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Sequence, genomic organization and expression of ghrelin receptor in grass carp, *Ctenopharyngodon idellus*

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ABSTRACT

The growth hormone secretagogue-receptor (GHS-R) is an endogenous receptor for the gut hormone ghrelin. Here we report the identification and characterization of GHS-R1a in grass carp, *Ctenopharyngodon idellus*. The full-length GHS-R1a cDNA contained a 1803-bp coding domain sequence which encoded a peptide of 360 amino acid residues. Comparison analysis revealed that the amino acid sequences of GHS-R1a were highly conserved in vertebrates and shared 97% amino acid identity with zebrafish (*Danio rerio*), 96% with jian carp (*Cyprinus carpio* var. Jian) and 93% with goldfish (*Carassius auratus*). The GHS-R1a showed the highest level of mRNA expression in the pituitary, followed by the brain and liver, and the lowest expression was observed in the hindgut. Intraperitoneally injected with grass carp ghrelin (50, 100 and 150 ng/g body weight (BW)), grass carp showed greater mRNA expression of GHS-R1a in the pituitary compared with saline injected at 0.5 h post-injection. It was observed that food deprivation could promote the expression of ghrelin and GHS-R1a in the pituitary, demonstrating that nutritional status can influence the expression of both ghrelin and GHS-R1a in the pituitary. After a 2- or 4-week fast, plasma growth hormone (GH) increased, was positively correlated with ghrelin and GHS-R1a mRNA expression levels in the pituitary. These results suggested that the involvement of ghrelin/GHS-R1a systems in mediating the effects of nutritional status and ghrelin on growth processes in grass carp.

1. Introduction

Growth hormone secretagogue receptor (GHS-R) belongs to the family of G-protein-coupled receptors containing seven transmembrane domains (TMD). Mammalian GHS-R gene is composed of two exons, and two types of GHS-R mRNAs, GHS-R1a and 1b, are generated by alternative transcription process of the gene (Howard et al., 1996). So far, GHS-Rs have been identified in mammals (Howard et al., 1996; Katayama et al., 2000; Kitazawa et al., 2011; Suzuki et al., 2012), avians (Geelissen et al., 2003; Tanaka et al., 2003) and several fish species (Palyha et al., 2000; Chan and Cheng, 2004; Olsson et al., 2008; Kaiya et al., 2009a,b; Small et al., 2009; Eom et al., 2014). In fish, GHS-R1a was first identified by Palyha et al. (2000) in pufferfish *Takifugu rubripes*. After that, Chan and Cheng (2004) identified two isoforms of GHS-R in black seabream *Acanthopagrus schlegeli*, sbGHSR-1a and sbGHSR-1b. It was the first report on the identification of a GHSR-1b transcript from species other than mammals and the demonstration that receptor interaction might provide a possible explanation for the existence and biological significance of the sbGHSR-1b transcript. Since then GHS-R has also been identified in the zebrafish *Danio rerio* (Olsson et al., 2008), orange-spotted grouper *Epinephelus coioides* (Chen et al., 2008), channel catfish *Ictalurus punctatus* (Small et al., 2009), rainbow trout *Oncorhynchus mykiss* (Kaiya et al., 2009a), Mozambique tilapia *Oreochromis mossambicus* (Kaiya et al., 2009b), goldfish *Carassius auratus* (Kaiya et al., 2010) and Atlantic salmon *Salmo salar* (Hevrøy et al., 2011).

Ghrelin was identified as the endogenous ligand of GHS-R and named for its potent growth hormone (GH)-secreting properties (Kojima et al., 1999). Several studies have also examined ghrelin's role in body weight homeostasis and as an important indicator of energy insufficiency (Kamegai et al., 2000; Tschop et al., 2000; Wisse et al., 2001; Wang et al., 2002; Asakawa et al., 2003; Yasuda et al., 2003). The central role of ghrelin in body weight homeostasis and the critical importance of GHS-R in transmitting ghrelin's energy balance and GH secretory messages are highlighted by recent studies on a GHS-R knock-out mouse model (Sun et al., 2004, 2008). In teleost, a goldfish that has four GHS-Ra that is divided into two types, 1a and 2a, and three of these four receptors (except 2a-2 receptor), were activated by goldfish ghrelin or GHS (Kaiya et al., 2010). Hevrøy et al. (2012) have reported that lower circulating ghrelin during negative energy homeostasis induces down-regulation of GHSR1a-LR, neuropeptide Y, and anorexigenic

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factors at transcriptional levels in the hypothalamus, which over time lead to a voluntary anorexia development in adult salmon held at 19 °C. The expression of the GH secretagogue receptors (sbGHSR-1a and sbGHSR-1b) was significantly increased in the hypothalamus of the food-deprived seabream (Zhang et al., 2008). However, the information about the physiological function on GHS-R of fish is still lacking.

Although accumulating evidence have demonstrated that ghrelin is a brain-gut peptide with multiple functions in fish like in mammals, the mechanism of its functions is still not well defined. Rainbow trout ghrelin stimulates GH release from the pituitary of juvenile rainbow trout in vivo and in vitro (Kaiya et al., 2003). Jönsson et al. (2007) have reported that rainbow trout ghrelin does not stimulate food intake when injected intraperitoneally. For further understanding how these physiological actions occur, it is necessary to identify and characterize ghrelin receptor in fish. The grass carp is one of the four major domesticated cyprinid fish species in China. This species has become the principal species for freshwater aquaculture and has been widely favored in China (Wang et al., 2008). Detailed study into the regulation of ghrelin and its receptors expression in different physiological states is anticipated to provide meaningful insight into grass carp ghrelin physiology. Therefore, the purpose of this study was to identify and characterize GHS-R1a in grass carp. We found that GHS-R1a was mainly expressed in pituitary. Tissue expression distribution of the receptor mRNA was determined by using quantitative real-time PCR, and investigated the effects of fasting and intraperitoneally injected with ghrelin on grass carp ghrelin and GHS-R1a mRNAs expression in the pituitary.

2. Materials and methods

2.1. Fish and experimental conditions and samples collection

The experimental grass carp (initial body mass: 43.91 ± 0.46 g) were provided by Wuhan Academy of Agricultural Science & Technology and kept in 1000-L tanks with a constant flow of filtered water. Prior to the initiation of the experiment, grass carp were fed twice a day at 8:00 h and 17:00 h for 2 weeks to acclimate to feeding conditions for 3 weeks. After acclimation, six grass carp were deeply anesthetized with MS-222 (200 mg L^{-1}), and killed by immediate spinal destroying for measure and dissection. The brain, pituitary, heart, spleen, foregut, midgut, hindgut, head kidney, liver, muscle, fat and kidney samples were collected and frozen immediately in liquid nitrogen and stored at −80 °C until used for tissue distribution analysis.

2.2. Experimental design

2.2.1. In vivo effects of grass carp ghrelin on pituitary GHS-R mRNA expression

In order to examine the physiological function of GHS-R in response to ghrelin, one group of experimental fish received IP injection of different doses of the synthetic ghrelin, and a control group received IP injection of the same volume of physiological saline (0.9% NaCl). The 19-amino-acid n-octanoic acid grass carp ghrelin peptide GT[S(N-OCTANOYL)]FLSPAQKPQGRRPPRV was synthesized by automated multiple solid-phase peptide synthesis (Syro, MultiSynTech, Bochum, Germany) by the orthogonal Fmoc/tert-butyl strategy (Shanghai Apeptide CO., LTD. Pudong District, Shanghai, China). Six fish per treatment were anesthetized with MS-222 (1:10 000) at 0, 0.5, 1, 3 and 6 h after IP injection, and pituitary tissues were rapidly excised from each fish, flash-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation, as previously described. GHS-R mRNA expression was determined by real-time quantitative PCR using the quantitative thermal cycler as previously described.

2.2.2. Effect of food deprivation on ghrelin and GHS-R mRNA expression levels in pituitary tissue and on plasma GH levels

After acclimation, the fish were divided into two groups: experimental group was starved, and one control group, which was fed twice a day at 8:00 h and 17:00 h. Six fish of experimental group and control group were anesthetized with MS-222 after 14-day, 21-day and 28-day fasting, pituitary tissues were isolated and frozen immediately in liquid nitrogen, and stored at −80 °C until RNA isolation as described. Blood samples were collected in syringes coated with heparin from the caudal vessels after 14-day, 21-day and 28-day fasting group and control group. Plasma was separated by centrifugation and stored at −80 °C until assayed for GH. Concentrations of plasma GH were determined using a homologous ELISA validated for quantifying GH in grass carp plasma (Chang et al., 1985). Ghrelin and GHR-S mRNA expression were measured by real-time (rt) quantitative PCR as previously described using primers (rt ghrelin-F and rt ghrelin-R; rt GHS-R1a-F and rt GHS-R1a-R) (Table 1).

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, USA), and then its purity and quantity were measured using protein and nucleic acid analyzer and agarose gel electrophoresis. cDNAs were obtained from 1 μg total RNA using the Revert Aid™ Reverse Transcriptase (Fermentas, Burlington, Canada) and an oligo-dT primer according to the manufacturer's instructions.

2.4. Cloning of GHS-R1a coding domain sequence

According to the genomic sequences of grass carp (College of Fisheries, Key Lab of Freshwater Animal Breeding, Ministry of Agriculture, Huazhong Agricultural University, Hubei Collaborative Innovation Center for Freshwater Aquaculture, Wuhan, Hubei 430070, China), primers GHS-R1a-forward(F)1 and GHS-R1a-reverse(R)1, GHS-R1a-F2 and GHS-R1a-R2 were designed to amplify the complete coding domain sequence of GHS-R (Table 1). The polymerase chain reaction (PCR) to get the fragment of GHS-R was conducted on Biometra Thermocyclers (Biometra, Germany) by using LA Taq DNA polymerase (TaKaRa, Japan), the PCR cycling parameters were denatured for 1 min at 94 °C; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension for 10 min at 72 °C. The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel for puring and cloned into the pGEM-T Easy Vector System (Promega, Nepean, ON, Canada). After transforming into the JM109 Competent Cells (Promega), the recombinants were identified through blue–white color selection in ampicillin-containing LB plates and confirmed by PCR. Positive clones were sequenced in both direction (Sangon, Shanghai, China), and resulting sequences were verified and subjected to cluster analysis in NCBI.

Table 1
Nucleotide sequences of the primers used for the cloning of GHS-R1a gene and RT-PCR of ghrelin and GHS-R1a.

Primers	Sequences(5′–3′)
Primers for partial fragment	
GHS-R1a-F1	ATGCCTACCTGGACGAACC
GHS-R1a-R1	TACCTTTGCCCTAAGAGGAAAAC
GHS-R1a-F2	TTTCCTCTTAGGGCAAAGGTAG
GHS-R1a-R2	TCACAGGCTGGCWGTAGATTC
Primers for real-time (rt) PCR	
rtGHS-R1a-F	GAGAAAGAGGGAGACGAT
rtGHS-R1a-R	GCACGAAGGCAAACA
rt ghrelin-F	CGCTCTTTACTTATGTCTCG
rt ghrelin-R	AGCACAGGACCGTATTTCT
rtβ-actin-F	GGCTGTGCTGTCCCTGTATG
rtβ-actin-R	GGTAGTCAGTCAGGTCACGGC