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Perturbation of gene expression and steroidogenesis with *in vitro* exposure of fathead minnow ovaries to ketoconazole

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Abstract

Ketoconazole is a fungicidal drug that inhibits function of cytochrome P450s in the synthesis of steroids. To examine if inhibition of P450 function affects gene expression in a dynamic manner, we conducted *in vitro* exposures of ovary tissue from fathead minnows (*Pimephales promelas*) to 0.5 μ M ketoconazole to investigate effects on steroid production and gene expression over time. Expression of four key steroidogenesis genes was examined at 1, 6, and 12 h of exposure. 11 β - and 20 β -hydroxysteroid dehydrogenases were down regulated at 1hr and Cytochrome P450 17 was down-

regulated at 12 h, consistent with the absence of steroid production. In contrast, cytochrome P450 19A was up-regulated at 6 h, indicating feedback regulation. Microarray analysis of 12 h exposures indicated enrichment of biological processes involved in neurotransmitter secretion, lymphocyte cell activation, sodium ion transport, and embryonic development. These data suggest that, with the exception of cytochrome P450 19A, these steroid metabolic genes are regulated in a feed forward manner and that the effects of ketoconazole may be broader than anticipated based on the mechanism of action alone.

Keywords: Gene expression profiling; Ketoconazole; Steroidogenesis.

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Ketoconazole (KTZ), an imidazole fungicidal drug, has been found to reduce production of 17 β -estradiol (E2) and testosterone (T) in fathead minnow ovaries *in vitro* and decrease egg production in adult fathead minnow suggesting that it may have potential impacts as an endocrine disruptor on the ecosystem (Villeneuve et al., 2007a; Ankley et al., 2007). Steroidogenesis, or sex steroid hormone synthesis, occurs principally through conversion of cholesterol to the hormones sequentially by cytochrome P450s (CYP) CYP11A, CYP 17 α -hydroxylase, 17,20-lyase (CYP17), and CYP 19 aromatase (CYP19A) in addition to the hydroxysteroid dehydrogenases (HSD) 3 β -HSD, 11 β -HSD, and 20 β -HSD. KTZ inhibits steroid synthesis by binding to the

heme iron of these CYPs (Kan et al., 1985; Weber et al., 1991). KTZ has also been found to affect expression of CYP11A and CYP19A in ovaries of fathead minnows exposed for 21-d and expression of CYP1A1 and CYP3A in rainbow trout liver (Ankley et al., 2007; Hegelund et al., 2004). Given that KTZ inhibits enzyme function over time, it is likely to affect expression of steroid metabolic genes in a dynamic manner along with a larger suite of genes beyond those involved in steroidogenesis. We tested this hypothesis by examining effects on CYP17, CYP19A, 11 β -HSD, and 20 β -HSD gene expression and steroid production over time and global gene expression at 12 h using *in vitro* exposures of fathead minnow ovary tissue to KTZ.

In vitro effects were examined by incubating 12 $\pm$ 5 mg ovary slices from six independent, Six month old, reproductively mature female fish, obtained from stocks at the US EPA, per exposure in culture medium with IBMX (0.1 mM), 25-hydroxycholesterol (1 μ g/ml), and 0.5 μ M KTZ, or solvent control (0.07% methanol) for intervals of 1 to 12 h (McMaster et al., 1995; Villeneuve et al., 2007a). Each fish provide a baseline 10 min exposure in media alone, one exposed and one control replicate per time point. The mean ($\pm$ SD) Gonado/Somatic Index of fish sampled was 14.34 ($\pm$ 3.26) %. E2 and T concentrations in media were measured by radioimmunoassay, normalized to tissue weight and analyzed by one-way ANOVA for treatment effects (Villeneuve et al., 2007b).

RNA was isolated from baseline, 1, 6 and 12 h tissues using Qiagen RNeasy[™] kits (Qiagen, Valencia, CA). Real-time (RT) PCR reactions for CYP17, CYP19A, 11 β -HSD, and 20 β -HSD (Villeneuve et al., 2007b) were performed using 60 ng randomly primed cDNA synthesized from 1 μ g RNA using Superscript III reverse transcriptase

(Invitrogen, Carlsbad, CA). RTPCR targets were amplified in 20 μ l using ABI SYBR Green PCR Master Mix with an ABI PRISM 7900 system (Applied Biosystems, Foster City, CA), quantified relative to control samples using the $\Delta\Delta C_t$ method, and normalized to ribosomal protein L8 gene expression (Applied Biosystems, Filby and Tyler, 2007).

Four individual samples from 12 h KTZ and control incubations were analyzed using 22,000 gene oligonucleotide microarrays (EcoArray, Alachua, FL and Agilent Technologies, La Jolla, CA) according to the manufacturer's recommendations. A reference design was used, where the reference sample of mixed RNAs from both female and male fathead minnows was labeled with Cy3 dye, while treated and control samples were labeled with Cy5 dye. Microarrays were analyzed by ANOVA followed by Tukey's test. Hierarchical clustering was performed on genes identified as differentially expressed ($p < 0.05$) and then z-transformed. Gene Ontology (GO) term enrichment tests were performed comparing the number of differentially expressed genes within a category to all possible genes within the category that were present on the array using Fisher's exact test.

To better understand the effects of KTZ we measured gene expression changes in the ovary concurrent with E2 and T production in the presence of the carrier solvent methanol. KTZ inhibited E2 and T production *in vitro* over time (Fig. 1) consistent with Villeneuve et al. (2007b). Hierarchical clustering demonstrated that exposed tissue is clearly different from controls (Fig. 2A). A total of 2042 genes were differentially expressed with 1744 up-regulated, while 298 were down-regulated at a cut off value of $p < 0.05$. Biological processes enriched by KTZ treatment provide a global view of potential effects of exposure on ovary tissues. The top six GO enriched terms in terms of

numbers of genes and low p values were regulation of neurotransmitter levels (8 significantly changed of 42 possible genes on array, $p=0.0033$), lymphocyte cell activation (6 of 28 genes, $p=0.0064$), sodium ion transport (10 of 71 genes, $p=0.0074$), embryonic development (20 of 200 genes, $p=0.0079$), neurotransmitter secretion (5 of 28 genes, $p=0.0237$), and defense responses to bacteria (3 of 11 genes, $p=0.0307$). These data indicate that the effects of KTZ may be broader than might be anticipated based on the mechanism of action alone.

Expression of key genes involved in steroidogenesis examined at baseline, 1, 6, and 12 h of exposure to KTZ revealed down regulation of HSDs 11β and 20β at 1 hr and CYP17 at 12 h (Fig. 2B). Down regulation of these genes is consistent with a feed forward regulatory model dependent upon the enzymes substrates reduced by KTZ's direct inhibition of enzyme activity. In contrast, CYP19A was up regulated at 6 hrs suggesting a regulatory feed back mechanism. Microarray and RTPCR data sets were in general agreement relative to controls at 12h: CYP17 (0.84 microarray vs. 0.33 RTPCR), CYP19A (0.82 vs. 0.45), 11β -HSD (1.60 vs. 1.08), and 20β -HSD (0.95 vs. 1.24).

Our results provide insight into the local, intraovarian, response to the chemical stressor KTZ. We have shown that the exposure to this compound affects gene expression in a specific and dynamic manner over time consistent with inhibition of CYPs involved in steroid hormone synthesis. Microarray analysis suggests that KTZ may have broader impacts on signaling and development of ovary tissues. Further studies should provide a deeper understanding of the effects of KTZ and other stressors in order to develop computational models that could be used for predictive ecotoxicology.

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Figure Captions

Fig. 1. Estradiol and testosterone production in media normalized to tissue weight for different time points of KTZ exposure (1 to 12h). Mean values $\pm$ SD for six independent ovary slices are shown with significant differences indicated (* $p < 0.1$; ** $p < 0.05$; *** $p < 0.005$).

Fig. 2. (A) Hierarchical clustering of genes significantly changed ($p < 0.05$) in ovaries of fathead minnows exposed to KTZ. (B) Real-time PCR analysis of CYP19A, CYP17, 11 β -HSD and 20 β -HSD expression in ovaries exposed *in vitro* to KTZ relative to expression in unexposed baseline tissues. Mean values $\pm$ SD for six independent ovary slices normalized to Ribosomal protein 18 expression are shown with significant differences indicated (* $p < 0.05$).

fig.1

Fig.2