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Cloning and expression characterization of peroxisome proliferator-activated receptors (PPARs) with their agonists, dietary lipids, and ambient salinity in rabbitfish *Siganus canaliculatus*

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ABSTRACT

Rabbitfish *Siganus canaliculatus* is the first marine teleost reported to have the ability of biosynthesizing $C_{20–22}$ long-chain polyunsaturated fatty acids (LC-PUFA) from C_{18} precursors, and thus provides a model for studying the regulatory mechanisms of LC-PUFA biosynthesis in teleosts. To investigate the possible roles of peroxisome proliferator-activated receptors (PPARs), critical transcription factors involved in the regulation of lipid metabolism, in the regulation of LC-PUFA biosynthesis in rabbitfish, the PPAR genes were cloned and their expression characterization with PPAR agonists, dietary lipid resource, and ambient salinity were examined. Three cDNA sequences respectively encoding 477, 516 and 519 amino acids of PPARα, PPARβ, and PPARγ isoforms were obtained. PPARα exhibited a wide tissue expression with its highest levels in the heart and brain; PPARβ was predominantly expressed in the gills, while PPARγ was highly expressed in the intestine and gills. In rabbitfish primary hepatocytes, both the PPAR agonists 2-bromopalmitate (2-Bro) and fenofibrate (FF) increased the expression of PPARγ, SREBP1c and Elovl5, whereas FF depressed the expression of Δ6/Δ5 Fad. Moreover, a higher hepatic PPARβ expression was observed in fish fed diets with vegetable oils (VO) than that with fish oil (FO), in the former the expression of PPARα, PPARβ, and PPARγ were increased at the low ambient salinity (10 ppt), where an increasing expression of Δ5/Δ6 Fad, Δ4 Fad and Elovl5 genes was previously reported. These results suggest that PPARs might be involved in the upregulation of LC-PUFA biosynthesis with dietary VO and low ambient salinity in rabbitfish.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic (EPA; 20:5n-3), arachidonic (ARA, 20:4n-6), and docosahexaenoic (DHA; 22:6n-3), play important roles in growth, development and reproduction of vertebrates, including maintenance of cellular membrane structure, energy metabolism, gene regulation and cellular signaling (Jaya-Ram et al., 2011; Vagner and Santigosa, 2011). In fish, LC-PUFA depends on the food supply or endogenous LC-PUFA biosynthesis from dietary C_{18} PUFA precursors such as α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6). In general, freshwater fish and salmonid species can convert ALA and LA into LC-PUFA through a series of desaturation and elongation of fatty acid desaturases (Fad) and elongases of very long-chain fatty acids (Elovl), while such ability is usually absent or low in marine teleosts except the recently reported rabbitfish *Siganus canaliculatus* (Li et al., 2008, 2010; Tocher, 2003, 2015). Therefore, potential mechanisms for improving the endogenous LC-PUFA biosynthesis capacity of fish have attracted considerable attention among researchers (Karagianni and Talianidis, 2015; Vagner and Santigosa, 2011).

The peroxisome proliferator-activated receptors (PPARs), which consist of three isotypes, namely, PPARα, PPARβ (δ), and PPARγ, are members of the superfamily of nuclear hormone receptor and act as key transcription factors involved in regulating hepatic carbohydrates and lipid metabolism in vertebrates, enabling adaptation to changing

Abbreviations: ARA, Arachidonic acid (20:4n-6); AF1, Activation function 1; AF2, Activation function 2; ALA, α-linolenic; cDNA, Complementary deoxyribonucleic acid; DHA, Docosahexaenoic acid (22:6n-3); DBD, DNA-binding domain; DMEM/F12, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; DMSO, Dimethyl Sulphoxide; Elovl, Elongases of very long-chain fatty acids; EPA, Eicosapentaenoic acid (20:5n-3); EtOH, Ethanol; Fad, Fatty acyl desaturases; FBS, Fetal bovine serum; FF, Fenofibrate; FO, Fish oil; HNF4α, Hepatocyte nuclear factor 4a; LA, Linoleic; LBD, Ligand binding domain; LC-PUFA, Long-chain polyunsaturated fatty acids; LXR, Liver X receptor; NAMBS, Nan Ao Marine Biology Station; ORF, Open reading frames; PCR, Polymerase chain reaction; PPARs, Peroxisome proliferator-activated receptors; PPREs, PPAR response elements; PUFA, Polyunsaturated fatty acids; RXR, Retinoid X receptor; SREBP, Sterol regulatory element binding proteins; SRE, Sterol regulatory element; VO, Vegetable oils; 2-Bro, 2-bromopalmitate; 15d-J2, 15-deoxy-D12,14-prostaglandin J2.

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energy requirement and nutritional status (Hummasti and Tontonoz, 2006; Jump et al., 2008; Kamalam et al., 2013). PPARs bind to the PPAR response elements (PPREs) of target genes with the retinoid X receptor (RXR) as heterodimers for transcriptional regulation after activation by natural or artificial ligands (Adeghate et al., 2011; Frazier-Wood et al., 2013). The natural ligands of PPARs include various fatty acids and fatty acid derivatives in vivo with a general preference for PUFA (Hostetler et al., 2006). Therefore, PPARs have been recognized as general fatty acid sensors and are master regulators of lipid metabolism in regulating the metabolic switch between the storage and mobilization of lipids through transcriptional regulation of target genes in mammals (Kersten, 2014; Varga et al., 2011). However, each isotype of PPARs has a distinct biological function. For example, mammalian PPARα is known for its role in fatty acid uptake, fatty acid activation and hepatic fat catabolism, and PPARβ is demonstrated to play a role in control of fatty acid oxidation in many tissues including adipose tissue and skeletal muscles, whereas PPARγ controls lipid storage and adipogenesis in adipose tissue (Hummasti and Tontonoz, 2006; Kersten, 2014).

Similar to other nuclear receptors, mammalian PPARα, PPARβ, and PPARγ are all composed of at least four functional domains: the activation function 1 (AF1) located in the N-terminal region (A/B domain), the DNA-binding domain (DBD, C domain), the ligand binding domain (LBD, E/F domain), and the activation function 2 (AF2) at the C-terminus of the receptor (Yu and Reddy, 2007). There is a highly flexible hinge region (D domain) linking the DBD and LBD. PPAR isotypes have been identified and cloned from several fish such as zebrafish (*Danio rerio*) (Ibabe et al., 2002), Atlantic salmon (*Salmo salar*) (Sundvold et al., 2010), brown trout (*Salmo trutta*) (Batista-Pinto et al., 2005), plaice (*Pleuronectes platessa*), gilthead sea bream (*Sparus aurata*) (Leaver et al., 2005), European sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004), Japanese seabass (*Lateolabrax japonicas*) (Dong et al., 2015), cobia (*Rachycentron canadum*) (Tsai et al., 2008), and red sea bream (*Pagrus major*) (Oku and Umino, 2008). Some studies suggested the functions of PPARs were associated with modulation of Δ6 Fad expression, the first rate-limiting enzyme of LC-PUFA biosynthesis in fish (Li et al., 2015; Morais et al., 2012; Vagner et al., 2009). However, the clear correlation between PPARs and LC-PUFA biosynthesis in fish was insufficiently elucidated.

Rabbitfish *S. canalicultus* is a euryhaline and herbivorous marine teleost widely distributed along the Indo-West Pacific including the coast of southeast China. Our recent studies indicated that rabbitfish is the first marine fish reported to possess all the key genes (Δ5/Δ6 Fad, Δ4 Fad, Elovl5, and Elovl4) with enzyme activities required for LC-PUFA biosynthesis and the ability to convert dietary C_{18} PUFA into LC-PUFA (Li et al., 2008, 2010; Monroig et al., 2012; Xie et al., 2015), which has been shown to be influenced by both dietary lipid resource and ambient salinity (Li et al., 2008; Xie et al., 2015), suggesting a repertoire of regulatory machinery responsible for LC-PUFA biosynthesis. Thus, these findings provide a foundation for using rabbitfish as a suitable model for studying the regulatory mechanism of LC-PUFA biosynthesis in fish. In the present study, three PPAR isotypes were isolated from rabbitfish and their structures, tissue distributions, and responses to PPAR agonists, ambient salinity, and dietary lipid resource were investigated. The response of other potential target and regulatory genes to the PPAR agonists in the primary hepatocytes of rabbitfish was also determined. The results will provide us further insight into the roles of PPARs in regulating LC-PUFA biosynthesis and the related pathways in rabbitfish and teleosts, and contribute to clarify the molecular regulation mechanism of LC-PUFA biosynthesis aiming at the optimization and/or enhancement of the LC-PUFA pathway in teleosts and at last to improve the nutritional quality of farmed fish.

2. Materials and methods

2.1. Feeding trials and sampling

Two isoproteic and isolipidic diets containing 35% crude protein and 8% crude lipids were prepared using either fish oil (FO, rich LC-PUFA) or vegetable oils, a blend of canola oil and perilla oil (VO, rich C_{18} PUFA and free LC-PUFA) as lipid sources. These diets were fed to rabbitfish juveniles at two ambient salinities (10 and 32 ppt) for 8 weeks. The dietary composition, experimental animal preparation, feeding trials, growth performance, biochemical composition, and lipid composition of fish have been previously described in detail (Xie et al., 2015). In brief, rabbitfish juveniles (body mass approximately 13 g) were captured in summer 2012 from the coast near Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China. The fish were kept in an indoor seawater pool (32 ppt) for 4 weeks by feeding a mixture of the FO and VO diets to adapt to the laboratory conditions. Subsequently, half of them were gradually acclimated to low-salinity water (10 ppt) over a period of 4 weeks while the remaining fish were still maintained in seawater (32 ppt), and then all fish were maintained under each ambient salinity (10 or 32 ppt) for a further 2 weeks prior to the initiation of the growth experiment. In the 8-week growth experiment, the fish reared under each ambient salinity (10 or 32 ppt) were fed the FO and VO diets, respectively. Each treatment had three replicate tanks with 20 fish per tank, and the total 12 tanks including 240 fish were involved in this experiment.

To determine the effects of diet and ambient salinity on gene expression in the liver, at the beginning of the growth experiment, liver tissues were collected from three seawater fish as the initial control samples. At the end of the trial, liver tissues were collected from two fish in each tank (six fish per dietary treatment per salinity). All samples were flash-frozen in liquid nitrogen and then stored at −80 °C for the determination of gene expression.

For the tissue expression analysis of rabbitfish PPARs, four fish in seawater during the initial indoor acclimation period were randomly selected, starved for 24 h, and dissected to collect the heart, spleen, brain, gill, muscle, eye, intestine, visceral fat depot, and liver tissues. All tissues were flash-frozen in liquid nitrogen and then stored at −80 °C for RNA extraction.

2.2. Isolation of rabbitfish primary hepatocytes and incubation with PPAR agonists

Rabbitfish primary hepatocytes were isolated as previously described (Zhang et al., 2014, 2016). Briefly, livers were isolated from 12 wild rabbitfish (body mass approximately 35 g) and sterilized by 70% ethanol. Then, chopped livers were pooled, followed by enzyme digestion using collagenase:hyaluronidase (0.1%:0.25%) (Sigma-Aldrich, St. Louis, USA) and filtered through a cell strainer (100 μm). Next, the isolated cells (viability ≥98%, evaluated through Trypan Blue dye exclusion) were seeded in 6-well plates at a density of 2×10^6 cells per well in the Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Life Technologies, USA) containing fetal bovine serum (FBS, 20%), penicillin (100 U/mL), and streptomycin (100 μg/mL), and cultured at 24 °C in an atmosphere with CO_2 (4%) for 24 h. The hepatocytes were incubated in an FBS-free DMEM/F12 medium for 3 h prior to chemical incubation, including 2-bromopalmitate (2-Bro, 3 and 30 μM), a dual PPARα and PPARβ agonist (Oku and Umino, 2008; Carmona-Antoñanzas et al., 2014); 15-deoxy-D12,14-prostaglandin J2 (15d-J2, 0.3 and 3 μM), a specific PPARγ ligand (Forman et al., 1997; Oku and Umino, 2008); and a specific PPARα ligand, fenofibrate (FF, 0.5 and 5 μM) (Oku and Umino, 2008). All the agonists were purchased from Sigma-Aldrich (St. Louis, USA). The corresponding treatment vehicles (DMSO for 15d-J2, and ethanol for 2-Bro and FF) and a control with no chemical supplement were also prepared. Each treatment was conducted within triplicate wells as technical replicates. After 6 h of incubation, the media were removed (Bionaz et al., 2012), and then cells were lysed in wells using lysis buffer (RNAprep pure cell/bacteria kit, Tiangen Biotech, Beijing, China), followed by RNA isolation according to the instruction of the commercial kit manual.