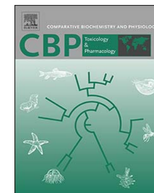
Comparative Biochemistry and Physiology, Part C 197 (2017) 8–18

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc



PPARα, PPARγ and SREBP-1 pathways mediated waterborne iron (Fe)-induced reduction in hepatic lipid deposition of javelin goby *Synechogobius hasta*

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ARTICLE INFO

Keywords:
Synechogobius hasta
Iron
Lipid deposition
Lipid metabolism
Signaling pathway

ABSTRACT

The 42-day experiment was conducted to investigate the effects and mechanism of waterborne Fe exposure influencing hepatic lipid deposition in *Synechogobius hasta*. For that purpose, *S. hasta* were exposed to four Fe concentrations (0 (control), 0.36, 0.72 and 1.07 μM Fe) for 42 days. On days 21 and 42, morphological parameters, hepatic lipid deposition and Fe contents, and activities and mRNA levels of enzymes and genes related to lipid metabolism, including lipogenic enzymes (6PGD, G6PD, ME, ICDH, FAS and ACC) and lipolytic enzymes (CPTI, HSL), were analyzed. With the increase of Fe concentration, hepatic Fe content tended to increase but HSI and lipid content tended to decrease. On day 21, Fe exposure down-regulated the lipogenic activities of 6PGD, G6PD, ICDH and FAS as well as the mRNA levels of G6PD, ACCa, FAS, SREBP-1 and PPARγ, but up-regulated CPT I, HSLa and PPARα mRNA levels. On day 42, Fe exposure down-regulated the lipogenic activities of 6PGD, G6PD, ICDH and FAS as well as the mRNA levels of 6PGD, ACCa, FAS and SREBP-1, but up-regulated CPT I, HSLa, PPARα and PPARγ mRNA levels. Using primary *S. hasta* hepatocytes, specific pathway inhibitors (GW6471 for PPARα and fatostatin for SREBP-1) and activator (troglitazone for PPARγ) were used to explore the signaling pathways of Fe reducing lipid deposition. The GW6471 attenuated the Fe-induced down-regulation of mRNA levels of 6PGD, G6PD, ME, FAS and ACCa, and attenuated the Fe-induced up-regulation of mRNA levels of CPT I, HSLa and PPARα. Compared with single Fe-incubated group, the mRNA levels of G6PD, ME, FAS, ACCa, ACCb and PPARγ were up-regulated while the CPT I mRNA levels were down-regulated after troglitazone pre-treatment; fatostatin pre-treatment down-regulated the mRNA levels of 6PGD, ME, FAS, ACCa, ACCb and SREBP-1, and increased the CPT I and HSLa mRNA levels. Based on these results above, our study indicated that Fe exposure reduced hepatic lipid deposition by down-regulating lipogenesis and up-regulating lipolysis, and PPARα, PPARγ and SREBP-1 pathways mediated the Fe-induced reduction of hepatic lipid deposition in *S. hasta*.

1. Introduction

Iron (Fe) is an essential micronutrient for vertebrates because it plays important roles in multiple metabolic processes, including oxygen transport, detoxification, electron transport, DNA synthesis and protein synthesis (Kwong and Niyogi, 2009). However, excessive Fe in the aquatic environments can be toxic, which has a devastating effects on fish species, affecting growth performance, Fe accumulation and absorption, and physiological response (Peuranen et al., 1994; Dalzell and Macfarlane, 1999; Lappivaara and Marttinen, 2005; Debnath et al., 2012; Glover et al., 2016).

The liver is the main site for Fe storage and also an important site for lipid metabolism. In mammals, studies indicated that variations in hepatic Fe stores modified lipid metabolism (Silva et al., 2008; Ahmed

Abbreviations: ACC, acetyl-CoA carboxylase; ANOVA, one-way analysis of variance; CF, condition factor; CPT, carnitine palmitoyltransferase; DMSO, dimethyl sulphoxide; F, fatostatin; FAS, fatty acid synthase; Fe, iron; G6PD, glucose 6-phosphate dehydrogenase; GW, GW6471; H & E, hematoxylin-eosin; HSL, hormone-sensitive lipase; HSI, hepatosomatic index; ICDH, isocitrate dehydrogenase; ICP-AES, inductively coupled plasma atomic emission spectrometry; ME, malic enzyme; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide; 6PGD, 6-phospho gluconate dehydrogenase; PPAR, peroxisome proliferators-activated receptor; qPCR, real-time fluorescence quantitative PCR; SEM, standard error of means; SREBP, sterol-regulator element-binding protein; T, troglitazone; TG, triglyceride; VSI, viscerosomatic index

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http://dx.doi.org/10.1016/j.cbpc.2017.04.003
Received 21 February 2017; Received in revised form 29 March 2017; Accepted 9 April 2017
Available online 11 April 2017
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et al., 2012; Wlazlo and van Greevenbroek, 2012). However, in fish, the studies on waterborne Fe exposure influencing lipid metabolism were poorly described. Fish use lipids as main energy reserves and lipids serve a vast array of functions in the life histories of fish (Sheridan, 1988). Recently, in our laboratory, Chen et al. (2016) pointed out that 1.128 μM of waterborne Fe exposure influenced Cu-induced changes of hepatic lipid deposition in *Synechogobius hasta*, a carnivorous and euryhaline fish species. However, in the study by Chen et al. (2016), only one Fe concentration (relatively high Fe concentration) was selected. At present, to our best knowledge, no other attempts have been made to demonstrate the relationship between different Fe exposure concentrations in water and lipid metabolism in fish.

Lipid accumulation results from the balance between synthesis of fatty acids (lipogenesis) and fat catabolism via β-oxidation (lipolysis), and many key enzymes and transcriptional factors are involved in these metabolic processes. These enzymes include lipogenic enzymes, such as glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS, and lipolytic enzymes, such as hormone-sensitive lipase (HSL) and carnitine palmitoyltransferase I (CPT I) (Elliott and Elliott, 2009). On the other hand, several transcription factors, such as peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) and sterol-regulator element-binding protein (SREBP-1), play an intermediary role in lipid homeostasis, by orchestrating the gene transcription of the enzymes involved in these pathways (Spiegelman and Flier, 2001). SREBP-1 and PPARγ are key transcriptional factors involved in lipogenesis whereas PPARα plays key roles in the catabolism of fatty acids (Spiegelman and Flier, 2001). Studies suggested that effects of metal elements (such as Cu and Zn) on enzymatic activities and expression of genes mentioned above were concentration-dependent in fish (Zheng et al., 2013, 2014; Chen et al., 2013a, 2013b, 2015; Huang et al., 2014, 2016; Wu et al., 2016a). In addition, investigation into toxicity of metal elements has focused on high-doses exposure, which is a very different situation from the low-doses exposure encountered in the environment by fish. Usually, under natural environment, fish often face the challenge from low waterborne concentration of metal elements, which poses the potential long-term influences on their physiological parameters. Theoretically, the effects of lower doses of Fe cannot be extrapolated from high doses and accordingly studies into the possible impacts of low-dose Fe exposure are needed. Thus, it is very meaningful and necessary to explore the effects of lower Fe concentration on lipid metabolism. Using the in vivo and in vitro experiments, our study was conducted to investigate the potential mechanism of low-dose Fe exposure influencing hepatic lipid deposition in *S. hasta*.

2. Materials and methods

We assured that the experiment performed on animals followed the ethical guidelines of Huazhong Agricultural University for the care and use of laboratory animals.

2.1. Experiment 1: in vivo study

2.1.1. Experimental procedures

S. hasta were obtained from a local marine water pond (Panjin, China). After acclimation for 2 weeks, they were transferred to indoor cylindrical fiberglass tanks (300-l in water volume). Subsequently, uniform-sized fish (mean weight: 23.0 ± 0.3 g, mixed sex) were exposed to four Fe concentrations (0 (control), 0.36, 0.72 and 1.07 μM), with triplicates for each concentration and 24 fish per tank. Fe concentrations in the test tanks were monitored twice every week by inductively coupled plasma atomic emission spectrometry (ICP-AES). During the trial, the measured Fe concentrations in the tank for four treatments were 2.14 ± 0.02 μM Fe (control), 2.51 ± 0.03 μM Fe, 2.86 ± 0.05 μM Fe, and 3.22 ± 0.05 μM Fe, respectively.

The experiment was conducted in semi-static aquarium system. Water sources are local seawater. During the experiment, all fish were fed with trash fish daily (6% of body weight). The measured Fe content of trash fish was 8.67 ± 0.30 mg/kg. In order to avoid polluting the water quality and influencing Fe concentration of the tanks, the remaining food was removed from the tanks after 15-min feeding. Water was completely changed daily to maintain good water quality. During the experiment, water quality parameters were followed: water temperature, 25.3–27.9 °C; pH, 8.30–8.50; salinity, 17.00–19.20‰; dissolved oxygen ≥ 6.00 mg/l; NH_4-*N* ≤ 0.080 mg/l. The experiment continued for 42 days and sampling occurred on day 21 and day 42, respectively.

2.1.2. Sampling

Before sampling, fish were starved for 24 h. On days 21 and 42, after euthanized with MS-222 (at 0.38 mM), three fish per tank were randomly selected and dissected on ice. The liver sample was obtained and used for the calculation of morphometric parameters, including condition factor (CF) [100 × (live weight, g)/(body length, cm)^3], hepatosomatic index (HSI) [100 × (liver weight)/(body weight)], viscerosomatic index (VSI) [100 × (viscera weight)/(body weight)]. Another four fish from each tank were used for histological and histochemical observation, enzyme activity and mRNA expression assays, as described in our previous study (Chen et al., 2016).

2.1.3. Enzymatic activity assays

For analysis of hepatic lipogenic enzymatic activities, 6PGD and G6PD activities were determined by the method of Barroso et al. (1999), ME activity following Wise and Ball (1964), ICDH activity according to Bernt and Bergmeyer (1974), and FAS activity according to the method of Chang et al. (1967) as modified by Chakrabarty and Leveille (1969). One unit of enzyme activity was defined as 1 μM of substrate converted to product per minute at 28 °C and was expressed as mU/(min × mg soluble protein). The protein content was measured following the method of Bradford (1976) with BSA as the standard.

2.1.4. mRNA expression analysis (qPCR)

Analyses on gene transcript levels were conducted by real-time quantitative fluorescence PCR (qPCR) method. Total RNA extraction, DNase treatment and cDNA synthesis were conducted as described in Chen et al. (2016). qPCR assays were carried out in a quantitative thermal cycler (MyiQ™ 2 Two-Color Real-Time PCR Detection System, BIO-RAD, USA) with a 20 μl reaction volume containing 2 × SYBR® Premix Ex Taq™ (TaKaRa, Japan) 10 μl, 10 mM each of forward and reverse primers 0.4 μl, 1 μl diluted cDNA template (10-fold), and 8.2 μl double distilled H_2O. Primers were given in Table 1. The qPCR parameters consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 57 °C for 30 s and 72 °C for 30 s. All reactions were performed in duplicates, and each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. A non-template control and dissociation curve were performed to ensure that only one PCR product was amplified and that stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA. The relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) when normalizing to the geometric mean (M) of the best combination of two genes as suggested by geNorm (Vandesompele et al., 2002). Prior to the analysis, we performed an experiment to check the stability of housekeeping genes (β-actin, GAPDH, RPL7, 18S-rRNA, HPRT, TBP and TUBA), from which GAPDH and TBP (M = 0.018) showed the most stable level of expression under the experimental conditions.

2.1.5. Measurement of lipid and Fe contents, and histological and histochemical observation

Lipid content of the liver samples were determined by the method of

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