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Dose-dependent compensation responses of the hypothalamic-pituitary-gonadal-liver axis of zebrafish exposed to the fungicide prochloraz

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ABSTRACT

Compensation responses and adaptability of hypothalamic-pituitary-gonadal (HPG) axis have been reported in fish exposed to model chemicals, however due to its importance in predictive toxicology further study was needed to elucidate details of the integrated responses to model chemicals. Transcriptional profiles of the hypothalamic-pituitary-gonadal (HPG) axis and concentrations of 17β-estradiol (E2) in plasma were measured in male and female zebrafish that had been exposed to one of seven concentrations of the fungicide, prochloraz: low (1, 3 or 10 μg/L), medium (30 or 100 μg/L) or high concentrations (300 or 1000 μg/L) for 4 days. In zebrafish exposed to the low and medium concentrations of prochloraz, compensation responses of the HPG axis through transcription, occurred in brain (up-regulation of *gnrh*, *gnrhr* and *lhβ*) and both brain and gonad (up-regulation of steroidogenic genes), respectively. Concentrations of E2 in plasma and expression of estrogen receptor 1 (*er1*) and vitellogenins (*vtgs*) in liver did not change. This result suggested that compensatory responses were successful in maintaining homeostasis. In zebrafish exposed to the two greatest concentrations, compensatory responses occurred in brain, gonad and liver through up-regulation of *er2β*, but it failed to maintain concentration of E2 in blood plasma and expression of *er1* and *vtgs* in liver. Collectedly, the results observed in this study allowed characterization of dose-dependent compensatory responses along the HPG axis and liver and identified key linkages between compensatory responses occurring in brain, gonad and liver after exposure to prochloraz.

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1. Introduction

As an imidazole fungicide, prochloraz is registered for various agricultural uses throughout the world (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=73665). This chemical can act as an inhibitor of cytochrome P450 (*CYP*) 14α-demethylase (*CYP51*), which is a key enzyme in the synthesis of ergosterol, and therefore inhibits growth of fungi (van den Bossche et al., 1987, 1982). However, prochloraz can also inhibit activities of other *CYP* enzymes, including cytochrome P450 c17α-hydroxylase/17,20-lyase (*CYP17*) and aromatase (*CYP19*) (Ankley et al., 2009). In vertebrates, the gene product of *CYP17* is responsible for synthesis of testosterone (T) and *CYP19* catalyzes conversion of T to 17β-estradiol (E2). Therefore, inhibition of activities of the two enzymes would decrease production of both T and E2. These effects have been previously confirmed experimentally, both in vitro and in vivo, in mammals, where exposure to prochloraz significantly inhibited activities of both *CYP17* and *CYP19* enzymes and decreased concentrations of T and E2 in blood plasma (Blystone et al., 2007; Mason et al., 1987; Noriega et al., 2005; Sanderson et al., 2002; Vinggaard et al., 2000). In fish, treatment with prochloraz decreased concentrations of T and E2 in blood plasma, and affected reproductive function (Ankley et al., 2005, 2009; Marca Pereira et al., 2011a, b; Liu et al., 2011; Skolness et al., 2011; Villeneuve et al., 2007; Zhang et al., 2008a,). Due to its effectiveness as an inhibitor of *CYP17* and *CYP19* enzymes, prochloraz has been used as a model chemical for studying the responses of

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hypothalamic-pituitary-gonadal (HPG) axes of vertebrates (Ankley et al., 2005, 2009; Marca Pereira et al., 2011a, b; Liu et al., 2011; Villeneuve et al., 2007; Zhang et al., 2008a).

The HPG axis is a dynamic endocrine system, that maintains physiological conditions of reproduction by various homeostatic feedback mechanisms during exposure to stressors including chemicals (Ankley et al., 2009). Compensatory responses of the HPG axis have been documented in fish exposed to model chemicals (Ankley et al., 2009; Liu et al., 2011; Skolness et al., 2011; Villeneuve et al., 2007; Zhang et al., 2008a, b, c). For example, results of several studies have demonstrated that exposure to fadrazol, which inhibits the enzymatic activity of *CYP19*, resulted in decreased concentrations of E2 in blood plasma and caused a time- and dose-dependent up-regulation of genes of HPG axis in small fish. Up-regulation of those genes was considered to be a compensatory mechanism to return concentrations of E2 in blood plasma to pre-exposure values (Villeneuve et al., 2009; Zhang et al., 2008c). Similarly, treatment with prochloraz significantly decreased concentrations of T and E2 in blood plasma and resulted in a comprehensive up-regulation of genes along the HPG axes of fishes (Ankley et al., 2009; Liu et al., 2011; Skolness et al., 2011; Zhang et al., 2008a). Furthermore, in two time-course studies, inhibitory effects of prochloraz or fadrozole on production of E2 in fathead minnow were transitory and did not persist during the 8-day exposure phase, which demonstrated the effectiveness of compensatory responses. Termination of the exposure resulted in recovery of expression of genes and concentrations of steroid hormones, including a brief period of "overcompensation" immediately after cessation of exposure (Ankley et al., 2009; Villeneuve et al., 2009). These results suggest key compensatory responses of the HPG axis after exposure to chemical stressors, and highlight the need to consider these compensatory responses when developing approaches to assess potential risks of chemicals (Ankley et al., 2009; Villeneuve et al., 2009).

To better understand compensatory mechanisms in fishes and more accurately predict effects of chemicals based on modes of action a more comprehensive characterization of these dose-dependent compensatory responses was still needed. Objectives of the present study were to: (1) examine dose-dependent expression behaviors of HPG axis and genes expressed in liver; (2) compare sensitivities of genes; and (3) identify linkages between responses occurring at different organs, including brain, gonad and liver of zebrafish.

2. Materials and methods

2.1. Chemicals and reagents

Prochloraz and TRIzol regent were obtained from Sigma (St. Louis, MO, USA) and Invitrogen (New Jersey, NJ, USA), respectively. Reverse transcription and SYBR Green kits were purchased from Takara (Dalian, Liaoning, China). 17β-estrogen (E2) enzyme immunoassay (EIA) kits were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All the other reagents used in this study were of analytical grade.

2.2. Fish and chemical exposure

Zebrafish were maintained in flow-through tanks at 28 ± 0.5 °C with a 12:12 light/dark cycle, and water pH, hardness and dissolved oxygen were routinely monitored. Before exposure, 5-month old males and females (sexual maturity) were acclimated in 15-L tanks filled with 10 L of carbon-filtered water for 1 week. After acclimation, fish were exposed to 0, 1, 3, 10, 30, 100, 300, 1000, 3000 or 10,000 μg/L (0, 0.0027, 0.0080, 0.027, 0.080, 0.27, 0.80, 2.7, 8.0 or 27 μM) prochloraz for 4 days. Concentrations were selected based on previous studies, where comprehensive compensation responses of HPG axis genes would be expected to occur (Ankley et al., 2009; Liu et al., 2011; Zhang et al., 2008a). Five females and five males were exposed in each of 2 replicated tanks for each concentration. One half of the water in each tank was replaced daily with fresh carbon-filtered water containing corresponding concentration of prochloraz. Both control and exposure groups received 0.01% DMSO since previous study demonstrated that such DMSO concentration did not affect reproductive function (Han et al., 2013). During the exposure period, survival was recorded. After exposure, fish were euthanized and blood was collected for plasma hormone analysis as described before (Liu et al., 2009). Tissues from brain (including hypothalamus and pituitary), gonad and liver were sampled and preserved in TRIzol reagent for subsequent RNA isolation. In this study, experimental procedures were carried out following the approved protocol by Institutional Animal Care and Use Committee (IACUC) of Huazhong Agricultural University.

2.3. Quantification of hormones

Briefly, plasma was obtained by centrifugation (5000 × *g* for 5 min at 4 °C) of whole blood. Plasma from 2 fish was pooled for quantification by use of a commercial EIA kit as described previously (Liu et al., 2009). Briefly, plasma (3 μL) from each pooled sample was diluted with 500 μL ultrapure water and extracted thrice with 2 mL of ethyl ether, and the ether phase was collected and evaporated. After that, residues were redissolved with EIA buffer provided in the kit and E2 was quantified following manufacturer's instructions. The limit of quantification was 6 pg/L, and the intra- and inter-assay coefficients of variance (CV) were <10%. Each sample was quantified 3–4 times. In order to determine effects of prochloraz on gene expression and hormone production, concentrations of E2 in plasma were expressed as fold-change relative to control.

2.4. Quantitative real-time PCR assay

In this study, quantitative real-time PCR was performed using minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (Bustin et al., 2009). The tissue of each sample employed for qRT-PCR was from one animal. The isolation of total RNA was performed using TRIzol regent following manufacturer's instructions. Purity of RNA was examined by measuring 260/280 nm ratios and 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining. Concentrations of RNA were estimated by determining absorbance at 260 nm. After measurement of concentration of total RNA, all RNA samples were diluted to 100 ng/μL, and equal volume of RNA (5 μL) was used for cDNA synthesis. First-strand cDNA syntheses and quantitative real-time PCR (qRT-PCR) were performed using commercial reverse transcription and SYBR Green kits (Takara, Dalian, Liaoning, China), respectively following manufacturer's instructions. Sequences of primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Table S1 in Supporting Information). Primer specificity was checked by NCBI BLAST, and melting curve was employed to check out purity and specificity of PCR productions in each assay. Selection of housekeeping gene was performed using previous method (Andersen et al., 2004). Transcription of three housekeeping genes (18S rRNA, *gapdh*, *β-action*) were tested, and expression of 18S rRNA kept unchanged in brain, gonad and liver of female and male fish after prochloraz exposure, therefore it was used as an internal control gene. Thermal cycling was set at 95 °C for 2 min, followed by 40–45 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression of target genes were

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