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Modulation of transcription of genes related to the hypothalamic–pituitary–gonadal and the hypothalamic–pituitary–adrenal axes in zebrafish (*Danio rerio*) embryos/larvae by androstenedione

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ABSTRACT

This study aimed to determine the effects of Androstenedione (AED) on the transcriptional expression of genes involved in the hypothalamic–pituitary–gonadal (HPG) and the hypothalamic–pituitary–adrenal (HPA) axes in the zebrafish embryos/larvae. Zebrafish embryos were exposed to 0, 4.0, 45.0, 487.0, and 980.0 ng/L of AED from the day of fertilization to 144 h post fertilization (hpf), during which the transcriptional profiles of key genes related to the HPG and HPA axes were examined daily using quantitative real-time PCR. The AED exposure significantly up-regulated several receptor signaling pathways and the key genes involved in various steps of the steroidogenic pathways were also affected. In addition, the AED exposure could significantly modulate the transcriptional profiles of the other target genes related to hypothalamic and pituitary hormones. The findings of this study suggest that AED, at environmentally relevant concentrations, affects the adrenal endocrine systems and the reproduction of zebrafish by interrupting the HPG and HPA axes.

1. Introduction

Endocrine disrupting chemicals (EDCs), such as natural and synthetic steroid hormones, are introduced into aquatic environments via fecal and urinary excretion of human and animals. This is a cause of global concern because of potentially adverse effects of EDCs on the reproduction and sexual differentiation of aquatic organisms, including both invertebrates and vertebrates (i.e., fish and amphibians) (Kloas et al., 2009; Fetter et al., 2015). There have been studies on the effects of these steroids on aquatic animals; however, most of these studies assessed the effects of the potent representatives of estrogens (Tyler et al., 1998) and progestin (Hogan et al., 2008; Fent et al., 2006; Hou et al., 2017). For androgens, such as androstenedione (AED), there have been very few studies on their environmental concentrations or on their effects on fish.

AED is mostly released in the environment through animal and human excretion as well as through wastewater discharged from the treatment plants, without the complete removal of AED (Liu et al., 2012b). It can be metabolized and converted to more potent androgens or estrogens, such as testosterone, estrone, or estradiol (Beamer et al., 1988). It has been found widely in aquatic habitats that acquire effluent release from pulp and paper factories, national sewage treatment plants, and livestock farms (Jenkins et al., 2001; Liu et al., 2012a, 2012b, 2012c). In addition, AED has been identified in the sewage from seven wastewater treatment plants in Beijing in concentrations ranging from 5 ng/L to 270 ng/L (Chang et al., 2011), in two different types of wastewater treatment plants in Guangdong Province in concentrations up to 1368 ± 283 ng/L (Liu et al., 2012b), and in natural waterways in Japan at concentrations up to 480 ng/L (Chang et al., 2008). The AED residues that have been introduced in the environment may pose significant risks to the aquatic organisms.

The adverse effects of AED on aquatic organisms have been described in previous studies; for example, laboratory and field studies revealed masculinization of female mosquitofish (*Gambusia holbrooki*) living in waters that were contaminated by pulp mills (Ellis et al., 2003) with androstadienedione (ADD) and AED (Denton et al., 1985; Jenkins et al., 2001; Parks et al., 2001). Masculinization of another female mosquitofish (*Gambusia affinis*) after acute exposure to AED at concentrations as low as 397 ng/L has also been reported (Stanko and Angus, 2007). Recently, it was found that masculinization of female

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mosquitofish in Dengcun River was due to higher concentrations of AED (Hou et al., 2011). Intriguingly, our recent study demonstrated that long-term exposure to AED, at an environmentally relevant concentration, could induce masculinization in female mosquitofish under the conditions tested (Hou et al., 2017). A previous study showed that exposure to AED caused masculinization of mature female mosquitofish in a comparatively small amount of time and substantial lengthening of the anal fin ray was noted after exposure (Stanko and Angus, 2007). However, few studies have reported on the toxicological effects of AED in fish, especially on the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–gonadal (HPG) axes and during the early stages of development.

In this context, we investigated the transcriptional impacts of AED on the HPG and HPA axes throughout the initial steps of zebrafish growth. Zebrafish embryos were exposed to AED in amounts ranging from 4 ng/L to 980.0 ng/L for 144 h post fertilization (hpf) and we examined the transcriptional profiles of the target genes linked to the receptor signaling and steroidogenic pathways and other target genes daily. Thus, the present study was conducted to determine whether the mRNA expression profiles of key genes in the HPG and HPA axes could be affected by AED exposure of the zebrafish larvae.

2. Materials and methods

2.1. Fish and embryos

All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou University, China. Wild type zebrafish (3-month old) were purchased from a local fish market (Guangzhou Huadiwan market, China) and maintained in the laboratory following the standard procedures (Liang et al., 2015). The zebrafish embryos were collected at the blastula stage from a single spawning of 20 parents and were subsequently maintained in beakers containing different freshly prepared media as described in Section 2.2.

2.2. Chemical exposure

AED (> 99.9% purity) was acquired from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions were established by dissolving the necessary quantities of AED in ethanol. Fresh working solutions were established each day by diluting the stock solutions 1000 × using MilliQ water to obtain the final concentrations. The embryos at the blastula stage were randomly divided into five groups and placed in beakers containing 500 mL of culture medium supplemented with 5, 50, 500, and 1000 ng/L AED (the treated groups) or 0.001% ethanol (the solvent control: SC). Each group had four replicates with 100 embryos in each. The experiment was conducted in a semi-static system for six days. The media were refreshed daily after the removal of dead embryos. At 24, 48, 72, 96, 120, and 144 hpf, 15 embryos/larvae were collected from each AED-treated or control group, immersed in Trizol (Invitrogen. China), and stored at − 80 °C until use. The embryos were incubated at 28 ± 1 °C under a 14-h light:10-h dark photoperiod in an incubator.

2.3. Measurement of AED concentration in the exposure solutions

The actual concentration of AED in the each exposure medium (500 mL) in all the groups was determined at the beginning of exposure (0 h) and before water renewal (24 h) in the first day. The extraction and analysis of AED was done as described in our previous report (Liu et al., 2011). Briefly, 500 mL water sample was collected from each breaker for solid phase extraction. AED was extracted using a CNWBOND LC-C18 SPE cartridge (200 mg, 3 mL) (Germany). The solution was loaded on a cartridge, which was then dried and eluted with 10 mL ethylacetate. The eluate was dried under nitrogen and redissolved in 1 mL methanol. The concentration of AED was determined via rapid resolution liquid chromatography-electrospray ionization tandem mass spectrometry (RRLC-MS/MS) (Agilent 1200 LC-Agilent 6460 QQQ, USA). The limit of detection (LOD) and the limit of quantitation (LOQ) of AED were 0.05 ng/L and 0.17 ng/L, respectively (Liu et al., 2011). The recovery of the analyte, determined by spiking the surface waters with known amounts of AED (5 and 100 ng/L), was 94.4% and 103%, respectively (Liu et al., 2011).

2.4. Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from the embryos/larvae with Trizol, following the protocol recommended by the manufacturer. Briefly, prior to RNA isolation, RNAlater was removed. 15 embryos/larvae per sample were homogenized and used for extraction of total RNA using Trizol reagent (Invitrogen). The extracted RNA was quantified using NanoDrop (Thermo Scientific). Then, the quality of total RNA was assessed using the electrophoresis on agarose gel stained with gelred (28 S/18 S rRNA intensity ratio about 2:1). Total RNA content was measured at 260 nm by using a SmartSpec™ Plus Spectrophotometer (Bio-Rad, USA) and the purity was determined using the absorbance ratios at 260 and 280 nm (range, 1.8–2.0). Each RNA sample was reverse transcribed with an oligo-dT primer using Transcriptor First Strand cDNA Synthesis Kit (Version 6.0; Roche), following the manufacturer's protocol. The RT-qPCR reaction mix was prepared using Lightcycler-Faststart DNA Master SYBR Green 1 and the reaction was performed on Lightcycler480 (Roche Applied Science). The specific primers for the target genes along with HPG and HPA axes (Table S1) were obtained from previous published zebrafish primers sequences (Liang et al., 2015). All primers were designed by using Primer Premier 5.0 program and synthetized by Invitrogen (China). The amplification efficiencies of all primers were between 95% and 107%. The genes assessed in the present study included those that are associated with both the receptor signaling pathways (*pgr*, *ar*, *mr*, *esr1*, *vtg1*, and *gr*) and the steroidogenic pathways (*star*, *cyp17*, *cyp19a1a*, *cyp11b*, *hsd3b*, *hsd20b*, *cyp11a1*, *hsd17b3*, and *hsd11b2*), along with some other target genes, namely *gnrh2*, *crh*, *gnrh3*, *fshb*, *lhb*, and *pomc*). The geometric mean of the expression levels of three housekeeping genes, namely *β-actin*, *rpl13a*, and *ef1a*, was used for normalization of the expression levels of the target genes. The $2^{-\Delta\Delta Ct}$ values were used to relate the expression levels of genes to their corresponding SC group (Han et al., 2014; Livak and Schmittgen, 2001).

2.5. Statistical analysis

The normality and homogeneity tests were performed on Origin 165 (version 8.0, OriginLab, Northampton, MA, USA) software and SPSS (version 13.0, IBM, Chicago, IL, USA). The differences between the groups were analyzed using one-way analysis of variance with specific mean comparisons done by Dunnett's test. Data are presented as means ± standard error (SE). A p value ≤ 0.05 was considered to be significantly different.

3. Results

3.1. Measured concentrations of AED

The measured AED concentrations in the exposure media were 4.0 ± 0.23, 45.0 ± 2.56, 487.0 ± 8.23, and 980.0 ± 9.65 ng/L for the nominal concentrations of 5, 50, 500, 1000 ng/L, respectively (Table S2); thus, the measured concentrations were greater than 80% of their respective nominal values. Androstenedione was not detected in the control medium. This indicated that AED concentrations remained relatively stable throughout the period of exposure. We have referred to the measured concentrations throughout the paper.