$ anchor primer and a CapFishing™ adaptor (Seegene).

Gene specific primers were selected from the PCR product obtained by RT-PCR in the present study. For the 3′ RACE, the 50 μl of PCR mixture contained 5 μl of 3′ RACE cDNA, 1 μl of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μl of 10 μM 3′ RACE sGnRH-specific primer (5′-GAG CTG GAG GCA ACC ATC AGA ATG ATG G-3′), 1 μl of 10 μM 3′ RACE sbGnRH-specific primer (5′-GCT GTC AGC ACT GGT CCT ATG GAC TG-3′), 1 μl of 10 μM 3′ RACE cGnRH-II-specific primer (5′-CAA GAG GGA GCT GGA CTC TTT TGG CAC-3′), and 25 μl of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: one cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by one cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μl of PCR mixture contained 5 μl of 5′ RACE cDNA, 1 μl of 10 μM 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μl of 10 μM 5′ RACE sGnRH-specific primer (5′-CCA TCA TTC TGA TGG TTG CCT CCA GCT C-3′), 1 μl of 10 μM 5′ RACE