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Research Paper

Preheating mitigates cadmium toxicity in zebrafish livers: Evidence from promoter demethylation, gene transcription to biochemical levels

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ABSTRACT

The working hypothesis for this study was that moderate heat stress would alleviate the deleterious effects of subsequent cadmium (Cd) exposure on fish. Thus, zebrafish (*Danio rerio*) were subjected to water maintained at 26 °C and 34 °C for 4 days, and then exposed to 0 or 200 μg/L Cd for 1 week at 26 °C. Multiple indicators were measured from livers of zebrafish at different levels, including DNA, RNA, protein and enzymatic activity associated with oxidative stress, inflammation and metal transport. The ameliorative effect of preheatinging on Cd toxicity was demonstrated. In the Cd-exposed groups, preheating decreased mortality and lipid peroxidation, increased activity levels of catalase (CAT) and copper/zinc-superoxide dismutase (Cu/Zn-SOD), and up-regulated mRNA levels of heat shock protein 70 (HSP70) and heat shock factor 2 (HSF2). Preheating also mitigated Cd-induced increases in protein and mRNA levels of metallothioneins (MTs), and mRNA levels of several inflammation-related genes. Furthermore, preheating alone dramatically up-regulated mRNA levels of genes related to antioxidant and immune defenses, zinc and copper transporters, protein folding, and reduced methylation levels in the HSF binding motif of the HSP70 promoter. Overall, preheating-induced accumulation of transcripts via demethylation might support the rapid defense responses at post-transcriptional levels caused by subsequent Cd exposure, indicating an adaptive mechanism for organisms exposed to one mild stressor followed by another.

1. Instruction

The pre-exposure to a mild stressor may lead to an enhanced tolerance to higher levels of that (or a different) stressor (Costantini, 2014). The phenomenon (termed conditioning or priming hormesis) is commonly observed in organisms across a wide range of taxa from plants to vertebrates, particularly in aquatic organisms including algae and fish (Adeyemi and Klerks, 2013; Dolci et al., 2014; Shrivastava et al., 2016; Tukaj and Tukaj, 2010; Zheng et al., 2016a, 2017). Among priming hormesis responses, thermal hormetic priming has received increasing attention in recent years, considering that heat waves have increased in frequency and severity in several regions of the world (Jentsch et al., 2007). Among metals, cadmium (Cd) has become one of the focuses in aquatic toxicology because of its widespread impact on redox balance (Onukwufor et al., 2016), metal homeostasis (Komjarova and Bury, 2014), immunity (Giri et al., 2016), protein folding and transport (Haap et al., 2015), and DNA modification (Pierron et al., 2014). Studies have shown that early conditioning to mild heat stress primed individuals to withstand subsequent episodes of lethal Cd exposure in microalgae (Tukaj and Tukaj, 2010), and mussels (Tedengren et al., 2000). Several *in vitro* experiments have indicated acclimation to elevated temperatures affected metal toxicity in fish (Olsvik et al., 2016; Sappal et al., 2015). However, to the best of our knowledge, little information is available from *in vivo* experiments on the effects of preheating on metal-induced toxicity in fish (Vergauwen et al., 2013a).

In conditioning hormesis responses, fish might develop a priming mechanism that facilitates a more rapid response to future stressors (Donelson et al., 2012). In this process, different pathways are involved, including antioxidant defense, metal transport, immunity, and protein folding (Shrivastava et al., 2016; Zheng et al., 2016a). In the antioxidant system, superoxide dismutase (SOD) and catalase (CAT) are regarded as the critical first line defenses against reactive oxygen

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species (ROS), because O^{2-} is changed to O_2 and H_2O_2 by SOD, which is converted to H_2O subsequently by CAT. Another major defense mechanism is associated with the regulation of metal homeostasis. When Cd accumulates in the liver, metallothioneins (MTs) are induced, which are involved in both transport and detoxification of metals through binding and removal of their redox potential, as well as scavenging of ROS (Hart et al., 2001). Metal transporters, such as copper transporter (CTR1), and Zir-, Irt-like proteins (ZIP8 and ZIP10), limit metal uptake, and other metal transporters, such as zinc transporter (ZnT1 and ZnT5) and Cu-transporting ATPases (ATP7A and ATP7B), eliminate excess intracellular metals. In the immune system, inducible nitric oxide synthase (iNOS) is early response gene in the inflammatory process and is commonly used as an inflammatory marker (Ramyaa et al., 2013). Proinflammatory cytokines act as modulators of immune responses, including interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α). These cytokines are functionally active in inflammation in teleosts (Bo et al., 2015; Grayfer et al., 2008; Karan et al., 2016; Zhang et al., 2016). Under environmental stress, in cooperation with other proteins, HSP70 prevents denaturation of other proteins and holds them in a state of folding or assembly to facilitate repair (Izagirre et al., 2014; Yokoyama et al., 2000), which is transcriptionally controlled by heat shock factors (HSFs) (Ostling et al., 2007). Multiple defense systems are dependent on distinct mechanisms that modulate either catalytic activity of pre-existing enzymes, protein expression, gene transcription, or DNA modification. Recently, the mechanisms underlying thermal hormetic priming have well been elucidated in aquatic organisms. They include mechanisms involved in transcriptional regulation of genes related to oxidative stress (Olsvik et al., 2016), increased protein and mRNA expression of HSP70 (Niu et al., 2008; Tedengren et al., 2000; Tukaj and Tukaj, 2010), and alterations in antioxidant enzymatic activity (Sappal et al., 2015), and body sodium regulation (Vergauwen et al., 2013a). To date, however, multiple defense pathways and associated regulation of DNA, RNA, protein and activity levels are largely unknown in thermal hormetic priming responses in aquatic organisms.

Zebrafish (*Danio rerio*) have become an important vertebrate model organism and are recommended for toxicity testing (OECD guideline 203, 1992). In a laboratory setting, zebrafish are typically kept at 25–28 °C and classified as eurythermal, having a particularly high temperature tolerance (Spence et al., 2008). Transcriptomic analysis suggests that zebrafish acclimation to 34 °C affects transcriptional levels of mounting genes involved in RNA processing, cellular metal ion homeostasis, cytokine signaling, and protein folding and transport (Long et al., 2012). Considering there is little information regarding the effects of preheating on metal-induced toxicity from *in vivo* experiments on fish and the thermal hormetic priming responses described above, the hypothesis for this study was that zebrafish that experienced heat stress early in life would develop increased tolerance and resistance to subsequent Cd exposure. The potential ameliorative effects of preheating on Cd toxicity were evaluated by determining survival rate and hepatic lipid peroxidation (LPO). To uncover associated molecular mechanisms, different pathways including antioxidant defenses, immunity, metal homeostasis and protein folding were examined in the liver of zebrafish. The detected indicators included mRNA and activity levels of Cu/Zn-SOD, CAT and iNOS, mRNA and protein levels of MTs, and gene transcriptional levels of Cu/Zn-SOD, CAT, iNOS, MTs, IL-6, IL-1β, TNF-α, ATP7A, ATP7B, CTR1, ZnT1, ZnT5, ZIP8, ZIP10, HSF1, HSF2, and HSP70. Furthermore, the methylation profiles in the HSF binding motif of the HSP70 promoter were analyzed in the liver of zebrafish.

2. Materials and methods

2.1. Fish maintenance and treatment protocol

Adult zebrafih (AB strain) were maintained in charcoal-filtered and aerated tap water at 26 °C with a photoperiod of 12L: 12D for 2 weeks. At the beginning of the trial, uniform-sized fish (initial body weight: 0.39 ± 0.05 g, mean ± SEM) were maintained at 26 °C and 34 °Cfor 4 days. Then both groups were exposed to 0 or 200 μg L^{-1} Cd (corresponding to 1.78 μM Cd) at 26 °C for 1 week, four tanks per level, with 100 fish in each tank. Temperatures were adjusted before 4-day heat exposure and 7-day Cd exposure in 1 °C steps each 12 h to final temperatures to reduce stress. Water was renewed 100% at 7 am every morning, Cd was added at the time of the water change. The fish were fed commercial diets two times daily at a rate of 1% of body weight. Dissolved oxygen and pH were 7.51 ± 0.27 mg L^{-1} and 7.47 ± 0.31, respectively. Water in each tank was sampled at 4 pm daily to detect water Cd levels. Cd concentrations for the control group and Cd exposed group were 0 and 191 ± 13 μg L^{-1}, respectively. The metal concentrations were measured using flame atomic absorption spectroscopy (FAAS).

At the end of the experiment, 24 h after the last feeding, fish were counted. all fish were euthanized by a 0.02% tricaine methanesulfonate solution (MS-222). Survival rate were assessed. Liver tissues from zebrafish () were separated and frozen in liquid nitrogen immediately, and stored at −80 °C till RNA extraction and biochemical detection. We ensured that all protocols with live fish adhered the Zhejiang Ocean University's ethical instructions for the care and use of lab animals.

2.2. Biochemical analysis

The preparation and extraction of homogenates and supernatants were preformed according to our previous study (Zheng et al., 2016b). The supernatants were maintained at 4 °C until being used for biochemical analysis. Ellman's reagent was used to measure liver MTs protein levels by a spectrophotometric assay (Viarengo et al., 1997). The thiobarbituric reactive species assay was adopted to measure the concentrations of malondialdehyde that reflects LPO (Livingstone et al., 1990). A spectrophotometric method was applied to measure Cu/Zn-SOD and CAT activity, according to the descriptions of Beauchamp and Fridovich (1971) and Beutler (1982) respectively. iNOS activity was determined using iNOS activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The Coomassie brilliant blue method was used to determine soluble protein content (Bradford, 1976). In the present study, five biological replicates and two technical replicates were used in the analyses. Detailed procedures were provided in Supplementary Material.

2.3. Expression levels of mRNA

RNA extraction, cDNA synthesis, and quantitative real-time PCR were carried out using RNAiso Plus Kit (Takara), PrimeScript® RT reagent Kit (Takara), and SYBR® Premix Ex Taq™ Kit (Takara) according to manufacturer instructions, respectively. The primer sequences of each gene used in this analysis are given in Supplementary Material. Each reaction was verified to contain a single product of the correct size using agarose gel electrophoresis and have no significant differences in amplification efficiencies. Abundances of transcripts of genes were calculated according to the $2^{-\Delta\Delta Ct}$ method, normalizing to the geometric mean of the best combination of β-Actin and GAPDH (Zheng, 2016c). Detailed procedures are provided in Supplementary Material.

2.4. Methylation analysis of HSP70

Genomic DNA was extracted from liver samples by using E.Z.N.A.®Tissue DNA Kit (Omega). DNA integrity was detected with 1% agarose gel electrophoresis, and the concentration was measured by a NanoDrop 2000c UV–vis Spectrophotometer (Thermo Fisher Scientific). 1 μg of DNA was bisulfite-modified using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instruction. The