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Research paper

Dynamic responses of prolactin, growth hormone and their receptors to hyposmotic acclimation in the olive flounder *Paralichthys olivaceus*

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ABSTRACT

Prolactin (PRL) and growth hormone (GH) play important roles in regulating salt and water balance through osmoregulatory organs in vertebrates. The aim of this study was to investigate the dynamic changes of GH/PRL hormone gene expressions in the pituitary gland and their receptors in gill and kidney, as well as the plasma osmolality when the olive flounder fish *Paralichthys olivaceus* were acclimated in freshwater (FW) conditions. After transfer from seawater (SW) to freshwater (FW), the osmolality of FW-adaption fish reached the lowest level at 1d which rose slightly afterwards. However, the hormone gene expression of PRL increased from 2d, reaching its peak at 5d, and then decreased at 14d. At this time, the value was still significantly higher than the control, showing a similar trend to the plasma hormone PRL. In contrast, the pituitary mRNA level of GH significantly decreased at 1d and then returned to normal levels. The mRNA levels of PRL receptor (PRLR) in both gill and kidney displayed a similar trend to the pituitary PRL. We also observed the synchronous expression trend of the renal PRLR with pituitary PRL (5d) and the asynchronous expression peaks between branchial (8d) and renal PRLR (5d). Significant responses of GH and its receptor (GHR) in both gill and kidney during the FW-acclimation were not observed. Nevertheless, the gene expression of GH receptor variant (GHR-V) in both gill and kidney declined at 2d, indicating unknown osmoregulatory functions of GHR-V. Collectively, our results provided more insights of the PRL, GH and their corresponding receptors in modulating osmoregulatory responses, representing an important aspect of FW–acclimation in flounder fish.

1. Introduction

Regulation of ion and water balance is an essential physiological process for most fish. In a freshwater (FW) environment, fish suffer excessive hydration and ion loss through their gill and body surface. By increasing ion absorption and producing dilute urine, fish can adapt to a hypotonic habitat. Euryhaline fish, such as flounders, swim between FW and seawater (SW) during their reproductive season (Allan et al., 1965; Raffaelli et al., 1990). Due to their perfect plasticity of gill and kidney, such euryhaline fish can effectively modulate their osmosis.

Endocrine systems have been universally acknowledged to play a critical role in regulating the homeostasis of salt and water balance in vertebrates (McCormick and Bradshaw, 2006). It also has been well established that growth hormone (GH) and prolactin (PRL) perform important moderating effects on "SW-adaption" and "FW-adaption" (Sakamoto and McCormick, 2006). The osmoregulatory capacity of GH was first reported in 1956 (Smith, 1956). Since then, GH has been deemed as a key factor in seawater adaptation which can stimulate gill Na+, K+-ATPase and change the number of chloride cell in fish (Bolton et al., 1987; Mccormick, 1996). It has been proven that the PRL and GH share a common structure and derive from a common ancestor (Kawauchi and Sower, 2006; Rand-Weaver et al., 1993). The osmoregulatory function of PRL in teleost was first described by Pickford and Phillips (1959). Later, further evidence suggested that PRL can function as a FW-adapting hormone in fish (Bole-Feysot et al., 1998). Some researchers measured the changes of gene expression, synthesis, secretion and plasma levels of PRL under FW exposure (Manzon, 2002; Miguel et al., 2002), others used the exogenous prolactin treatment to investigate the changes of FW-type ionocytes which are responsible for ion absorption (Watanabe et al., 2016).

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GH-PRL family hormones are cytokine signaling molecules that interact with the single-transmembrane receptors located on the surface of the target cells (Bole-Feysot et al., 1998). These receptors are composed of an extracellular ligand binding domain, a transmembrane domain, and an intracellular domain containing second messenger signaling elements. This hormone binds to two receptors that initiate signal transduction through the JAK/STAT pathway (Bole-Feysot et al., 1998). The osmoregulatory function of growth hormone receptor (GHR) and prolactin receptor (PRLR) in gill and kidney have been well established (Auperin et al., 1995; Gray et al., 1992; Weng et al., 1997). Previous studies have found two GHRs and one PRLR in the olive flounder *P. olivaceus* (Higashimoto et al., 2001; Nakao et al., 2004). One of these GHRs contains 641 amino acid residues and the other contains 329 amino acid residues (called growth hormone receptor variant, GHR-V), and they play different roles in endocrine function (Nakao et al., 2004).

The olive flounder is a marine demersal species found along the coasts of Japan, Korea and China (Sabate et al., 2008; Tomiyama et al., 2008). This flounder migrates from deep-sea to shallow-sea for reproduction, so osmoregulation is an important way to keep its internal environment steady. In this study, to better understand the dynamic responses of the GH and PRL as well as the role of different receptors in relevant tissues when the olive flounder *P. olivaceus* acclimates in FW, we investigated the dynamic changes on osmolality, GH and PRL in plasma, gene expression levels of GH and PRL in pituitary, and also their corresponding receptors in gill and kidney during low salinity acclimation in this species. We hypothesized that the ability of the olive flounder to adapt to a hyposmotic environment is correlated with the dynamic responses of systemic hormone levels and the gene expression of GH/PRL-family receptors in osmoregulatory tissues.

2. Material and method

2.1. Fish and ethics approval

The gynogenetic olive flounder is very popular for aquaculture in China. There may be some sexual dimorphisms when fish is subject to hyposmotic stress, thus gynogenetic fish were chosen to reduce the potential influence in this study. Gynogenetic olive flounder were produced as previously described (Liu et al., 2012) and reared in SW (30‰) recirculating aquaculture systems at the Central Experimental Station of Chinese Academy of Fisheries Sciences (Beidaihe, Hebei, China). The experiment was conducted at the same location in September 2014. A total of 72 gynogenetic olive flounders (body weights: 500 ± 50 g) were randomly distributed in 12 tanks with flow-through, filtered seawater (30‰) systems at 20 ± 1 °C for more than 2 weeks. Black plastic lightproof curtains surrounded each set of tanks and artificial illumination was provided with white fluorescent lamps. Mean light intensity was approximately 40 lux measured centrally at the bottom of each seawater tank. Fish were starved throughout the experiment, in order to reduce the influence of feeding (Miguel et al., 2002). The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (SHOU), Shanghai, China, and abides by the Guidelines on Ethical Treatment of Experimental Animals established by the Ministry of Science and Technology, China.

2.2. Hypotonic experiments

Fish were divided into two treatment groups, and acclimated to seawater (SW; n = 36) and freshwater (FW; n = 36) conditions for two weeks, respectively. Fish were sampled in both SW (n = 6, 1 each tank) and FW (n = 6, 1 each tank) treatments at 0 h (immediately prior to FW treatment), 1, 2, 5, 8 and 14d after transfer. At each sampling point, fish were anesthetized with 2-phenoxyethanol (0.2 ml/L, Sigma, St. Louis, MO), and blood was collected from the caudal vasculature by a syringe treated with ammonium heparin (200 U/ml, Sigma). Plasma was separated by centrifugation for 20 min at 3000 rpm and stored at −80 °C until the measurement of plasma hormones by radioimmunoassay. The pituitary gland, gill and kidney were removed and instantly frozen in liquid nitrogen for the later analysis of gene expression. All samples were taken during the day time.

2.3. Plasma measurements

Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5200, Logan, UT, USA). Plasma levels of PRL and GH were quantified by radioimmunoassay described by Yada et al. (1994).

2.4. Relative quantitative RT-PCR

The pituitary, gill and kidney mRNA expression levels were analyzed by quantitative real-time PCR on ABI 7500 Real-Time PCR System (Applied Biosystems, Singapore). Relative quantification of the target gene transcripts was analyzed using β-actin gene expression as the reference gene (Zou et al., 2016). Sequences of PRL, GH, PRLR, GHR, GHR-V were obtained from GeneBank. The primers were designed using Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, CA), and synthesized commercially (Sangon Biotech, Shanghai, China) (Table 1). The optimization and validation of primers and probes were performed using standard ABI protocols.

Total RNA was extracted from the tissues of each individual by RNAiso Plus (TaKaRa, Japan). One μg total RNA was treated by PrimeScript™ RT reagent kit with gDNA Eraser. Briefly, quantitative real-time PCR assays were run using FastStart Universal SYBR Green Master kit (Roche, USA), in 20 μl reaction volume, under a standard amplification procedure (2 min at 50 °C, 10 min at 95 °C and then 40 cycles of the following process: 15 s at 95 °C and 30 s at 60 °C).

2.5. Statistics

The $2^{-\Delta\Delta}$ Ct method was used to analyze the real-time PCR data (Livak and Schmittgen, 2001), and amplified transcripts were expressed as the fold change relative to the mean value of the standard sample. Data were tested for normality by the Shapiro-Wilk's test and homogeneity of variance by Levene's test, and expressed as means ± SEM. Significant differences among sampling time points were tested by one-way ANOVA, followed by the Student–Newman–Keuls multiple comparison test (SNK). Significant

Table 1
Gene specific primers for β-actin, PRL, GH, PRLR, GHR and GHR-V of Japanese flounder *P. olivaceus*.

Gene	GenBank	Primers(5′-3′)
β-actin	HQ386788.1	F: GGAAATCGTGCGTGACATTAAG
		R: CCTCTGGACAACGGAACCTCT
PRL	AF047616.1	F: TCTCCTCCCTGCCCTTCA
		R: ATCTCAGGTTGCGTGTTTGC
GH	D29737.1	F: GGAGGATCAACGTCTTCTCAA
		R: ACTGCGGCTGTTACTTATTCA
PRLR	AB047922.1	F: GGAACCGCCAAACAAAGC
		R: CCTGACCGCAGACTGAAAATA
GHR	AB058418.1	F: CCACCAGTTCCTGCACCC
		R: CACGTCGATGAACTCAACCCA
GHR-V	AB110985.1	F: CCACCAGTTCCTGCACCC
		R: GCCATGATACTCTATCACTGGAGTC

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