Effect of Methoxychlor and Estradiol on Cytochrome P450 Enzymes in the Mouse Ovarian Surface Epithelium

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Although the ovarian surface epithelium (OSE) is responsive to hormones and endocrine-disrupting chemicals, little information is available on the metabolizing capabilities of the OSE. Thus, we tested the hypothesis that the OSE is capable of expressing genes regulating phase I metabolism of estrogen and the estrogenic endocrine disruptor methoxychlor (MXC). To test this hypothesis, we isolated mouse OSE cells and cultured them with vehicle (dimethylsulfoxide; DMSO), 3 μM MXC, or 0.1 μM 17β-estradiol (E2) ± the anti-estrogen ICI 182,780 (1 μM) for 14 days. After culture, the cells were subjected to quantitative real-time polymerase chain reaction for cytochrome P450s (CYPs) 1A1, 1B1, 2C29, and 1A2, and estrogen receptor α (ERα). Our results indicate that E2 and MXC did not alter the expression of CYP1A1 or CYP1A2. In contrast, E2 significantly increased expression of CYP1B1 compared to controls (DMSO = 0.93 ± 0.1, E2 = 3.12 ± 0.64 genomic equivalents (GE), n = 4, p ≤ 0.01). The E2-induced increase in CYP1B1 was abolished by co-treatment with ICI 182,780 (0.41 ± 0.17 GE). MXC treatment did not affect CYP1B1 expression. Both MXC and E2 increased expression of CYP2C29 (DMSO = 0.02 ± 0.003; MXC = 0.04 ± 0.008; E2 = 0.46 ± 0.03 GE, n = 4, p ≤ 0.05). MXC- and E2-induced elevations in CYP2C29 were abolished by co-treatment with ICI 182,780 (0.02 ± 0.005; 0.02 ± 0.07 GE). In addition, E2 increased ERα expression 15-fold compared to controls (DMSO = 1.10 ± 0.09, E2 = 15.0 ± 3.60 GE, n = 3, p ≤ 0.05), and ICI 182,780 abolished the E2-induced increase in ERα expression (1.85 ± 1.09 GE). MXC treatment did not affect ERα expression. These data indicate that the OSE expresses enzymes known to metabolize native and xenoenestrogens and that MXC and E2 modulate expression of some of them through ER-linked mechanisms.

Key Words: ovarian surface epithelium; methoxychlor; estradiol; cytochrome P450; estrogen receptor.

INTRODUCTION

The ovarian surface epithelium (OSE) is a distinct layer of cuboidal cells with mesothelial lineage. It serves as a protective barrier that separates the ovary from the peritoneal cavity. Several lines of evidence indicate that the OSE is a functioning component of the reproductive tract with attributes of an estrogen-responsive tissue. First, the rodent OSE has been shown to contain estrogen receptor alpha (ERα) by immunohistochemistry (Pelletier et al., 2000) and by real-time polymerase chain reaction (PCR) analysis (Symonds et al., 2005). Second, the OSE has been shown to proliferate in response to estrogen in the rabbit (Bai et al., 2000) and in response to the xenoestrogen methoxychlor (MXC) in the mouse (Symonds et al., 2005). Third, the OSE is known to undergo proliferative changes in the vicinity of the ovulation site via a mechanism that may be hormonally coordinated (Osterholzer et al., 1985). In addition, the OSE has the potential to undergo morphologic transformation to an endometrial phenotype in response to hormones (Okamura et al., 2003) and to transform to neoplastic Mullerian phenotypes such as morphologically typical endometrial carcinoma (Auersperg et al., 2001).

Ovarian toxicants have the ability to target specific cell types in the ovary differentially. For example, MXC has been shown to induce proliferation of the OSE (Symonds et al., 2005), but to cause atresia of antral ovarian follicles in the mouse (Borgeest et al., 2004; Miller et al., 2005). The reasons for the differential effects of MXC on ovarian cell types are unknown, but they might be the result of distinct receptor activities in ovarian cell types: MXC may exhibit agonist effects when it binds to ERα, the predominant ER isoform found in the OSE, but it may exhibit antagonistic effects when it binds to ERβ, the predominant ER isoform found in the ovarian follicle (Gaido et al., 1999). Alternatively, these effects might be the result of different metabolic capacities of ovarian cells. Several studies indicate that phase I cytochrome P450 (CYP) enzymes are important in the metabolism of estrogens (Hayes et al., 1996; Lee et al., 2003) and xenoestrogens such as MXC (Stresser and Kupfer, 1998). The estrogenic metabolites that result from CYP metabolism may have different biologic activity than the parent compound, and thus they may exert varying degrees of toxicity in ovarian tissues.

Despite the potential for the OSE to respond to toxicants in a variety of ways, very little is known about the metabolic capabilities of the OSE. Specifically, little is known about which CYP enzymes are present in the OSE. Further, little is...
known about whether CYP enzymes in the OSE can be induced or inhibited by estrogens and xenoestrogens and, if so, whether CYP induction/inhibition occurs through estrogen receptor (ER)-linked mechanisms. Therefore we used an OSE isolation technique (Symonds et al., 2005) to test the hypothesis that the OSE is capable of expressing enzymes with known ability for phase I metabolism of estrogen and the estrogenic endocrine disruptor MXC. We treated OSE cells with 17β-estradiol (E2) and MXC, and we measured the expression of several CYP enzymes using real-time PCR. We elected to measure CYP1A1 and CYP1B1 expression because these CYPs are known to metabolize estrogen (Lee et al., 2003). We also chose to investigate CYP2C29, the murine homolog of CYP2C19, which, with CYP1A2, is active in human metabolism of MXC (Stresser and Kupfer, 1998). In addition, we treated parallel isolates simultaneously with each agent and the anti-estrogen ICI 182,780 to assess whether the ER mediated any changes in CYPs in response to estradiol or MXC.

MATERIALS AND METHODS

Animals. Female FVB mice were used in all experiments. The mice were maintained at the University of Maryland Central Animal Facility, provided food and water ad libitum, and euthanized prior to all experiments between postnatal days (PD) 60 and 90. Temperature was maintained at 22 ± 1°C, and animals were subjected to 12-h light/dark cycles. The University of Maryland School of Medicine Institutional Animal Use and Care Committee approved all procedures involving animal care, euthanasia, and tissue collection.

Primary cultures of OSE cells. Primary cultures were isolated using previously reported techniques (Symonds et al., 2005). In brief, ovaries were excised from mice aseptically and individually placed in 0.5 ml of Medium 199(E) (BioSource, Rockville, MD) with 500 units of crude collagenase (Type XI, Sigma-Aldrich Inc., St. Louis, MO). After 60 min of incubation at 37°C, each ovary was vortexed for 2 min to remove the ovaries, and the suspension was vortexed again for 2 min to disaggregate cell clusters. The suspension was diluted with additional Medium 199(E) containing 15% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) and an anti-microbial solution (10 μg/ml) containing 10 mg/ml streptomycin, 10 μg/ml penicillin G, and 25 μg/ml amphotericin (Sigma-Aldrich Inc.). Aliquots containing 200–500 cells/100 μl from a single ovary were pipetted into 96-well plates (Corning Costar Corp, Cambridge, MA) with frequent mixing during aliquoting. Control and treatment wells were from the same suspension. Cultures were incubated at 37°C with 5% CO2 for 3 days to allow for attachment.

Treatments of cultures. After cell attachment, cultures were incubated with 150 μl of Medium 199(E) containing 15% fetal calf serum, anti-microbial solution, and one of the following: vehicle (dimethylsulfoxide: DMSO); ICI 182,780 (1 μM); MXC (3 μM); MXC (3 μM) + ICI 182,780 (1 μM); 17β-estradiol (0.1 μM) or 17β-estradiol (0.1 μM) + ICI 182,780 (1 μM). Incubation was carried out for 14 days, with renewal of incubation solution every third day. Purified MXC was obtained from ChemService (West Chester, PA). ICI 182,780 was obtained from Tocris-Cookson (Ellisville, MO), and 17β-estradiol was obtained from Sigma-Aldrich. Doses of MXC and ICI 182,780 were based on our previous study, which indicated that the selected dose of MXC increases OSE proliferation and inhibits OSE apoptosis, and the selected dose of ICI 182,780 inhibits the ability of MXC to induce proliferation of the OSE (Symonds et al., 2005). The dose of 17β-estradiol was based on published work indicating that the selected dose affects cell growth in vitro (Bai et al., 2000).

RNA isolation for real-time polymerase chain reaction (PCR). Cells grown for 14 days with the above treatment schedule were used for RNA isolation. Total RNA was isolated using the RNeasy procedure from Qiagen (Valencia, CA) according to the manufacturer’s protocol. Cells were detached in lysis buffer by scraping with a pipette tip. The lysates from six wells were collected and pooled. Homogenization was performed with a QiShredder column (Qiagen) and a syringe containing a 21-gauge needle. Reverse-transcriptase generation of cDNA was performed with 0.4 μg of total RNA using an Omniscript RT kit (Qiagen) with random primers according to the manufacturer’s protocols. Subsequent PCR analysis was carried out on 3 μl of the cDNA product as described below.

Real-time PCR analysis of CYP1A1, CYP1B1, CYP1A2, CYP2C29, and ERs. Real-time PCR analysis was performed as previously described (Symonds et al., 2005) using a MJ Research (OPTICON) real-time PCR machine and accompanying software according to the manufacturer’s instructions (MJ Research, Waltham, MA). The OPTICON quantifies the amount of PCR product generated by measuring a dye (SYBR green) that fluoresces when bound to double-stranded DNA. A standard curve was generated from five serial dilutions of purified PCR product. Primer sequences and temperature conditions are listed in Table 1. For each primer set, a melting curve was performed. An initial incubation of 95°C for 10 min was followed by 40 cycles of melting (T1), annealing (T2), and extension temperature (T3), each at 10-s intervals, followed by a final extension (T4) at temperatures as described in Table 1. Primers for CYP1A1 (Zhang et al., 2003) and CYP1B1 (Xu and Miller, 2004) were identified and used as published in BLAST (www.ncbi.nlm.nih.gov). Primers for CYP1A2 were designed using Blast 2 sequence analysis comparing human CYP1A2 (NM_000761) and mouse CYP1A2 (NM_009993). Primers for CYP2C29 were identified through a search in PrimerBank (http://pga.mgh.harvard.edu/primers/index.html) (Wang and Seed, 2003). The PrimerBank identification number for mouse CYP2C29 is 31982435a1, based on GenBank Accession Number NM_007815. Mouse CYP2C9 (NM_007815) was found to be 79% homologous with human CYP2C19 (NM_007699), a CYP enzyme responsible for MXC metabolism in the human liver (Stresser and Kupfer, 1998). Expression values were calculated for the experimental samples based on a standard curve. β-Actin mRNA was measured in each sample and used to normalize ratios between samples. Data were collected from at least three independent experiments. Results are expressed in normalized β-actin ratios (genomic equivalents; GE) ± standard error.

Statistical analysis. Data were analyzed using SPSS statistical software (SPSS, Inc., Chicago). Analysis of variance (ANOVA), followed by a Scheffe post hoc test, was used for multiple comparisons between treatment groups. Data are presented as means ± standard error of the mean. A p value ≤ 0.05 was considered significant.

<p>| TABLE 1 | Polymerase Chain Reaction Conditions |</p>
<table>
<thead>
<tr>
<th>CYP</th>
<th>F/R</th>
<th>Primer sequence</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
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<tr>
<td>CYP1A1</td>
<td>F 5′ agatcaggaggagaactagac 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>78°C</td>
<td>82°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5′ ccctcctgctacttccgtgc 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>78°C</td>
<td>82°C</td>
<td></td>
</tr>
<tr>
<td>CYP1B1</td>
<td>F 5′ tggaccctagaggagaactc 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>78°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5′ ggtgctctgtagggagag 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>78°C</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
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<td>57°C</td>
<td>72°C</td>
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<tr>
<td></td>
<td>R 5′ ctgagctgtgacctcgacg 3′</td>
<td>94°C</td>
<td>57°C</td>
<td>72°C</td>
<td>77°C</td>
<td></td>
</tr>
<tr>
<td>CYP2C29</td>
<td>F 5′ atcgcctgctactcagcg 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>78°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5′ aagttcagccagcccaaatc 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>78°C</td>
<td></td>
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<tr>
<td>ERα</td>
<td>F 5′ aactgcagcatactgagcgc 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>78°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 5′ gttcctcaactctcctcctc 3′</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>78°C</td>
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Note: F = forward; R = reverse; T1 = melting temperature; T2 = annealing temperature; T3 = first extension temperature; T4 = final extension temperature.
RESULTS

E2, MXC, and ICI 182,780 treatment did not significantly alter expression of CYP1A1 compared to DMSO controls (DMSO = 0.31 ± 0.07; MXC = 0.41 ± 0.14; E2 = 0.56 ± 0.09; ICI 182,780 = 0.46 ± 0.28; MXC + ICI 182,780 = 0.11 ± 0.03; and E2 + ICI 182,780 = 0.30 ± 0.08 genomic equivalents (GE), n = 3). Similarly, E2, MXC, and ICI 182,780 treatment did not significantly alter the expression of CYP1A2 from control levels (DMSO = 1.03 ± 0.24; MXC = 1.05 ± 0.24; E2 = 1.06 ± 0.37; ICI 182,780 = 1.16 ± 0.43; MXC + ICI 182,780 = 1.32 ± 0.41; and E2 + ICI 182,780 = 1.14 ± 0.40 GE, n = 4).

In contrast, E2 treatment markedly increased expression of CYP1B1 compared to controls (DMSO = 0.93 ± 0.1, E2 = 3.12 ± 0.64 GE, n = 4, p < 0.01; Fig. 1). In addition, co-treatment with E2 and ICI 182,780 abolished the E2-induced increase in expression of CYP1B1 (0.42 ± 0.17 GE; Fig. 1). MXC did not affect the expression of CYP1B1 compared to DMSO (Fig. 1).

Both MXC and E2 significantly increased the expression of CYP2C29 compared to DMSO, but the increase in CYP2C29 was much greater with E2 treatment than with MXC treatment (DMSO = 0.02 ± 0.003; MXC = 0.04 ± 0.008; E2 = 0.46 ± 0.03 GE, n = 4, p < 0.05; Fig. 2). In both MXC- and E2-treated cells, ICI 182,780 abolished the increased expression of CYP2C29 (Fig. 2).

E2 treatment increased ERα expression 15-fold compared to controls (Fig. 3). Moreover, co-treatment with E2 and ICI 182,780 abolished the E2-induced increase in expression of ERα (1.85 ± 1.09 GE; Fig. 3). MXC treatment did not affect ERα expression (Fig. 3).

DISCUSSION

This study demonstrates that the mouse OSE expresses CYP enzymes (CYP1A1, CYP1B1, CYP1A2, CYP2C29) that are capable of metabolizing E2 and MXC. It also demonstrates that E2 significantly increases the expression of CYP1B1 and that both E2 and MXC significantly increase the expression of CYP2C29. Further, this study indicates that E2- and MXC-induced increases in CYP expression occur via ER-linked...
mechanisms. The reproductive consequences of altered CYP expression, however, may be significantly different for E2 and MXC. In the case of estrogen, CYP1B1 catalyzes hydroxylation, which leads to decreased estrogenic activity. Further action of CYP1B1 results in the formation of a catechol estrogen that may have serious toxicological effects (Tsuchiya et al., 2004). In the case of MXC, phase I metabolism by CYP2C19 results in the formation of estrogenic derivatives of MXC, namely the mono- and bis-demethylated derivatives (Stresser and Kupfer, 1998). Demethylation of MXC is necessary to confer estrogenic characteristics (Ousterhout et al., 1981).

In the mouse OSE, we found that E2 upregulates CYP1B1, but not CYP1A1. These data differ from those reported for the human OSE, in which exposure to E2 did not alter expression levels of CYP1A1 or CYP1B1 of non-neoplastic cells (Leung et al., 2005). The reasons for the different findings in our study compared to human studies are unknown, but such variations may stem from species differences in CYP expression and responsiveness. Further, treatment of human OSE in the latter report was much shorter (24 h) than in our studies, which may account for differences in expression of CYP1A1 and CYP1B1 mRNA.

Our findings related to CYP1B1 expression are similar to those of Tsuchiya et al. (2004), who showed that E2 induces CYP1B1 expression in MCF-7 cells (an ERα-positive cell type), and that E2 regulation of CYP1B1 was controlled by ERα. We showed that E2 induces CYP1B1 and that the upregulation of CYP1B1 by E2 is abrogated by co-treatment with ICI 182,780. Interestingly, we found that E2 upregulates the expression of CYP2C29 more strongly than MXC, a finding that may be explained by the ability of E2 to regulate several related gene families simultaneously (Waters et al., 2001).

The ability of the OSE to upregulate CYP1B1 and ERα in response to E2 stimulation may have implications for ovarian carcinogenesis. Multiple lines of evidence implicate estrogen in the neoplastic transformation of the OSE (Ho, 2003). Catechol derivatives of estrogen, formed by action of CYP1B1, have been found to be carcinogenic in the Syrian hamster kidney, possibly by forming quinones, which can generate reactive oxygen species (Yager, 2000). Mutagenic 8-oxoguanine adducts of DNA found at the time of ovulation repair have been implicated as potential initiating events in ovarian carcinogenesis (Murdoch and McDonnel, 2002). Our results indicate that the OSE has the ability to convert E2, but not MXC, to metabolic products capable of initiating this sequence. The OSE may be particularly vulnerable to such toxic by-products because of the formation of inclusion cysts shaped by sequestered islands of epithelial cells at the time of ovulation, thus exposing epithelial cells to metabolites for a prolonged period.

In conclusion, this work indicates that the mouse OSE expresses CYP enzymes known to be capable of metabolizing native estrogens as well as xenoestrogens. Furthermore, this work shows that regulation of specific CYP enzymes (CYP1B1 and CYP2C29) by estradiol or methoxychlor occurs through ER-linked mechanisms. Further studies should examine whether the selected CYP enzymes and their ability to metabolize estrogens play a role in ovarian carcinogenesis.

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