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Molecular characterization of carnitine palmitoyltransferase IA in *Megalobrama amblycephala* and effects on its expression of feeding status and dietary lipid and berberine

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ABSTRACT

Carnitine palmitoyltransferase I (CPT I, EC 2.3.1.21) controls the main regulatory step of fatty acid oxidation, and hence studies of its molecular characterization are useful to understand lipid metabolism in cultured fish. Here, a full-length cDNA coding CPT I was cloned from liver of blunt snout bream *Megalobrama amblycephala*. This cDNA obtained covered 2499 bp with an open reading frame of 2181 bp encoding 726 amino acids. This CPT I mRNA predominantly expressed in heart and white muscle, while little in eye and spleen. The phylogenetic tree constructed on the basis of sequence alignments among several vertebrate species suggests that this blunt snout bream CPT I sequence belongs to the CPT IA family. In order to investigate the characterization of CPT IA mRNA expression, post-prandial experiment and feeding trial were conducted. The results showed that CPT IA mRNA expression was unchanged from 2 to 12 h, and then significantly increased at 24 h post-feeding in liver and heart. Berberine, an alkaloid, was identified as a promising lipid-lowering drug. In order to elucidate the effect of berberine on CPT I expression, fish were fed for 8 weeks with three diets (low-fat diet (LFD, 5% fat), high-fat diet (HFD, 15% fat), and berberine-supplemented diet (BSD, 15% fat). The results showed that HFD could decrease the expression of CPT IA and PPARα, while BSD increased those expressions.

1. Introduction

Lipids are the major sources of metabolic energy in fish (Sargent et al., 2002). The β-oxidation of fatty acids plays a central role in the production of energy, and most oxidation occurs in the mitochondria (Bartlett and Eaton, 2004; Lu et al., 2014). However, the long-chain fatty acids cannot directly cross the mitochondrial inner membrane, and their entry to the mitochondrion is a major point for control and regulation of the β-oxidation flux (Bartlett and Eaton, 2004). The carnitine palmitoyltransferase system consists of carnitine palmitoyltransferase I and II (CPT I and CPT II), which mediates the uptake of long-chain fatty acids into mitochondria (McGarry and Brown, 1997). CPT I, located in outer membranes of mitochondria, controls the flux through β-oxidation and is a rate-limiting enzyme of fatty acid oxidation (Skiba-Cassy et al., 2007). Therefore, the study of CPT I characterization has implication for the understanding of the β-oxidation in fish.

In mammals, CPT I exists in three isoforms: a liver isoform (CPT IA or L-CPT I), a muscle isoform (CPT IB or M-CPT I), and a brain isoform (CPT IC) (Esser et al., 1996; Price et al., 2002; Treber et al., 2003). These three isoforms vary in their tissue distribution and enzymatic properties (Skiba-Cassy et al., 2007). CPT IA is expressed in most tissues such as liver, intestine, and heart. CPT IB is mainly expressed in muscle, while CPT IC expression is restricted to the central nervous system (Zheng et al., 2013a). Moreover, CPT IA and CPT IB are 62% identical in amino acid sequence and exhibit different sensitivity to malonyl-CoA inhibition (Treber et al., 2003). To date, the full-length cDNA encoding CPT I has been cloned and sequenced in several carnivorous fish species, including gilthead sea bream (*Sparus aurata*) (Boukouvala et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Gutières et al., 2003), yellow catfish (*Pelteobagrus fulvidraco*) (Zheng et al., 2013a), and Japanese seabass (*Lateolabrax japonicas*) (Zheng et al., 2012). Moreover, the study shows that CPT I displayed different properties between carnivorous and herbivorous fish (Zheng et al., 2013b). However, there is little information available on the tissue expression, protein structure, or kinetics of CPT I in the herbivorous fish.

Blunt snout bream (*Megalobrama amblycephala*) is an herbivorous freshwater fish native to China (Zhou et al., 2008). Due to its fast growth, tender flesh, and high disease resistance, this species has been widely favored for aquaculture in China (Li et al., 2012). However, compared to a number of other commercially produced fishes, its artificial

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rearing is often associated with the occurrence of fatty liver disease, which correlates closely with a high rate of mortality or poor growth (Lu et al., 2013a, 2013b). The attenuated β-oxidation capacity owing to decreased catalytic efficiency of CPT I is closely related to the cause of fatty liver (Lu et al., 2014). One of the projects in our laboratory is to identify the Chinese herb to supplement into feeds in order to improve the lipid metabolism of fish. Berberine, an alkaloid isolated from huanglian (*Coptis chinensis*), has been extensively used in traditional Chinese medicine (Holy et al., 2009). Recently, berberine was identified as a promising lipid-lowering drug to reduce hepatic fat content and could remarkably elevate the expression of key fatty acid oxidation genes such as CPT I and peroxisome proliferator-activated receptors (PPARs) in rats (Brusq et al., 2006; Chang et al., 2010; Kim et al., 2009; Kong et al., 2004). PPARs can also regulate expression of genes encoding enzymes involved in β-oxidation (Mandard et al., 2004; Wang et al., 2003). In addition, it is currently well established that physiological conditions such as feeding and fasting affect the CPT I expressions (Brandt et al., 1998). Therefore, some researchers tried to lower fat level in liver by starvation in fish, which is very practical in aquaculture (Nagai, 1971; Du et al., 2003). In order to gain more information concerning β-oxidation and CPT I expression in blunt snout bream, the present study was conducted to (1) achieve the molecular characterization of a cDNA of CPT I from the liver tissue, (2) examine hepatic CPT I mRNA expression after feeding, and (3) elucidate effect of CPT I mRNA expression of dietary lipid and berberine. These results may have important implications for our understanding of lipid metabolism in cultured herbivorous fish.

Table 1
The primers used for cloning and expression analysis.

Primers	Sequence(5′—3′)	Use
Primers used for RACE		
CPT I5-R	GATGGCAATCCACAGA	Synthesis of the first-strand cDNA for 5′RACE
CPT I5-R1	CACCCCCAACTAACATCTGC	Used with Oligo(dT)16AP for first PCR of 5′RACE
CPT I5-R2	GCCAAGTTTGACGAACAG	Used with AP for nested PCR of 5′RACE
CPT I3-F1	TGATGCTCCAGTTGTTGGCCACCTC	Used with AP for first PCR of 3′RACE
CPT I3-F2	CTGCCTGGACCTCAGAGACTCCAAT	Used with RACE3-R for nested PCR of 3′RACE
Oligo(dT)16AP	CTGATCTAGAGGTACCGGATCC$(T)_{16}$	Synthesis of the first-strand cDNA for 3′RACE
AP	CTGATCTAGAGGTACCGGATCC	Used with CPT I3-F1 or CPT I5-R2
RACE3-R	CTAGAGGTACCGGATCCTT	Used with CPT I3-F2
Primers for real-time PCR		
CPT I-F	TACTTCCAAAGCGGTGAG	Real-time PCR
CPT I-R	AGAGGTATTGTCCGAGCC	
PPARα-F	GTGCCAATACTGTCGCTTTCAG	Real-time PCR
PPARα-R	CCGCCTTTAACCTCAGCTTCT	
PPARβ-F	CATCCTCACGGGCAAGAC	Real-time PCR
PPARβ-R	CACTGGCAGCGGTAGAAG	
PPARγ-F	AGCTTCAAGCGAATGGTTCTG	Real-time PCR
PPARγ-R	AGGCCTCGGGCTTCCA	
β-actin-F	CGGACAGGTCATCACCATTG	Real-time PCR
β-actin-R	CGCAAGACTCCATACCCAAGA	

2. Materials and methods

2.1. Fish and sample collection

Juvenile blunt snout bream (*Megalobrama amblycephala*) was obtained from the fish hatchery of Wuhan (Hubei, China). Fish were maintained in a recirculating aquaculture system of laboratory under the following conditions: water temperature, 25–27 °C; DO, 5.0–6.0 mg/L; pH, 7.2–7.6; photoperiod, 12:12 h (dark: light). Fish were fed a commercial diet (31% protein, 7% lipid) for 2 weeks to acclimate to the experiment conditions. Before the sampling, fish (weight: 30 ± 1 g) were starved 24 h and then euthanized by 100 mg/L MS-222 (tricaine methanesulfonate; Sigma, USA). Tissue samples (heart, liver, spleen, kidney, white muscle, intestine, brain, gills, adipose tissue, and eye; n = 6) were immediately collected and frozen in liquid nitrogen and then stored at −80 °C.

2.2. Molecular cloning

Total RNA was extracted from the liver tissue using RNAiso Plus (Takara Co. Ltd, Japan). RNA samples were treated by RQ1 RNase-Free DNase prior to RT-PCR (Takara Co. Ltd, Japan) to avoid genomic DNA amplification. Quantity and purity of isolated RNA were later determined by absorbance measures at 260 and 280 nm, and its integrity was tested by electrophoresis in 1.0% formaldehyde denaturing agarose gels. The partial fragment of CPT I was obtained by the high-throughput sequencing in a previous study (our unpublished data). According to the sequence information of this fragment, gene-specific primers were designed for 3′ RACE and 5′ RACE (Table 1). Rapid amplification of the 5′ end was performed using the 5′ RACE System of Invitrogen. Briefly, 2 μg sample RNA was performed with CPT I5-R to obtain the first-strand cDNA. After RNase treatment, an Oligo(dC) at the 5′ end was added using terminal deoxynucleotidyl transferase. The resulting product was used as a template for the first PCR amplification at 94 °C for 2 min and 30 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and 72 °C for 7 min. Then, the first PCR product was used as a template for the nested PCR. The nested PCR product was eluted from 1% agarose gel and delivered to Shanghai Sangon Biotech Service Co. Ltd. (Shanghai, China) for sequencing. Rapid amplification of the 3′ end was performed using the 3′-full RACE Core Set (TaKaRa, Dalian, China) following the manufacturer's instructions. The primers used for 3′ RACE were shown in Table 1. After the first and nested PCRs (consisted of 25 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 68 °C), the PCR product was eluted from 1.0% agarose gel and sequenced following the procedures detailed above.

2.3. Sequence analysis

The sequence was edited and analyzed using the program EditSeq in DNAStar Package to search open reading frame (ORF), and then translated into amino acid (AA) sequence using standard genetic codes. The molecular weight (MW) and isolectric point (PI) of CPT I protein were both predicted using the compute pI/Mw software at http://cn.expasy.org/tools/pi_tool.html. Putative trans-membrane regions were predicted with HMMTOP v2.0 at http://www.enzim.hu/hmmtop/. Protein sequence alignments were made by MegAlign program in DNAStar Package (version 5.01) using the ClustalW method. The phylogenetic tree was constructed based on the deduced AA sequences using the neighbor-joining (N-J) algorithm within MEGA 5.0. Functional motifs were identified by a PROSITE search at http://au.expasy.org/prosite.

2.4. Quantitative real-time PCR (qPCR)

Extraction of total RNA from fish tissues and first-strand cDNA synthesis were performed as described above. Real-time PCR was employed to determine mRNA levels based on the SYBR Green I fluorescence kit. Primer characteristics used for real-time PCR are listed at Table 1. Real-time PCR was performed in a Mini Option real-time detector (BIO-RAD, USA). The fluorescent quantitative PCR reaction solution consisted of 12.5 μl SYBR® premix Ex Taq™ (2×), 0.5 μl PCR forward primer (10 μM), 0.5 μL PCR reverse primer (10 μM), 2.0 μL RT reaction (cDNA solution), and 9.5 μL dH_2O. The reaction conditions were as follows: 95 °C for 3 min followed by 45 cycles consisting of 95 °C for 10 s and 60 °C for 20 s. The fluorescent flux was then recorded, and the reaction continued at 72 °C for 3 min. The dissociation rate was measured between 65 and 90 °C. Each increase of 0.2 °C was maintained for 1 s,