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Transcriptional regulatory dynamics of the hypothalamic–pituitary–gonadal axis and its peripheral pathways as impacted by the 3-beta HSD inhibitor trilostane in zebrafish (*Danio rerio*)

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ABSTRACT

To study mechanisms underlying generalized effects of 3β hydroxysteroid dehydrogenase (HSD3B) inhibition, reproductively mature zebrafish (*Danio rerio*) were exposed to trilostane at two dosages for 24, 48, or 96 h and their gonadal RNA samples profiled with Agilent zebrafish microarrays. Trilostane had substantial impact on the transcriptional dynamics of zebrafish, as reflected by a number of differentially expressed genes (DEGs) including transcription factors (TFs), altered TF networks, signaling pathways, and Gene Ontology (GO) biological processes. Changes in gene expression between a treatment and its control were mostly moderate, ranging from 1.3 to 2.0 fold. Expression of genes coding for HSD3B and many of its transcriptional regulators remained unchanged, suggesting transcriptional up-regulation is not a primary compensatory mechanism for HSD3B enzyme inhibition. While some trilostane-responsive TFs appear to share cellular functions linked to endocrine disruption, there are also many other DEGs not directly linked to steroidogenesis. Of the 65 significant TF networks, little similarity, and therefore little cross-talk, existed between them and the hypothalamic–pituitary–gonadal (HPG) axis. The most enriched GO biological processes are regulations of transcription, phosphorylation, and protein kinase activity. Most of the impacted TFs and TF networks are involved in cellular proliferation, differentiation, migration, and apoptosis. While these functions are fairly broad, their underlying TF networks may be useful to development of generalized toxicological screening methods. These findings suggest that trilostane-induced effects on fish endocrine functions are not confined to the HPG-axis alone. Its impact on corticosteroid synthesis could also have contributed to some system wide transcriptional changes in zebrafish observed in this study.

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1. Introduction

Over the past decade there has been an increasing emphasis on the potential harmful effects of endocrine disrupting chemicals (EDCs) on humans and wildlife (WHO, 2002; Diamanti-Kandarakis et al., 2009). Investigation of the role and function of the hypothalamic–pituitary–gonadal axis (HPG-axis), and more specifically, receptors and enzymes involved in steroidogenesis, is critical to an improved mechanistic understanding of chemical effects on endocrine function (Ankley et al., 2009). While the identities of many HPG-axis components targeted by various chemicals are known, information concerning how these genes/proteins function in a wider biological context remains limited. A better understanding of the relationship between the HPG-axis and the transcription factor (TF) networks/signaling pathways it interacts with would facilitate the development of mechanistically based indicators/endpoints and enhance the extrapolation of toxic effects across species and chemical structures. This should provide a basis for a more informative and efficient assessment of EDC exposures, adverse effects, and risks (Ankley et al., 2009).

Conceptually, perturbing the HPG-axis in a targeted manner over a series of experiments can help reveal its transcriptional regulatory dynamics (Ankley et al., 2009). One potential target for perturbation is 3β hydroxysteroid dehydrogenase (HSD3B, EC 1.1.1.145), a well-characterized enzyme catalyzing key steps in formation of corticosteroids and sex steroids (Simard et al., 2005). The HSD3B gene (family) is conserved across vertebrate species

Abbreviations: Cy3, Cyanine 3; Cy5, Cyanine 5; DEG, differentially expressed gene; GSEA, gene set enrichment analysis; E-GSEA, extended-GSEA; EDC, endocrine disrupting chemical; FDR, false discovery rate; GO, gene ontology; HPG, hypothalamic–pituitary–gonadal; HSD3B, 3β hydroxysteroid dehydrogenase; IPA, Ingenuity Pathway Analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; TF, transcription factor

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(Simard et al., 2005) and is typically present as multiple isozymes with tissue-specific expression. Inhibition of this enzyme activity should disrupt steroidogenesis, thereby affecting different biological pathways, including those within the HPG-axis. Indeed, impaired spawning, vitellogenesis, and in vitro steroid production has been demonstrated in fathead minnows (and their tissues; *Pimephales promelas*) exposed to the HSD3B inhibitor, trilostane (Villeneuve et al., 2008). An elucidation of the genes and corresponding TF networks/signaling pathways responsive to HSD3B inhibition could yield significant insights into the transcriptional regulatory control of steroidogenesis and the HPG-axis, and contribute to a better overall understanding of mechanisms of endocrine disruption.

While the impact of HSD3B inhibition on the overall transcriptional regulatory dynamics of steroidogenesis/HPG-axis is not well understood, considerable knowledge exists with regard to TFs and cytokine signaling molecules implicated in the regulation of HSD3B gene expression (Payne and Hales, 2004; Simard et al., 2005; Lavoie and King, 2009). These include interleukin (IL)-4, IL-13, insulin-like growth factor (IGF)-1, members of nuclear hormone receptor family NR4A, NR5A, DAX-1, STAT proteins (signal transducers and activators of transcription; STAT5, 6), epidermal growth factor (EGF), GATA protein family (GATA4, 6), and transforming growth factor TGF-β. A better biological context for understanding the role of these TFs and cytokines, relative to HSD3B specifically and the HPG-axis more broadly, could be established by systematically examining them, along with many other potential TFs and target genes, in a genome-wide framework of interacting TF networks/signaling pathways. Advances in "-omics" technologies and computational biology in the past decade have made construction of such a tentative framework possible in a model species like zebrafish (Wang et al., 2010). In the present study, networks/pathways involved in the functions of steroidogenesis/HPG-axis were explored by perturbing zebrafish via exposure to trilostane, followed by whole genome expression profiling. Trilostane is a relatively specific competitive inhibitor of HSD3B that was originally developed for treatment of Cushing's syndrome in humans (Komanicky et al., 1978; Potts et al., 1978; Touitou et al., 1984).

The data presented here are part of a larger integrated project investigating mechanisms of endocrine disruption using model chemicals with known or hypothesized impacts on HPG-axis function (Ankley et al., 2009). The specific objective of this investigation was, through studying the effect of the model compound trilostane on the transcriptional regulatory dynamics, to identify candidate TFs, HPG-axis members, TF networks, and signaling pathways impacted by exposure to a HSD3B inhibitor. Resulting insights can be used to formulate specific, testable hypotheses for future studies of endocrine disruption and search for mechanistically based molecular indicators. Using previously reverse-engineered TF networks (Wang et al., 2010), the linkage between trilostane exposure and TF networks/pathways impacted was examined by Gene Set Enrichment Analysis (GSEA, Subramanian et al., 2005), Extended-GSEA (E-GSEA, Lim et al., 2009; Wang et al., 2010), and IPA (Ingenuity Pathway Analysis, www.ingenuity.com).

2. Materials and methods

All laboratory procedures involving animals were reviewed and approved by the Animal Care and Use Committee at the US EPA Mid-Continent Ecology Division (Duluth, MN) in accordance with Animal Welfare Act and Interagency Research Animal Committee guidelines. Details and rationale regarding the overall experimental design, zebrafish exposure to trilostane, and gene expression profiling, including microarray data preprocessing and analyses, are presented elsewhere (Ankley et al., 2009; Wang et al., 2010). Only a brief overview is provided here.

2.1. Exposure and tissue sampling

Reproductively mature male and female zebrafish were exposed to a continuous flow of trilostane (two exposure concentrations) dissolved in sand-filtered, UV-treated Lake Superior water (with no solvent), for 24, 48, or 96 h (h). Concentrations of trilostane in these treatments (and a corresponding Lake Superior water control) were determined using high-pressure liquid chromatography with diode-array detection (Villeneueve et al., 2008) on each sampling day. Measured concentrations averaged 488 and 2367 μg trilostane/liter, and no trilostane was detected in the control tanks. At the end of each exposure period, fish were sacrificed in a buffered solution of tricaine methanesulfonate (MS-222; Finquel, Argent, Redmond WA, USA) and gonad, liver, and brain tissues were collected, snap frozen in liquid nitrogen, and stored at −80 °C until extracted for analysis. Total RNA isolated from gonadal tissue samples only was labeled with either Cyanine-5 (Cy5, treated) or Cy3 (control) and hybridized in pairs to individual Agilent two-color 4 × 44k zebrafish microarrays (G2519F, Agilent Technologies, Santa Clara, CA 95051, United States) by an Agilent certified contract laboratory (Cogenics, Morrisville, North Carolina 27560, USA). Microarray data were generated for a total of six treatment (time/exposure) conditions.

2.2. Microarray data analysis

Trilostane data from 30 microarrays, representing six conditions and five replicates each: 24, 48, and 96 h testis high dose, 96 h testis low dose, 96 h ovary high dose, and 96 h ovary low dose, were analyzed for the present investigation. Each treatment condition consisted of ten unique biological samples (five treated and five control), with a treated and control sample hybridized as a pair to a single Agilent two-color microarray. Unless specified otherwise, all expression data were analyzed in the form of $\log_2$ (Cy5/Cy3) ratios. After preprocessing, differentially expressed genes (DEGs) were identified for individual conditions by one class *t*-tests corrected for false discovery rate (FDR; Benjamini and Hochberg, 1995) in GeneSpring GX10 (Agilent Technologies). Where N is the total number of genes and $p(k)$ the kth smallest P-value (out of N sorted from low to high), FDR for gene k is defined as

$$N \times p(k)/k, \quad k = 1 \text{ to } N$$

FDR could be interpreted as the expected fraction of false positives among the genes identified as significant (Benjamini and Hochberg, 1995). The concept of FDR is critical to microarray data analysis due to a large number of genes/tests involved. Due to the relatively small sample size (e.g., $n=5$) per condition (Pawitan et al., 2005), FDRs determined in the present study were widely variable and their cutoffs had to be set between 15% and 80% in order to identify a reasonable number of DEGs for individual treatment conditions. The DEGs with FDRs $>30\%$ were dropped from further analysis. Given the excellent congruence in gene expression determination between quantitative-PCR and microarray in a pilot study (Wang et al., 2008), no additional independent validation of selected DEGs was repeated here.

To lower FDRs and increase confidence in the DEGs identified, DEGs were subsequently discovered by pooling the individual conditions to different degrees: trilostane (all six conditions), trilostane ovary (two conditions), and trilostane testis (four conditions). While this pooling approach may obscure some condition-specific responses (i.e., those observed at a specific dose or time point), the increased statistical power enhanced the detection of genes modulated by trilostane treatment. For an examination of individual DEGs, only those conditions/pooled conditions with FDR cutoffs $\leq 30\%$ and a fold change of treated/control ≥ 1.3 were considered. While selection of 30% FDR threshold was arbitrary, selection of 1.3 as a minimum fold-change criterion was based on the evaluation of the technical noise of the array platform and two-color design used for the present work (Wang et al., 2008). Mapping to orthologous HMR (human–mouse–rat) pathways by IPA was based on DEGs (FDR $\leq 5\%$, treated/control ≥ 1.3 fold) from pooled conditions of trilostane ovary and trilostane testis, at a P-value threshold of ≤ 0.05. Probes (Agilent zebrafish annotation release on June 17, 2007 for designs 013223 and 015064) were first mapped to their human orthologs, which were then searched against Ingenuity Knowledge Base as a reference set for significant associations with HMR pathways.

Besides IPA mapping, several additional approaches were utilized to provide a biological context for the evaluation of the transcriptomic impact of trilostane on zebrafish gonad. First, GO terms associated with individual DEGs, as provided by Agilent in its zebrafish gene annotations, were examined. Second, an enrichment analysis of GO terms among groups of DEGs by various treatment conditions was conducted using GoMiner (http://discover.nci.nih.gov/gominer/htgm.jsp, Zeeberg et al., 2005) at a FDR $\leq 5\%$. And third, previously constructed TF networks, along with a group of compiled HPG-axis genes (Villeneuve et al., 2007) and publically available KEGG pathways (as of October, 2008; Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/kegg), were treated as gene sets and associated with individual trilostane conditions by GSEA and its variant, E-GSEA at a FDR threshold of 25% (Wang et al., 2010). To assess the inter-relationships of selected networks, those determined to be significantly impacted by trilostane exposure were overlaid on a clustering dendrogram composed of the entire set of TF networks/pathways