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Impairment of the cortisol stress response mediated by the hypothalamus–pituitary–interrenal (HPI) axis in zebrafish (*Danio rerio*) exposed to monocrotophos pesticide

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ABSTRACT

In teleosts, an important component of the stress response is coordinated by the hypothalamic–pituitary–interrenal (HPI) axis. Environmental contaminants might disrupt the stress axis and consequently affect the stress response in fish. To investigate the effect of monocrotophos (MCP) pesticide on the stress response of fish and its potential mechanisms, adult zebrafish (*Danio rerio*) were exposed to 0, 1, 10, and 100 μg/L of a 40% MCP-based pesticide for 21 d, after which time fish were subjected to a 3-min air-exposure stressor. Concentrations of the whole-body cortisol were measured by radioimmunoassay and abundances of transcripts of proteins involved in the HPI axis were determined using quantitative real-time PCR. Results showed that 100 μg/L of MCP pesticide decreased whole-body cortisol levels of female zebrafish in response to an acute stressor, but without any effect on the cortisol response in males. 100 μg/L MCP pesticide reduced POMC and GR expression in the brain, MC2R and $P450_{11\beta}$ expression in the head kidney, but enhanced 20β-HSD2 expression in the head kidney, suggesting that MCP damaged the HPI axis involving acting at pituitary regulatory levels, inhibiting cortisol synthesis and stimulating cortisol catabolism, or disturbing the negative feedback regulation. Additionally, MCP depressed liver GR transcription but did not affect phosphoenolpyruvate carboxykinase and tyrosine aminotransferase expression in zebrafish, suggesting a role for this pesticide in reducing target tissue responsiveness to cortisol. Considered together, the reduced ability to elevate cortisol levels in response to an acute stress may be an endocrine dysfunction occurring in zebrafish subchronically exposed to MCP pesticide.

1. Introduction

The stress response is a highly conserved adaptation among vertebrates to maintain internal homeostasis in a variable environment (Ings et al., 2011; Tort, 2011). In teleosts, an important component of the stress response is regulated by the hypothalamic–pituitary–interrenal (HPI) axis, which controls the circulating levels of glucocorticoid cortisol (Wendelaar Bonga, 1997; Flik et al., 2006). In response to a stressor, corticosteroid-releasing factor (CRF) is released from the hypothalamus and stimulates the anterior pituitary to release the pro-opiomelanocortin (POMC)-derived adrenocorticotropic hormone (ACTH) (Huising et al., 2004; Metz et al., 2004). ACTH binds to the melanocortin 2 receptor (MC2R) on the steroidogenic interrenal cells of the head kidney, and activates the corticosteroid biosynthesis (Aluru and Vijayan, 2008). Several proteins/enzymes are involved in the conversion of cholesterol to cortisol in the interrenal cells including steroid acute regulatory protein (StAR) and 11β-hydroxylase ($P450_{11\beta}$), while limiting the activity of cortisol involves the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) and 20β-hydroxysteroid dehydrogenase 2 (20β-HSD2). Circulating cortisol levels are also tightly regulated by a negative feedback loop, including glucocorticoid receptor (GR) signaling in the brain, inhibiting the release of trophic hormones (CRF and/or ACTH) in response to elevated steroid levels (Wendelaar Bonga, 1997; Mommsen et al., 1999).

Any adverse effect on the functioning of the HPI axis would compromise the ability of fish to mount an adequate response to stressors (Hontela, 1998; Mommsen et al., 1999) and studies have shown that fish exposed to chronic chemical stressors display a decreased corticosteroid response to a second acute stressor. For instance, Benguira et al. (2002) reported that treatment with *o,p′*-dichlorodiphenyldichloroethane for 14 days reduced the normal capacity of rainbow trout (*Oncorhynchus mykiss*) to elevate plasma cortisol in response to an acute stress. Monocrotophos (MCP; CAS number, 6923-22-4), an organophosphorus pesticide, has been shown to exert endocrine-disrupting effects in teleost

Abbreviations: HPI, hypothalamic–pituitary–interrenal; CRF, corticosteroid-releasing factor; POMC, pro-opiomelanocortin; ACTH, adren-corticotropic hormone; MC2R, melanocortin 2 receptor; StAR, steroid acute regulatory protein; $P450_{11\beta}$, 11β-hydroxylase; 11β-HSD2, 11β-hydroxysteroid dehydrogenase 2; 20β-HSD2, 20β-hydroxysteroid dehydrogenase 2; GR, glucocorticoid receptor; MCP, monocrotophos; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase; RIA, radioimmunoassay; 11β-HSD3, 11β-hydroxysteroid dehydrogenase 3.

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fish (Tian et al., 2010; Zhang et al., 2013). Our recent study showed that after 21 days of exposure to the MCP pesticide, concentrations of cortisol were significantly decreased in plasma of female goldfish (*Carassius auratus*) (Zhang et al., 2014). However, it is still unclear whether sub-chronic exposure to MCP pesticide would affect the integrated stress response to a secondary stressor.

During stress, elevated cortisol levels mediate crucial physiological processes such as glucose plasma concentrations and various other stress responses for coping with stress. In hepatocytes, glucocorticoid hormones facilitate the process of gluconeogenesis by transcriptional activation of gluconeogenesis enzymes including phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (TAT). GR signaling is thought to play a key role in mediating the stress effects of cortisol. Cortisol up-regulates mRNA abundance of hepatic PEPCK mediated *via* the GR, resulting in increased glucose production by hepatocytes (Aluru and Vijayan, 2007). Joshi and Rajini (2012) demonstrated a 2-hour MCP exposure induced increase in plasma corticosterone levels with concomitant increase in blood glucose and activities of liver TAT in rats (*Rattus norvegicus*). Accordingly, it is speculated certain stressor-mediated liver metabolic capacity for coping with stress might also be disturbed by the MCP pesticide.

The objective of this study was first to investigate the influence of a 21-d MCP exposure on the stress responsiveness of adult zebrafish by analyzing whole-body cortisol levels as indicators of stress. By including a second air exposure stressor after exposure of MCP, we could test if MCP-exposure caused a dysfunctional response in the established pathways, implying that fish would be incapable of responding to an additional stressor when in a MCP-polluted environment. Second, gene expression of CRF, POMC, and GR in the brain and MC2R, StAR, $P450_{11\beta}$, 11β-HSD2, and 20β-HSD2 in the interrenal tissue was determined to investigate possible mode of actions of MCP on the HPI axis. At last, liver GR, PEPCK, and TAT transcripts were used as markers of target tissue cortisol responsiveness.

2. Materials and methods

2.1. Animals

Sexually mature Tu wild-type zebrafish (*Danio rerio*) of both sexes (8–10 months-old; 0.33 ± 0.04 g wet weight and 2.73 ± 0.07 cm standard length for males, 0.63 ± 0.05 g wet weight and 2.68 ± 0.09 cm standard length for females) were obtained from a local commercial distributor and housed at a density of up to five animals per liter in 50 L tanks with dechlorinated tap water. Fish were continuously aerated (7.0 ± 1.0 mg O_2/L) at 28 ± 1 °C, under a 14/10 h light/dark controlled photoperiod. Fish were fed twice a day with freshly hatched brine shrimp (*Artemia nauplii*) and were maintained healthy and free of any signs of disease according to the National Institute of Health Guidelines for Handling and Care of Experimental Animals. The animal utilization protocol was approved by the Institutional Animal Care and Use Committee of the Ocean University of China.

2.2. Experimental design

2.2.1. MCP pesticide exposure

MCP pesticide (3-hydroxyl-*N*-methyl-*cis*-crotonamidedimethyl phosphate, 40% water-soluble preparation) was purchased from the Qingdao pesticide factory in China. The concentration on the label was 40%, which was consistent with the actual concentration determined by gas chromatography (40 ± 0.1%) (Ru et al., 2003). The half-life of MCP is approximately 66 days at pH 7.0 and 20 °C (Wang and Zhang, 1989).

Fish were acclimated for at least two weeks before the experiments, then sexes were kept separate in 5 L glass beaker containing 2.5 L dechlorinated tap water. Eight male (or female) fish were assigned to each glass beaker and exposed to MCP pesticide (1, 10, and 100 μg/L) dissolved in water for 21 d. Triplicate beakers were used for each treatment group and two control groups with dechlorinated tap water (to one of which no air-exposure stress would be administered) were included in the exposure design. Fish exposure were conducted as a semi-static toxicity test (all water/MCP pesticide of the beaker was changed after each 24 h of exposure in order to keep the treatment concentrations constant and water fresh) and other conditions were the same as those described in Sect. 2.1. Fish were fed with freshly hatched brine shrimp twice daily. A total of 240 fish (including 120 males and 120 females) were used for this study and no deaths were observed in any of the treatment groups during experimentation.

2.2.2. Air exposure

No food was provided 24 h before the end of the 21-d MCP exposure. At 22nd day according to Ramsay et al. (2009), both the fish and the water contained in the beaker were poured into a net, leaving the fish suspended in the air for 3 min. Then all fish were immediately returned to a prepared 50-L tank containing 30 L dechlorinated tap water with the same temperature, allowed to swim freely in the tank, and sampled at 3 min post-air exposure-stress. All sampling commenced at approximately the same time of day (9:00–10:00 h) in order to minimize the possibility of fluctuations in cortisol due to natural circadian rhythms (Schreck, 1981).

2.2.3. Sampling procedures

Upon sampling, fish were euthanized with a lethal dose of MS-222 (500 mg/L), blotted on paper towels to remove excess water, and then weighed. One subgroup of six fish per sex from each treatment group was immediately frozen in liquid nitrogen, and stored at −80 °C for whole-body cortisol analysis. Tissues of the rest of the fish including brain, liver, and head kidney were removed, 3 brains (livers or head kidneys) were pooled to obtain 6 samples per treatment, placed in RNase-free tubes, and stored at −80 °C for RNA extraction.

2.2.4. Whole-body cortisol extraction and measurement

Whole-body cortisol was extracted using a modification of a method described by Sink et al. (2007). Individual whole, frozen zebrafish were thawed, homogenized, and extracted twice with 2 mL diethyl ether. After freezing the solution with liquid nitrogen immediately, the unfrozen portion (ethyl ether containing cortisol) was decanted. The ethyl ether was transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol. The extract was reconstituted with 0.3 mL phosphate-buffered saline with 1% BSA, vortexed for 30 s, and stored at −20 °C until the radioimmunoassay (RIA) was conducted.

Cortisol in the whole-body extracts was measured using an RIA using commercially available kits, following the protocols provided by the manufacturer (Beijing North Institute of Biological Technology, Beijing, China). The RIA kit for human cortisol was validated for use with zebrafish samples by demonstrating parallelism between a series of diluted and spiked samples in relation to the standard curve attached to the assay kits. Standards and samples were added to the test tubes in duplicate. The assay detection limits were 2 ng/mL and the inter- and intra-assay coefficients of variation were <15% and <10%, respectively.

2.3. Gene expression analysis

Total RNA was isolated from the liver, brain, and head kidney from a pool of three fish using the phenolic reagent TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and then was measured by spectrometry at $OD_{260/280}$. Equal amounts of RNA (1 μg) were reverse-transcribed into cDNA using the PrimerScript RT reagent Kit with gDNA Eraser (Perfect Real Time) as described by the manufacturer (Takara, Dalian, China). Separate samples were identically treated without the addition of reverse transcriptase (PrimeScript RT Enzyme Mix

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