Molecular characterization of peroxisome proliferator activated receptor gamma (*PPARγ*) in loach *Misgurnus anguillicaudatus* and its potential roles in fatty acid metabolism *in vitro*

Yun Cui[a,b], Xiao Liang[a,b], Xiaojuan Cao[a,b], Jian Gao[a,b,*]

[a] *College of Fisheries, Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Huazhong Agricultural University, Wuhan, 430070, China*
[b] *Key Lab of Freshwater Animal Breeding, Ministry of Agriculture, Huazhong Agricultural University, Wuhan, 430070, China*

ARTICLE INFO

ABSTRACT

Peroxisome proliferator activated receptor gamma (*PPARγ*) was firstly investigated in loach *Misgurnus anguillicaudatus* by RACE (rapid amplification of cDNA ends) and qPCR (real-time quantitative PCR). An *in vitro* cell culture experiment was then performed and showed that loach *PPARγ* positively affected expressions of four fatty acid metabolism related genes, namely *fatty acid desaturase 2, lipoprotein lipase, elongase of very long chain fatty acids 5* and *sterol regulatory element-binding protein-1c*. At last, effects of C18:2n-6 (linoleic acid, LA) and C18:3n-3 (linolenic acid, LNA) on loach *PPARγ* expression were investigated. Loach fin cells cultured with 50 μM and 100 μM of LA or 100 μM LNA exhibited significantly lower *PPARγ* transcriptions than those cultured with 0 μM LA and LNA, showing that high-dose LA/LNA suppressed *PPARγ* expression in loach fin cells. Results of the two *in vitro* experiments suggested that *PPARγ* played an important role in fatty acid metabolism of the loach.

1. Introduction

The peroxisome proliferator activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors [1]. There are three subtypes of PPARs, namely PPARα, PPARβ and PPARγ. Gene sequences and ligands for *PPARα* and *PPARβ* in vertebrate share high homology, but those for *PPARγ* show low homology with them. It suggests that *PPARγ* may have discrepant functions [2]. Thus, *PPARγ* has been the most intensively studied gene among the three *PPAR* subtypes.

So far, *PPARγ* has been cloned in a variety of fish species such as Atlantic salmon (*Salmo salar*) [2], zebrafish (*Danio rerio*) [3], brown trout (*S. trutta*) [4] and yellow catfish (*Pelteobagrus fulvidraco*) [5]. *PPARγ* has been demonstrated to play a crucial role in regulating lipid metabolism and adipocyte differentiation [6]. It is well known that C18:2n-6 (linoleic acid, LA) and C18:3n-3 (linolenic acid, LNA) are essential fatty acids for freshwater fish. Several previous studies have declared that dietary plant oils which were high in polyunsaturated fatty acids (such as LA and LNA) could result in hepatic lipid deposition in freshwater fish [7,8]. However, the correlation between *PPARγ* and LA/LNA supplement was insufficiently elucidated in fish.

Dojo loach (*Misgurnus anguillicaudatus*), a demersal freshwater teleost, is expansively distributed over East Asia. Its advantages including omnivorous food habit, fast growth, high tolerance in oxygen depleted water [9] and high traditional Chinese medicine value [10] determine high aquaculture and market demand. In this study, we firstly cloned *PPARγ* from the loach liver, and then its molecular characterizations and expression profiles in different tissues and early life stages were investigated. At last, two *in vitro* experiments were respectively performed to study the relationship between loach *PPARγ* and other four fatty acid metabolism related genes, namely *fatty acid desaturase 2* (*fads2*), *lipoprotein lipase* (*lpl*), *elongase of very long chain fatty acids 5* (*elovl5*) and *sterol regulatory element-binding protein-1c* (*serbp1c*), and effects of LA and LNA on loach *PPARγ* expression. This study could be foundation of exploring the functional characterization of *PPARγ* in further studies.

2. Materials and methods

2.1. Compliance with ethics guidelines

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong Agricultural University. All efforts were made to

---

minimize suffering of the loaches.

2.2. Fish and sample collections

Loaches were obtained from Baishazhou fish market (Wuhan, China). All loaches were subjected by a ploidy analyser (Partec, Germany) to determine their ploidy levels. Diploid loaches were used here. Livers from three loaches were used for cloning *PPARγ*. Ten tissues including spleen, muscle, heart, brain, gill, kidney, liver, intestine, female gonad and male gonad (n = 3) were collected from adult loaches for studying tissues' expressions of *PPARγ*. All samples were frozen at −70 °C prior to RNA extraction.

A pair of parents was used for reproduction. Offsprings were collected at different early life stages, including unfertilized spermatozoa (US), unfertilized ovum (UO), oosperm, 2 cell, 8 cell, 32 cell, blastula, gastrula, neurula, tail-bud forming, heart-beating stage, hatching, and second day after hatching (2nd), tenth day after hatching (10th), twentieth day after hatching (20th) and thirty fifth day after hatching (35th), frozen at −70 °C prior to RNA extraction. The feeding regimes of the loach offsprings were performed according to the method in our laboratory [11]. Samples of each early life stage were collected from 15 loaches and each five loaches presented as a biological repeat.

2.3. Cloning of PPARγ in loach

Total RNA was isolated from the liver tissues using RNA isoPlus (TaKaRa, Japan). Quantities and qualities of isolated RNAs were ascertained by electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). Total RNA was reverse-transcribed to cDNA with oligo-dT primers and a cDNA Synthesis Kit (TaKaRa), on the basis of manufacturer instructions. The product was used to amplify the conserved regions. The universal amplified primers of loach *PPARγ* were designed based on the multiple alignments of grass carp (*Ctenopharyngodon idella*), zebrafish, and blunt snout bream (*Megalobrama amblycephala*). The PCR program was set as follow: initial denaturing for 5 min at 94 °C, 32 cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 72 °C, and extra elongation for 10 min at 72 °C. The amplified fragments were purified by SanPrep Column DNA Gel Extraction Kit (Sangon, China), and then loaded into the pMD 19-T cloning vector (TaKaRa).

In addition, total RNA was also inverse transcribed by the SMART RACE Kit (Clontech, USA). Following the manufacturer's instructions, the specific primers were performed for the amplifying of 5′ and 3′ RACE. PCRs were carried out at 94 °C (30 s), 62 °C (30 s), and 72 °C (60 s) for 20 cycles with an additional initial 3 min denaturation at 94 °C and a 10 min final extension at 72 °C.

The examined positive results reaching the expectant sizes were sequenced (Sangon). Primers mentioned above are all shown in Table 1.

2.4. Sequence and phylogenetic analysis

The fragments of loach *PPARγ* were assembled by BioEdit. The putative amino acid sequences of loach *PPARγ* were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Other vertebrate PPARγ amino acid sequences for alignments and constructing phylogenetic tree were obtained from NCBI and the identities of these sequences were blasted by Blastp (NCBI). The method of neighbor-joining (NJ) (bootstrap method: 1000 replications) was used to construct the phylogenetic tree by MEGA 5.0 program [12]. PredictProtein predicted the secondary structure of PPARγ (http://www.predictprotein.org/), and then the three-dimensional (3D) structure of PPARγ was constructed by SWISS-MODEL [13].

2.5. Cell culture and PPARγ treatment

Fin cell line of diploid loach (LF cells) was kindly given by Prof. Xia

Table 1
Nucleotide sequences of the primers for PCR.

Target genes	Primer sequences (5′-3′)	Tm(°C)
Primers for partial cDNA cloning		
PPARγ [a]-central-F	ATACACAARAAGAGCMGCAACAA	58.0
PPARγ-central-R	ATCAGGGTCCCGTCYTTRTTCAT	58.0
Primers for 3′-RACE		
PPARγ-3′ outer	ACTCTCAGGAAAGACCAGCGACA	62.0
PPARγ-3′ inner	CGCAAGCCCTTCTGTGAAATGATGGA	62.0
Primers for 5′-RACE		
PPARγ-5′ outer	CGAAAGGGGCATTGTCGCTGGTC	62.0
PPARγ-5′ inner	GCTCTGCGGCGGGCTCGGCTCATACT	62.0
Primers for qPCR [b]		
β-actin-F	TTACCCACACCGTGCCCATCTAC	59.0
β-actin-R	TACCGCAAGACTCCATACCCA	59.0
GAPDH [c]-F	ACCAACTGCTTGGCTCCCC	59.0
GAPDH-R	GGAATGACTTTGCCCACG	59.0
PPARγ-F	TGGCTTTCACTATGGCGTTCA	59.0
PPARγ-R	GCATTTGTTGCGACTCTTCTTG	59.0
Fads2 [d]-F	CACAGGTTCGGCACTTACAC	59.0
Fads2-R	TCGCATCTTCTCCAGCATAATG	59.0
Lpl [e]-F	GAGCCGACGAGCGAGTCTACAAC	59.0
Lpl-R	CAGTGACCGTCCATCCGTGAATA	59.0
Elovl5 [f]-F	CACACTAATCTTGCTCTTCAC	59.0
Elovl5-R	GCTGCTCTACTACATTGGTT	59.0
Srebp1c [g]-F	ATACCAGAAGCAGCAGAGT	59.0
Srebp1c-R	CCAGAAGAGGACCGATGAT	59.0

[a] *PPARγ*: Peroxisome proliferator activated receptor gamma.
[b] qPCR: Real-time quantitative PCR.
[c] *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.
[d] *Fads2*: Fatty acid desaturase 2.
[e] *Lpl*: Lipoprotein lipase.
[f] *Elovl5*: Elongase of very long chain fatty acids 5.
[g] *Srebp1c*: Sterol regulatory element-binding protein-1 c.

Li, Dalian Ocean University, China. They were cultured in a constant condition at 28 °C with DMEM/F12 (Hyclone, USA) containing 15% fetal bovine serum (Gibco, USA), 10U/ml penicillin and 10 μg/ml streptomycin (Invitrogen, China).

LF cells were trypsinized and placed in 6-well cell culture plates (Biologix, China) for *PPARγ* treatment. When the complete monolayer was formed, *PPARγ* activator (rosiglitazone, Selleck, USA) and inhibitor (T0070907, Selleck) were respectively added into different wells. Both rosiglitazone and T0070907 were dissolved in dimethyl sulfoxide (DMSO) and their final concentrations were 50 μM. LF cells cultured with normal culture medium containing DMSO were served as control. After 24-h culture, all the cell samples were washed with phosphate buffer saline (PBS) and then collected for RNA extraction. Expressions of *PPARγ* and four fatty acid metabolism related genes, namely *fads2*, *lpl*, *elovl5* and *serbp1c*, were then determined.

2.6. Fatty acids induction experiment

LF cells were placed in a 6-well cell culture plate (Biologix) at a density of 1×10^5 cells per insert. The cells were maintained until they adhered, and then the culture medium was replaced by DMEM/F12 containing 2% bovine serum albumin (BSA) (WAKO, Japan). After 24-h metabolism, 50 μM and 100 μM LA and LNA were respectively added into the culture medium. LF cells cultured with 0 μM LA and LNA were considered as the control. One day later, all the cells were harvested for RNA extraction. Expressions of *PPARγ* were evaluated.

2.7. Real-time quantitative PCR (qPCR)

The stored samples were prepared for extracting total RNA by RNA isoPlus (TaKaRa) and one microgram of total RNA of each sampling was used to synthesize the first-strand cDNA. Quantification was performed in a Mini opticon real-time detector (BIO-RAD, Hercules, CA USA). The