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Reactive oxygen species participate in liver function recovery during compensatory growth in zebrafish (*Danio rerio*)

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ABSTRACT

Compensatory growth (CG) is defined as a phase of accelerated growth when the disadvantageous environment is improved, accompanied by metabolic adjustment. Here, we report that hepatic oxidative phosphorylation (OXPHOS) activity was enhanced during compensatory growth in zebrafish. Mitochondrial metabolism enabled the generation of reactive oxygen species (ROS), which activated the nrf2 (nuclear factor-erythroid 2-related factor 2) signaling pathway, as well as the mTOR signaling pathway. Tempol (a superoxide dismutase mimetic) treatment blocked ROS signaling in the liver as well as CG in zebrafish. These results demonstrated that mitochondrial ROS signaling are essential for the occurrence of compensatory growth in zebrafish.

1. Introduction

Compensatory growth has been reported in many species, especially in fish [1]. Most fish have the ability of continuous growth, making these organisms suitable models for growth studies [2]. In general, the CG of fish is characterized by great appetite, high feed conversion efficiency and high specific growth rate [3,4], which could be applied to aquaculture and reduce farming costs. CG improves the digestive capacity of the digestive system [5], regulates the metabolic hormone levels in the circulatory system [6], and improves growth.

Based on our preliminary results, zebrafish could trigger compensatory growth after 3 weeks of fasting, accompanied by hepatic enlargement (unpublished data). The liver is critically involved in metabolism and a variety of physiological functions, the augmentation of the liver during CG might imply active metabolic activities. The liver is sensitive to nutrient intake and responds earlier than other tissues when feeding is regained after fasting [7]. In rainbow trout, liver mTOR signaling was activated after feeding was regained for up to 5 h after fasting for 60 h [8]. A previous microarray study also showed alterations in genes associated with cellular growth and proliferation in the hepatic tissue of steers following restricted feeding [9]. The author also emphasized that mitochondrial function improvement contributed to the accomplishment of compensatory growth. However, the precise role of mitochondrial metabolism in regulating compensatory growth is not fully understood. In the present study, hepatic oxidative phosphorylation (OXPHOS) activity was detected to verify the active metabolic activity in the early refeeding of zebrafish. The participation of reactive oxygen species and the subsequent biological process were investigated by a combination of biochemical and pharmacological techniques.

2. Materials and methods

2.1. Fish culture and experiment station

Female zebrafish, approximately 3 months old, were fed to satiation with a commercial diet twice daily for two weeks as acclimatization, and then starved for 3 weeks. When the fasting ended, food was re-supplied. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl radical) (Sigma, US) was administered during refeeding at a dose of 1.5 μmol/L to the ponds that

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raised zebrafish. Tempol-supplemented water was changed every day for 2 weeks. Liver and muscle samples were obtained when refed for 3 and 7 days. Body weight was detected weekly for 2 weeks during refeeding. The experiment was performed in 24-L ponds with a photoperiod maintained at a constant 12 h/day (8 a.m.-8 p.m.). All fish rearing was performed at the fish culture experiment station in Sun Yat-sen University, Guangdong Province, China. All animal experiments were performed in accordance with the guidelines and approval of the Sun Yat-Sen University Animal Care and Use Committee.

2.2. Mitochondria isolation

The liver mitochondria were prepared with the Mitochondria Isolation Kit (Beyotime, China), according to the manufacturer's protocol. The mitochondria isolated using this method present intact inner membranes and respiratory complexes [10]. Muscle mitochondria were extracted as previously described [11]. The mitochondria were prepared fresh for each experiment and used within 4 h of isolation. For mitochondria function verification, 100 μg of mitochondria were incubated at 25 °C in the reaction buffer (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM potassium phosphate, and 1 mg/ml defatted BSA) with respective substrate mix in a total volume of 100 μL.

2.3. Oxygen consumption

Aerobic respiration of isolated mitochondria was measured by MitoXpress probe (Luxcel, Ireland), according to the manufacturer's protocol [12]. Briefly, 100 μg of mitochondria were incubated in the reaction system with 2.5 mM of glutamate, malate and 100 μM ADP. Next, 100 μL of pre-warmed mineral oil was added to each well to block ambient oxygen from the mitochondria. With an excitation wavelength of 380 nm and emission wavelength of 650 nm, a reading was acquired every 3 min for 1 h using a time-resolved fluorescence microplate reader (Perklin Elmer Vivtor X5, US).

2.4. ATP production

Mitochondrial ATP synthesis was determined as previously described [13]. Briefly, 100 μg of mitochondria were incubated in the reaction system with 1 mM pyruvate, 1 mM malate and 100 μM ADP. Then, 0.4 μg oligomycin was added as the background light emission. A total of 100 μL of firefly luciferase and luciferin, obtained from the ATP Assay Kit (Beyotime, China), were added to the reaction system. The emitted light was measured every 30 s after incubation at 25 °C. The cellular ATP level was measured by a commercial firefly luciferase assay kit (Beyotime, China) according to the manufacturer's protocol.

2.5. Measurement of ROS production

ROS levels were measured with the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Sigma, US), as previously described [14]. A total of 100 μg of fresh mitochondria were incubated in the reaction buffer with 2.5 mM malate and 2.5 mM glutamate at 25 °C for 30 min. Dichlorofluorescein (DCF) fluorescence intensity was detected at 488 nm excitation and 525 nm emission by PerkinElmer Vivtor X5, and the mean fluorescence intensity represented the amount of ROS.

2.6. Lipid oxidation detection

Zebrafish liver samples were homogenized and sonicated in RIPA buffer on ice. Then, the tissue lysates were centrifuged at 12,000 g for 15 min at 4 °C to collect the supernatant. The liver tissue proteins were diluted to appropriate concentrations and subjected to MDA assay as described in the Lipid Peroxidation MDA assay kit (Beyotime, China). The MDA levels were detected by using PerkinElmer Vivtor X5 at an absorbance of 532 nm.

2.7. Protein oxidation detection

The OxyICC™ Oxidized Protein Detection Kit was purchased from Merck Millipore, Germany. The carbonyl groups in the protein side chains were derivatized to DNP-hydrazone by reaction with DNPH according to the manufacturer's instructions. After the derivatization of the protein sample, 15% SDS-PAGE was performed. The proteins were transferred to PVDF membranes. After incubation with anti-DNP antibody, the blot was developed using a chemiluminescence detection system (Tanon, China).

2.8. Proteasome activity assay

A commercially available indirect enzyme-based luminescent assay was modified (Promega, US) to detect proteasome activity [15]. Proteasome activity was assayed using 5 μg of protein extract (1 μg for the trypsin-like activity) from zebrafish liver in the presence of the specific luminogenic substrates. To calculate the proteasomal activity, dual measurements with or without the addition of 30 μM of the irreversible and highly specific proteasomal inhibitor adamantane-acetyl-(6-aminohexanoyl)3-(leucinyl)3-vinyl-(methyl)-sulfone (AdaAhx3L3VS) (Merck Millipore, Germany) were performed.

2.9. RNA extraction and real-time PCR

Gene expression was analyzed by real-time PCR (RT-PCR). Total RNA prepared from zebrafish livers was extracted with Trizol (Omega, US). Complementary DNA generated by M-MLV Reverse Transcriptase (Thermo Fisher Scientific, US) was analyzed by quantitative PCR using an SYBR Premix Ex Taq (TOYOBO, Japan). All samples were run in duplicate and normalized to eEF1a1a expression. The sequences of the oligonucleotide primers used in the present study are described in Supplemental Table 1.

2.10. Western blot

Western blot of zebrafish liver samples was performed as previously described [16]. Primary antibodies against GAPDH (glyceraldehyde-3-phosphate dehydrogenase), total Akt, phospho-Akt (Ser473), total p70 S6 Kinase and phospho-p70 S6 Kinase (Thr389) were purchased from Cell Signaling Technology (US). Goat anti-rabbit secondary antibodies were purchased from Boster (China). Immunoblots were visualized using an enhanced chemiluminescence (ECL) detection kit (Tanon, China).

2.11. Hematoxylin and eosin (H&E) staining

Intestine samples were obtained after refeeding for 3 days, while muscle samples were obtained after refeeding for 10 days, as muscle tissues required more time for noteworthy recovery. H&E staining was performed as previously described [17]. The sections were then observed and photographed with an optical microscope (Olympus, Japan). The fiber cross-sectional area (FCSA) of the fast muscle around the spinal cord was directly determined using the OLYMPUS microscope image processing software cellSens, and at least 150 cells were counted in 3 samples in total.

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