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Quantitative analysis of in-vivo responses of reproductive and thyroid endpoints in male goldfish exposed to monocrotophos pesticide

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ABSTRACT

Cross-regulation occurs at many points between the hypothalamic-pituitary-gonad (HPG) and hypothalamic-pituitary-thyroid (HPT) axes. Monocrotophos (MCP) pesticide could disrupt HPG and HPT axes, but its direct target within the endocrine system is still unclear. In the present study, hormone concentrations and transcriptional profiles of HPG and HPT genes were examined in male goldfish (*Carassius auratus*) exposed to 0, 4, 40, and 400 μg/L MCP for 2, 4, 8, and 12 d. In vivo data were analyzed by multiple linear regression and correlation analysis, quantitatively indicating that MCP-induced plasma 17*β*-estradiol (E_2) levels were most associated with alteration of *cyp19a* transcription, which was also a potential point indirectly modulated by the MCP-altered thyroid hormones (THs) status; disturbance of THs pathways was most related with effect of MCP on regulation of the hypothalamic-pituitary hormones involved in the thyroid system, and the increased E_2 levels might enhance the impact of MCP on HPT axis by modulating hepatic deiodinase expression. Our finding, based on these correlational data, gave a whole view of the regulations, especially on the cross-talk between sex hormone and thyroid hormone pathways upon exposure to chemicals with unknown direct target in vivo, and cautions should be exercised when developing adverse outcome pathway networks for reproductive and thyroidal endocrine disruption.

1. Introduction

Multiple endocrine hormones including thyroid hormones (THs), sex hormones, growth hormones, and glucocorticoids play important roles in regulation of growth, development, reproduction, and metabolism in fish. Of the endocrine system, THs, including 3,3′,5-triiodo-$_L$-thyronine (T_3) and 3,3′,5,5′-$_L$-thyroxine (T_4), are synthesized and secreted by the thyroid follicles under the control of the hypothalamic-pituitary-thyroid (HPT) axis (reviewed by Carr and Patiño (2011)), and the synthesis and release of sex steroids, such as 17*β*-estradiol (E_2) and testosterone (T), is regulated by the hypothalamic-pituitary-gonad (HPG) axis (Villeneuve et al., 2007). Accordingly, many exogenous chemicals with endocrine disrupting effects have been proved to disturb homeostasis of THs or sex hormones by interfering with the HPT or HPG axis, respectively. It is also worth to note that several compounds could elicit adverse effects on both THs and sex steroids pathways. For instance, DE-71 reduced plasma E_2 levels in female zebrafish (*Danio rerio*) but elevated T levels in males, and it could also decrease T_4 levels in the larvae (Yu et al., 2010; Yu et al., 2014); perfluorooctane sulfonate has been shown to increase concentrations of T_3 in zebrafish and elevate T levels in fathead minnow (*Pimephales promelas*) (Ankley et al., 2005; Shi et al., 2009). Previous studies in our lab demonstrated that a 21-d exposure to the organophosphate pesticide monocrotophos (MCP) caused elevated E_2 levels and decreased concentrations of T and T_3 in plasma of male goldfish (*Carassius auratus*) (Tian et al., 2010; Zhang et al., 2013).

Recently, many researchers have taken interactions among major endocrine systems into consideration when studying endocrine disrupting effects of exogenous chemicals. Evidence has shown that chemical-induced alterations on one endocrine axis will indirectly lead to changes in other endocrine axes. For example, by using in vivo waterborne exposure and in vitro brain culture, Liu et al. (2011) found a well-

Abbreviations: THs, thyroid hormones; T_3, 3,3′,5-triiodo-$_L$-thyronine; T_4, 3,3′,5,5′-$_L$-thyroxine; HPT, hypothalamic-pituitary-thyroid; E_2, 17*β*-estradiol; T, testosterone; HPG, hypothalamic- pituitary-gonadal; MCP, monocrotophos; CRH, corticotropin-releasing hormone; GtHs, gonadotropins; LH, luteinizing hormone; cyp19, cytochrome P450 aromatase; FSH, follicle-stimulating hormone; RIA, radioimmunoassay; qPCR, quantitative real-time polymerase chain reaction; UPLC-MS/MS, ultra performance liquid chromatography-tandem quadrupole mass spectrometry; *vtg*, vitellogenin; sGnRH, salmon gonadotropin-releasing hormone; *cGnRH-II*, chicken-II gonadotropin-releasing hormone; TSH, thyroid-stimulating hormone; TRH, thyrotropin-releasing hormone; TTR, transthyretin; *dio1*, type I deiodinase; *dio2*, type II deiodinase; *dio3*, type III deiodinase; TRs, thyroid hormone receptors

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described endocrine-disrupting chemical prochloraz (300 μg/L) decreased plasma E_2 and T levels and indirectly affected the HPT axis by down-regulating corticotrophin-releasing hormone (CRH). However, for most kinds of chemical substances that occur in the environment, the direct target axis in the endocrine system is largely unknown. Like MCP, Tian et al. (2010) reported that the 21-d MCP exposure elevated plasma E_2 levels in male goldfish by increasing pituitary follicle-stimulating hormone *β* subunit (*fshβ*) and gonadal cytochrome P450 aromatase (*cyp19a*) mRNA expression, then Zhang et al. (2013) found that MCP could decrease plasma T_3 levels via interference with deiodination conversion of THs mainly in the liver. Since cross-regulation between the HPG and HPT axes has been proved in fish (e.g., Bagamasbad and Denver, 2011; Cyr and Eales, 1996; Duarte-Guterman et al., 2014; Flood et al., 2013; Vasudevan et al., 2002), it was hypothesized that the mechanism underlying MCP-caused disruption of sex hormones and THs could be not merely explained by internal factors along either the HPG or HPT axis, but also related to cross-regulation between these hormones.

In teleosts including goldfish, THs have been shown to impact reproductive function through their involvement in regulating pituitary gonadotropins (GtHs) production, steroidogenesis, and steroid receptor expression (as reviewed by Habibi et al. (2012)). Nelson et al. (2010) reported that T_3 treatment decreased the expression of pituitary luteinizing hormone *β* subunit (*lhβ*) and *cyp19a* mRNA levels in the testis of goldfish. In addition, effects of sex steroids primarily E_2 on synthesis and conversion of THs have also been reported. For example, E_2 depressed criteria of thyroidal status and plasma T_3 levels, and it could also suppress conversion of T_4 to T_3 in peripheral tissues (Cyr and Eales, 1996). From this point, THs might play a modulatory (inhibitory) role in goldfish reproduction by interfering with key factors along the HPG axis, and vice versa. Accordingly, based on results of our previous study, we proposed hypothesis that changes of one hormone would aggravate the effect of MCP on the other hormone by modulating key factors along the endocrine axis. Indeed, the hormone homeostasis, regulated by endocrine axes, is a dynamic process that depends on the balance among hormone synthesis, transport, and metabolism. One-time point study would not allow us to delineate the temporal relationship between these events upon MCP exposure (Ankley et al., 2009; Skolness et al., 2011), and thus a designed time-course study with multiple end-points will help address the cross response of these two axes.

In the present study, concentrations of plasma steroids (including E_2 and T) and THs (including T_3 and T_4) were determined by radioimmunoassay (RIA) in male goldfish after an exposure to 0, 4, 40, and 400 μg/L MCP for 2, 4, 8, and 12 d. In addition, transcriptional level changes of several important HPG genes and HPT genes following the exposure were examined by quantitative real-time polymerase chain reaction (qPCR). Finally, these in vivo data were put into multiple linear regression models to select potential target genes involved in the HPG and HPT axis responsible for disturbed steroids and THs, respectively, and Pearson's correlation coefficient was used to explain the interplay of these two axes upon MCP exposure. Considering that the complicated interactions among the endocrine axes could be hardly mimic in vitro, this study was utilizing in vivo data together with the physiological cross-talk between hormones, from both internal regulation of each axis and interplay between different axes, quantitatively identifying the most direct reason for MCP-induced imbalance of sex hormones and THs. This study will provide new insights in characterizing and predicting interactions among major endocrine systems in vivo underlying effects of chemicals, especially those with unknown initiating targets, on the endocrine system.

2. Materials and methods

2.1. Fish exposure and sample protocol

Sexually mature male goldfish (2 years old) in late recrudescence to prespawning (March) stages, were obtained from a local dealer in Qingdao, PR China, with body weight and length of 18.2 ± 2.1 g and 8.1 ± 0.2 cm, respectively. The fish were maintained in 70 L aquaria containing 50 L of dechlorinated tap water at ambient temperature (18 ± 2 °C), with a dissolved oxygen content of 7.0 ± 0.1 mg/L, and they were fed a pelletized diet daily. In addition, the fish were handled according to the National Institute of Health Guidelines for the 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.

MCP (CAS number 6923-22-4, purity ≥ 99.3%) was obtained from Sigma (Shanghai, China). Following acclimation in the laboratory for two weeks, the fish were exposed to nominal 4, 40, and 400 μg/L MCP [based on our previous study Tian et al. (2010) and Zhang et al. (2013)]. The experiments were conducted in 70 L aquaria containing 50 L of dechlorinated tap water, using a semi-static toxicity test (20 L of water renewal daily to keep the MCP concentration constant). For each treatment and control group, 24 fish were randomly and evenly distributed between three tanks (eight fish/tank). No mortality was observed in any of the treatments during the exposure experimentation.

At 2, 4, 8, and 12 d after starting the MCP exposure, two fish were randomly removed from each exposure tank, yielding a total of six fish per treatment at each sampling time, and then anesthetized in 75 mg/L of MS-222 (Sigma, Shanghai, China). Considering the possible diurnal fluctuations in hormone levels, the fish were sampled between 8:00 and 11:00 a.m. (e.g., Leiner et al., 2000). Blood was collected from the caudal vein with chilled heparinized syringes and was kept on ice. Plasma samples were obtained after centrifugation at 1000*g* for 10 min and were stored at −20 °C until the hormone assay could be performed. The liver, gonad, and certain brain tissues (including hypothalamus and pituitary) were each dissected, frozen in liquid nitrogen, and stored at −80 °C for the quantification of gene expression analysis by qPCR.

2.2. Quantification of MCP in exposure solutions

Since exposure solutions were renewed daily, water samples were collected once after the last time of exposure solution renewal. According to Zhang et al. (2013), the MCP was analyzed using the ultra performance liquid chromatography-tandem quadrupole mass spectrometry (UPLC-MS/MS) on a Shimadzu Nexera UPLC system interfaced to a Shimadzu LCMS-8030 quadrupole mass spectrometer (Kyoto, Japan). Detection limit was 0.5 μg/L.

2.3. Hormone assay

E_2, T, T_3, and T_4 levels were measured in plasma samples by RIA using commercially available kits, following the protocols provided by the manufacturer (Beijing North Institute of Biological Technology, Beijing, China). As described by Zhang et al. (2013), the RIAs for E_2, T, T_3, and T_4 have been validated for use with goldfish 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 1 pg/mL for E_2, 0.01 ng/mL for T, 0.05 ng/mL for T_3, and 2 ng/mL for T_4. The inter- and intra-assay coefficients of variation for all of the above hormones were < 10% and < 15%, respectively.

2.4. Gene expression analysis

Total RNA was isolated from each tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's