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Molecular characterization and nutritional regulation of carnitine palmitoyltransferase (CPT) family in grass carp (*Ctenopharyngodon idellus*)

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ABSTRACT

The carnitine palmitoyltransferase (CPT) gene family plays an essential role in fatty acid β-oxidation in the mitochondrion. We identified six isoforms of the CPT family in grass carp and obtained their complete coding sequences (CDS). The isoforms included CPT 1α1a, CPT 1α1b, CPT 1α2a, CPT 1α2b, CPT 1β, and CPT 2, which may have resulted from fish-specific genome duplication. Sequence analysis showed that the predicted protein structure was different among the CPT gene family members in grass carp. The N-terminal domain of grass carp CPT 1α1a, CPT 1α1b, CPT 1α2a, and CPT 1α2b contained two transmembrane region domains and two acyltransferase choActase domains that exist in human and mouse proteins also; however, only one acyltransferase choActase domain was found in grass carp CPT 1β. The grass carp CPT 2 had two acyltransferase choActase domains. The grass carp CPT 1α1b, CPT 1α2a, CPT 1α2b, and CPT 1β contained 18 coding exons, while CPT 1α1a and CPT 2 consisted of 17 coding exons and 5 coding exons, respectively. The mRNA of the six CPT isoforms was expressed in a wide range of tissues, but the mRNA abundance of each CPT showed tissue-dependent expression patterns. The expression of CPT 1α1a, CPT 1α2a, and CPT 1β at 48 h post-feeding was significantly increased in the liver ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). The diverse responses of multiple isoforms in the liver during nutritional limitation suggest that they may play different roles in fatty acid β-oxidation.

1. Introduction

The carnitine palmitoyltransferase system is an essential step in the β-oxidation of long chain fatty acids (Lopes-Marques et al., 2015), because it allows for subsequent movement of the acyl carnitine from the cytosol across the intermembrane space of mitochondria. The carnitine palmitoyltransferase (CPT) family, a mitochondrial transferase enzyme (EC 2.3.1.21) family, plays an indispensable role in fatty acid β-oxidation via catalyzing the conversion of fatty acid-CoAs into fatty acid–carnitines. Specifically, CPT 1 is anchored in the mitochondrial outer membrane while CPT 2 in the mitochondrial inner membrane (Kerner and Hoppel, 2000). The CPT 1 has a pivotal effect on catalyzing the synthesis of long-chain fatty acylcarnitine, which is a rate-limiting step in mitochondrial beta-oxidation (McGarry and Brown, 1997). Then, the long-chain fatty acylcarnitine is broken down by CPT 2 into long-chain fatty acids, a β-oxidation process to supply energy, and is reconverted to fatty acyl-CoA, liberating carnitine that can be recycled (Glatz et al., 2010).

Mammals possess four paralogs of CPT: CPT 2 is found in all tissues, and tissue-specific CPT 1 paralogs are found in the liver (CPT 1α), muscle (CPT 1β), and brain (CPT 1γ) (Price et al., 2002; van der Leij et al., 2000). The regulatory mechanism of the CPT enzyme system consists of transcriptional regulation, malonyl-CoA-dependent, and malonyl-CoA-independent regulation in mammals (Kerner and Hoppel, 2000, 2005). The gene expression and activity of the CPT family could be up-regulated by fasting and insulin, because of changes in the sensitivity of CPT 1 to malonyl-CoA (Ryu et al., 2005; Skiba-Cassy et al., 2007). Similarly, in fish, the isoforms CPT 1α and CPT 1β were identified and characterized in previous studies on trout and sea bream (Boukouvala et al., 2010; Morash et al., 2008; Morash and McClelland, 2011). Interestingly, teleosts possess multiple isoforms of the CPT gene family, including CPT 1α1a, CPT 1α1b, and CPT 1α2a (Wu et al., 2016; Zheng et al., 2013), which might result from a unique evolutionary history in fish that involves genome duplication (Lopes-Marques et al., 2015; Morash et al., 2010).

Mechanisms involving the CPT gene family in fish energy metabolism have not been studied in depth, even if their multiple functions in

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fish resulting from genome duplication could be more complicated than mammals. Excess energy intake may result in lipid accumulation in fish species in production, therefore, understanding the regulation of CPT genes in energy metabolism may contribute to the elucidation of its underlying mechanisms. Morash et al. (2008) found that the CPT family played a key role in controlling the fatty acid flux through β-oxidation in vertebrates, and it might be the main regulatory enzyme family of fatty acid β-oxidation. The fatty acids are a source of metabolic energy in fish, and are utilized for growth, reproduction, movement, and migration (Tocher, 2003). Given the significant role of the CPT family enzymes in energy metabolism, it is important to understand their characteristics, including their regulation and structure. Food intake limitation and starvation are direct threats to the survival of animals, that could lead to the stimulation of oxidation of stored fats to meet the body's energy demands (McCue, 2012). The fasting test is an important approach to observe and study the nutritional and metabolic regulation of activities of the CPT enzymes.

Grass carp (*Ctenopharyngodon idella*), a herbivorous freshwater fish, is an important farmed fish in China, known for its delicious meat and high market value. It is considered a good model for the study of lipid metabolism because grass carp stores excess fat in liver and adipose tissue. Additionally, the draft genome of the grass carp, recently released, is a useful tool for identifying genomic structure of genes involved in lipid metabolism. In this study, we identified six isoforms of the CPT family and obtained their complete coding sequences (CDS) in grass carp. We then molecularly characterized the isoforms and analyzed their tissue expression pattern. To investigate the roles of these multiple isoforms in fatty acid β-oxidation, we examined their mRNA expressions during fasting in vivo and in vitro.

2. Materials and methods

2.1. Experiment 1: carnitine palmitoyltransferase (CPT) family gene cloning and tissue expression pattern

2.1.1. Fish and sampling

The experimental grass carps (*Ctenopharyngodon idellus*) were selected from a group provided by a fish farm. They were cultured in a recirculating fresh water system at a constant water temperature (26–28 °C), natural photoperiod (light 12 h and dark 12 h), and the sufficient dissolved oxygen (5.0–6.0 mg/L) for approximately two weeks. Six randomly selected grass carps (weight: 48.06 ± 6.09 g) starved for 24 h were euthanized by immersion in MS222 (Sigma, St. Louis, MO, USA). Tissue samples (heart, liver, spleen, kidney, white muscle, red muscle, brain, and adipose tissue) for RNA collection were removed on the ice and quickly frozen in liquid nitrogen until RNA extraction.

2.1.2. Identification and cloning of carnitine palmitoyltransferase (CPT) gene family

Sequences of grass carp CPT 1α1a, CPT 1α1b, CPT 1α2a, CPT 1α2b, CPT 1β, and CPT 2 were searched by gene annotation in our transcriptomic database (accession number: SRP044769). All the expressed sequence tags (ESTs) were assembled in silico into a consensus sequence containing the complete open reading frame (ORF) using the SeqMan program of DNAstar software. To amplify and confirm the whole length of these cDNAs, PCR specific primers were designed (Table 1).

The total RNA was extracted from the liver of the grass carp using RNAiso Plus (TaKaRa, Dalian, PR China) according to the manufacturer's instructions and the RNA integrity was measured through agarose gel electrophoresis and by using a spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, USA) at 260 and 280 nm. Corresponding cDNAs were synthesized by reverse transcription using the M-MLV reverse transcriptase (TaKaRa, Dalian, PR China). The PCR parameters were 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min, with an additional initial 4-min denaturation at 94 °C and a 10-min final extension at 72 °C. The products of the PCR were electrophoresed on 1.5% agarose gel. Bands with the expected size were purified using a PCR Purification Kit (TaKaRa, Dalian, PR China). The purified products were then ligated into a PMD18-T vector following the manufacturer's instructions (TaKaRa, Dalian, PR China) and propagated in *E. coli* DH5α for confirming the intact sequence.

Table 1
Specific Primers used for CDS cloning and qPCR of CPT family from grass carp.

Primers	Sequences (5′-3′)	Size (bp)	Usage
CPT1α1a-F	CAGTCAGCATGGCAGAAGCG	2401	CDS cloning
CPT1α1a-R	GAAGAGACAGATCACTTGTTGTTGG		
CPT1α1b-F	AGCGATCCAGTATGGCAGAAG	2341	CDS cloning
CPT1α1b-R	ACCAGTCAAATCACTTTGGGTC		
CPT1α2a-F	AGAAATGGCAGAAGCCCATCA	2396	CDS cloning
CPT1α2a-R	CCGTCATGGGCAATCTCTTCT		
CPT1α2b-F	CAGAGATGGCAGAGGCTCAC	2372	CDS cloning
CPT1α2b-R	TGCATCCAGTCTTCCTTTCAGA		
CPT1β-F	GCCTGTATAATGGCAGAGGCT	2327	CDS cloning
CPT1β-R	AGGAGTCACATCTTCTTCTCTTTC		
CPT2-F	GGCAGGGTTGCTCTCTACAC	2049	CDS cloning
CPT2-R	CTAGTCCACATGCCAAGTGCT		
CPT1α1a-F	CAGACACATCGCCGTATTC	113	qPCR
CPT1α1a-R	CCACCAAGATCCTTTCAATCT		
CPT1α1b-F	GACCTGGAAACTTCGACTATG	129	qPCR
CPT1α1b-R	CTTCTCATGGTGTCCTTCAC		
CPT1α2a-F	CAGTCCAGATGCCTTCATTC	102	qPCR
CPT1α2a-R	CGGAACAAACGGGTCATAG		
CPT1α2b-F	GCGTTTCAGTTCACCATCT	111	qPCR
CPT1α2b-R	CCTGCTGATGCGTTTCTT		
CPT1β-F	GCGAATGAGCAGAGGTTAG	117	qPCR
CPT1β-R	CACAACATAGAGGCAGAAGAG		
CPT2-F	GATGAGGAGGTGATGAGAGA	109	qPCR
CPT2-R	CGTCCTTAGCCAAGATGATAC		
β-actin-F	CGTGACATCAAGGAGAAG	115	qPCR
β-actin-R	GAGTTGAAGGTGGTCTCAT		

2.1.3. Sequence and phylogenetic analysis

Sequences were analyzed using the BLAST network service at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple protein sequence alignments were conducted using the ClustalW program. The predicted protein sequence was analyzed by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Deduced amino acid sequences were aligned using the ClustalW program using the sequences in Supplementary Table 1. The protein structures of the CPT gene family were predicted using the conserved domains profile (http://smart.embl-heidelberg.de/).

2.2. Experiment 2: fasting treatment in vivo and in vitro

2.2.1. Fasting treatment in vivo

For the fasting treatment, fish were assigned randomly to one of five treatment groups in 150 L circular tanks (approximately 16–20 fish per tank) with a flow-through water supply at 28 °C under a 12 L:12D photoperiod. A total of six fish (3 per tank) were randomly collected at 0, 6, 12, 24, and 48 h after feeding them by immersion in MS222 (Sigma, St. Louis, MO, USA). Samples of liver were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.2.2. Fasting treatment in vitro

The hepatocytes of grass carp were bought from the China Center for Type Culture Collection (CCTCC). The hepatocytes were placed into M199 cell culture medium (Thermo Scientific, Wilmington, USA) with 10% fetal bovine serum (FBS) and incubated at 28 °C in a 5% CO_2 humidified atmosphere, and the medium was refreshed every two or three days until cell confluence. The confluent hepatocytes were then placed into M199 cell culture medium with 400 μL