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Molecular characterization of AMP-activated protein kinase α2 from herbivorous fish *Megalobrama amblycephala* and responsiveness to glucose loading and dietary carbohydrate levels

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ABSTRACT

This study aimed to characterize the full-length cDNA of AMPKα2 in *Megalobrama amblycephala*, and evaluate its potential role in glucose homeostasis and carbohydrate metabolism. The cDNA obtained covered 1942 bp with an open reading frame of 1635 bp encoding 545 amino acids. Multiple alignments and phylogenetic analysis revealed a high homology (91–100%) among most fish and higher vertebrates. This AMPKα2 mRNA predominantly expressed in muscle, liver and brain, while little in gill and intestine. Then, the AMPKα2 expressions were determined in the muscle, liver and brain of fish subjected to a glucose load (injected intraperitoneally with 0, 1.67 and 3.34 g glucose per kg body weight) and after a 12-week feeding trial (fed two dietary carbohydrate levels: 30% and 43%), respectively. After the glucose load, plasma glycemia peaked at 1 h in fish. Thereafter, it decreased significantly to the basal level at 8 h. However, AMPKα2 expression in muscle, liver and brain all decreased significantly during the first 2 h, then returned to the basal value at 24 h. Unlikely, tissue AMPKα2 expression of fish receiving saline solution increased significantly during the whole sampling period. Additionally, high-carbohydrate diet enhanced its expression in liver and muscle, but not that in brain. These findings indicated that the AMPKα2 gene shared a high degree of conservation with that of the other vertebrates. Muscle, liver and brain AMPKα2 expressions were highly induced by glucose administration. Furthermore, high dietary carbohydrate modified its expressions in these tissues.

1. Introduction

Glucose is an excellent source of energy and carbon for mammalian cells. A constant supply of glucose could ensure against hypoglycemia and the potentially catastrophic effect on cells of the nervous system (Pilkis and Granner, 1992). Unlike the case of mammals, it is not usually regarded as the panaceas for fish (Wilson, 1994). Although a continual ingestion of carbohydrate-enriched diet or glucose loading can provide the essential metabolic energy, most fish species appear to have an impaired glucose tolerance and often display a prolonged postprandial hyperglycemia (Kamalam et al., 2016). Additionally, the nutritional value of carbohydrates varies greatly in different fish species. Indeed, the great capability to utilize carbohydrate has been observed in herbivorous fish, such as grass carp (*Ctenopharyngodon idellus*) (Lin, 1991) and blunt snout bream (*Megalobrama amblycephala*) (Li et al., 2013); but high carbohydrate contents also result in poor growth and feed utilization (Mohapatra et al., 2003). Until now, the underlying mechanisms for the poor carbohydrate utilization in fish are still poorly understood. Recently, several studies suggested that the ineffective utilization of glucose in fish may be attributed to the poor postprandial supervision of certain energy metabolic sensors, which are closely involved in glucose metabolism (Polakof et al., 2012; Kamalam et al., 2016). Considering this, investigations regarding the molecular characterization and nutritional regulation of these energy metabolic sensors in fish deserve our special attention.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine protein kinase. Being a key energy metabolic sensor, it regulates energy homeostasis through multiple metabolic pathways coordinately, as helps organisms and cells adapt to different energy statuses (Foretz and Viollet, 2011). In addition, it is also regarded as a fuel-sensing protein kinase, and plays a central role in both insulin-sensitive tissues and pancreatic β cell, thus regulating glucose homeostasis (Kurth-Kraczek et al., 1999; Rutter and Leclerc, 2009). In mammals, AMPK is a heterotrimeric complex, which consists of one catalytic (α) and two regulatory subunits (β and γ) (Hardie and Carling, 1997). Among them, the α catalytic subunit, as a characteristic

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structure of AMPK, has two separate subtypes (α1 and α2). These two subunits are characterized by distinct physiological properties: (1) the α1 subunit is located in the cytoplasm, exhibiting an activity 5–10 folds higher than that of the α2 subunit; (2) the α2 subunit displays sub-nuclear localisation, thus regulating the expressions of relevant genes (Salt et al., 1998; Da et al., 2000). Although both subunits are implicated in the control of carbohydrate metabolism by acting as a glucose sensor, the latter appears to be more sensitive (Sun et al., 2010). In mammals, knockout of the AMPKα2 gene makes body easily exposed to insulin resistance and glucose intolerance, whereas the inactivation of the α1 subunit does not result in significant metabolic abnormalities (Viollet et al., 2003a; Jørgensen et al., 2003). In addition, the liver kinase B1 (LKB1), which also is an AMP-activated protein kinase, could vastly up-regulate the basal activity of AMPKα2 but not AMPKα1 (Sakamoto et al., 2006). All these evidence suggest that AMPKα2 might play a more important role in glucose metabolism than AMPKα1. To date, both the function and regulation of AMPKα2 have been extensively characterized in mammals (Lee-Young et al., 2011; Phoenix et al., 2012). However, such information is still quite limited in aquatic species. Recently, the AMPK gene has been characterized molecularly in several aquatic species, including channel catfish *Ictalurus punctatus* (Vides et al., 2011), rainbow trout *Oncorhynchus mykiss* (Polakof et al., 2011), turbot *Scophthalmus maximus* (Zeng et al., 2016) and pacific white shrimp *Litopenaeus vannamei* (Xu et al., 2016b). In addition, its expressions and activities have been both reported to be affected by a large number of factors, such as AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide), metformin, insulin, food intake, salinity stress, etc. (Vides et al., 2011; Polakof et al., 2011; Xu et al., 2016b; Zeng et al., 2016). However, its potential roles in intermediary glucose metabolism are still barely understood. In addition, previous studies mainly focus on carnivorous and omnivorous species, the molecular investigation of AMPK in herbivorous fish is still lacking, as warrants further studies.

Blunt snout bream (*Megalobrama amblycephala*), an herbivorous freshwater carp, is one of the most economically important aquaculture species in China. In recent years, in order to maximize the profit, diets formulated for this fish in China usually contain large amounts of carbohydrates to reduce feed cost. Although previous studies showed that the optimal dietary carbohydrate content for juvenile *M. amblycephala* is approximately 30%, high-carbohydrate feeding usually causes a severe metabolic burden of this species coupled with the compromised glucose homeostasis (Zhou et al., 2013; Li et al., 2014). Therefore, it is quite urgent to investigate the intermediary carbohydrate metabolism of this species, which unfortunately is still unavailable. Considering the crucial role of AMPKα2 in intermediary glucose metabolism, the present study was conducted (1) to clone the full-length cDNA of the AMPKα2 gene from the liver of *Megalobrama amblycephala*; (2) to determine its tissue distribution; and (3) to elucidate its transcriptional responses to glucose loading and dietary carbohydrate levels, respectively. The results obtained here will facilitate our understanding of the carbohydrate metabolism of fish. It is also helpful for the development of low-protein and high-energy food for fish.

2. Material and methods

2.1. Fish and the sampling procedures

Juvenile blunt snout bream (average weight: 63.6 ± 0.4 g) were obtained from the national fish hatchery farm (Yangzhou, Jiangsu province, China). Fish were fed three times daily with a commercial diet containing 32% protein and 31% nitrogen-free extract (Shuaifeng Feed Co., Ltd., Nanjing, Jiangsu province, China) in a slight excess of satiation for one week. In this diet, fish meal, soybean meal, rapeseed meal and cottonseed meal served as protein sources, whereas wheat middlings was adopted as the main carbohydrate source. During this period, water temperature was maintained at 28 ± 2 °C; dissolved oxygen was above 5.0 mg/L; pH ranged from 7.0 to 7.4; and total ammonia nitrogen was <0.04 mg/L. After acclimation, fish were starved for 24 h. To analyze full-length cDNA cloning and tissue-specific expression of AMPKα2 gene, 4 fish were rapidly anesthetized in diluted MS-222 at the concentration of 100 mg/L and sampled for nine organs/tissues (gill, liver, spleen, intestine, trunk kidney, muscle, heart, brain and perivisceral fat). All of the sampled tissues were kept at −80 °C until analyzed.

2.2. Full-length cDNA cloning of the AMPKα2 gene

Total RNA was isolated from the liver of blunt snout bream using Trizol reagent (Invitrogen, CA, USA) and then was treated with DNase I using RQ1 RNase-free Dnase kit (Promega, Madison, USA) to degrade genomic DNA. Spectrophotometric analysis (the 260/280 nm ratio) and 1.0% agarose gels electrophoresis were used to assess the quality and quantity of extracted RNA.

The cDNA was synthesized using total RNA (1 μg) from the liver as template and Oligo(dT)$_{18}$ as primer, and was amplified using an AMV First Strand cDNA Synthesis Kit (GeneCopoeia, Maryland, USA) following the manufacturer's instructions. Degenerated primer pairs of AMPKα2F/AMPKα2R (Table 1) were designed based on highly conserved regions from the available sequences of various invertebrate and vertebrate species. PCR amplification was performed with 2 μL of RT reactions in a total volume of 50 μL and 2.5 U of Platinum Taq DNA Polymerase (Invitrogen). The PCR cycling conditions were one cycle of 94 °C for 4 min, 30 cycles of 94 °C for 40 s, 52 °C for 40 s, and 72 °C for 60 s, followed by one cycle of 72 °C for 7 min. The purified PCR products separated by electrophoresis using 1.0% agarose gels with a molecular size marker and were delivered to Shanghai Sangon Biotech Service Co. Ltd. (Shanghai, China) for sequencing. Sequencing was performed in both forward and reverse directions by using an ABI PRISM® 377 DNA automated sequencer (Applied Biosystems). The forward and reverse sequences were assembled using SeqMan II software in DNAStar Package version 5.01, through which the core fragment of AMPKα2 was obtained. According to the sequence information of this fragment, gene-specific primers were designed for the 3′ RACE and 5′ RACE.

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. Firstly, total RNA (2 μg) from liver was reverse-transcribed using Oligo(dT)$_{16}$AP

Table 1
Primers used for the cDNA cloning of AMPKα2 and RT-PCR.

Primers	Sequence (5′ → 3′)	Use
AMPKα2-F	GCCCTCTGGATGCTCT	Used with AMPKα2-R for RT-PCR of core fragment
AMPKα2-R	GGGTCGGGTAGTGTTG	Used with AMPKα2-F
Oligo(dT)$_{16}$AP	CTGATCTAGAGGTACCGGATCC(T)$_{16}$	Synthesis of the first-strand cDNA for 3′ RACE
AMPKα2,3-F1	CTCAACACTACCCGACCCA	Used with AP for first PCR of 3′ RACE
AP	CTGATCTAGAGGTACCGGATCC	Used with AMPKα2,3-F1 or AMPKα2,5-R2
AMPKα2,3-F2	TCAACACTACCCGACCCA	Used with RACE3-R for nested PCR of 3′ RACE
RACE3-R	AACAGCCACGCTCGCAGA	Used with AMPKα2,3-F2
AMPKα2,5-R	ATGATGTGCGGATGTCTAA	Synthesis of the first-strand cDNA for 5′ RACE
AMPKα2,5-R1	ATGATGTGCGGATGTCTAA	Used with Oligo(dT)$_{16}$AP for first PCR of 5′ RACE
AMPKα2,5-R2	ACGGCTTCCTCATCTACCA	Used with AP for nested PCR of 5′ RACE
q AMPKα2-F	ACAGCCCTAAGGCACGATG	Used with q AMPKα2-R for RT-PCR of tissue distribution
q AMPKα2-R	TGGGTCGGGTAGTGTTGAG	Used with q AMPKα2-F
EF1α-F	CTTCTCAGGCTGACTGTGC	Used with EF1α-R as internal standard
EF1α-R	CCGCTAGCATTACCCTCC	Used with EF1α-F