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The inhibition of GSK-3β promotes the production of reactive oxygen species via β-catenin/C/EBPα signaling in the spleen of zebrafish (*Danio rerio*)

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ABSTRACT

In this study, the mechanism that the inhibition of glycogen synthase kinase-3β (GSK-3β) promotes the production of reactive oxygen species (ROS) via β-catenin/CCAAT/enhancer binding protein α (C/EBPα) signaling was investigated in the spleen of zebrafish (*Danio rerio*). The results demonstrated that the inhibition of GSK-3β induced the mRNA expression of β-catenin and C/EBPα by lithium (Li) treatments or GSK-3β RNA interference. The levels of hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and hydroxy radical (·OH) as well as the activity of superoxide dismutase (SOD) were increased, while the activities of catalase (CAT) and glutathione peroxidase (GSH-PX) were decreased in the spleen and ZF4 cells of zebrafish by Li^+ treatments. In addition, GSK-3β RNA interference increased ROS levels and decreased the activities of CAT and GSH-PX in the spleen. The fluorescence intensity of ROS was increased but the mitochondrial membrane potential (MMP) was decreased by Li^+ treatments in ZF4 cells labeled with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and Rhodamine-123, respectively. The results of present study indicated that the inhibition of GSK-3β promoted the ROS production via β-catenin/C/EBPα signaling in the spleen of zebrafish, and the balance between ROS and antioxidants could be destroyed by the GSK-3β/β-catenin/C/EBPα signaling. The results may be a valuable contribution to understanding the modulatory mechanism of GSK-3β/β-catenin/C/EBPα signaling on the antioxidant system in fish species.

1. Introduction

Fish are constantly exposed to various pathogens and stress factors in water, which easily stimulate the inflammatory responses and tissue injuries [1,2]. Fish immunity is tightly associated with the normal structural and function of organs as well as their antioxidant status [3,4]. It is known that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and hydroxy radical (·OH), are mainly generated by electrons escaping from the mitochondrial electron transport chain and by NADPH-oxidase. However, the inordinate accumulation of ROS is harmful and may cause serious tissue damage to the aquatic animals. The protein carbonylation, lipid peroxidation, and/or DNA modifications can be induced by the excessive ROS [5–7]. The antioxidant defense mechanisms, including antioxidant molecules and antioxidant enzymes, are involved in reducing the damage from oxidative stress. The antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), play a key role in defensing against ROS-mediated cellular injury in fish [8,9].

As a serine/threonine protein kinase, glycogen synthase kinase-3β (GSK-3β) participates in regulating numerous biological processes, including cell differentiation, cell cycle, and apoptosis [10,11]. In the intracellular signaling systems, GSK-3β is also a fascinating enzyme with an astoundingly diverse number of actions. Thus the control of GSK-3β activity is a key component in regulating complex signals, and dysregulation of GSK-3β has been observed in multiple diseases such as cancer, diabetes, hepatic, and neurological diseases [10]. Moreover, GSK-3β regulates diverse transcription factors and consequently the mRNA expression of various genes, which further contribute to regulate apoptosis and cell survival [12,13].

In addition, GSK-3β plays a significant role in regulating the accumulation of β-catenin. β-catenin can be phosphorylated by GSK-3β and

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leads to ubiquitin-mediated degradation [14,15]. However, GSK-3β inactivation causes the cytosolic β-catenin accumulation, which consequently translocates into the nucleus and activates the transcription of ROS-responsive genes related to cell proliferation and differentiation [16,17]. Furthermore, β-catenin is involved in regulating the expression of CCAAT/enhancer binding protein α (C/EBPα) and cellular processes [18,19]. Until recently, there is little information on the regulation and molecular mechanism of GSK-3β inhibition on oxidative stress in fish species.

For the function of GSK-3β on the production of ROS in fish remains unknown, it is essential to investigate whether GSK-3β inhibition is involved in regulating the levels of ROS. As an attractive target for inhibiting β-catenin signaling, the activity of GSK-3β kinase can be inhibited by lithium and thereby causes the activation of β-catenin signaling [20]. By targeting GSK-3β, lithium induces apoptosis, cell growth arrest, and terminal differentiation [21]. For GSK-3β plays a significant role in regulating physiological processes, in this study, lithium was used to inhibit GSK-3β activity and GSK-3β RNA was interfered to investigate the mechanism that the inhibition of GSK-3β participates in regulating ROS production via β-catenin/C/EBPα signaling in the spleen of zebrafish (*Danio rerio*).

2. Materials and methods

2.1. Animals and experimental conditions

Danio rerio were obtained from Zibo (Shandong, China) and transported to Shandong University of China (Zibo, China). Firstly, 108 fish (0.36 ± 0.05 g) were randomly distributed into nine 3.0 L glass tanks. Animals were acclimated for 15 days and fed twice daily with a commercial diet (Sanyou Beautification Feed Tech Co., Ltd, China). All tanks were maintained with a natural photoperiod and the continuous aeration. The water temperature was 29.0 ± 1 °C, dissolved oxygen was 6–8 mg/L, and the ammonia-nitrogen and nitrite was 0.07–0.1 mg/L. Triplicate tanks were used for fish treated by per concentration of lithium ion (Li^+). The different concentration of LiCl was added into tanks and the final concentration of Li^+ was 0, 25, and 50 mg/L, respectively. Animals were fed twice daily with the commercial diet. After two weeks, three independent spleen samples were collected after fish were anesthetized with MS222 (0.1 g/L), of which each sample consisted of spleens from six fish. The samples were immediately frozen in liquid nitrogen and then stored at −80 °C for molecular biology analysis. The other three spleen samples were collected for the subsequent biochemical analysis. All animal procedures were approved by Shandong 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. GSK-3β RNA interference

Animals and experimental conditions were the same as the lithium treatments, and 72 fish (0.36 ± 0.05 g) were randomly distributed into six 3.0 L glass tanks. Triplicate tanks were used for the control and GSK-3β RNA interference group, respectively. The open reading frame (ORF) region of GSK-3β gene (NM_131381.1) was cloned according to the primers: F1, 5′-CTG GTG AGC AGT AGG GTG-3'; R1, 5′-CGG ATT CGT TCA AGA CAA-3'. The double-T7-stranded RNA was further synthesized according to the primers: F2, 5′-GAT CAC TAA TAC GAC TCA CTA TAG GGC GGC ATT CGG CAG CAT GAA AG-3'; R2, 5′-GAT CAC TAA TAC GAC TCA CTA TAG GGG CAC GGC TGT GTC TGG GTC CA-3'. Reactions were performed in water at 37 °C in a solution containing 100 mM DTT, 5 × transcription buffer, RNase Inhibitor (Takara), 2.5 mM rNTP, T7 RNA polymerase (Promega) and the template. For the experimental group, the double-stranded RNA (dsRNA) was diluted to 8 ng/μL and one fish received intraperitoneal injection of 25 μL dsRNA RNA (200 ng/per fish). The fish of control group received 25 μL DEPC water. Three days later, the spleen was sampled as the lithium treatments for the subsequent biochemical analysis and molecular biology analysis.

2.3. ZF4 cell culture and lithium treatment

The ZF4 cells of Zebrafish were obtained from China Zebrafish Resource Center (CZRC) (Wuhan, China), and maintained at 27 °C with 5% CO_2 in DMEM:F12 (Gibco) supplemented with 10% fetal bovine serum. ZF4 cells were cultured in basal culture medium containing 0, 1, 5, and 10 mmol/L LiCl for 48 h, respectively. Triplicate culture dishes were used for each concentration, then cells were collected for gene expression analysis. The cell treatments for biochemical analysis was the same as the gene expression analysis.

2.4. Assay the levels of ROS and the activities of antioxidant enzymes

The spleen samples were homogenized with a glass homogenizer, and the ZF4 cell samples were washed with PBS and treated with 0.2% Triton X-100. Then the samples were centrifuged at 4000 × g for 15 min at 4 °C and supernatants were collected for biochemical analysis. The levels of H_2O_2, ·OH, and $O_2^{\cdot-}$, the activities of SOD, CAT, GSH-PX, and the protein concentrations of supernatants were measured according to the analytical procedures specified in the commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

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

Total RNA was extracted from spleen and ZF4 cell samples using Trizol reagent (Invitrogen, USA). Then total RNA was transcribed to cDNA with a PrimeScript™ RT Reagent Kit (Takara, Japan), and SYBR® Premix Ex Taq™ II was used to quantify the expression level of genes (Takara, Japan). The primer sequences for GSK-3β, β-catenin, C/EBPα, and β-actin were designed and listed in Table 1. Real-time PCR was carried out in a quantitative thermal cycle (ROCHE, Lightcycler96, Switzerland). The real-time quantitative PCR program was as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. 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 using β-actin as the internal reference gene according to the method of Livak and Schmittgen [22].

2.6. Immunofluorescent microscopy

ZF4 cells were cultured in basal culture medium containing various concentrations of LiCl (0, 1, 5, and 10 mmol/L) for 48 h. Then cells were

Table 1
Real-time quantitative PCR primers for genes of zebrafish.

Target gene	Forward (5′-3′)	Reverse (5′-3′)	GenBank
GSK-3β	TCTGCTCACCGTTTCCTTTC	CTCCGACCCACTTAACTCCA	NM_131381.1
β-catenin	GGAGCTCACCAGCTCTCTGT	TAGCTTGGGTCGTCCTGTCT	NM_001001889.1
C/EBPα	CACAACAGCTCCAAGCAAGA	AATCCATGTAGCCGTTCAGG	BC063934.1
β-actin	CCGTGACATCAAGGAGAAGC	TACCGCAAGATTCCATACCC	AF057040.1