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Wnt/β-catenin signaling participates in the regulation of lipogenesis in the liver of juvenile turbot (*Scophthalmus maximus* L.)

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ABSTRACT

In this study, the mechanism that Wnt/β-catenin signaling inhibits lipogenesis was investigated in the liver of juvenile turbot (*Scophthalmus maximus* L.) by LiCl or XAV939 treatment. Wnt/β-catenin signaling was activated by LiCl treatment or inhibited by XAV939 treatment through regulating the expression of glycogen synthase kinase-3β (GSK-3β) and Wnt10b. In addition, the expression of lipoprotein lipase (LPL), fatty acid synthetase (FAS), peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer binding protein α (C/EBPα) was inhibited by LiCl treatment, but induced by XAV939 treatment. In the plasma of juvenile turbot, the level of nonesterified fatty acid (NEFA), glycerol, triglyceride (TG), total cholesterol (TC), and low density lipoprotein cholesterol (LDL-C) was decreased by LiCl treatment, which was related to the decrease of the activity of LPL and FAS. Thus the inhibitory effect of Wnt/β-catenin signaling on lipogenesis was associated with the expression of key enzymes and transcriptional factors. Wnt/β-catenin signaling may participate in inhibiting lipogenesis by inhibiting the expression of PPARγ and C/EBPα in the liver of juvenile turbot.

1. Introduction

Wnt subfamilies are an early development in the evolution of metazoan, which likely occurred about 650 Ma ago (Kusserow et al., 2005). At least 11 Wnt subfamilies have been found in the sea anemone (*Nematostella vectensis*) (Kusserow et al., 2005). In mammals, Wnt genes are classified into 12 subfamilies (Nusse, 2001; Prud'homme et al., 2002), in which Wnt10b specifically activates Wnt/β-catenin signaling (Barbolina et al., 2011; Wend et al., 2012). Wnt/β-catenin signaling is one of the negative signaling pathways on adipogenesis (Bennett et al., 2002; Prestwich and Macdougald, 2007), and β-catenin is involved in cell adhesion and transcriptional regulation (Ross et al., 2000). The expression level of β-catenin is associated with a destruction complex, mainly including glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC), and axin (Ross et al., 2000). In the absence of Wnt signals, β-catenin is phosphorylated by GSK-3β and degraded by ubiquitin (Aberle et al., 1997; Huelsken and Behrens, 2002). Nevertheless, Wnt ligands cause the inactivation of GSK-3β and cytosolic β-catenin accumulation, which will induce the target gene expression in the nucleus (Huelsken and Behrens, 2002). Moreover, the transcriptional factors CCAAT/enhancer binding proteins (C/EBP) and peroxisome proliferator-activated receptors (PPARs) play a significant role in the transition of pre-adipocytes into adipocytes (MacDougald and Lane, 1995). It has been found that Wnt/β-catenin signaling inhibits adipocyte differentiation by decreasing the expression of C/EBPα and PPARγ in 3T3-L1 preadipocytes (Ross et al., 2000; Huelsken and Behrens, 2002; Moldes et al., 2003).

Fish is a potential model for studying lipid metabolism due to its relatively high lipid accumulation in the liver and muscle (Robinson and Mead, 1973; Sheridan, 1988). Fatty acid synthetase (FAS) and lipoprotein lipase (LPL) play a key role in the regulation of hepatic lipid deposition (Nilsson-Ehle et al., 1980; Kerner and Hoppel, 2000; Richard et al., 2006). In addition, PPARγ participates in lipid homeostasis by orchestrating the gene transcription of the lipogenesis enzymes (Spiegelman and Flier, 2001). In fish species, PPARγ has been found in the Atlantic salmon (*Salmo salar*) (Ruyter et al., 1997), European sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004), and gilthead sea bream (*Sparus aurata* L.) (Leaver et al., 2005). For the excessive accumulation of lipid that causes production losses, flesh quality alteration, and lipoid liver disease in fish, the regulation of lipid metabolism has become a notable point of interest in aquaculture research (Seierstad et al., 2005; 2009; Benedito-Palos et al., 2008; Saera-Vila et al., 2009). Since Wnt/β-catenin signaling affects adipogenesis, it is essential to investigate whether Wnt/β-catenin signaling is involved in the process of lipogenesis in fish species.

Juvenile turbot (*Scophthalmus maximus* L.) is an economically important flatfish species. In Wnt/β-catenin signaling, GSK-3β is an attractive target and lithium could activate Wnt/β-catenin signaling by inhibiting the activity of GSK-3β kinase (Terstappen et al., 2006). However, XAV939 displays nanomolar potency and antagonizes Wnt/β-catenin signaling by stabilizing Axin1 (Huang et al., 2009; Karlberg

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et al., 2010). The result of the previous study has shown that XAV939 blocks Wnt/β-catenin signaling in a mouse model (Yang et al., 2013). In this study, Wnt/β-catenin signaling was studied in the liver of juvenile turbot (*S. maximus* L.) by LiCl or XAV939 treatment. Furthermore, the mechanism that Wnt/β-catenin signaling regulates the expression of lipogenesis enzymes and transcriptional factors was investigated in the liver of juvenile turbot.

2. Materials and methods

2.1. Animals and experimental conditions

Juvenile turbot were obtained from a commercial farm (Qingdao, China) and transported to the indoor facilities in the experimental station of the Ocean University of China (Qingdao, China). At the beginning of the experiment, 135 fish (average body weight 50.32 ± 0.05 g) were randomly distributed into nine 400-L circular fiberglass flat-bottom tanks. In addition, another 10 fish were put in the tenth tank for tissue distribution analysis of Wnt signaling-related genes. Animals were fed once daily with a commercial diet (Qihao Biotech Co. Shandong, China) and acclimated for 15 days. All rearing tanks were maintained with a continuous aeration and a natural photoperiod. The water temperature was 18.0 ± 1 °C, the dissolved oxygen was approximately 7 mg/L, and the ammonia–nitrogen and nitrite were lower than 0.1 mg/L. All animal procedures were approved by the Ocean University of China's Institutional Animal Care Committee in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of China).

2.2. Tissue distribution of Wnt signaling-related genes

After the acclimatization period and fasted for 24 h, five juvenile turbot (approximately 50.0 g) from the tenth tank were randomly sampled and immediately anesthetized. Then the tissue samples (stomach, intestine, spleen, heart, brain, kidney, liver, and muscle) were removed and frozen in liquid nitrogen to analyze the gene expression related to Wnt signaling.

2.3. Injection experiment

Juvenile turbot were injected intraperitoneally with 50 mg/mL LiCl (0.1 mg/g body weight), or 2 mg/mL XAV939 (4.0 μg/g body weight), or 0.9% sodium chloride (2 μL/g body weight) two times a week. LiCl (Sigma, America) or XAV939 (Medchem Express, America) was dissolved in 0.9% sodium chloride, respectively. Fish injected with sodium chloride were treated as the control. Three tanks were randomly used for the treatments of LiCl, XAV939, or the control group.

2.4. Sample collection

After two weeks, twelve fish from each tank were randomly sampled and anesthetized. In each tank, the livers from eight fish were removed, frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis (the livers of five fish were pooled for biochemical analysis, and the livers of three fish were pooled for gene and protein expression analyses). Subsequently, the blood samples from another three fish were pooled with heparinized syringes, and plasmas were obtained after centrifugation for 10 min at 1400 × *g* (4 °C). Finally, the liver of the twelfth fish was fixed for 24 h in a solution of 4% paraformaldehyde dissolved in 0.1 mol/L sodium phosphate buffer (PBS, pH 7.4, 4 °C) for oil red O staining.

2.5. Biochemical analysis

Liver samples from each tank were homogenized in ice-cold 0.7% NaCl, and the supernatants were collected for biochemical analysis after centrifugation at 1400 × *g* for 10 min. For the nonesterified fatty acid (NEFA) that could bind with copper ion, NEFA content was assayed by determining the content of copper ion according to the analytical procedures in the nonesterified free fatty acid assay kit. Glycerol was measured based on the reaction of Trinder according to the analytical procedures in the glycerol assay kit. TG could be decomposed into glycerol and NEFA by LPL, and LPL activity was assayed based on the NEFA level according to the analytical procedures in the lipoprotein lipase kit. In addition, FAS catalyzes CoA and NADPH to generate long chain fatty acids, and FAS activity was assayed by determining the content of NADPH according to the procedures in the fatty acid synthase kit. All kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Finally, the protein concentration of liver supernatants was detected by the method of Coomassie Brilliant Blue G250 staining.

The level of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C) in plasma was measured according to the procedures of the total cholesterol assay kit, triglyceride assay kit, Low-density lipoprotein cholesterol assay kit, and high-density lipoprotein cholesterol assay kit (Nanjing Jiancheng Bioengineering Institute, China), respectively. The content of NEFA and glycerol in plasma was measured as the method described in the liver analysis.

2.6. Oil red O staining

The fixed liver tissues were washed three times with PBS (pH 7.4) and sectioned at a thickness of 20 μm in a cryostat (Leica CM1850, Germany), followed by mounting on the chromalum-gelatin-coated glass slides. Afterwards, the tissues were stained according to the procedures of oil red O staining kit (Nanjing Jiancheng Bioengineering Institute, China). Then the slides were re-dyed with hematoxylin and observed under a microscope.

2.7. RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted from the liver using TRIzol reagent (Invitrogen, USA) and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. Then 3.0 μg total RNA was subjected to reverse transcription with EasyScript First-Strand cDNA Synthesis SuperMix Kit (TransGen, Beijing, China), and SYBR® Premix Ex Taq™ II was used to quantify the expression level of genes (TransGen, Beijing, China). The partial coding sequences for Wnt10b, GSK-3β, and β-catenin were cloned by PCR using degenerate primers deduced from the published sequences of other fish species (Table 1). The primer sequences for LPL, FAS, PPARγ, C/EBPα, Wnt10b, GSK-3β, β-catenin, and reference gene (β-actin) were designed following the published sequences and listed in Table 2. Real-time PCR was carried out in a quantitative thermal cycle (Mastercycler® ep realplex; Eppendorf, Germany). The real-time PCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. The reaction was carried out with three duplicates for each sample. The $2^{-\Delta\Delta CT}$ method was employed to analyze the differences of relative gene expression in each sample using β-actin as the internal reference gene (Livak and Schmittgen, 2001).

Table 1
Degenerate primers for gene clone of Wnt signaling in juvenile turbot.

Target gene	Forward (5′–3′)	Reverse (5′–3′)
Wnt10b	GGSAGCTGCCAGTTYMAGA	CTCHTCRCASAKCACATA
GSK-3β	TYCGYCTGCGHTACTTCT	AWGGRTGGGCCTTRATCT
β-Catenin	AACCARGCCARCTGATTGCTGT	TGGAGGGWGTKCGTATGGAGG