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Cloning and characterization of SREBP-1 and PPAR-α in Japanese seabass *Lateolabrax japonicus*, and their gene expressions in response to different dietary fatty acid profiles

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ABSTRACT

In the present study, putative cDNA of sterol regulatory element-binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptor α (PPAR-α), key regulators of lipid homoeostasis, were cloned and characterized from liver of Japanese seabass (*Lateolabrax japonicus*), and their expression in response to diets enriched with fish oil (FO) or fatty acids such as palmitic acid (PA), stearic acid (SA), oleic acid (OA), α-linolenic acid (ALA), and n − 3 long-chain polyunsaturated fatty acid (n − 3 LC-PUFA), was investigated following feeding. The SREBP-1 of Japanese seabass appeared to be equivalent to SREBP-1a of mammals in terms of sequence feature and tissue expression pattern. The stimulation of the mRNA expression level of SREBP-1 in liver of Japanese seabass by dietary fatty acids significantly ranked as follows: PA, OA > SA, ALA, and n − 3 LC-PUFA > FO. A new PPAR-α subtype in Japanese seabass, PPAR-α2, was cloned in this study, which is not on the same branch with Japanese seabass PPAR-α1 and mammalian PPAR-α in the phylogenetic tree. Liver gene expression of PPAR-α1 of Japanese seabass was inhibited by diets enriched with ALA or FO compared to diets enriched with PA or OA, while the gene expression of PPAR-α2 of Japanese seabass was up-regulated by diets enriched with ALA or n − 3 LC-PUFA compared to diets enriched with OA or FO. This was the first evidence for the great divergence in response to dietary fatty acids between PPAR-α1 and PPAR-α2 of fish, which indicated probable functional distribution between PPAR-α isotypes of fish.

1. Introduction

Sterol regulatory element binding proteins (SREBP) belong to the basic helix–loop–helix-leucine zipper (bHLH-Zip) family of transcription factors. The mammalian SREBP subfamily is encoded by two genes, SREBP-1 and SREBP-2, which are major regulators of mammalian fatty acid/lipid and cholesterol biosynthetic genes, respectively, including genes of long-chain polyunsaturated fatty acids (LC-PUFA) synthesis (Horton, 2002; Horton et al., 2002, 2003). The 5′ end of the mRNA encoding mammalian SREBP-1 exists in two forms, designated 1a and 1c. SREBP-la may be responsible for maintaining basal levels of cholesterol and fatty acid synthesis in vivo, while a number of studies in mammals have demonstrated that SREBP-1c predominantly acts to increase the expression of genes involved in fatty acid synthesis, including fatty acid synthase (FAS), fatty acid desaturase (Fad), and elongase of very long chain fatty acid (Elovl) genes (Matsuzaka et al., 2002; Kumadaki et al., 2008; Qin et al., 2009). In fish, however, so far only one single form of SREBP-1 gene has been characterized (Minghetti et al., 2011), and it is unknown whether it has functions of both SREBP-1a and c genes in mammals. Limited studies on fish have implied that SREBP-1 are important transcriptional regulators of salmon Δ6 Fad (Zheng et al., 2009) and the gene expression of salmon SREBP-1 could be regulated by DHA and EPA (Minghetti et al., 2011).

The peroxisome proliferator-activated receptors (PPAR) are members of the nuclear receptor superfamily of ligand activated transcription factors, which can bind to a specific DNA regulatory element (peroxisome proliferator response elements, PPREs) located in the promoter region or intronic sequence of the target genes (Desvergne and Wahli, 1999). Three PPAR isotypes, α, β (also known as δ), and γ, have been identified and these PPAR subtypes display distinct but overlapping expression and functions (Poulsen et al., 2012). Among the three PPAR isotypes, PPAR-α plays critical roles in fatty acid homeostasis, regulating the peroxisomal and mitochondrial fatty acid oxidation, and expressions of fatty acid desaturases, Δ5 and Δ6 Fad, i.e., having pleiotropic functions in the regulation of lipid metabolic pathways (Escher et al., 2001; Matsuzaka et al., 2002; Mandard et al., 2004; Nakamura et al., 2004; Oosterveer

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et al., 2009). In fish, two orthologs of human PPAR-α have been observed in fugu, zebrafish, Japanese medaka, turbot, and grass carp (Robinson-Rechavi et al., 2001; Maglich et al., 2003). However, in some fish such as olive flounder only one single form of PPAR-α gene has been observed (Cho et al., 2012). The exact number of genes and/or the presence of distinct PPAR-α isotypes, as well as the functional comparison of different PPAR-α in fish, have not been determined.

Based on the roles of SREBP-1 and PPAR-α in lipid/fatty acid metabolism, SREBP-1 and PPAR-α serve as major sensors of fatty acids, in particular n-3 PUFA, and thus have emerged as key mediators of gene regulation by fatty acids (Nakamura and Nara, 2003; Nakatani et al., 2003; Nakamura et al., 2004; Howell et al., 2009; Poulsen et al., 2012). PUFA are reported to be ligands of PPAR-α (Price et al., 2000) and suppressors of SREBP (Morton and Shimomura, 1999) in mammals and humans. In fish, considering that lipid homeostasis, in particular LC-PUFA homeostasis, is so important to fish products, and that the present expansion of aquaculture requires alternative lipid sources due to the decline in commercial fisheries and consequently the decreasing supply of fish oil, the research into the regulation of SREBP-1 and PPAR-α by dietary fatty acids will be critical for elucidating the mechanisms involved in the regulation of lipid homeostasis of fish, in particular the degradation and synthesis of LC-PUFAs, by dietary lipid sources. However, up to now, limited information is available about the expression pattern of fish SREBP-1 and PPAR-α in response to dietary fatty acids. It is also not clear whether fatty acids act simply by regulating SREBP/PPAR-DNA binding or if they can also regulate the biosynthesis of SREBP/PPAR themselves and consequently regulate the nuclear abundance of these nuclear factors (Di Nunzio et al., 2009).

The present study was aimed to clone and characterize the gene of SREBP-1 and PPAR-α in an important aquaculture species Japanese seabass *Lateolabrax japonicus*, as well as to investigate the gene expression patterns of Japanese seabass SREBP-1 and PPAR-α in response to different dietary fatty acid profiles. The liver was used as the tissue to extract and assay the quantitative expression of Japanese seabass SREBP-1 and PPAR-α mRNA considering its importance in lipid homoeostasis and the high expressions of these transcription factors in liver (Shimomura et al., 1997). A greater understanding of the molecular basis of lipid and fatty acid homeostasis in fish will enable efficient and effective use of sustainable dietary oils while maintaining the nutritional quality of farmed fish. Since it is still not clear whether SREBPs and PPARs have similar roles in teleosts and mammals and to what extent the regulation of SREBP and PPAR are shared between the two species (Rakhshandehroo et al., 2009), this study on Japanese seabass SREBP and PPAR-α will provide new information on the evolution of structure and function of these receptors, as well as a clearer understanding of the involvement of SREBPs and PPARs in lipid homeostasis of fish.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from liver using TransZol (TransGen, Beijing, China) and then electrophoresed on a 1.5% agarose gel to test the integrity. Then, 1 μg total RNA was subjected to TransScript ™ One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China) in 20 μl volume for reverse transcription and DNA erasure.

2.2. Cloning and sequencing of SREBP-1 and PPAR-α2 cDNA fragment

Four specific primers (SREBP-1F, SREBP-1R, PPAR-α2F, and PPAR-α2R) (Table 1) were designed based on highly conserved regions from the genes of other fish available in the GenBank database and synthesized by Biosune (Shanghai, China). Liver cDNA were used as the template for amplification. PCR amplifications using the primers and Taq DNA Polymerase (Takara, Dalian, China) were performed with an initial denaturation at 95 °C for 3 min and 35 cycles of "95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min", followed by a final extension at 72 °C for 10 min. All PCR products were run on a 1.5% agarose gel, and then purified by SanPrep PCR Purification Kit (Sangon Biotech, Shanghai, China). PCR products were cloned into pEASY-T1 simple cloning vector (TransGen, Beijing, China) and sequenced in BioSune (Shanghai, China).

2.3. Rapid amplification of cDNA ends (RACE)

Four gene-specific primers of each gene were designed based on the known sequences of the internal fragments of SREBP-1 and PPAR-α2 cDNA singlet to clone the 3′- and 5′-end by rapid amplification of cDNA ends (RACE) using the SMARTer™ RACE cDNA Amplification Kit (Clontech, California, USA) (Table 1). The 3′- and 5′-end cDNA templates were synthesized according to the user's manual. For 3′ RACE, the first and nested PCR rounds were performed using primers SREBP-1-3F1, SREBP-1-3F2, PPAR-α2-3F1, and PPAR-α2-3F2, and for 5′ RACE, SREBP-1-5R1, SREBP-1-5R2, PPAR-α2-5R1, and PPAR-α2-5R2 were used (Table 1). All PCR products were purified, cloned into vector, and sequenced as described in Section 2.2.

2.4. Real-time quantitative-polymerase chain reaction (RT-qPCR) analysis and semi-quantitative PCR (Semi-qPCR)

Real-time fluorescent quantitative PCR (RT-qPCR) was used to assay the relatively quantitative mRNA expression of SREBP-1 and PPAR-α in tissues of Japanese seabass. β-actin (GenBank accession no. HE577671.1) was selected as the reference gene from a number of commonly used reference genes in Japanese seabass such as 18 s rRNA, β-actin, TUBA, ODC, GAPDH, and EF1α based on preliminary tests using geNorm (version 3.5) and NormFinder algorithms (Vandesompele et al., 2002; Andersen et al., 2004). Specific primers for SREBP-1 (SREBP-1-qF and SREBP-1-qR), PPAR-α1 (PPAR-α1-qF and PPAR-α1-qR), PPAR-α2 (PPAR-α2-qF and PPAR-α2-qR), and β-actin (β-actinF and β-actinR) were designed using Primer 5.0 based on the partial cDNA sequences of SREBP-1 and PPAR-α2 obtained previously, and the complete cDNA sequences of PPAR-α1 (GenBank accession no. FJ208989.1) and β-actin from NCBI (Table 1). To determine the amplification efficiency and linear range of the real-time PCR assay, standard curves of the template cDNA were generated. The generated standard curves showed linearity over the entire quantitation range (The coefficients of linear regression (R2) were more than 0.99). The amplification efficiency was 94.17%, 95.79%, 98.48%, and 92.42% for β-actin, SREBP-1, PPAR-α1, and PPAR-α2, respectively.

First strand cDNA was synthesized as described previously in Section 2.2 and then diluted by 4 times using sterilized double-distilled water. The real-time PCR was carried out in a quantitative thermal cycler (Mastercyclerep *realplex*, Eppendorf, Germany) in a final volume of 25 μl containing 2 × TransStart™ Green qPCR SuperMix (TransGen, Beijing, China), primer pairs, and cDNA. The program was 95 °C for 2 min followed by 40 cycles of "95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s". Melting curve (1.85 °C increment/min from 58 °C to 95 °C) was performed after the amplification phase for confirmation. Each sample was run in triplicate. The gene expression levels of putative SREBP-1, PPAR-α1, and PPAR-α2 were studied by RT-qPCR method: $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Semi-qPCR was also used to assay the mRNA expression of SREBP-1, PPAR-α, and β-actin in tissues of Japanese seabass. Semi-qPCR was performed with 1 μl of the first-strand cDNA and 12.5 μl TaKaRa Ex Taq mix (TaKaRa, Dalian, China). Semi-qPCR was carried out with an initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gel. The expression of the housekeeping gene β-actin was used as internal control to check the efficiency of cDNA synthesis