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Effects of gonadotropin-releasing hormone analog (GnRHa) on steroidogenic factor-1 (SF-1) and estrogen receptor β (ERβ) gene expression in the black porgy (*Acanthopagrus schlegeli*)

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Abstract

We examined effects of GnRHa on expression of steroidogenic factor-1 (SF-1) and estrogen receptor β (ERβ) in the pituitary and gonad of protandrous black porgy (*Acanthopagrus schlegeli*). Fish were intraperitoneally injected with 0.2 μg GnRHa/g fish and then pituitary, gonad and plasma were sampled at 0, 6, 12, 24 and 48 h after injection. In gonad, the mRNA levels of the SF-1 were high at 6 h post injection, and then continuously decreased until 24 h; high expression of ERβ mRNA levels was only observed at 12 h. In contrast, pituitary SF-1 mRNA levels were very low during the experimental period. GnRHa stimulation caused a significant increase of plasma testosterone (T) and estradiol-17β (E_2) after 24 h. We suggest that SF-1 and ERβ play an important role in the development of gonad and these genes are involved with sex change in fish.
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Keywords: Black porgy; *Acanthopagrus schlegeli*; GnRHa; SF-1; ERβ; Expression

1. Introduction

Many transcriptional factors in the orphan nuclear receptor superfamily have been cloned. The nuclear transcription factors, steroidogenic factor-1 (SF-1), adrenal 4-binding protein and mammalian homolog of *fushi tarazu* factor-1 (FTZ-F1) are expressed in the adrenal gland and gonads (Honda et al., 1993). In particular, the orphan nuclear receptors are known to regulate gene expression related to gonadotropins (GTHs) and steroidogenesis (Barnhart and Mellon, 1994; Ikeda et al., 1994; Luo et al., 1994; Dréan et al., 1996; Halvorson et al., 1996).

SF-1 is known to regulate the cell-specific expression of cytochrome P450 steroid hydroxylase, in order to convert cholesterol into steroids (Morohashi et al., 1992; Honda et al., 1993), and to bind with the GTH promoter to activate the transcription of GTH and GTH-releasing hormone (GnRH)-receptor genes (Barnhart and Mellon, 1994; Halvorson et al., 1996; Keri and Nilson, 1996; Duval et al., 1997; Liu et al., 1997; Ngan et al., 1999). In addition, SF-1 not only regulates the hypothalamus–pituitary–gonad axis, but is also expressed in steroidogenic tissues and gonadotrope cells in the hypothalamus and pituitary (Ikeda et al., 1995; Haisenleder et al., 1996).

In vertebrates, including fish, estrogen plays an important role in sexual maturation and differentiation (Wu et al., 2003), and is also known to function in non-reproductive tissues, such as bones, brain and heart (Ishibashi and Kawashima, 2001). Synthesized in the gonads, estrogen has an impact on the transcription of specific genes through estrogen receptors (ERs) that exist in the nuclei of target cells.

ERs are members of a superfamily of transcription factors that induce expression of target genes by binding to *cis*-acting enhancer elements located in the promoter region of responsive genes. More recently, a second type of ER widely expressed in the male reproductive tract was found to be present in rat (Kuiper et al., 1996). These receptors are not isoforms of each other, but rather are the products of distinct genes located on separate chromosomes.

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ERα and ERβ subtypes have also been reported in fishes (Chang et al., 1999; Guiguen et al., 1999; Patino et al., 2000; Lassiter et al., 2002; Choi and Habibi, 2003; Halm et al., 2004).

ERs regulate various biological functions of estrogen (Lewandowski et al., 2002), and are expressed in the hypothalamus, pituitary and gonad (Gustafsson, 1999). While it is known that ERα induces the expression of target genes by binding with *cis*-acting enhancer elements located in the promoter part of a specific gene (Choi and Habibi, 2003), the exact role of ERβ has not yet been clarified.

GnRH is secreted by the hypothalamus in the brain of vertebrates, including fish, and regulates the synthesis and secretion of GTH in the pituitary. It is also known that the synthesis and secretion of steroids regulate the development and maturation of the gonads (Andrews et al., 1988; Wierman et al., 1989).

Therefore, this study investigated SF-1 and ERβ gene expression in the pituitary and gonad, as well as the steroid level in the plasma, when GnRHa treatment was applied to 1-yr-old protandrous black porgy. This research aimed to explain the roles of these genes in the development and maturation of the gonads, and to determine the relationships between the genes.

2. Materials and methods

2.1. Experimental fish

We used one year old black porgy (*Acanthopagrus schlegeli*, Perciformes: Sparidae; average length: 16.4±0.8 cm, mass: 66.7±10.9 g) from the culture cages of the Marine Science Technology Center (Pukyong National University, Korea), were placed into four units of 220 L circulation filter tanks in the laboratory at 30 fish per tank. During the experimental period, the water temperature was kept at 20±1 °C and the photoperiod was 12-h light/12-h dark. No food was given during the experimental period.

2.2. Hormone treatment

Black porgy were anesthetized with tricaine methane sulfonate (MS-222, 200 mg/L). GnRHa (des Gly^{10}-[D-Ala^6] LHRH ethylamide, Sigma, USA) was initially dissolved in ethanol and was then diluted in physiological saline. Each fish was given an injection of GnRHa (0.2 μg/g, body mass (BW)) at volume of 1 μL per g BW. After injection, blood and tissues were sampled from five fish at each of the following time periods: 0, 6, 12, 24 and 48 h. All fish survived the experimental period.

2.3. Sampling and hormonal analysis

After anesthetizing the experimental fish with MS-222 (200 mg/L), blood was collected from the caudal vasculature using a heparin-treated syringe (3 mL). Plasma was obtained through centrifugation (4 °C, 1500 *g*, 5 min) and stored at −80 °C until the hormonal analysis. The pituitary and gonads were collected, immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction. Plasma testosterone (T) and estradiol-17β (E_2) levels were analyzed by radioimmunoassay (RIA) using T RIA kit (DSL, USA) and E_2 RIA kit (ADLTIS, Italy), respectively.

2.4. Polymerase Chain Reaction (PCR)

Complementary DNA (cDNA) was synthesized after extracting total RNA from the pituitary and gonads using the total RNA Extraction Kit (Promega, USA). PCR was used for the amplification of SF-1 and ERβ cDNA with the following primers: SF-1 forward primer, 5′-ATG CTG CCC AAA GTC GAG ACT-3′; SF-1 reverse primer, 5′-TGG CGT GGA GCA TTT CGA T-3′; ERβ forward primer, 5′-TGC TCG CCA GAG AAG GAT CA-3′; and ERβ reverse primer, 5′-ACT CGC AGR TTC TGG CCA CA-3′. The degenerate primers were designed using highly conserved regions, with reference to the known sequences of other species. For PCR, the Perfect Premix Kit Ver. 2.0 (Takara, Japan) was used to perform denaturation for 2 min at 94 °C, denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C, a total of 35 extensions for 1 min in 72 °C and a final extension for 7 min at 72 °C.

The β-actin mRNA was amplified in each RT reaction as a loading control. The β-actin primers were 5′-TCG AGC ACG GTA TTG TGA CC-3′ for the forward primer and 5′-ACG GAA CCT CTC ATT GCC GA-3′ for the reverse primer. Amplification of β-actin was carried out with 27 cycles; 94 °C for 30 s for denaturing; 55 °C for 30 s for primer annealing, and 72 °C for 1 min for extension.

The amplified PCR products were processed by electrophoresis with agarose gel containing 1% ethidium bromide (0.5 μg/μL).

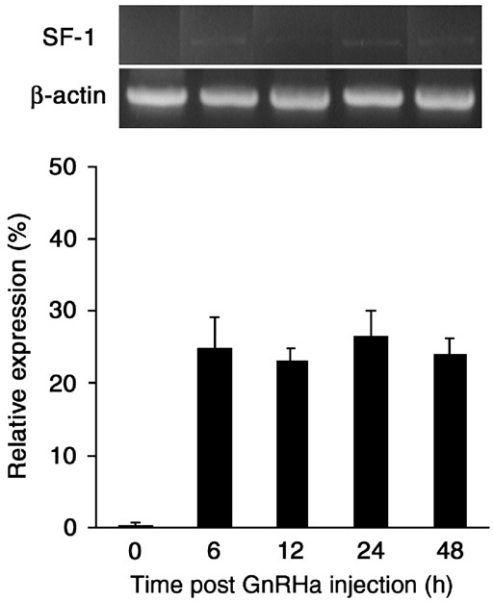

Fig. 1. Time-related effect of GnRHa (0.2 μg/g) on SF-1 mRNA levels in the black porgy (*Acanthopagrus schlegeli*) pituitary. Total RNA was extracted 0, 6, 12, 24 and 48 h after treatment, and 1 μg was used for PCR. The values are percentage increase relative to control value. The expression level of each sample was normalized with respect to the β-actin signal, and expressed as relative expression level. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means±SD of these three experiments. Each experimental group consisted of five fish.

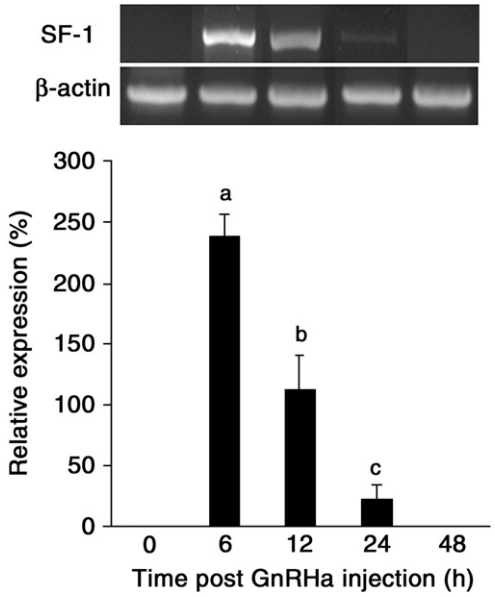

Fig. 2. Time-related effect of GnRHa (0.2 μg/g) on SF-1 mRNA levels in the black porgy (*Acanthopagrus schlegeli*) gonad. Total RNA was extracted 0, 6, 12, 24 and 48 h after treatment, and 1 μg was used for PCR. The values are percentage increase relative to control value. The expression level of each sample was normalized with respect to the β-actin signal, and expressed as relative expression level. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD of these three experiments. Each experimental group consisted of five fish.

The quantification of RT-PCR amplified fragments was conducted with a high-resolution scanner, and band densities were estimated as described previously (Kermouni et al., 1998).

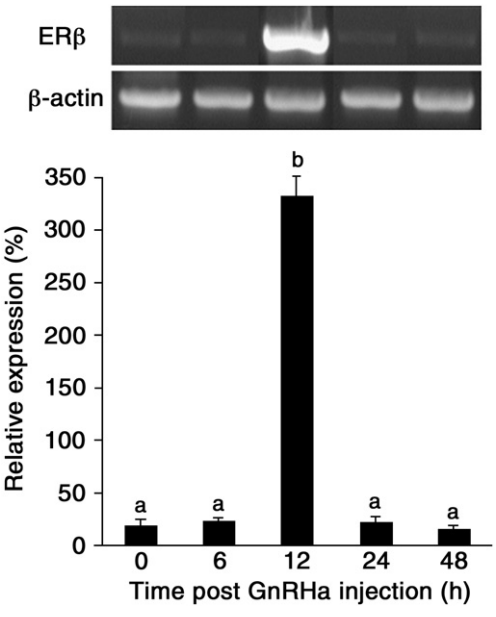

Fig. 3. Time-related effect of GnRHa (0.2 μg/g) on ERβ mRNA levels in the black porgy (*Acanthopagrus schlegeli*). Total RNA was extracted 0, 6, 12, 24 and 48 h after treatment, and 1 μg was used for PCR. The values are percentage increase relative to control value. The expression level of each sample was normalized with respect to the β-actin signal, and expressed as relative expression level. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD of these three experiments. Each experimental group consisted of five fish.

2.5. Sequence analysis

The amplified SF-1 and ERβ fragments were ligated into a pGEM-T Easy Vector (Promega), and the colony formed by transformation was cultivated in DH5α in order to purify the plasmid DNA with a LaboPass Mini Plasmid DNA Purification Kit (Cosmo). Based on the reaction of the purified plasmid DNA, the sequence was determined using an ABI DNA Sequencer (Applied Biosystems, USA). The DNA sequence was analyzed using the GENETYX-WIN (Software Development Co., Japan) software package.

2.6. Statistical analysis

The existence of significant differences between the data obtained from each experiment was tested using one way analysis of variance (ANOVA; Tukey's test or LSD test) with the SPSS statistical package (version 9.0) at a significance level of $P<0.05$.

3. Results

3.1. Expression of SF-1 mRNA

After GnRHa treatment, SF-1 mRNA was not expressed in the pituitary at the start of the experiment and only weak expression was observed after 6 h ($P>0.05$) (Fig. 1). SF-1 mRNA was also not expressed in the gonad at the start of the experiment; however, the expression level rapidly increased after 6 h of treatment and a significant decrease was observed thereafter (Fig. 2).

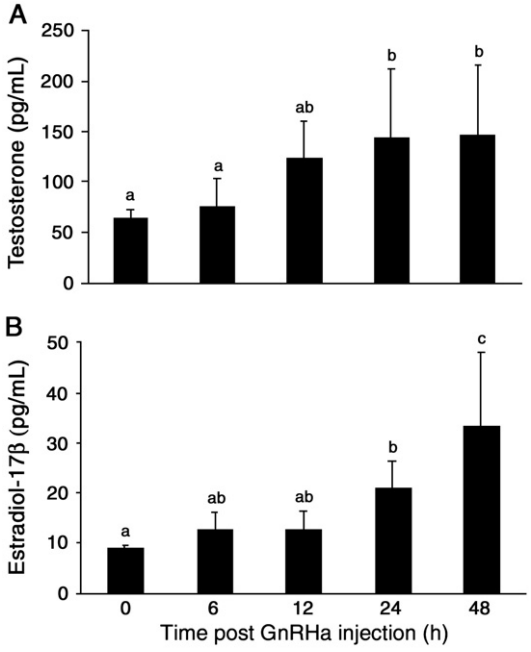

Fig. 4. Time-related effect of GnRHa (0.2 μg/g) on testosterone (A) and 17β-estradiol (B) plasma levels in the black porgy (*Acanthopagrus schlegeli*). Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means ± SD of these three experiments. Each experimental group consisted of five fish.

3.2. Expression of ERβ mRNA

The expression level of gonadal ERβ mRNA was similar to that of SF-1 mRNA: it rapidly increased up to 12 h and decreased thereafter to control levels (Fig. 3).

3.3. Plasma T and E_2 levels

The changes in plasma T and E_2 caused by GnRHa treatment are shown in Fig. 4. The plasma T level, which was 65.0±7.1 pg/mL at the start of the experiment, approximately doubled to 144.0±67.3 pg/mL after 24 h ($P<0.05$), and reached a maximum level of 146.0±68.8 pg/mL at the end of the experiment (after 48 h). The plasma E_2 level was 9.0±0.7 pg/mL at the start of the experiment, increased to 21.1±5.4 pg/mL after 24 h, and reached a maximum level of 33.3±14.7 pg/mL at the end of the experiment (after 48 h); it therefore displayed a similar pattern to that seen in the plasma T level.

4. Discussion

The current study measured the SF-1 and ERβ mRNA expression levels in 1-yr-old protandrous black porgy fish after exogenous GnRHa treatment.

While SF-1 showed relatively weak expression 6 h after the start of GnRHa treatment in the pituitary, it showed a rapid increase in expression after 6 h in the gonad. This led to the conclusion that SF-1 expression might be induced by GnRHa. However, differences in the expression levels displayed in the pituitary and gonads could be explained as follows. Firstly, in 1-yr-old protandrous black porgy, SF-1 might induce more active transcription in the gonads than in the pituitary. Secondly, the SF-1 expression level in the pituitary might be sufficient to activate the GTH gene. In fact, previous experiments showed a significant increase in the expression of the GTH Iβ gene 6 h after the start of GnRHa treatment (Choi et al., 2005). Generally, although SF-1 is known to regulate the LHβ gene through binding with the LHβ promoter in mammals (Brown and Mecneilly, 1997), no connection with FSHβ has yet been described. However, from the above experimental results, it can be concluded that SF-1 in 1-yr-old protandrous black porgy is more directly involved with the expression of the FSHβ gene than that of the LHβ gene.

High levels of SF-1 mRNA expression have been reported in the gonads of maturing frogs (Kawano et al., 1998) and rats (Lala et al., 1992). In addition, the level of SF-1 expression was reportedly higher in the testes of mature goldfish than in those of immature goldfish (Choi, 2003). In the chum salmon, a high level of SF-1 expression was observed immediately before ejaculation (Higa et al., 2001). The current study showed that the expression level of SF-1 mRNA significantly increased 6 h after the start of GnRHa treatment in the gonad. After 24 h, the plasma T and E_2 levels were also found to increase. Based on these results, it can be concluded that SF-1 has a direct function in steroid synthesis for the development and maturation of gonads.

ER expression occurs at the time of sexual differentiation and maturation, and plays an important role in the activation of estrogen-mediated gene expression (Guiguen et al., 1999). In the current study, ERβ mRNA was weakly expressed before GnRHa treatment. Although the expression level had increased by approximately 10 times after 12 h of treatment compared with the start of the experiment, it gradually decreased thereafter, and had returned to the initial level after 24 h. This was presumed to be the result of both direct and indirect promotion of ERβ mRNA expression by GnRHa for up to 12 h. Because of the decreased level of exogenous GnRHa, it may have failed to exert an influence on the expression of ERβ mRNA after 24 h.

The high levels of plasma E_2 displayed by 3-yr-old male black porgy during the spawning period are related to sex change (Chang et al., 1994, 1997; Chang and Lin, 1998, Lee et al., 2000). Moreover, wrasses and eels are induced to change sex (from female to male) by sGnRHa or LHRH treatment (Kramer et al., 1993; Tao et al., 1993) and 1-yr-old black porgy undergo sex change when subjected to E_2 treatment (Chang et al., 1994). These facts, together with the increase of E_2 induced by GnRHa treatment in the current study, suggest that a sex change from male to female could occur in one year old black porgy because the E_2 level is increased by continuous treatment with GnRHa, or is activated by the high level of ERβ expression. In particular, cytochrome P450 aromatase (an important enzyme that catalyzes the conversion of androgens into estrogens) is closely associated with sex change. SF-1, as mentioned earlier, regulates the transcription of cytochrome P450 aromatase in the gonads, and therefore ultimately activates estrogen (Gardner et al., 2005).

Activation of the reproductive axis can be identified through the levels of SF-1 and ERβ expression in the pituitary and gonads of one year old protandrous black porgy after GnRHa treatment; consequently, the degree of gonadal development and maturation can be determined. The possibility of sex change can also be investigated through expression of the SF-1 and ERβ genes. It can be proposed that GnRH in the hypothalamus stimulates expression of the SF-1 gene in the pituitary and gonads, in order to enable the expression of ERβ and E_2 by cytochrome P450 aromatase; alternatively, GnRH might directly trigger the expression of ERβ and E_2 to make sex change possible in the gonads. Further studies will be required to verify these hypotheses.

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