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Methoxychlor affects multiple hormone signaling pathways in the largemouth bass (*Micropterus salmoides*) liver

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Abstract

Methoxychlor (MXC) is an organochlorine pesticide that has been shown to have estrogenic activity by activating estrogen receptors and inducing vitellogenin production in male fish. Previous studies report that exposure to MXC induces changes in mRNA abundance of reproductive genes in the liver and testes of largemouth bass (*Micropterus salmoides*). The objective of the present study was to better characterize the mode of action of MXC by measuring the global transcriptomic response in the male largemouth liver using an oligonucleotide microarray. Microarray analysis identified highly significant changes in the expression of 37 transcripts ($p < 0.001$) (20 induced and 17 decreased) in the liver after MXC injection and a total of 900 expression changes ($p < 0.05$) in transcripts with high homology to known genes. Largemouth bass *estrogen receptor alpha* (*esr1*) and *androgen receptor* (*ar*) were among the transcripts that were increased in the liver after MXC treatment. Functional enrichment analysis identified the molecular functions of steroid binding and androgen receptor activity as well as steroid hormone receptor activity as being significantly over-represented gene ontology terms. Pathway analysis identified c-fos signaling as being putatively affected through both estrogen and androgen signaling. This study provides evidence that MXC elicits transcriptional effects through the estrogen receptor as well as androgen receptor-mediated pathways in the liver.

Keywords

toxicogenomics; estrogen; androgen; insulin-like growth factor

Introduction

The organochlorine pesticide methoxychlor (MXC) (1,1,1-trichloro-2,2-bis(p-methoxyphenyl)ethane) was used primarily as a substitute for banned DDT in the USA in 1970 until MXC itself was banned for all uses between 2000 and 2003 (US EPA, MXC

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RED Publication No. EPA 738-R-04-010, accessed 2010-07-21). The primary reasons for substituting MXC for DDT were the rapid metabolism of MXC and its lack of bioaccumulation in non-target organisms (Magliulo et al., 2002; Palanza et al., 2001). MXC is metabolized into two estrogenically active metabolites: mono-demethylated 2-(p-hydroxyphenyl)-2-(p-methoxyphenyl)-1,1,1-trichloroethane (OH-MXC) and bis-demethylated 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). In channel catfish (*Ictalurus punctatus*), Stuchal et al. (2006) demonstrated that both CYP1 and CYP3 family enzymes are responsible for hepatic biotransformation of MXC into OH-MXC and HPTE and Blum et al. (2008a) showed that administration of MXC stimulated cytochrome P450, family 3, subfamily A (CYP3A) activity in largemouth bass (*Micropterus salmoides*, LMB) liver by 24 hours after administration. However, despite rapid biotransformation of MXC into these metabolites, there can be adverse biological effects associated with the MXC degradation byproducts.

MXC stimulates expression of estrogen receptor (ER) transcription in the murine uterus similarly to 17 β -estradiol (E₂) (Eroschenko et al., 1996), suggesting that MXC, and/or its breakdown products, are estrogenic. In the liver of channel catfish, MXC is metabolized into the mono- and bis-demethylated metabolites, which bind to and activate ER more effectively than the parent compound but much less effectively than endogenous E₂ (Schlenk et al., 1998). For example, OH-MXC (IC₅₀ = 1.8 μ M) had approximately 43-fold higher affinity for hepatic ER binding than MXC by measuring displacement of 17 β -[³H]-estradiol. In another study in teleost fish, Denny et al. (2005) determined that MXC weakly binds to the ERs of both rainbow trout *Onchorynchus mykiss* and fathead minnow (FHM, *Pimephales promelas*) with approximately 1000-fold less affinity than 17- α ethinylestradiol (EE₂). However, the rainbow trout study was performed with MXC, rather than the relatively more active demethylated metabolites. Overall, these studies suggest that MXC functions as a weak environmental estrogen in both mammals and fish.

There is also evidence to suggest that MXC can also act as an anti-androgen, however most of these data were collected in mammalian studies. *In vitro* evidence includes MXC weakly blocking dihydrotestosterone (DHT) activation of human androgen receptor (AR) (Sonneveld et al., 2005) and OH-MXC and HPTE decreasing both human AR activity and ER β (ESR2) activities while increasing ER α (ESR1) activity (Gaido et al., 2000). In *in vitro* rat studies, HPTE decreases testosterone (T) production in cultured fetal Leydig cells. However, this result is not observed with classic anti-androgens such as hydroxyflutamide and vinclozolin (Muroso and Derk, 2005). A proposed mechanism for decreased T production in mammals is inhibition of P450 side-chain cleavage P450_{scc} (CYP11A1) activity, which has been demonstrated in *ex vivo* rat Leydig cells (Muroso et al., 2006) as well as *ex vivo* rat ovarian cells, where it also decreases progesterone production (Akgul et al., 2008). While limited studies have been performed in fish, it has been demonstrated that MXC decreases T production in LMB cultured ovaries, while having no effect on E₂ production (Borgert et al., 2004).

In addition, acute exposure to MXC negatively impacts normal reproductive processes in fish (Ankley et al., 2001) and mammals (Eroschenko et al., 1996; 2000), as well as birds (Gee et al., 2004) and amphibians (Fort et al. 2004). However, the mechanisms leading to impaired reproduction are not well understood. Waterborne exposure to MXC in teleost fish elicits both physiological and genomic effects similar to endogenous E₂. Adult male sheepshead minnows (*Cyprinodon variegatus*) continuously exposed to greater than 2.5 μ g MXC/L showed a rapid, dose-dependent mRNA and plasma increase in the E₂-responsive egg yolk precursor protein vitellogenin (VTG) (Hemmer et al., 2001). In FHM, exposure to 5.0 μ g MXC/L caused a significant induction of plasma VTG in males (Ankley et al., 2001). In LMB, Blum et al. (2008a) showed that the expression of *vtg1* mRNA in the liver was

significantly increased after 24 hours by intraperitoneal (IP) injections of 2.5, 10, and 25 mg MXC/kg. Furthermore, hepatic *esr1* expression in LMB was significantly increased by 10 and 25 mg MXC/kg treatment after 48 hours. Both *vtg1* and *esr1* mRNA in the liver are highly induced by estrogens and xenoestrogens and increased levels of *vtg1* and *esr1* in response to MXC treatments suggest that MXC is acting as an estrogen in the liver.

The objective of the present study was to further characterize the hepatic response of male LMB to MXC using microarray analysis in order to better elucidate the underlying molecular response affected by this OCP. In order to extract the most informative patterns from genomic data produced in this microarray study, we performed pathway analysis, as well as functional enrichment analysis based on gene ontology (GO) terms. This study has increased the mechanistic understanding of MXC toxicity in the LMB liver and identified steroid production and binding as major biological processes disrupted by MXC.

Materials and Methods

2.1. Experimental animals

All animal care and use procedures were approved by the University of Florida Institutional Animal Care and Use Committee. LMB liver RNA used in this experiment was extracted from fish used in a previous study by Blum et al. (2008a). Briefly, male LMB were treated with a single IP injection of either acetone/sesame oil vehicle (n = 9), 1 mg E₂/kg (n = 9) as a reference estrogen, 2.5 mg MXC/kg (n = 18), 10 mg MXC/kg (n = 18), or 25 mg MXC/kg (n = 18), held in freshwater flow-through tanks, and sampled after 24, 48, or 72 h. One third of the animals from each treatment group were collected at each of the time points. The fish were anaesthetized using buffered tricaine methanesulfonate, (MS-222), and euthanized by blunt head trauma. Dissected livers and testes were immediately frozen in liquid nitrogen and stored at -80°C until RNA was extracted.

2.2. Microarray analysis

Microarray profiling was performed in LMB liver from the 48 hour, 25 mg MXC/kg treatment group. This time point and dose were chosen because Blum et al. (2008a) observed a significant induction of both *esr1* and *estrogen receptor beta (esr2b)* mRNA in male LMB liver after 48 hours, similar to the induction observed after a 1 mg E₂/kg injection. Please note that throughout the manuscript and in an effort to simplify, genes appear in italicized lower case letters and proteins appear in upper case letters. The liver was investigated because it is the major site for detoxification of compounds such as MXC and its metabolites (James et al., 2008) and is the site for the synthesis of VTG prior to translocation to the ovaries for incorporation into the eggs. Therefore, the liver has a major role in the normal reproductive process of female teleost fish of many egg-laying species and the liver can be responsive to estrogens in males after exposure by producing VTG.

2.2 Gene expression profiling in the liver of largemouth bass after IP injection of MXC

The LMB 4 × 44K microarray used in this study (GEO accession #GPL6527) has previously been described and validated (Garcia-Reyero et al., 2008). Gene expression analyses were performed using four biological replicates each from the livers of control and MXC-treated male LMB. Frozen LMB liver was homogenized on ice, and total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA) as per the manufacturer's protocol. RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and total RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the RNA 6000 Nanochip (average RIN = 8.3). Hybridization was performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol using Cyanine 3 (Cy 3) (Agilent publication G4140-90040

v5.7). One microgram of total RNA for each sample was used as starting material. After amplification/labeling and purification, samples were evaluated for yield and incorporation of Cy3 dye using the NanoDrop spectrophotometer. Each sample had a specific activity ≥ 9.0 pmol Cy3/ μ g cRNA. 1.65 μ g Cy3-labeled cRNA was used for microarray hybridizations. Samples were fragmented according to the manufacturer's protocol and hybridized to microarrays for 17 hours at 65°C. Microarrays were washed according to the supplemental Agilent protocol to reduce the susceptibility of the Cy3 dye to ozone degradation, using Agilent's Stabilization and Drying Solution. Microarrays were scanned on the Agilent G2505B Microarray Scanner at 5 μ m resolution, and signals were extracted using the Agilent Feature Extraction Software v9.5. The quality of microarray data were manually evaluated using the quality control reports and were deemed to be of high quality.

Raw expression data were imported into JMP Genomics v3.2 (SAS, Cary, NC). Raw intensity data for each microarray were normalized using Loess normalization with a smoothing factor of 0.2. Following normalization, data were re-evaluated for quality by distribution analysis, correlation analysis, and principal components analysis to ensure microarrays were effectively normalized across individuals. After verification of successful normalization of microarrays, differentially regulated transcripts were identified using one-way analysis of variance (ANOVA), with a False Discovery Rate statistic (FDR=5%) in JMP Genomics (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). Raw microarray data for this experiment were deposited into GEO (accession #GSE12700), in accordance with MIAME standards (Brazma et al., 2001).

Following identification of differentially regulated probes, hierarchical clustering was performed using normalized signals for each of the eight arrays on all 233 unique, annotated probes identified by one-way ANOVA as being significantly different between control and treatment ($p < 0.01$, FDR=5.0%). Twenty-four duplicate probes and 340 probes with no annotation were removed prior to this analysis. Distance calculations were made using the program Cluster (Eisen et al., 1998). Normalized values were mean-centered for each row (gene) and column (array), and complete linkage clustering analysis was performed based on centered correlation. The resulting cluster was visualized using the Java TreeView program (Saldanha, 2004).

2.3 Functional Enrichment and Pathway Studio® analyses of microarray data

Functional enrichment analysis was performed using the Column Enrichment function in JMP Genomics v4. This analysis was used to determine over-represented GO term categories in the MXC-regulated gene set compared to all transcripts on the LMB microarray. All differentially expressed genes with a significance of $p < 0.05$ were used in the functional enrichment analysis and GO terms from this list were compared to the remaining annotated genes on the LMB microarray platform ($p < 0.05$, FDR=5.0%).

In addition to the GO analysis, for transcripts identified as differentially regulated in the microarray study with a significance value of $p < 0.01$, pathway analysis was done using Pathway Studio v7.1 operating on the Resnet7 database (Ariadne Genomics, Rockville, MD, USA) (Nikitin et al. 2003) to further characterize putative cellular processes regulated by MXC. Human homologs for LMB genes were manually obtained by finding the human sequence with the closest match to LMB genes using the BLAST alignment tool in the National Center for Biotechnology Information (NCBI). Entrez Gene identifiers for human homologs were retrieved using the ID mapping service in Pathway Studio to MD Anderson GeneLink (University of Texas, Houston, TX, USA). If a human homolog could not be confidently identified, the LMB transcript was not included in the pathway analysis. First, a pathway was built with all genes with human homologues that were differentially regulated ($p < 0.01$). The program was queried to identify all direct interactions among the gene

products in the list. Second, only the genes identified by hierarchical clustering as having similar patterns of expression to *esr1* and *ar* were increased in mRNA abundance. For this comparison, the program was queried to find all of the shortest known paths between these gene products, as well as links to cellular processes in which they are involved. This was done to gain additional information about whether genes within an expression cluster are involved in common pathways or affect common cell processes.

2.4 Real-time PCR of select differentially regulated genes in the liver

RNA from LMB liver samples was prepared as described in section 2.2. First-strand cDNA was synthesized from 3 µg of DNase-treated RNA using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

The web utility Primer3 (Rozen and Skaletsky, 2000) was used to design primers for real-time PCR. The real-time PCR primer set for LMB *androgen receptor* (*ar*) is previously reported in Garcia-Reyero et al. (2006). Real-time primer sets for *insulin-like growth factor I* (*igf1*), *glutathione S-transferase* (*gst*), and *Cu/Zn superoxide dismutase* (*sod1*) were designed from the following LMB sequences in the gene database maintained by the National Center for Biotechnology Information (NCBI); *igf1* (DQ666526), *gst* (AY335905), and *sod1* (FJ030929). For *igf1* (*Micropterus salmoides*), there were four probes on the microarray that showed a consistent increase in this gene of +2.94 ($p=0.052$), +2.04 ($p=0.069$), +2.52 ($p=0.074$), and +2.32 ($p=0.10$). Therefore, it was chosen for real-time PCR. The decision to investigate *gst* with real-time PCR was based on the oxidative stress response to MXC in the LMB liver after 48 hours, according to the microarray, which identified changes in transcripts such as *manganese superoxide dismutase* (+2.0-fold; $p<0.01$), *gst* (+2.0-fold; $p<0.05$), *catalase* (+2.8-fold; $p<0.05$), and *glutathione peroxidase* (-2.0-fold; $p=0.06$). *Sod1* was not significantly affected based on the microarray data and we tested this transcript to validate a gene that did not show changes in expression levels but was also involved in the general stress response.

Real-time primer sets produced amplicons in the range of 66-268 base pairs (bp) with optimal annealing temperature ~60 °C (Table 1). Amplicons were verified for sequence specificity as per previous methods by Martyniuk et al. (2009). Sanger sequencing was performed at the Interdisciplinary Center for Biotechnology Research (ICBR; University of Florida).

Real-time PCR analysis of gene expression was performed using 1× iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 µl of each gene-specific primer (stock 10 mM), and 100 ng first-strand cDNA derived from DNase-treated RNA samples. Cycling parameters for real-time PCR and method for generation of standard curves for quantitation are provided in Martyniuk et al. (2009). For the genes examined, linearity of eight-point standard curves (1×10^9 through 1×10^2 copies/reaction) showed $R^2>0.991$; efficiencies ranged from 93-108%. Each real-time reaction plate contained negative control samples that included pooled RNA samples ($n=2$) that received no reverse transcriptase during cDNA synthesis to assess efficiency of DNase treatment. In addition, negative control samples ($n=2-4$) that included water instead of template cDNA were used to ensure there was no genomic contamination in real-time PCR reagents.

A one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test for multiple hypothesis testing was performed on log transformed real-time PCR expression data (JMP® Genomics v3.2) ($\alpha=0.05$). All results were plotted as mean $\pm$ SEM of untransformed data ($n=3-6$). Sample size for each treatment group is provided in the figure legend.

Results

3.1 Candidate genes regulated by MXC in the liver 48 hours post-injection

Microarray analysis identified 3039 probes differentially regulated by 25 mg MXC/kg in the liver ($p < 0.05$, FDR=5%), 900 of which were unique (after removing duplicate probes) and had annotation to known genes. The complete list of data is provided in Appendix 1 and provides the LMB ProbeName on the microarray, Gene Title, accession number and e-value for closest sequence matches in the NCBI NR and NT libraries, p-value for ANOVA (FDR-adjusted), and fold change of transcript relative to control. When multiple probes were present for a single transcript (for example, *esr1* was identified as differentially expressed with five probes on the microarray), the fold change reported in this manuscript was calculated from the average of all probes. Table 2 lists the transcripts that were highly significantly altered by MXC in the liver ($p < 0.001$), arranged in order of decreasing fold change. Hierarchical clustering of unique, significant probes ($p < 0.01$) with annotation is presented in Figure 1. Mean-centered intensity values for each spot range from very low relative to row mean (blue) to very high relative to row mean (red) in the figure. Clusters containing *esr1* and *ar* have been enlarged (inset) to identify other genes that show corresponding expression patterns across microarrays in order to identify transcripts that may be regulated by MXC in a similar manner to *esr1* and *ar*.

3.2 Functional enrichment analysis of Gene Ontology (GO) terms

Functional enrichment analysis based on differentially regulated transcripts ($p < 0.05$) showed that the biological processes glycolysis (GO:0006096), prostate gland development (GO:0030850), and sex differentiation (GO:0007548) were over-represented among differentially regulated transcripts in our dataset (Table 3). Additionally, the molecular function GO terms steroid binding (GO:0005496), androgen receptor activity (GO:0004882), and steroid hormone receptor activity (GO:0003703) were over-represented in the list of MXC-regulated genes (Table 3). We identified specific genes from biological processes that included steroid biosynthesis/steroid metabolism or have a molecular function that includes steroid hormone receptor activity or steroid binding (Table 4). Note that the mRNA levels of the nuclear steroid hormone receptors *esr1*, *esr2b*, *ar*, and *glucocorticoid receptor (nr3c1)* were increased by MXC.

3.3 Pathway analysis of differentially regulated genes: Relationship between ER and AR signaling

The results of the pathway analysis indicate a high level of connectivity between *esr1* and *ar*, with a number of direct effects on cellular oncogene *fos (c-fos)*, which is also differentially regulated (Figure 2A). Interestingly, both *esr1* and *ar* mRNA levels are increased while *c-fos* mRNA is down-regulated by MXC treatment at 48 hr. *Esr1* and *ar* each interact with the *proline-, glutamic acid-, and leucine-rich protein 1 (pelp1)*, a known co-activator of human *esr1* (Vadlamudi et al., 2001) that is down-regulated in this study. The clusters containing *esr1* and *ar*, indicate numerous non-genomic routes connecting ESR1 and AR via proteins with which they interact (Figure 2B), indicating that the exact pattern of gene regulation by MXC may be driven by a combination of genes for which transcription changes (red for up and blue for down) and those for which transcription is unchanged (gray).

3.4 Real-time PCR validation of regulated transcripts

We utilized additional liver samples to compare to microarray data and to explore dose- and time-dependent effects of MXC and E_2 treatments on select transcripts. LMB *igf1* mRNA was significantly decreased by 1 mg E_2 /kg at 24 hours post-injection but not at 48 hours.

MXC did not affect transcript levels of *igf1* at 24 hours but significantly induced *igf1* mRNA at all three doses of MXC at 48 hours (2.5, 10, and 25 mg MXC/kg) (Figure 3A). LMB *ar* mRNA was significantly decreased by E₂ treatment at 24 hours. At 24 hours, there was a trend for decreased *ar* expression by 25 mg MXC/kg but this was not significant (p=0.06) (Figure 3B). LMB *gst* mRNA was significantly decreased in the liver following 1 mg E₂/kg at 24 hours post-injection but was not significantly altered by E₂ at 48 hours or by MXC at any time point or dose (Figure 3C). With respect to the Cu/Zn superoxide dismutase, *sod1*, real-time PCR data indicated that there were no significant differences between control and treatment groups at 24 or 48 hour post-injection (Figure 3D), as was the case with the microarray data. Blum et al (2008a) previously reported that both *esr1* and *esr2b* mRNA were significantly increased in the LMB male liver at 48 hours with 25 mg MXC/kg, a finding that is also confirmed by the microarray data. Therefore, there was high correlation between microarray and real-time PCR data.

Discussion

MXC affects the processes of steroid receptor binding/signaling and steroidogenesis

While it is clear that MXC negatively impacts fish reproduction (Ankley et al., 2001; Schlenk et al., 1998) and MXC and/or its metabolites interact with the binding site of fish ERs (Nimrod and Benson, 1997; Blum et al. 2008b), the multiple modes of action by which MXC exerts its effect in fish is unclear. For example, MXC may differ from E₂ in that it is both weakly estrogenic and is also capable of competitive inhibition of more potent ER activators. These microarray data support the hypothesis that MXC can act through ER-mediated pathways and suggests that MXC affects other steroid receptor pathways in the LMB liver, including androgen- and glucocorticoid-receptor mediated pathways.

Transcripts with a role in steroid receptor binding, androgen receptor activity, and steroid hormone receptor activity were significantly impacted by MXC. Other transcripts such as *steroid acute regulatory protein-related (star)* (decreased), *3 β -hydroxysteroid dehydrogenase* (decreased), and *17 β -hydroxysteroid dehydrogenase isoform 8* (increased) are involved in steroid hormone synthesis and were affected by MXC. The role(s) of genes involved in steroid synthesis in the liver is not well understood, and historically, studies have focused largely on the gonad and other reproductive tissues which are the major sites of sex hormone-related steroidogenesis. In adult male rats exposed to MXC, there was a significant decline in the activities of testicular *3 β -hsd* and *17 β -hsd* at 6h as well as reductions in StAR protein at 6 and 12 hours (Vaithinathan et al., 2008). In the male LMB testis, *star* mRNA expression is inhibited in a time-dependent manner by both E₂ and MXC (Blum et al., 2008a). Thus, there may be common gene responses of the steroidogenic pathway in both fish and mammals and in different tissues such as the gonad and liver.

MXC genomic effects are both similar to and different from other xenoestrogens

We observed an increase in *esr1* and *esr2b* expression in the LMB liver. Auto-regulation of ERs expression by estrogens is known and previous data have shown that gene expression profiles can be comparable between MXC and known xenoestrogens. For example, Larkin et al. (2003) examined the effects of MXC and E₂, as well as ethinylestradiol, diethylstilbestrol, *p*-nonylphenol, and endosulfan on mRNA levels via a cDNA macroarray in sheepshead minnow and identified common estrogen responsive transcripts. However, this does not account for the unique responses to individual chemicals in *in vitro* assays and there are almost certainly more difference between the *in vivo* effects of MXC and E₂ than the strength of their binding to ER. It is important to note that transcriptional differences are driven by factors other than just a difference in estrogenic potency and interactions between

MXC and estrogen signaling are complex and likely involve additional molecular pathways, some of which are identified in the present study.

Both gene expression data and transient transfection experiments with LMB ERs in HepG2 cells suggests that MXC has genomic effects in LMB that parallel endogenous estrogens as well as other xenoestrogens (Blum et al., 2008 a,b; Sabo-Attwood et al., 2007). This is based upon data demonstrating that MXC and two of its metabolites, OH-MXC and HPTE, are agonists of LMB ERs driving them to activate gene transcription. However, differences exist in the downstream responses of the LMB ERs to MXC activation. Mammalian steroid hormone receptors also appear to have unique responses to MXC. The direct effects of MXC may be a function of the rate of metabolism of MXC in a particular tissue (i.e. liver versus ovary) resulting in differences in active metabolite production and biological effects. This is supported by a study in channel catfish, *Ictalurus punctatus* in which differences in MXC metabolism between liver and intestine were observed (Stuchal et al., 2006). However, each tissue likely has different co-activators that also affect how the activated ERs function and affect downstream gene expression.

Increased expression of liver androgen receptor mRNA after MXC treatment

It is difficult to predict the effects of endocrine-active compounds over a range of doses and time points. In our microarray study, both *esr1* and *ar* mRNA were up-regulated by a relatively high-dose IP injection of MXC after 48 hours. Noted was that the effect of MXC on steady-state levels of *ar* mRNA varied considerably with both dose and time. Twenty-four hours after the high-dose MXC injection, *ar* mRNA levels were lower than in control fish but greater than in E₂-treated fish. The difference between MXC and E₂ at 48 hours is inconsistent with the idea that MXC acts solely as an estrogen or anti-androgen. At least, this calls to attention the complex differences in downstream effects caused by an increase in the endogenous estrogen E₂ compared to an exposure to a weak xenoestrogen with the potential to antagonize the estrogen receptors.

ESR1 and AR interact directly and indirectly in numerous ways, mostly characterized in mammals. ESR1 is known to negatively regulate AR expression (Frasor et al., 2003). Further, E₂-activated ERs induce transcription of c-FOS signaling (Hennessy et al., 2005). AR is negatively regulated by c-FOS (Bubulya et al., 1996), but the impact of this secondary connection, if any, on *ar* expression after 48 hours in the present study is difficult to predict. PELP1/MNAR is a co-activator of mammalian ER alpha and beta that is also induced by E₂-activated ERs and down-regulated by the anti-estrogen ICI 182,780 (Mishra et al., 2004); the same molecule is known to bind to and increase non-genomic activity of AR leading to activation of MAPK (Haas et al., 2005). In our 48-hour microarray data, no molecule known to directly increase transcription of AR is significantly induced ($p < 0.01$) and activation of ESR1 by MXC would not directly lead to up-regulation of AR. Therefore, it is plausible that 1) AR transcription is directly increasing as a result of MXC acting as an androgen or 2) as a result of a complex temporal signaling cascade stemming from MXC binding estrogen receptors, which is a more likely scenario based on the weight of evidence from previous studies. In particular, given the knowledge that the MXC metabolite HPTE is a partial agonist/antagonist of LMB ESR1 (Blum et al., 2008b), binding of HPTE to ESR1 could theoretically result in decreased transcription of PELP1/MNAR, c-FOS, or other ESR1 targets, as observed in this study. This could alter the signaling events between *esr1* and *ar* expression, leading to the increased *ar* transcription observed after 48 hours. However, this is an untested hypothesis.

Further complicating the interpretation of these results, ESR1 and AR are connected by a number of non-genomic effects. Pathway analysis of transcripts with patterns of expression highly similar to *esr1* and *ar* found connections between some of these transcripts, mostly

through other genes that either were not differentially regulated or were not present on our microarray. Among the many connections identified, note that rat ESR1 and AR, in the presence of estrogen, have been shown to colocalize, possibly forming nuclear heterodimers (Ochiai et al., 2004). This and other binding effects could certainly affect the activity of ESR1 and AR as transcription factors following acute exposure to a partial agonist of ESR1 such as MXC.

Microarray and real-time PCR analysis suggested that LMB *ar* mRNA is differentially regulated by E₂ and MXC. Estrogenic treatments have been shown to modulate *ar* steady state levels in tissues in teleost fish. In FHMs, there are different hepatic genomic responses in fish exposed to 320 µg flutamide/L (an anti-androgen) or 10 ng 17α-ethinylestradiol (EE₂)/L for 21 days. For example, flutamide reduced liver *ar* mRNA in female minnows, but not in males, while EE₂ had no effect on hepatic *ar* mRNA in either sex (Filby et al., 2006). In the same study, flutamide and EE₂ treatments decreased *ar* mRNA equally in the gonad, by 35 percent in males and by 32-42 percent in females. Brain *ar* mRNA levels have been shown to decrease in response to 5, 50, and 500 ng EE₂/L after 7 days in male medaka (*Oryzias latipes*) while being relatively unaffected in the liver after EE₂ treatments (Zhang et al., 2008). An estrogenic or anti-androgenic effect of MXC or its metabolites may be sufficient to indirectly alter *ar* mRNA expression in the liver. However, studies in the gonad of neonatal rats (Muroso and Derk, 2005) and LMB *ex vivo* ovarian cultures (Borgert et al., 2004) also suggest that MXC impairs steroidogenesis based upon a significant reduction in T production. Therefore, the reduction in gene expression and activity of key enzymes for vertebrate steroidogenesis may contribute in part to the declines in T in the vertebrate testis that occur after MXC treatments (Muroso and Derk, 2005) and to the reduction in circulating plasma concentrations of T and E₂ observed in male and female fish after *in vivo* waterborne MXC treatments (Ankley et al., 2001). Reduced circulating T, in turn, might result in the increased expression of *ar* mRNA observed in the present study. While T levels were not measured for these fish, it is known that MXC treatment reduces T at least in the ovary of LMB (Borgert et al., 2004).

MXC increases the expression of hepatic *igf1* mRNA

The gene expression data support the hypothesis that MXC exerts a stimulatory effect on *igf1* transcript levels in the LMB liver. LMB *igf1* was induced between 3-4-fold at all doses of MXC examined after 48 hours. This effect appears to be novel; there is evidence suggesting that estrogens and anti-androgens decrease *igf1* mRNA expression in hepatic tissue of fish whereas androgens increase *igf1* mRNA. In male FHMs exposed to 35 ng E₂/L, there was a significant decrease in *igf1* mRNA of approximately 40% after 14 days of exposure (Filby et al., 2006). In a second study, Filby et al. (2007) exposed male and female FHMs to 320 µg flutamide/L or 10 ng EE₂/L for 21 days. In the liver, both flutamide and EE₂ decreased *igf1* mRNA levels in males by 48% and 7% compared to control animals. In another study, sexually mature female tilapia (*Oreochromis mossambicus*) and E₂-injected male tilapia (5 µg/g) showed decreased mRNA expression of growth hormone receptors (*ghr1* and *ghr2*), *igf1*, and *igf2* (females only) in the liver (Davis et al., 2008). In addition, there was also a significant reduction of approximately 10% in plasma IGF-I in males after E₂ treatments. Taken together, these studies suggest that E₂ and flutamide decrease *igf* mRNA levels in the liver.

In contrast to estrogens, administration of the androgenic compound 17α-methyl-dihydrotestosterone, resulted in an induction in *igf1* mRNA at all doses examined (0.1, 0.7, and 4.9 µg) in the liver of female zebrafish at both 24 and 168 hours (Hoffmann et al., 2008). The increase in *igf1* mRNA in the LMB liver in the present study may be due in part to the anti-estrogenic effects of MXC or its metabolites that have been reported in other studies (Eroschenko et al., 2000; Gaido et al. 1999). Of interest, there is evidence in the

human hepatoma cell line Hep3B transfected with a reporter construct containing the *igf1* promoter that there can be differential regulation of *igf1* expression by the ER subtypes (Fournier et al., 2001). Transfected cells showed increased *igf1* activation from anti-estrogens, such as ICI 182,780, but not for other treatments such as 4(OH)-tamoxifen in combination with E₂. Therefore, the mechanisms by which MXC affects *igf1* mRNA expression are unclear at present and it is difficult to determine if modulation of *igf1* mRNA is due to estrogenic effects of MXC or to a non-estrogenic mode of action.

Conclusion

This study demonstrates that MXC induces genomic responses in the liver that are both similar to, and different from, the molecular responses to the endogenous steroid E₂. Unique gene expression profiles from other weak xenoestrogens compared to E₂ have been previously characterized in teleost fish (Benninghoff and Williams, 2008; Larkin et al., 2002; Moens et al., 2007; Sabo-Attwood et al., 2007). There is evidence to suggest that MXC has estrogenic, anti-estrogenic, and also anti-androgenic activities (Blum et al., 2008b; Eroschenko et al., 1996, 2000; Folmar et al., 2002; Larkin et al., 2003; Schlenk et al., 1998) and in the present study, MXC alters transcript levels associated with multiple receptor-mediated signaling pathways.

Lastly, this study demonstrates that signaling pathways other than steroid hormone receptor-mediated pathways (e.g. c-fos) may also be impacted by MXC. This may result in the unique genomic responses observed with MXC when compared to other xenoestrogens and may account for the MXC effects observed in other aquatic organisms that differ from E₂ effects (Ingermann et al., 1999). However, it could also be a result of cross-talk between hormone signaling cascades, for example between estrogen signaling and growth factor signaling. It is currently difficult to speculate on the interactions occurring between receptor signaling pathways and how these systems are integrated to modulate LMB liver physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hierarchical clustering for each control and MXC microarray sample. Distance calculations were made using Cluster (Eisen et al., 1998) and visualized using Java TreeView (Saldanha, 2004). Clusters containing *esr1* and *ar* are enlarged to identify genes with similar patterns of expression. These genes are labeled with their annotated gene title, or, where no gene title has been assigned, their probe name on the LMB microarray (UF_Msa_AF_#). Note that the color represents intensity of the single dye channel with red being high probe intensity and green being low probe intensity. Steady state mRNA levels of both *esr1* and *ar* transcripts are higher in the MXC cluster than control cluster.

Figure 2.

Pathway Studio® (Ariadne Genomics) was used to identify known relationships among human homologues of the genes identified as differentially regulated by ANOVA. Blue indicates a down-regulation in the gene and red indicates an induction in gene expression. In panel A, all direct relationships were identified between any two differentially regulated transcripts ($p < 0.01$). In panel B, the shortest known connections were identified between any two transcripts from the enlarged clusters from figure 1 (i.e. transcripts regulated in a fashion similar to ER α or AR). Symbols include transcription factors, nuclear receptors, kinases, phosphatases, membrane receptors and ligands.

Abbreviations follow mammalian nomenclature rules (all letters capitalized) because these pathways are primarily based upon mammalian literature: ABL1, c-abl oncogene 1 receptor tyrosine kinase; ACAD11, acyl-Coenzyme A dehydrogenase family, member 11; ACTB, actin, beta; ACTN1, actinin, alpha 1; ADFP, adipose differentiation-related protein; AKT1, similar to protein kinase N2; APOA1, apolipoprotein A-I; AR, androgen receptor α ; AURKB, aurora kinase B; BAZ1A, bromodomain adjacent to zinc finger domain, 1A; CDC20, cell division cycle 20 homolog; DCXR, dicarbonyl/L-xylulose reductase; DDIT3, DNA-damage-inducible transcript 3; DDX5, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5; ENO1, enolase 1, (alpha); ENO2, enolase 2 (gamma, neuronal); ESR1, estrogen receptor 1; FLOT1, flotillin 1; FOS, v-fos FBJ murine osteosarcoma viral oncogene homolog; FOXO3, forkhead box O3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPT, hypothetical protein LOC793673; GTF2B, general transcription factor IIB; HDAC1, histone deacetylase 1; HDAC3, histone deacetylase 3; INS, insulin; LDHB, lactate dehydrogenase B; LEF1, lymphoid enhancer-binding factor 1; MAPK8, mitogen-activated protein kinase 8; MAPK14, mitogen-activated protein kinase 14; MCL1, myeloid cell leukemia sequence 1; MCM2, minichromosome maintenance complex component 2; MYC, v-myc myelocytomatosis viral oncogene homolog (avian); MYLK, myosin light chain kinase; P2RX7, purinergic receptor P2X ligand-gated ion channel 7; PCDH1, protocadherin 1; PDPK1, 3-phosphoinositide dependent protein kinase-1; PELP1, proline glutamate and leucine rich protein 1; PNPO, pyridoxamine 5'-phosphate oxidase; POLR2A, polymerase (RNA) II (DNA directed) polypeptide A, 220kDa; POLR2F, polymerase (RNA) II (DNA directed) polypeptide F; POLR2G, polymerase (RNA) II (DNA directed) polypeptide G; PPARA, peroxisome proliferator activated receptor alpha; RB1, retinoblastoma 1; RNF4, ring finger protein 4; SIRT4, sirtuin (silent mating type information regulation 2 homolog) 4; SLC25A37, solute carrier family 25 member 37; SNAP25, synaptosomal-associated protein 25; SORBS2, sorbin and SH3 domain containing 2; SRPR, signal recognition

particle receptor; STXBP1, syntaxin binding protein 1; TNKS, tankyrase TRF1-interacting ankyrin-related ADP-ribose polymerase; UBE2A, ubiquitin-conjugating enzyme E2A (RAD6 homolog); UXT, ubiquitously-expressed transcript; VDAC1, voltage-dependent anion channel 1; ZNF143, zinc finger protein 143.

Figure 3.

Real-time PCR expression data for A) *igf1* mRNA B) *ar* mRNA C) *gst* mRNA, and D) *sod1* mRNA. In addition to the samples used for microarray analysis, samples for 24 hours (control, n=4; 1 mg E₂/kg, n=3; and 25 mg MXC/kg, n=4) and a dose-response at 48 hours (control, n=4; 1 mg E₂/kg, n=2; and 2.5, 10, and 25 mg MXC/kg, n=4, 4, 6 respectively) were included in the analysis. Asterisk denotes significant difference (p<0.05) based upon ANOVA followed by post hoc Dunnett's pairwise multiple comparison on log transformed expression data.

Table 1

Primers used in real-time PCR assays. Note that the forward primer is given in the 5' to 3' orientation in relation to the 5' to 3' coding strand and the reverse primer is 5' to 3' in relation to the complementary strand.

Gene	Forward 5' to 3'	Reverse 5' to 3'	base pairs
<i>ar</i>	CAC CAC AGA GAA TGT GCC TGA	CAG GTG AGT GCG CCG TAA	66
<i>igf1</i>	TGT GTG GAG AGA GAG GCT TTT ATT	CTT GTC TGC TGC TGT GCT GTC	268
<i>gst</i>	AAC TTT TCG CTG GCT GAT GT	TCT TGT CCC TGT GGG TTC TC	173
<i>sod1</i>	TGG CAA GAA CAA GAA CCA CA	CCT CTG ATT TCT CCT GTC ACC	167
<i>tpst8</i>	CGG CTA CCA CAT CCA AGG AA	CCT GTA TTG TTA TTT TTC GTC ACT ACC T	86

Table 2
Genes differentially expressed in the LMB liver following MXC treatment after 48 hours (p<0.001; FDR=5%).

ProbeName	NR_Accession	GeneTitle	Fold Change	p-value
Up-regulated				
UF_Msa_AF_104788	AAG44622	estrogen receptor alpha (<i>esr1</i>)	11.28	0.0003
UF_Msa_AF_104888	CAG09113	novel protein containing a phosphotyrosine interaction domain	7.96	0.0004
UF_Msa_AF_106753	CAI21213	low density lipoprotein receptor adaptor protein 1	5.05	0.0006
UF_Msa_AF_102841	CAF98203	phosphoribosyl pyrophosphate synthetase 1A	3.16	<0.0001
UF_Msa_AF_107128	AAQ97825	Zgc:63470	2.68	0.0004
UF_Msa_AF_102957	XP_692005	proline synthetase co-transcribed bacterial homolog protein	2.55	<0.0001
UF_Msa_AF_108822	CAF95950	FCH domain only 2	2.47	0.0001
UF_Msa_AM_116969	AAH89254	fdx1l protein	2.43	0.0001
UF_Msa_AF_102970	XP_692484	CG3184-PA	2.03	0.0008
UF_Msa_AF_103496	AAH55669	hypothetical protein Wt:Jf80h11	1.80	<0.0001
UF_Msa_AF_108230	CAF91784	chromosome 21 open reading frame 55	1.79	0.0010
UF_Msa_AM_110869	CAF88943	tetraspanin 13	1.69	0.0005
UF_Msa_AF_107024	CAG08451	hypothetical protein FLJ38663	1.67	0.0009
UF_Msa_AF_104927	CAF99130	butyrophilin, subfamily 3, member A3	1.61	0.0009
UF_Msa_AF_109448	CAF93873	GIY-YIG domain containing 2	1.57	0.0008
UF_Msa_AF_105423	CAF89943	x-prolyl aminopeptidase 1, soluble	1.52	0.0010
UF_Msa_AF_108227	AAH95670	Zgc:112102	1.47	0.0008
UF_Msa_AF_102011	CAG02122	serine peptidase inhibitor, kunitz type, 2	1.43	0.0007
UF_Msa_AF_108095	No Hit	triple functional domain	1.26	0.0009
UF_Msa_AF_102602	CAH73826	hypothetical protein FLJ14547	1.05	<0.0001
Down-regulated				
UF_Msa_AF_107532	AAH44416	ubiquitin-conjugating enzyme E2A	-1.26	0.0008
UF_Msa_AF_104297	XP_707239	ring finger protein 4	-1.34	0.0004
UF_Msa_AF_108375	NP_701289	ubiquitin specific protease 12	-1.37	0.0008
UF_Msa_AF_103737	CAF93254	Zgc:55292	-1.52	0.0007
UF_Msa_AF_101703	CAG10000	cDNA sequence BC021395	-1.55	0.0002
UF_Msa_AF_105439	XP_698729	52 KDa repressor of the inhibitor of the protein kinase	-1.57	0.0008

ProbeName	NR_Accession	GeneTitle	Fold Change	p-value
UF_Msa_AM_111555	NP_001002118	Zgc:86811	-1.60	0.0001
UF_Msa_AM_111169	CAG06267	glyoxylate reductase/hydroxypyruvate reductase	-1.67	0.0002
UF_Msa_AF_109567	BAD98461	RAD51 homolog	-1.79	0.0008
UF_Msa_AM_115244	XP_694020	thyroid hormone receptor interactor 12	-1.88	0.0008
UF_Msa_AF_102993	CAF95833	elongation of Very Long Chain Fatty Acids-like 1	-1.91	0.0001
UF_Msa_AF_110046	CAF93178	zona pellucida glycoprotein 3	-1.98	0.0002
UF_Msa_AM_114357	CAF94428	nucleoporin like 1	-2.08	0.0007
UF_Msa_AF_103691	AAQ94618	zinc finger protein 143	-2.14	0.0006
UF_Msa_AF_100229	CAI51981	proline, glutamic acid and leucine rich protein 1	-2.15	0.0001
UF_Msa_AM_111062	AAH46037	tumor necrosis factor receptor superfamily, member 9	-2.26	<0.0001
UF_Msa_AM_112084	CAG00514	DEAD box polypeptide 52	-2.47	0.0001

Table 3

Functional enrichment analysis for biological process, molecular function, and cellular component (FDR-adjusted $p < 0.05$ for multiple tests). Number regulated is the number of probes in that GO category that were differentially regulated at $p < 0.05$ while number not regulated is the number of probes in that GO category that were not differentially regulated by MXC but present on the LMB microarray. Over/Under indicates whether the term was overrepresented or underrepresented based on Fisher's exact test.

Gene Ontology	GO Term	GO ID	# DR	# not DR	FDR p-value	Over/Under
Biological Process	glycolysis	GO:0006096	26	92	<0.001	over
	embryonic development (sensu Mammalia)	GO:0001701	5	0	<0.001	over
	male gonad	GO:0008584	5	0	<0.001	over
	prostate gland development	GO:0030850	5	0	<0.001	over
	sex differentiation	GO:0007548	6	7	0.021	over
	protein biosynthesis	GO:0006412	31	737	0.022	under
Molecular Function	steroid binding	GO:0005496	14	18	<0.001	over
	oxidoreductase activity...	GO:0016712	11	13	<0.001	over
	androgen receptor activity	GO:0004882	5	0	0.001	over
	general RNA polymerase II TF activity	GO:0016251	6	2	0.001	over
	metal ion binding	GO:0046872	9	15	0.004	over
	structural constituent of ribosome	GO:0003735	21	617	0.006	under
	steroid hormone receptor activity	GO:0003707	13	41	0.010	over
	oxidoreductase activity	GO:0016491	77	635	0.019	over
	phosphopyruvate hydratase activity	GO:0004634	8	18	0.023	over
	carbamoyl-phosphate synthase activity	GO:0004086	5	5	0.023	over
	viral envelope	GO:0019031	10	0	<0.001	over
	transcription factor TFIIF complex	GO:0005674	6	2	<0.001	over
	intracellular	GO:0005622	35	845	0.015	under
Cellular Compartment	phosphopyruvate hydratase complex	GO:0000015	8	18	0.015	over
	ribosome	GO:0005840	17	510	0.015	under

MXC-regulated transcripts ($p < 0.05$) summarized here are involved in biological processes that include steroid biosynthesis or steroid metabolism or have a molecular function that includes steroid hormone receptor activity or steroid binding.

Table 4

UF ProbeName	NR Annotation	Gene	Fold change	p-value
UF_Msa_AF_101900	NP_001017545	nuclear receptor subfamily 1, group H, member 3	-2.9	0.048
UF_Msa_AM_111655	AAW62970	3beta-hydroxysteroid dehydrogenase	-2.0	0.049
UF_Msa_AF_109013	AAK82981	steroid acute regulatory protein-related	-1.6	0.006
UF_Msa_AF_102201	BAD93257	17beta-hydroxysteroid dehydrogenase type 8	1.4	0.036
UF_Msa_AF_101323	CAG07990	sterol O-acetyltransferase	1.6	0.049
UF_Msa_AF_100686	AAU09477	androgen receptor	1.7	0.003
UF_Msa_AF_101561	CAG00687	7-dehydrocholesterol reductase	1.7	0.024
UF_Msa_AM_115234	AAO39210	estrogen receptor beta (esr2b)	2.1	0.037
UF_Msa_AF_108892	XP_699049	transthyretin	2.2	0.036
UF_Msa_AF_100843	AAH76007	sulfotransferase family 1, cytosolic sulfotransferase 3	2.6	0.048
UF_Msa_AF_105079	O73673	glucocorticoid receptor	3.1	0.039
UF_Msa_AF_106753	CAI21213	low density lipoprotein receptor adaptor protein 1	5.1	0.001
UF_Msa_AF_104788	AAG44622	estrogen receptor alpha (esr1)	11.3	<0.001