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Cloning, mRNA expression and transcriptional regulation of five retinoid X receptor subtypes in yellow catfish *Pelteobagrus fulvidraco* by insulin

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ABSTRACT

Retinoid X receptors (RXRs) are members of the nuclear receptor superfamily and mediate development, reproduction, homeostasis and cell differentiation processes in vertebrates. In this study, full-length cDNA sequences of five *rxr* subtypes from yellow catfish *Pelteobagrus fulvidraco* were cloned. Their mRNA expression patterns in different tissues and transcriptional regulation by insulin were determined. Five *P. fulvidraco rxr* (*Pf-rxr*) subtypes differed in the length of cDNA sequence and the open reading frame, but shared the similar domain structures as in typical nuclear receptors. Phylogenetic analysis revealed that the five *Pf-rxr* subtypes were paralogous genes, and that *Pf-rxrβa* and *Pf-rxrβb* had arisen during a teleost-specific genome duplication event. Five subtypes of *Pf-rxr* were detected in all the tested tissues. Overlapping and distinct expression patterns were found for different *Pf-rxr* subtypes, suggesting functional redundancy and divergence of these duplicates. Intraperitoneal insulin injection and incubation reduced the mRNA expression of *Pf-rxrgb*, but not other subtypes, in the liver and hepatocytes of *P. fulvidraco*, respectively, suggesting that *Pf-rxrgb* is the dominant *rxr* subtype involved in the insulin signaling pathway in *P. fulvidraco*.

1. Introduction

Retinoid X receptors (RXR; NR2B), the members of the nuclear receptor (NR) superfamily, form homodimers or heterodimers with other nuclear receptors, including retinoic acid receptors, thyroid-hormone receptors, liver X receptors and pregnane X receptor (Chawla et al., 2001). RXR participates in many important biological functions in vertebrates, such as development, reproduction, homeostasis and cell differentiation. In mammals, studies have been conducted to determine the structure and function of RXRs (Aranda and Pascual, 2001; Merki et al., 2005), and three different RXR family members have been characterized, named RXRα (NR2B1), RXRß (NR2B2) and RXRg (NR2B3) (reviewed by Chawla et al. (2001)). In teleost fish, additional *rxrβ* paralogs have been characterized that were retained from a teleost-specific whole genome duplication (Tallafuss et al., 2006). For example, in zebrafish *Danio rerio*, four *rxr* subtypes were initially reported (Jones et al., 1995). In kelpfish *Sebastiscus marmoratus*, He et al. (2009) cloned partial sequences of three *rxr* subtypes (*rxrα*, *-β*, *-g*). *rxr* sequences are also found in NCBI database for other fish species, such as: medaka *Oryzias latipes rxra* (accession number: ABQ09280), *rxrβ* (BAD93255), Japanese flounder *Paralichthys olivaceus rxrα* (BAB71758), goldfish *Carassium auratus rxrα* (AAO22211), striped trumpeter *Latris lineate rxrα* (ABO15684), turbot *Scophthalmus maximus rxrβ* (AAK76400). Similar to other members of the nuclear receptor family, *rxrs* are highly conserved in structure and composed of at least four functional domains, such as the A/B domain, DNA-binding domain (DBD), D domain and ligand binding domain (LBD) (Aranda and Pascual, 2001). Regarding their tissue expression profiles, studies have provided transcript accumulation of four *rxr* subtypes during zebrafish development (Tallafuss et al., 2006) and embryonic expression profiles of three *rxr* subtypes in *S. marmoratus* (He et al., 2009). However, tissue expression analysis of *rxrs* in adult fish were limited.

Insulin is a peptide hormone that stimulates cell growth and differentiation, and promotes the storage of substrates in tissues by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown (Saltiel and Kahn, 2001). Several RXR agonists such as LG100268, LG1069 and CBt-PMN have been reported to improve insulin sensitivity and glucose tolerance in rats (Mukherjee et al., 1997; Guleria et al., 2013; Kakuta et al., 2012), suggesting that the

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regulation of some aspects of insulin signaling could be mediated by RXRs. Sugita et al. (2011) demonstrated that over-expression of RXRγ in skeletal muscle of mice led to higher glucose disposal and insulin sensitivity. However, no studies have examined the transcriptional regulation of *rxr* subtypes by insulin in fish.

Yellow catfish *Pelteobagrus fulvidraco*, an omnivorous freshwater fish, is regarded as a good candidate for freshwater culture in China for its delicious meat and high market value (Chen et al., 2013). It is considered as a good model for the study of lipid metabolism because *P. fulvidraco* store excess fat in liver and adipose tissue. In this study, the full-length cDNA sequences of five *rxr* subtypes were cloned and characterized, and their tissue-specific expressions were determined. The transcriptional regulation of various *rxr* subtypes by insulin was evaluated using both *in vivo* and *in vitro* model systems. The present study provided an initial basis for further exploring the biological roles of different RXR family members in *P. fulvidraco*.

2. Materials and methods

Two experiments were conducted. The first involved cloning and sequences analysis of *rxr* cDNAs. The second evaluated the mRNA expression patterns and transcriptional regulation of *rxrs* by insulin *in vivo* and *in vitro*. All experiments followed the ethical guidelines of Huazhong Agricultural University.

2.1. Experiment 1: cloning and sequences analysis of rxr genes

2.1.1. Fish culture and sampling

P. fulvidraco (body weight: 89.7 ± 3.4 g) were purchased from a local fish dealer (Wuhan, China). They were maintained in indoor cylindrical fiberglass tanks (70 cm height, 80 cm diameter and 300-L water volume) with a natural dark/light cycle for one month. Water quality parameters were monitored twice a week in the morning. The parameters were as follows: water temperature, 25 ± 0.8 °C; pH, 7.83 ± 0.27; dissolved oxygen, 6.35 ± 0.38 mg/l; hardness, 73.5 ± 3.4 mg/l as $CaCO_3$. Fish were fed a commercial pellet diet (crude protein: 42.6%; crude lipid: 9.4%) twice a day and provided with continuous aeration to maintain the dissolved oxygen level near saturation. Meantime, water was renewed 80% daily to ensure good water quality. At the end of acclimatization, fish were fasted for 24 h before sampling. After being anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222, 10 mg/l), liver and mesenteric fat were removed on ice, rapidly frozen in liquid nitrogen and stored at −80 °C for RNA isolation.

2.1.2. RNA isolation and first-strand cDNA synthesis

Frozen liver and mesenteric fat samples were powdered in a liquid nitrogen-chilled mortar and pestle. Total RNA was extracted using TRIzol RNA reagent (Invitrogen, USA) based on the acid guanidinium thiocyanate-phenol–chloroform extraction method. The integrity of total RNA was confirmed by agarose gel electrophoresis. The purity of total RNA was measured by using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA) for the determination of OD_{260}, OD_{280} and OD_{230} ($OD_{260}/_{280} \geqslant 1.8$, $OD_{260}/_{230} \geqslant 1.5$). Total RNA was reverse-transcribed to cDNA with oligo-dT primers and a cDNA Synthesis Kit (TaKaRa, Japan), according to manufacturer instructions.

2.1.3. Cloning and sequencing of rxr cDNAs

Degenerate primers (Supplementary Table 1), designed based on the most conserved regions of the teleost *rxr* sequences available in the GenBank and Ensembl database, were used to amplify partial cDNA fragment of *rxrs*. The PCR program consisted of initial denaturation for 4 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final elongation step at 72 °C for 5 min. The target fragments were purified using the EZNA gel extraction kit (Omega, USA), and then sub-cloned into the pMD 19-T cloning vector (TaKaRa, Japan). Positive clones containing inserts of an expected size were sequenced by the Sanger method (Sangon, China).

In order to obtain the 3′ and 5′ end sequences of *rxrs*, nested 3′ and 5′ RACE PCR were performed with a SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, CA, USA) using DNase treated total RNA. In the first PCR, the cDNA was amplified with two outer primer sets (Supplementary Table 1) and Universal Primer Mix (provided in the kit) using Advantage® 2 PCR Kit (Clontech). In the second PCR, inner primer sets (Supplementary Table 1) and Nested Universal Primer (provided in the kit) were used. The PCR parameters were 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with an additional initial 3-min denaturation at 94 °C and a 10-min final extension at 72 °C. The RACE products were sequenced as described above.

2.1.4. Sequence analysis

The core fragment, 3′ end and 5′ end sequences were assembled using SeqMan II software in DNASTAR PACKAGE to obtain full-length *rxr* cDNAs. The sequences were edited and analyzed using the program EDITSEQ of DNASTAR package to search for the open reading frame (ORF) and then translated into amino acid sequences using standard genetic codes. The nucleotide sequences were compared with DNA sequences present in the GenBank database BLAST through NCBI (http://blast.ncbi.nlm.nih.gov/) (Johnson et al., 2008). Sequence alignments and percentage of amino acid conservation were assessed with the Clustal-W multiple alignment algorithm (Larkin et al., 2007). For phylogenetic analysis, vertebrate *rxr* amino acid sequences were downloaded from GenBank and Ensembl database. Multiple sequence alignments of *rxrs* were made with MAFFT (Katoh et al., 2002) using an amino acid model through the GUIDANCE web-server (http://guidance.tau.ac.il/) (Penn et al., 2010), which pruned unreliably aligned regions by rejecting columns with confidence scores below 0.995. A Neighbor Joining (NJ) phylogenetic tree was constructed with MEGA 5.0 (Tamura et al., 2011). The best-fitting model of amino acid substitution (JTT + G) was selected using maximum likelihood (ML) in Mega 5.0 on the basis of Bayesian Information Criterion statistics. The NJ tree was performed using JTT + G after setting the gamma distribution parameter at the value estimated by ML (Gamma Distributed 0.48). The confidence of each node was assessed by 1000 bootstrap replicates. Additionally, ME and ML analyses were also performed with JTT + G (Gamma Distributed 0.48) as the substitution model in MEGA 5.0. The sequence alignment and accession numbers are provided in Supplementary Figs. 1 and 2.

2.2. Experiment 2: insulin administration in vivo and in vitro

Bovine insulin (Sigma, USA) was used here. The insulin administration experiment was divided into two parts. In part 1, *P. fulvidraco* were intraperitoneally injected with bovine insulin. In part 2, primary hepatocytes of *P. fulvidraco* were incubated with insulin.

2.2.1. Intraperitoneal injection of insulin

For intraperitoneal injection of insulin, 90 uniformly-sized *P. fulvidraco* (mean body weight: 21.6 ± 2.5 g) were stocked in 6 fiberglass tanks with 15 fish in each tank. The farming condition was similar to those mentioned above. Prior to injection, they were fasted for 24 h. Then they were anesthetized (MS-222 at 10 mg/l) and intraperitoneally injected with 1 μl/g fish body weight (BW) of PBS (control) or with insulin (dosage: 1 μg/g BW) according to Zheng et al. (2015). After injection, fish were returned to their