The pesticide methoxychlor (MXC) is a reproductive toxicant that targets antral follicles of the mammalian ovary. Cytochrome P450 enzymes metabolize MXC to mono-OH MXC (1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane [mono-OH]) and bis-OH MXC (1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane [HPTE]), two compounds that are proposed to be more toxic than the parent compound, can interact with the estrogen receptor (ER), and are proposed to be responsible for ovarian toxicity. Thus, this work tested the hypothesis that MXC metabolites may be responsible for inducing antral follicle–specific toxicities in the ovary and that this toxicity may be mediated through ER-regulated pathways. Mouse antral follicles were isolated and exposed to mono-OH (0.01–10 μg/ml), HPTE (0.01–10 μg/ml), or MXC (100 μg/ml) alone or in combination with ICI 182,780 (ICI; 1μM) or 17β-estradiol (E2; 10 and 50nM) for 96 h. Follicle diameters were measured at 24-h intervals. After culture, follicles were morphologically evaluated for atresia. Both mono-OH and HPTE (10 μg/ml) inhibited follicle growth and increased follicle atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia. The antiestrogen, ICI, did not protect antral follicles from MXC or metabolite toxicity in regard to follicle growth or atresia.
toxicity may be mediated through ER-regulated pathways. Using an in vitro follicle culture assay, we exposed antral follicles to dose-response concentrations of mono-OH and HPTE and evaluated follicle growth and atresia. In addition, we investigated the potential role of ER in mediating the toxicity of MXC and its metabolites in antral follicles through cotreatment with the antiestrogen ICI 182,780 (ICI) to block the effect of these chemicals and protect against the chemically induced follicle growth inhibition and atresia, or cotreatment with E2 to observe whether the added E2 would override the toxicity of MXC and its metabolites and support follicle growth and cell survival. Our results show that MXC metabolites do have a toxic effect on antral follicle growth and atresia and that E2 regulation of ER pathways may play a role in mediating the ovarian toxicity of MXC and its metabolites.

MATERIALS AND METHODS

Chemicals. MXC (99% pure) was purchased from ChemService (West Chester, PA). Mono-OH and HPTE were purchased from Cedra Corporation (Austin, TX). Stock solutions of MXC, mono-OH, and HPTE for in vitro dosing were prepared using dimethylosulfoxide (DMSO) (Sigma, St Louis, MO) as a solvent and in various concentrations (0.0133, 0.133, 1.33 mg/ml) that allowed an equal volume to be added to culture wells for each treatment group to control for solvent concentration. Final concentrations of MXC, mono-OH, and HPTE in culture were 100, 10, 1, 0.1, and 0.01 μg/ml (ppm). These doses were selected for in vitro studies because they have been shown to affect proliferation and gene expression in ovarian cells and uterine leiomyoma cells (Chedrese and Feyles, 2001; Hodges et al., 2000). In addition, we have observed that MXC causes antral follicle atresia in vivo at concentrations of 32 and 64 mg/kg/day (32 and 64 ppm/day), 10 and 100 μg/ml MXC compromises follicle growth in culture, and 100 μg/ml MXC causes antral follicle atresia in culture (Borgeest et al., 2002, 2004; Miller et al., 2005). The selected in vitro doses are in the same range as the previously used in vivo doses. The in vitro doses are also environmentally relevant in that environmental levels of MXC range from 40–160 ppm in waters downstream of MXC-sprayed areas (Wallner et al., 1969) to 0.1–4.0 ppb/day exposures of humans via the diet (Agency for Toxic Substances and Disease Registry, 2002). ICI was obtained from Tocris-Cookson (Ellisville, MO). Stock solutions of ICI were prepared in DMSO (2mM) for a final dose in culture of 1μM. E2 was obtained from Sigma. Stock solutions of E2 were prepared in DMSO (20 or 100μM) for a final dose in culture of 10 or 50nM. Concentrations of ICI and E2 were chosen based on dose-response evaluations performed in our laboratory (Symonds et al., 2006; Barnett and Flaws, unpublished results). Unless otherwise specified, all reagents were obtained from Sigma.

Animals. Female mice in a FVB background were used from breeding colonies currently maintained by our laboratory at the University of Maryland Central Animal Facility. Mice were housed (five animals per cage) at the University of Maryland Central Animal Facility and provided food and water ad libitum. Temperature was maintained at 22 ± 1°C, and animals were subjected to 12L:12D cycles. The University of Maryland School of Medicine Institutional Animal Use and Care Committee approved all procedures involving animal care, euthanasia, and tissue collection.

Follicle culture. Female mice were sacrificed on postnatal days 30–35 and their ovaries removed. Antral follicles were isolated mechanically from the ovary based on relative size and cleaned of interstitial tissue using fine watchmaker forceps. Sufficient numbers of antral follicles for experimental significance were isolated from unprimed mouse ovaries; follicles from 2–4 mice were isolated per day with approximately 20–25 antral follicles from each mouse. Upon isolation, follicles were placed individually in wells of a 96-well culture plate with unsupplemented α-minimal essential media (α-MEM) prior to treatment. Each experiment contained a minimum of eight follicles per treatment. Supplemented α-MEM was prepared with 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml human recombinant Follicle-stimulating hormone (Dr A. F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA), and 5% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA). A dose-response regimen of mono-OH (0.01–10 μg/ml), HPTE (0.01–10 μg/ml), and DMSO controls was independently prepared in supplemented α-MEM with an equal volume of chemical added for each dose to control for the amount of vehicle in each preparation. For cotreatment experiments, ICI (1μM) or E2 (10 or 50nM) was prepared alone and in combination with MXC (100 μg/ml), mono-OH (10 μg/ml), or HPTE (10 μg/ml) in supplemented α-MEM. For treatment, unsupplemented α-MEM was removed from each well and replaced with 150 μl supplemented α-MEM containing vehicle or chemicals of interest. Follicles were then incubated for 0–96 h at 37°C in 95% air and 5% CO2. DMSO concentrations in all experiments were kept below 0.075% for dose-response follicle growth experiments and less than 0.125% for coinubation experiments using ICI and E2. These levels that solubilized MXC, mono-OH, and HPTE in aqueous media without overt changes in growth or atresia.

Analysis of follicle growth. Antral follicles were cultured as described above for 96 h. Follicle growth was examined at 24-h intervals by measuring follicle diameter on two perpendicular axes with an inverted microscope equipped with a calibrated ocular micrometer. Antral follicles were considered as those having diameters of 200 μm or greater (Smits and Cortvindt, 2002), which correlates with the histological appearance of these follicles. At least three separate culture experiments were performed for each chemical treatment. Follicle diameter measurements were averaged among treatment groups and plotted to compare the effects of chemical treatments on growth over time.

Histological evaluation of atresia. At the end of each follicle culture, supplemented α-MEM was removed from each well and Dietrick’s solution was immediately added to fix follicles. Follicles were fixed for at least 24 h in Dietrick’s solution and transferred in histology cassettes to 70% ethanol. The tissues were dehydrated, embedded in Paraplast (VWR Scientific, West Chester, PA), serially sectioned (5 μm), mounted on glass slides, and stained with Weigert’s hematoxylin and methyl blue-picric acid. Each follicle section was examined for level of atresia as evidenced by the presence of pyknotic bodies and reported at the highest level observed throughout the tissue. Follicles were rated on a scale of 1–5 for the presence of pyknotic bodies: 1 = healthy; 2 = less than 10% pyknotic bodies (early); 3 = 10–30% pyknotic bodies (mild); 4 = greater than 30% pyknotic bodies in an isolated area (late); and 5 = greater than 30% pyknotic bodies over the entire follicle (late and widespread). Representative photographs of atresia ratings have been previously published (Miller et al., 2005). In addition, follicles were evaluated based on size: small follicles (200–349 μm starting diameter) versus large follicles (350 μm and larger starting diameter). This was done to compare atresia in small and large follicles in response to MXC because studies indicate that small and large antral follicles may respond differently to toxicants (Hirshfeld, 1988; Roby, 2001). At least three separate culture experiments and atresia analyses were performed for each chemical treatment. Ratings were averaged and plotted to compare the effect of chemical treatments on atresia levels.

Statistical analysis. All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). For all comparisons, statistical significance was assigned at p ≤ 0.05. For multiple comparisons between DMSO and MXC-, mono-OH-, and HPTE-treated follicles and E2- or ICI-cotreated follicles, we used analysis of variance, along with a Tukey post hoc test, multiple regression analysis, or Mann-Whitney nonparametric tests. At least eight follicles per treatment per growth experiment were evaluated, and the results of at least three separate experiments were combined for data analysis.
RESULTS

Exposure of mouse antral follicles in vitro to MXC (10 and 100 µg/ml) has been shown to inhibit follicle growth after 72 and 96 h in culture and increase atresia over control levels after 96 h in culture (Miller et al., 2005). To investigate whether metabolites of MXC would induce the same toxicities, we exposed antral follicles in vitro to either mono-OH or HPTE (0.01–10 µg/ml) and evaluated their growth over time and resulting atresia. Mono-OH significantly inhibited antral follicle growth over control levels at both 72 and 96 h after exposure (Fig. 1A). In addition, mono-OH (10 µg/ml) increased atresia of small and large antral follicles over control levels (Figs. 1B and 1C). HPTE (10 µg/ml) only inhibited antral follicle growth at 96 h postexposure (Fig. 2A) and increased atresia in small antral follicles but not large antral follicles (Figs. 2B and 2C).

To determine whether MXC or its metabolites induce toxicity in antral follicles via an ER-regulated mechanism, we cotreated antral follicles in culture with the antiestrogen ICI (1µM) or E2 (10 or 50nM) and MXC (100 µg/ml), mono-OH (10 µg/ml), or HPTE (10 µg/ml) and evaluated follicle growth and atresia. Antral follicles treated with ICI alone grew in culture in a manner similar to controls; however, ICI cotreatment with MXC, mono-OH, or HPTE did not reverse the growth inhibition due to MXC, mono-OH, or HPTE and still inhibited follicle growth without any significant difference from chemical alone at all time points examined (0–96 h) (Figs. 3A–3C). As for atresia, ICI alone did not induce atresia over controls in either small or large antral follicles and levels remained comparable to DMSO (Figs. 4A–4F). ICI cotreatment with MXC or mono-OH did not change the levels of follicle atresia in either small or large antral follicles compared to atresia levels with chemical alone.

Antral follicles treated with E2 alone (10 and 50nM) grew in culture in a manner similar to controls, however, E2 cotreatment with MXC, mono-OH, or HPTE was not sufficient to reverse the growth inhibition due to MXC, mono-OH, or HPTE and still inhibited follicle growth without any significant difference from chemical alone at all time points examined (0–96 h) (Figs. 5A–5C). E2 (10nM) was able, however, to reduce the amount of atresia induced by MXC in small antral follicles after 96 h and, at a concentration of 50nM, was able to reduce the amount of atresia induced by mono-OH in small antral follicles after 96 h (Figs. 6A and 6C). E2 did not have any effect on the amount of atresia induced by HPTE in these experiments (Figs. 6E and 6F).

FIG. 1. Effect of in vitro mono-OH exposure on antral follicle growth and atresia. Antral follicles were exposed in vitro to 0.01–10 µg/ml mono-OH for 96 h. (A) Antral follicle growth analysis over 96 h (n = 23–30 follicles per treatment). (B) Small antral follicle atresia after 96 h mono-OH exposure (n = 5–7 follicles per treatment). (C) Large antral follicle atresia after 96 h mono-OH exposure (n = 14–22 follicles per treatment). DMSO = dimethylsulfoxide vehicle control. OH = mono-OH. Graphs represent means ± SE from three separate experiments. *p ≤ 0.05. **p ≤ 0.001.

DISCUSSION

The present studies were conducted to test the hypothesis that MXC metabolites may be responsible for inducing antral follicle–specific toxicities in the ovary and that this toxicity may be mediated through ER-regulated pathways. To begin, we have shown that the MXC metabolites mono-OH and HPTE are both directly toxic to antral follicles, similar to the toxicity found with the parent compound (Miller et al., 2005). Mono-OH inhibits antral follicle growth to the same degree as MXC at both 72 and 96 h, while HPTE also inhibits antral follicle
growth, but only after 96 h exposure. In addition, mono-OH induces atresia of small and large antral follicles in the same manner as MXC but at a lower concentration (10 μg/ml mono-OH vs. 100 μg/ml MXC), while HPTE (10 μg/ml) induces atresia of small antral follicles only. Therefore, one possible mechanism of MXC toxicity in the ovary could be through
metabolism of the parent compound by the liver and/or ovary to these metabolites that would then cause toxicity.

While both metabolites cause antral follicle toxicity, our results indicate that mono-OH may be slightly more toxic than HPTE in our antral follicle culture system as follicle growth inhibition occurs at an earlier time point, and the rating of atresia in small antral follicles tended to be slightly higher with mono-OH exposure than HPTE. Specifically, HPTE 10 exposure only induces atresia in small antral follicles at ratings between 2.1 and 2.4, which indicate 10% pyknotic bodies or less, while mono-OH 10 induces atresia in this same population of follicles at ratings between 3.2 and 4.0, which indicate 10–30% or higher pyknotic bodies. Many studies have primarily focused on HPTE as the primary toxic metabolite of M XC.

![Graphs showing the effect of various treatments on atresia ratings.](image)

**FIG. 4.** Effect of in vitro ICI cotreatment with MXC, mono-OH, and HPTE exposure on antral follicle atresia. Antral follicles were exposed in vitro to MXC (100 µg/ml), mono-OH (10 µg/ml), or HPTE (10 µg/ml) for 96 h either alone or in combination with ICI (1 µM). Small (A) and large (B) antral follicle atresia after 96 h MXC and ICI exposure (n = 9–13 follicles per treatment). Small (C) and large (D) antral follicle atresia after 96 h mono-OH and ICI exposure (n = 7–13 follicles per treatment). Small (E) and large (F) antral follicle atresia after 96 h HPTE exposure (n = 7–13 follicles per treatment). DMSO = dimethylsulfoxide vehicle control. M = MXC. OH = mono-OH. H = HPTE. Graphs represent means ± SE from three separate experiments. *p ≤ 0.05. **p ≤ 0.001.
(Bulger et al., 1978; Gaido et al., 1999, 2000; Waters et al., 2001); therefore, the finding that mono-OH may be more toxic than HPTE in our system is novel. HPTE has been shown to have a higher affinity for ER than mono-OH or MXC and has been primarily investigated as responsible for the endocrine-disrupting capabilities of MXC (Bulger et al., 1985; Gaido et al., 1999, 2000; Ousterhout et al., 1981). Studies by Ohyama et al. (2005), however, help to support our findings of the toxicity of mono-OH as they have shown that there is sex-specific metabolism of MXC. In rat liver slices, MXC is primarily metabolized to mono-OH versus HPTE in female rats (60/40 mono/bis ratio), while in male rats, MXC is primarily metabolized to HPTE versus mono-OH (5/95 mono/bis ratio). Female rodents may then be more susceptible to mono-OH toxicity since this is a primary metabolite formed upon MXC metabolism in the female rodent liver. Alternatively, many of the aforementioned studies with HPTE were performed in uterine tissue and HepG2 cells, possibly indicating tissue-specific toxicity of mono-OH versus HPTE. In a study by Waters et al. (2001), differential HPTE-regulated gene expression was observed in the uterus versus ovary. Therefore, it is possible that mono-OH may regulate genes with a greater effect than HPTE in the ovary.

MXC has been shown to be an ERα agonist and ERβ antagonist (Gaido et al., 1999, 2000), and HPTE has been shown to regulate a variety of genes through ER mechanisms (Waters et al., 2001). Therefore, we examined whether the inhibition of antral follicle growth and increase in atresia that we observe in antral follicles upon MXC and metabolite exposure were mediated by ER mechanisms and could be blocked by cotreatment with ICI or E2. The antiestrogen ICI was unable to protect antral follicles treated with MXC, mono-OH, or HPTE from decreased follicle growth in culture and was also unable to protect MXC- and mono-OH–exposed antral follicles from atresia. Thus, it appeared that the toxicity of MXC could not be reduced by an ERα- or Eβ-mediated mechanism. When antral follicles were cotreated with E2, along with MXC, mono-OH, or HPTE, there was no protection from decreased follicle growth in culture; however, E2 was able to protect MXC- and mono-OH–exposed small antral follicles from atresia. As a result, E2 is able to inhibit MXC and metabolite toxicity in small antral follicles, though the mechanism may not be ER dependent as we had initially hypothesized. It is important to note that we do not think that the metabolism of MXC is compromised in the presence of E2 as E2 does not significantly inhibit CYP activities in human liver microsomes exposed to MXC in culture (Laine et al., 2003). Moreover, treatment of male channel catfish with E2 does not affect the conversion of MXC to mono-OH or HPTE, indicating that E2 exerts its effect downstream of metabolism (Schlenk et al., 1997).

One possible mechanism of estrogen-regulated ER-independent MXC toxicity could involve E2 regulation of apoptotic pathways through nongenomic mechanisms. In

---

**FIG. 5.** Effect of *in vitro* E2 cotreatment with MXC, mono-OH, and HPTE exposure on antral follicle growth. Antral follicles were exposed *in vitro* to MXC (100 μg/ml), mono-OH (10 μg/ml), or HPTE (10 μg/ml) for 96 h either alone or in combination with E2 (10 or 50nM). (A) Antral follicle growth analysis over 96 h MXC and E2 exposure (n = 22–24 follicles per treatment). (B) Antral follicle growth analysis over 96 h mono-OH and E2 exposure (n = 23–24 follicles per treatment). (C) Antral follicle growth analysis over 96 h HPTE and E2 exposure (n = 31–32 follicles per treatment). Data are expressed as change in diameter from 0 h time point. DMSO = dimethylsulfoxide vehicle control. OH = mono-OH. H = HPTE. Graphs represent means ± SE from three to four separate experiments. *p ≤ 0.05. **p ≤ 0.001.
support of this mechanism, MCF-7 breast cancer cells exposed to E2 showed increased levels of antiapoptotic Bcl-2 protein without any changes in apoptotic Bax protein levels (Kandouz et al., 1996). In addition, E2 has also been shown to phosphorylate cAMP response element–binding protein to then upregulate bcl-2 through a phosphatidyl inositol 3-kinase/Akt–dependent pathway (Honda et al., 2001). This is further supported by evidence that E2 regulation of bcl-2 gene expression does not require direct ER binding to the promoter region but involves E2-induced activation of cAMP (Dong et al., 1999). These nongenomic mechanisms of E2-regulating apoptotic signaling are highly likely since we have previously shown that overexpression of Bcl-2 in the ovary protects from MXC-induced growth inhibition and atresia in antral follicles.
(Miller et al., 2005). A second possible mechanism of toxicity through ER-independent pathways may be involved also in that E2 possesses antioxidant activity (Behl et al., 1995). E2 could be acting as an antioxidant to reduce cell injury and apoptosis caused by the generation of oxidative stress through MXC metabolism in the ovary (Latchoumycandane and Mathur, 2002).

In conclusion, these studies have shown that the MXC metabolites mono-OH and HPTE directly inhibit antral follicle growth and induce atresia, and that this toxicity may be mediated by estrogen-regulated pathways. Future studies to investigate the protection from MXC toxicity by nongenomic actions of E2 will be helpful in developing ways to prevent toxicity by similar environmental chemicals and preserve ovarian health and fertility.

ACKNOWLEDGMENTS

The authors would like to acknowledge funding by NIH RO1 ES012893, NIH R21 ES1306, T32 ES07263-13, and a Colgate-PalmoIve Postdoctoral Fellowship in In Vitro Toxicology. In addition, the authors would like to thank Janice Babus for her assistance with histology and Lynn Lewis for her assistance with formatting the manuscript.

REFERENCES


