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Stimulation of transactivation of the largemouth bass estrogen receptors alpha, beta-a, and beta-b by methoxychlor and its mono- and bis-demethylated metabolites in HepG2 cells

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Abstract

The purpose of this study was to determine the mechanisms by which the pesticide, methoxychlor (MXC), acts as an environmental endocrine disruptor through interaction with the three largemouth bass (*Micropterus salmoides*) estrogen receptors (ERs) α , β a, and β b. MXC is a less-environmentally persistent analog of DDT that behaves as a weak estrogen. Using transient transfection assays in HepG2 cells, we have previously shown that each receptor is responsive to the endogenous ligand 17 β -estradiol (E_2) in a dose-dependent manner. The parent compound, MXC, showed dose-dependent stimulation of transcriptional activation through all three ERs. In addition to the parent molecule, each of the metabolites was also estrogenic with all three ERs. The order of potency for ER α and ER β b was HPTE > OH-MXC > MXC, while the opposite order was seen for ER β a. HepG2 cells did not substantially metabolize MXC to the active metabolites, thus the activity of MXC was not due to metabolism. When examining the effects of increasing concentrations of MXC at a fixed concentration of E_2 , all three ERs show increased activity compared to that with E_2 alone, showing that the effects of MXC and E_2 are additive. However, when this experiment was repeated with increasing concentrations of HPTE at a fixed concentration of E_2 , the activity of ER α was decreased, that of ER β b was increased, while that of ER β a was unaffected compared to E_2 alone. These experiments suggest that HPTE functions as an E_2 antagonist with ER α , an E_2 agonist with ER β b and does not perturb E_2 stimulation of ER β a. While it is clear the ER β subtypes are the products of different genes (due to a gene duplication in teleosts) the differences in their responses to MXC and its metabolites indicate that their functions diverge, both in their *in vivo* molecular response to E_2 , as well as to their interaction with endocrine disrupting compounds found in the wild.

Keywords

largemouth bass; estrogen receptors; methoxychlor

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1. Introduction

The estrogen receptors (ERs) are ligand-dependent transcription factors that function to stimulate or repress the expression of target genes [1]. As such, these receptors play pivotal roles in the orchestration of reproductive function and behavior, in particular under the control of endogenous ligands. Chemicals from the environment with the capacity to interact with these receptors may cause disruption to the orderly sequence of events to lead to dysfunction of reproduction. Decreasing sperm counts and fertility, as well as increasing incidences of cancers of the reproductive organs, like the uterus and prostate, have been linked with increasing numbers of synthetic chemicals found in the environment. This has raised concern that these compounds are affecting the health of not only of wildlife species, but also of humans [2].

Methoxychlor (MXC) is an environmental contaminant that interacts with the ERs. MXC is an organochlorine pesticide structurally similar to DDT that was introduced as a less persistent DDT analogue. The structural differences between the molecules, changing of the p,p'-chlorine atoms for methoxy groups, caused a sharp decrease in both the biological and environmental half-lives (from 2-15 years to 120 days) [3,4] of the compound. MXC, and its demethylated metabolites, monohydroxymethoxychlor (OH-MXC) and 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), have been shown to cause ER-mediated signaling disruption in rodents [5-7], frogs [8], and various fish species [9,10]. These effects are thought to occur both through direct interaction with the ERs or indirectly by altering enzymes such as CYP3A, involved in the metabolism of endogenous E₂ [11], or through changes in steroid biosynthesis [12,13] possibly through ER-independent pathways [14,15]. Understanding how the ERs are directly affected has been studied through analysis of E₂ displacement from the ERs by these compounds [16], and through transfection assays using ERs from human [17,18], rat [17], and a rainbow trout chimera [16]. For human and rat ERs, HPTE has been shown to act both as an agonist for ER α and an antagonist for ER β [17]. In addition to its effects on the ERs, HPTE has also been shown to inhibit androgen receptor-mediated transcription [19].

In vivo studies using ovariectomized mice have shown that administration of MXC or its metabolites stimulates uterine hypertrophy in comparison to vehicle or sham-treated controls [20] and the effect is blocked by the ER-antagonist ICI 162,780 [14]. Many studies have shown stimulation of Vtg 1 synthesis in the liver of fish following MXC treatment [9,10,21-24], but these studies have not clearly shown which ER may be causing this effect.

Since it is possible that the effects on uterine growth or Vtg 1 synthesis were the result of changes in other steroid hormones, like estrogens or androgens, known to be mimicked or antagonized by MXC [12,13], it may also be possible that the effects observed for Vtg 1 and CYP3A may have occurred by some other indirect mechanism. So in order to provide more evidence of the link that MXC and/or MXC metabolites may have on gene transcriptional activation through the ERs, it is necessary to show direct stimulation of activity on the ERs. With this focus, the aims of this study were the following: 1) to measure the transactivation of the largemouth bass (LMB) ERs by MXC, along with the MXC metabolites, OH-MXC and HPTE; and 2) to determine whether co-treatment of MXC or HPTE with E₂ affects overall transactivity.

2. Materials and methods

2.1 Chemicals

17 β -estradiol (E₂), MXC, and DMSO were purchased from Sigma. MXC metabolites were synthesized by boron tribromide-catalyzed demethylation of MXC [25,26], and purified by

column chromatography and recrystallization. [^{14}C]-MXC was purchased from Sigma and purified, as described in Stuchal et al. [26].

2.2 Culture and Transfection of HepG2 Cells

HepG2 cells were obtained from the American Type Culture Collection. They were cultured in 100 mm tissue culture plates (Corning) in a humidified incubator at 37°C in an atmosphere of 5% CO_2 :95% air. Cells were maintained in phenol red-free Eagle's Minimum Essential Medium (EMEM) (Sigma) supplemented with 10% normal fetal bovine serum (FBS) (Hyclone), 1 mM sodium pyruvate (Hyclone), 2 mM L-glutamine (Hyclone), and 1% antibiotic/antimycotic (Sigma). Cells were passaged weekly.

One hundred thousand cells were plated per well of a 24-well plate in a total of 500 μl of normal culture medium. Twenty-four hours later the cells were transfected using Fugene 6 (Roche) reagent used at a ratio of 3 μl of Fugene 6 per microgram of DNA. The transfection reactions were performed as directed by the manufacturer.

The reaction mixtures for the transfections were prepared in serum-free medium (SFM) by adding Fugene 6 and the mixture was equilibrated at room temperature for 5 minutes. The DNA (purified using GenElute HP plasmid maxiprep kit, Sigma) was then added to the mixture (0.5 μg of ER expression vector plus 1 μg 2x-ERE-luciferase construct per well [26,27]) and incubated at room temperature for 15 minutes. A control expression vector for renilla luciferase (pRL-TK-renilla, Promega) was made up in a separate tube using 0.2 μg pRL-TK-renilla per well using the same 3:1 ratio as above. Each of the transfection mixtures were made up to a final volume of 100 μl per well.

While the transfection mixtures were incubating, the cell culture medium was aspirated and the cells were rinsed once with Hank's balanced salt solution (Sigma) and then 800 μl culture medium containing charcoal-stripped FBS (CS) medium (CSM) (made up as for normal medium) were added per well. The CS was made by combining dextran-coated charcoal (DCC, 20 g/l, Sigma) and incubating on a shaker overnight at 4°C. The following morning the DCC was removed by filtering through a 0.22 micron cellulose acetate filter unit (Corning).

Each of the transfection mixes was added successively in a dropwise manner to each well (final well volume was approximately 1 ml) and the transfections were allowed to proceed for 18 hours. After the transfection, the medium was removed and replaced with 500 μl CSM containing the respective treatment or vehicle (DMSO) not exceeding 0.1% (for single chemical exposures) or 0.2% (for two-chemical treatments) total volume. The treated cells were cultured for 48 hours at which time the medium was removed, cells rinsed once with PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.4) and then lysed with 100 μl 1X passive lysis buffer (Promega) per well. Cell lysates were then frozen until they were analyzed using Promega's Dual Luciferase Assay System on an Lmax II 384 dual channel luminometer (Molecular Devices).

2.3 In vitro [^{14}C]-MXC Metabolism by HepG2 Cells

HepG2 cells were plated as described for the transfections. The day following plating, the culture medium was removed, and the cell monolayers were rinsed once with HBSS and then cultured with serum-free medium containing 10 μM [^{14}C]-MXC (9.6 mCi/mmol) in DMSO. Forty eight hours following treatment, the culture medium was collected and the cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $\text{Na}_2\text{-EDTA}$, 1% Triton X-100) for 15 minutes. The lysates were collected and the wells were rinsed once with 1 ml PBS to ensure all of the lysate was collected and pooled with the rest of the lysate. Both the media and the lysates were extracted twice with water-saturated ethyl acetate, and the two

extracts were pooled, dried, and dissolved in methanol. The extracts were then applied to silica gel thin-layer chromatography (TLC) plates and separated by a mobile phase of n-heptane:diethyl ether (1:1, vol:vol). The TLC plates were imaged by electronic autoradiography and quantities of each metabolite measured by comparison with known standards [26].

2.4 Statistical Analysis

Each firefly luciferase activity value was normalized to its corresponding renilla luciferase value by computing the ratio of unit firefly luciferase per unit of renilla luciferase. The normalized vehicle means were calculated and used as the denominator to calculate normalized firefly luciferase fold-change for each experiment. The fold-changes between different chemical concentrations were compared by ANOVA. The main effects analyzed by ANOVA were concentration and experiment (each ER was tested in at least three different experiments for each chemical and concentration) as well as for the interaction of these main effects. When the concentration effect was found to be significant by ANOVA ($p < 0.05$), differences between concentrations were determined by Duncan's multiple range test with $\alpha = 0.05$ using the Statistical Analysis System 8.1 [29]. Data are represented as means $\pm$ SEM. Sigmoidal dose-response curves and EC_{50} values were generated using SigmaPlot 9.0.

3. Results

3.1 MXC is an Agonist for all Three LMB ERs

In order to measure the activation of the LMB ERs by MXC, HepG2 cells were co-transfected with each ER individually along with a luciferase reporter construct driven by a 2x tandem repeat of the ERE from the *Xenopus* Vtg A2 promoter. The results of these experiments are shown in Figure 1. Each of the three LMB ERs was responsive in a dose-dependent manner. The magnitude of the activation compared to vehicle was greatest for $ER\beta$, followed by $ER\alpha$, and then by $ER\beta$. The response curves for both $ER\alpha$ and $ER\beta$ did not reach a clear plateau at the concentrations tested, and higher concentrations proved toxic to the cells. $ER\alpha$ and $ER\beta$ each required 1 μ M MXC before becoming distinguishable from vehicle control, while $ER\beta$ was more sensitive, becoming different by 0.5 μ M. The calculated EC_{50} values for each of the receptors were 3.5 μ M for $ER\alpha$ (95% CI of 1.6-7.7 μ M), 0.6 μ M for $ER\beta$ (95% CI of 0.1-2.7 μ M) and 4.5 μ M for $ER\beta$ (95% CI of 2.7-7.5 μ M). In comparison to the EC_{50} values measured for E_2 , which were 0.107 μ M, 0.128 μ M, and 0.135 μ M, for each ER, respectively [28], the EC_{50} values for MXC were approximately 32.8 times higher for $ER\alpha$, 4.8 times higher for $ER\beta$, and 33.4 times higher for $ER\beta$.

3.2. HepG2 cells are Capable of Metabolizing MXC

From the published literature, we were not expecting the response to MXC that was seen in Figure 1. Reports using mammalian ERs [17,18] have shown that it is the demethylated MXC metabolites that are responsible for the estrogenic activity of MXC. In addition to this, it has also been shown that the relative binding affinity of MXC to either human $ER\alpha$ or $ER\beta$ is less than 0.01% of that seen with E_2 [30]. In the present study, the potency of MXC on $ER\alpha$ activation is about 3.1% of that seen with E_2 [28] and about 28.6% of the efficacy. On the other hand, the activation of bass $ER\beta$ (which is more like human $ER\beta$) by MXC also exhibited about 3.0% of the potency of E_2 , but had 59% greater efficacy than E_2 . Since HepG2 cells are liver-derived, we considered if the responses to MXC by the LMB ERs were simply due to demethylation of the parent compound, or if the responses were truly due to MXC itself.

Cells were incubated with 10 μ M [14 C]-MXC (9.6 mCi/mmol) for 48 h and the culture medium was removed from the cells, which were then lysed. After separating the organic phase from the aqueous phase for both the medium and cellular lysates, they were separated by TLC and

quantified. An example from an experiment where the cells were treated in serum-free medium is shown in Figure 2. The figure shows that the HepG2 cells can metabolize MXC and produce both demethylated metabolites. They were also able to export these metabolites out of the cells as most of each metabolite was found in the culture medium (this was also seen in experiments using charcoal-stripped serum medium, data not shown). The measured starting amount of [^{14}C]-MXC added to the cells was 3,300 pmol per well. A net average (quantity found in treated cells minus the amount found in cell-free wells) of 32 pmol of OH-MXC was produced per well, with 62% found in the culture medium, while a net average of 7 pmol of HPTE was produced per well, all of which was found in the culture medium. The percent conversion of the parent compound was 0.97% to OH-MXC and 0.02% to HPTE, representing a total of 0.99% conversion of the parent compound to either estrogenic metabolite in 48 h. Thus, 99.01% of the original parent compound remained, suggesting that the transactivation activity seen by MXC in Figure 1 was due to the parent compound and not due to the metabolites produced by the cells.

3.3. OH-MXC is an Agonist for all Three LMB ERs

Figure 3 shows the results of treating the transfected cells with OH-MXC. As seen with the parent compound, ER β b produced the greatest magnitude of response compared to vehicle, followed by ER α , and ER β a. In order to see a difference from vehicle, ER α and ER β b each required 0.5 μM concentration, while ER β a was not different until the concentration was between 1.0 and 5.0 μM . The EC₅₀ value for each ER was 1.1 μM for ER α (95% CI of 0.63-2.0 μM), 1.6 μM for ER β a (95% CI of 0.11-25.0 μM), and 1.0 μM for ER β b (95% CI of 0.24-4.7 μM).

3.4. HPTE is an Agonist for all Three LMB ERs

Figure 4 shows the results of treatment of the transfected cells with HPTE. As with MXC and OH-MXC, each of the ERs was responsive in a dose-dependent manner. The magnitude of induction was greatest for ER β b, followed by ER α , and then by ER β a. Both ER α and ER β b responses became significantly different from vehicle with 0.1 μM , while ER β a was not different until the concentration reached 5.0 μM . The EC₅₀ value for ER α was 0.3 μM (95% CI of 0.18-0.51 μM), for ER β a the EC₅₀ value was 6.5 μM (95% CI 1.01-41.86 μM), and for ER β b the EC₅₀ value was 0.72 μM (95% CI 0.32-1.64 μM).

3.5. Co-treatment of Transfected Cells with E₂ and MXC is Stimulatory for ER Activity

Since exposure *in vivo* to any endocrine disruptor does not occur on its own, it was of interest to see how the addition of MXC would affect the response of E₂. These data are found in Figure 5. Each of the ERs was stimulated by the addition of increased amounts of MXC. ER α required 0.5 μM to get significantly greater activity than E₂ alone (equal amounts of each ligand) after which no further differences were detected. ER β a required 0.1 μM MXC (20% of E₂ concentration) to get increased transactivation, while increasing MXC beyond this level did not result in further agonist activity. ER β b was also stimulated by the addition of 0.1 μM MXC, which stayed constant until the addition of 5.0 μM MXC (10-fold over E₂) where further stimulation was seen.

3.6. HPTE has Mixed Effects on the ERs when Treated in Combination with E₂

Figure 6 shows how the ERs responded to co-treatment of E₂ (0.5 μM) with increasing concentrations of HPTE. ER α activity showed a decrease in activity when 0.5 μM HPTE was present (equal to E₂ concentration); increasing HPTE further did not result in a further decrease in receptor activity. ER β a was unaffected by the addition of HPTE at any of the concentrations tested. Finally, ER β b activity was significantly increased by the addition of 0.1 μM HPTE

(20% concentration of E₂) and remained steady until the addition of 5.0 μM HPTE (10-fold E₂ concentration) which increased overall activity further.

4. Discussion

Previously, our lab described the presence of three ERs in LMB [31]. In that report, the authors characterized the partial tissue distribution of the three ERs, then known as ERs alpha, beta, and gamma (ERα, ERβb, and ERβa, respectively, in this report). When the DNA-binding domains of the LMB ERs were compared with that of human ERα, it was found that the receptors share greater than 90% homology between the species [28,32]). The consequences of these close homologies would suggest that there would be little difference in DNA-binding site preference based on the DNA-binding domains.

On the other hand, a similar comparison of the ligand-binding domains showed far greater differences. Assuming the ligand-binding domains of the LMB ERs are similarly placed compared to their human counterparts, which share about 59% amino acid identity, LMB ERα has only 58% and 57% conservation with ERβa and ERβb, respectively. The two ERβs are 76% similar to one another [28,32]. The residues important to E₂ binding are 100% conserved with those identified in human ERα [33,34]. Amino acids believed to be involved with binding to other ligands as described by Hawkins et al. [35] do show many differences among the LMB ERs. The homologies suggest similar activation by E₂, but that receptor activation by other types of ligands, like endocrine disrupting compounds may be different.

Determining how MXC and its metabolites interact with the estrogen receptors is important to understand how these molecules are able to cause their deleterious effects *in vivo*. The background knowledge that MXC is able to stimulate the expression and synthesis of Vtg 1 in the liver of LMB treated with MXC by intraperitoneal injection [32,36] is why we chose to use a liver cell model. HepG2 cells were chosen because they are known to express very little, if any endogenous ERs, and yet offer a liver cellular background allowing interactions with other transcription factors. Another advantage of using these cells is that comparison to the work already done in this area with mammalian ERs is readily achieved [17].

We were surprised to find that MXC itself was in fact able to stimulate ER-mediated promoter activity through ERα and ERβb in light of work done using the human and rat ERs where it is clear that MXC itself only had a relative potency of 0.01% to transactivate ERα compared with E₂ [18]. One possibility we considered was that the HepG2 cells could quickly biotransform MXC to its more potent demethylated metabolites, OH-MXC and HPTE. To test this possibility, we measured the capacity of the cells to biotransform MXC. This is the first report to show that HepG2 cells can metabolize MXC. While the cells were indeed capable of producing small amounts of each metabolite, the concentrations produced were not adequate to stimulate the responses seen, compared to studies in which the cells were treated with each metabolite alone. The sum of the concentrations of the metabolites found in the culture medium and in the cells themselves were 64 nM and 13 nM for OH-MXC and HPTE, respectively. These concentrations were about 10% of the concentration necessary to reach the lowest observable effect level for either metabolite. In addition, these concentrations were far lower than the concentrations necessary to reach the EC₅₀ values found for the ERs. Therefore, we are confident that the MXC parent compound, rather than the metabolites resulting from cellular biotransformation, is responsible for the observed transactivation.

We showed that MXC, in combination with E₂, produces clear additive effects for each ER, with ERβb (the receptor which appears to function more similarly compared to human ERβ) showing greater sensitivity than the other receptors. Gaido et al. [18] showed that MXC was an agonist of human ERα when used alone, but when the HepG2 cells were co-treated with

E₂, along with increasing concentrations of MXC, they found it was an antagonist, with a tendency to decrease overall activity. They also showed that MXC was not an agonist of ER β activity when used alone or in combination with E₂ (0.1 μ M).

When co-treating the transfected cells with HPTE and E₂, ER α decreased in activity as the amount of HPTE was increased, while the activity of ER β b increased compared to E₂ alone. We did not see any effect on ER β a, probably because the maximum concentration of HPTE used in these experiments fell below the calculated EC₅₀ value of 6.5 μ M for this receptor. In a similar experiment using the human ERs, Gaido et al. [18] found that HPTE added in combination with E₂ (0.1 μ M) caused a stimulation of ER α activity, whereas with human ER β they saw a dramatic decrease in receptor activity. This difference in effects may be due to the differences found among the LMB ERs and the human ERs in their ligand-binding domains or other regions important for both ligand recognition as well as dimerization with other co-activators required for ER activity.

With regard to the EC₅₀ values determined for the LMB ERs, the EC₅₀ of MXC for ER α activity is about 3.5 μ M (all EC₅₀ values for LMB ERs are summarized in Table 1). Lemaire et al. [37] reported a similar EC₅₀ of 4.0 μ M using a cell line derived from HeLa cells that was stably transfected with human ER α . They did not determine a value for human ER β . Legler et al. [38] stably transfected a 3x repeat of an ERE-luciferase reporter in T47D (breast cancer tumor) cells and reported an EC₅₀ of about 5.7 μ M. There appears to be a lack of information in the literature on EC₅₀ values reported for MXC activation of human ER β activity, since most reports find little to no activity from this chemical. There are no other reported EC₅₀ values for fish ERs tested with MXC.

For the demethylated metabolites, we found EC₅₀ values that are within the range reported for human ERs. The EC₅₀ of OH-MXC on LMB ER α was about 1.1 μ M. Gaido et al. [18] found an EC₅₀ value for human ER α to be about 0.2 μ M, suggesting that human ER α is more sensitive to this metabolite than LMB ER α . LMB ER β yielded an EC₅₀ value of about 1.6 μ M, which is similar to the estimated EC₅₀ value of about 1.3-2.0 μ M from Figure 2B of Gaido et al. [18]. The EC₅₀ value for HPTE activation of LMB ER α was about 0.3 μ M, a value similar to the 0.51 μ M reported by Gaido et al. [18] for human ER α . No reports of an EC₅₀ value for activity through human ER β can be found in the literature.

Upon direct comparison of the EC₅₀ values for the LMB ERs, we found that for both ER α and ER β b, an increase in potency comes with increasing degree of demethylation: HPTE>OH-MXC>MXC. Conversely, the potency series for ER β a was MXC>OH-MXC>HPTE. The biological meaning for this difference is not immediately clear. Data from the co-transfection experiments in Sabo-Attwood et al. [28], suggest that both LMB ER β s can interact with ER α and modulate its activity. In those experiments, ER β a led to a greater degree of decrease in activity than was seen with ER β b. Perhaps one of the functions of ER β a *in vivo* in the liver is to regulate ER α activity as a repressor, rather than as a transcription factor in its own right. It may have been an evolutionary advantage for teleost fish (other fish groups have not been reported to have a second ER β) to limit ER α activity in this way, perhaps even to limit the effects of non-endogenous ligands taken up by the fish from its environment. However, the LMB ERs appear to have distinct expression levels in different tissue types [31]. This idea would need further study to obtain an accurate measure of the ERs in various tissues at the protein level in the fish, but the idea of combinatorial regulation of the function of the ERs is an interesting one. Further study with other xenoestrogens, both *in vitro* and *in vivo*, to measure the interactions that occur among the ERs themselves and other transcriptional regulators is necessary. Additionally, the use of ER ligands other than E₂ can be diagnostic of structural differences among the receptors and may be used to understand how they differ in function.

Potentially, the use of knock-out or knock-down experiments using transgenic fish could be one way of ascertaining the true functional role of each ER in fish.

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Figure 1.

MXC-stimulated transactivation of the LMB ERs. HepG2 cells were transfected with each ER along with a reporter luciferase construct driven by a 2x tandem repeat of the Vtg A2 ERE. Data are means of the fold-change in luciferase activity versus vehicle (DMSO) control $\pm$ SE from three independent experiments (n=3 per experiment). A) Activation of ER α . B) Activation of ER β a. Inset is a re-plot of the data on a different scale to better visualize the dose response. C) Activation of ER β b. Differences between activities for MXC concentrations were determined by Duncan's multiple range test.

Figure 2.

Representative image of the TLC plates run following treatment of HepG2 cells with [^{14}C]-MXC. The cells were treated with a concentration of 10 μM [^{14}C]-MXC for 48 h. Following treatment the culture medium was collected and the cells were lysed and both the culture medium and cell lysates were extracted with water-saturated ethylacetate. The organic phase was dried the residue was dissolved in methanol and spotted onto the TLC plates. The metabolites were separated with a mobile phase of n-heptane:diethylether (1:1). OH-MXC was found in both the cellular and medium phases while HPTE was only found in the medium.

Figure 3.

OH-MXC-stimulated transactivation of LMB ERs. HepG2 cells were treated following transfection. Data are means of the fold-change in luciferase activity versus vehicle control $\pm$ SE from three independent experiments ($n=3$ per experiment). A) Activation of ER α . B) Activation of ER β a. Inset is a re-plot of the data on a different scale to better visualize the dose response. C) Activation of ER β b. Differences between activities for OH-MXC concentrations were determined by Duncan's multiple range test.

Figure 4. HPTE-stimulated transactivation of the LMB ERs. Data are means of the fold-change in luciferase activity versus vehicle control $\pm$ SE from three independent experiments ($n=3$ per experiment) using HepG2 cells. A) Activation of ER α . B) Activation of ER β a. Inset is a re-plot of the data on a different scale to better visualize the dose response. C) Activation of ER β b. Differences between activities for HPTE concentrations were determined by Duncan's multiple range test.

Figure 5.

MXC and E₂ cotreatment effect on ER activation in HepG2 cells. Following transfection, HepG2 cells were treated as described in materials and methods. Data are means of the fold-change in luciferase activity versus vehicle alone $\pm$ SE from three independent experiments (n=3 per experiment). A) Additive effects on ER α . B) Additive effects on ER β a. C) Additive effects on ER β b. Differences between treatment groups were determined by Duncan's multiple range test.

Figure 6.

HPTE and E₂ cotreatment of HepG2 cells with HPTE and E₂. Data are means of the fold-change in luciferase activity versus vehicle alone $\pm$ SE from three independent experiments (n=3 per experiment). A) Antagonistic effects on ER α . B) Effects on ER β a. C) Additive effects on ER β b. Differences between treatment group were determined by Duncan's multiple range test.

Table 1EC₅₀ values for transactivation of LMB ERs by MXC and its metabolites (μM)

	ERα	ERβa	ERβb
MXC	3.5 (1.6-7.7)	0.6 (0.1 – 2.7)	4.5 (2.7-7.5)
OH-MXC	1.1 (0.7-2.0)	1.6 (0.1-25.0)	1.0 (0.2-4.7)
HPTE	0.3 (0.2-0.5)	6.5 (1.0-41.9)	0.7 (0.3-1.6)

Values in parentheses are the 95% confidence intervals of the EC₅₀ concentrations