Identification and structural characterization of two peroxisome proliferator activated receptors and their transcriptional changes at different developmental stages and after feeding with different fatty acids

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ABSTRACT

Peroxisome proliferator activated receptors beta1 (PPARβ1) and beta2 (PPARβ2) were investigated in loach (*Misgurnus anguillicaudatus*). The PPARβ1 and PPARβ2 were widely distributed in loach tissues. Multiple alignments of deduced amino acid sequences revealed homologous characteristics of the two subtypes of PPARβ with 88% identity. PPARβ1 was markedly expressed in the liver, about 100-fold higher than liver PPARβ2. The two subtypes in unfertilized ovum (UO) showed the highest transcriptions in early life stages, and there were great divergences in expression between unfertilized and fertilized stages. The regulation of PPARβ1 and PPARβ2 in response to dietary fatty acids was studied in liver of loach fed with diets containing fish oil (FO, rich in n − 3 highly unsaturated fatty acid) or soybean oil (SO, rich in 18:2n − 6) for 75 days. Results showed that hepatic transcription of PPARβ1 in the SO group was higher than in the FO group. However, PPARβ2 expression was similar. The differences of molecular characterization, tissue expressions in early life stages, and transcriptional regulation by lipid resources indicated that PPARβ1 and PPARβ2 were functionally different. This is the first report of differential expression of PPARβ1 and PPARβ2 in various tissues and early life stages of loach are regulated by lipid resources. These results will stimulate further studies to better understand the functional characterization of PPARβ1 and PPARβ2.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) consist of PPARα, PPARβ and PPARγ, valuable ligand-activated transcription factors (Kliewer et al., 1992). Previous studies have reported the PPARβ gene in human (*Homo sapiens*) (Michalik et al., 2003), mouse (*Mus musculus*) (Larsen et al., 2002), Nile crocodile (*Crocodylus Niloticus*), turtle (*Trachemys scripta elegans*) (Hughes et al., 1999), African clawed frog (*Xenopus laevis*) (Dreyer et al., 1992), jungle fowl (*Gallus gallus*) (Takada et al., 2000) and several fish species including gray mullet (*Mugil cephalus*) (Ibabe et al., 2004), grass carp (*Ctenopharyngodon idella*) (He et al., 2012) and cobia (*Rachycentron canadum*) (Tsai et al., 2008). Although some studies discovered four PPARβ subtypes in salmon (*Salmo salar*) (Leaver et al., 2007) and two PPARβ subtypes in zebrafish (*Danio rerio*) (Robinson-Rechavi et al., 2001), the existence of distinct PPARβ subtypes in many fish species is still unclear.

Previous studies have indicated that PPARβ serve as an important nuclear hormone receptor factor, which is linked to lipid homeostasis (Schmuth et al., 2004; Lee et al., 2006) and cell proliferation (Westergaard et al., 2001). Reilly and Lee (2008) reported that PPARβ regulated fat oxidation, glucose consumption and lipid accumulation in a range of tissues including adipose tissue thus confirming its role in lipid and glucose homeostasis. Tachibana et al. (2005) demonstrated that PPARβ plays a metabolically active role in various tissues especially in the control of genes involved in fatty acid (FA) metabolism.

Dietary lipids are an important nutrient for fish growth, acting as a source of energy and essential fatty acid (EFA) (Sargent et al., 2002). Fish oil (FO), is rich in n − 3 long chain polyunsaturated fatty acids (LC-PUFA) such as 20:5n − 3 (eicosapentaenoic acid, EPA) and 22:6n − 3 (docosahexaenoic acid, DHA). In contrast, soybean oil has a low concentration of n − 3 LC-PUFA but rich in C18 fatty acids like 18:2n − 6 (linoleic acid, LA) and 18:3n − 3 (linolenic acid, LNA). It is well-documented that freshwater fish have an innate capacity to convert LA and LNA to n − 6 and n − 3 LC-PUFA, respectively. Unsaturated fatty acids are not only important lipid sources in fish but also the essential ligands of PPARβ (Forman et al., 1997; Clarke et al., 1999). However, limited information

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is available on the effects of different dietary lipid sources on PPARβ subtypes in fish.

Dojo loach (*Misgurnus anguillicaudatus*), a Cypriniform species, is a widely distributed demersal cobitidae fish in eastern Asia. In recent times, its market value has increased because of its high nutritional and medicinal value (Wang et al., 2010; Gao et al., 2012). In this study, we firstly cloned two isoforms of PPARβ (PPARβ1 and PPARβ2) from loach (*M. anguillicaudatus*) liver, and the differences in molecular characterization, tissue and early life stages expressions, and transcriptional regulation by dietary lipid sources between PPARβ1 and PPARβ2 were determined. This study could be the foundation of exploring the functional characterization of PPARβ1 and PPARβ2 in further studies.

2. Materials and methods

2.1. Fish

The wild-adult loach (body mass about 20 g) was obtained from Ezhou City in China and kept at stable temperature (26 ± 2 °C) in aquaria. The ploidy levels of all loaches were estimated by flow cytometry (Beckman Coulter, Brea, CA, USA). Red blood cells were obtained by caudal venepuncture and stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, USA). Chicken (Gallus domesticus) erythrocytes were used as the reference cells (Zhu et al., 2012).

2.2. Cloning of PPARβ1 and PPARβ2 cDNAs in loach

Total RNA was isolated from loach liver (40–60 mg) using RNA isoPlus (TaKaRa, Japan). Quantity and quality of isolated RNA were ascertained by electrophoresis and spectrophotometry (Nanodrop 2000, Thermo Scientific, USA). Then RNA was dissolved in 40 μl RNase free water and stored at −70 °C.

Reverse transcription was conducted with PrimeScript®RT reagent Kit with gDNA Eraser (TaKaRa, Japan), and the 3′ and 5′ ends of cDNAs were cloned using the RACE-PCR with the SMART RACE Kit (Clontech,USA). The universal amplified primers of loach PPARβ1 and PPARβ2 were respectively designed on distinct conserved regions from multiple alignments of various fish including zebrafish (*D. rerio*), salmon (*S. salar*), medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*). The primers to amplify the partial sequences are listed in Table 1. Parameters for PCR are as follows: 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C, an initial 94 °C denaturation for 5 min and a 72 °C extension for 8 min. After that, RACE primers were designed on the basis of the amplified fragments (Table 1) and PCR was performed according to the manufacturer's instructions (Clontech, USA). The annealing temperature was 62 °C for extending sequences. The PCR products described above were purified from 2.0% agarose by SanPrep Column DNA Gel Extraction Kit (Sangon, China) and loaded into the pMD 19-T cloning vector (TaKaRa, Japan). Then the positive transformants were selected and sequenced (Sangon, China).

2.3. Sequence and phylogenetic analysis

The sequences of PPARβ1 and PPARβ2 were assembled through BioEdit, and then translated by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The PPARβ sequences from vertebrates for phylogenetic tree and alignments were obtained from the GenBank and Protein databases at NCBI and the identity of sequences were analyzed by Blast (http://blast.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed by MEGA 5.0 program with the neighbor-joining method, which was evaluated by the bootstrap method with 1000 replications (Tamura et al., 2011). For deduced amino acid analysis, the calculated molecular weight and theoretical isoelectric point were obtained by ProtParam tool (http://web.expasy.org/protparam/). The amino acid sequences were performed with multiple alignments by BioEdit software,

Table 1
Nucleotide sequences of the primers for PCR.

Target genes	Primer sequences (5′-3′)	Tm(°C)
Primer for partial cDNA cloning		
PPARβ1-central-F	GAYAARGCHTCRGGRTTCCACT	58.0
PPARβ1-central-R	TTBCYYTCTCYTCCTCNGGGCAT	
PPARβ2-central-F	AAGCCTCWGGNTTYCAYTAYGG	58.0
PPARβ2-central-R	CCARCARVCCRTCYTTRTTCAT	
3′-RACE		
PPARβ1-3′ outer	AAGCATCAGGATTTCACTATGGTGTTCA	62.0
PPARβ1-3′ inner	CGGTATGGACGGATGCCCGAGTCAGAGAA	
PPARβ2-3′ outer	CTGGTGGCGGGTTTGCTCGCAGGGGA	62.0
PPARβ2-3′ inner	TATGAATAAGGACGGGCTGCTGGTAG	
5′-RACE		
PPARβ1-5′ outer	CTTCTCTGACTCGGGCATCCGTCCATAC	62.0
PPARβ1-5′ inner	ATTCCAACTTCATACGGATGGTCCTTCT	
PPARβ2-5′ outer	TTGAGGTTCTTCAGGTAGGCTGTGTT	62.0
PPARβ2-5′ inner	TTCTCCCCTGCGAGCAAACCCGCCACC	
qPCR[a]		
GAPDH-F[b]	ACCAACTGCTTGGCTCCCC	60.0
GAPDH-R[b]	GGAATGACTTTGCCCACG	
β-actin-F	TTACCCACACCGTGCCCATCTAC	60.0
β-actin-R	TACCGCAAGACTCCATACCCA	
PPARβ1-F	CATCATTGCTGCTCGTTGGA	60.0
PPARβ1-R	CATTCTGTTGTTCTTGTGTCTCTG	
PPARβ2-F	CTCCGCAGCCAGACTTGAA	60.0
PPARβ2-R	TCCGTTCCACTTCCGTTCTTA	

[a] qPCR: Real-time quantitative PCR.
[b] *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

whose secondary structures were noted. Then the secondary and three-dimensional (3D) structures of PPARβ1 and PPARβ2 were predicted by PredictProtein (http://www.predictprotein.org/) and SWISS-MODEL (Arnold et al., 2006), respectively. The 3D models constructed were selected from the templates through multiple alignments with known ones.

2.4. Samples collection

The tissues (spleen, muscle, heart, brain, gill, kidney, liver, intestines, gonads) were dissected from three adult loaches, stored at −70 °C for RNA extraction later to study the distribution of PPARβ1 and PPARβ2.

Samples at 16 different developmental stages (i.e., unfertilized spermatozoa (US), unfertilized ovum (UO), fertilized egg, 2 cells, 8 cells, 32 cells, blastula, gastrula, neurula, tail-bud forming stages (organ formation stage), hatching, and second day (2nd,) (5.64 ± 0.58 mm), tenth day (10th) (9.81 ± 1.29 mm), twentieth day (20th) (17.59 ± 3.05 mm) and thirty fifth day (35th) (31.41 ± 5.16 mm) after hatching) were frozen at −70 °C prior to RNA extraction. The feeding regimes of the stock larvae were based on the methods from our laboratory described by Gao et al (2014).

2.5. Real-time quantitative PCR (qPCR)

Total RNA was extracted as described above and 1 μg of total RNA of each sampling was used for reverse transcription. Specific primers of target genes and reference genes (Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin) are listed in Table 1. Quantification was performed in a Mini opticon real-time detector (BIO-RAD, Hercules, CA USA). The qPCR reaction solutions were as follows: 5 μl SYBR® premix Ex Taq™ ($2\times$), 0.5 μl PCR forward primer (10 μM), 0.5 μl PCR reverse primer (10 μM), 1.0 μl RT reaction (cDNA solution with 5-fold diluting), and 3.0 μl ddH$_2$O. The reaction was performed with 95 °C for 30 s followed by 40 cycles consisting of 95 °C for 5 s, and 57 °C for 30 s. The fluorescent flux was recorded and the reaction continued at 72 °C 6 s and 95 °C for 5 s. The absolute quantification was performed through the standards of parallel set of reactions. So the copy numbers