Methoxychlor Disrupts Uterine Hoxa10 Gene Expression

Xiaolan Fei, Hajin Chung, and Hugh S. Taylor

Division of Reproductive Endocrinology, Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale University School of Medicine, New Haven, Connecticut 06520

Methoxychlor (MXC) is a pesticide that has adverse effects on reproductive capability in mice. MXC and its metabolites bind the estrogen receptor and function as endocrine disruptors. MXC diminishes the uterine decidual cell response, necessary for the support of pregnancy. Hoxa10 is an estrogen-regulated gene that is an essential mediator of the decidual response and necessary for pregnancy. Here we demonstrate that a mechanism by which MXC disrupts uterine function is by suppressing Hoxa10 expression. MXC treatment of mice produced a mild uterotropic response as measured by increased uterine weight and epithelial height. MXC treatment of uterine Ishikawa cells in vitro induced Hoxa10 expression. Estrogen receptor (ER) binding to the HOXA10 estrogen response element (ERE) was promoted by treatment with estradiol (E2); however, MXC disrupted E2/ER/ERE complex formation and gel shift. MXC alone allowed weak ER/ERE binding. In vivo MXC blocked the effect of E2 on Hoxa10 expression. Neonatal MXC treatment resulted in an immediate suppression and cellular restriction of Hoxa10 expression as well as a permanent generalized decrease in expression that persisted in the adult. MXC inhibited the expression of Hoxa10, a gene necessary for uterine development and function. One common mechanism by which endocrine disrupting chemicals produce lasting reproductive tract defects is through permanent alteration of developmental gene expression. (Endocrinology 146: 3445–3451, 2005)
MXC has both ER agonist and antagonist properties in vivo. MXC is metabolized in vivo into mono- and bisphenol demethylated derivatives that demonstrate estrogenic activity in vivo. Hydroxylation also occurs in some cell types in vitro.

Prenatal or postnatal MXC exposure in mice impairs the female reproductive system; the ovaries are atrophic or cystic, whereas the uterus is hypertrophied and lacks the normal decidual response necessary for implantation (27, 28). Because Hox genes are necessary for decidualization, we examined the uterine response to MXC and its effect on Hoxa10 expression in adult female and newborn mice; we also examined the effect of in vitro treatment on HOXA10 expression in Ishikawa cells.

Materials and Methods

Chemicals

Methoxychlor was purchased from Sigma-Aldrich (St. Louis, MO).

Animals

CD-1 mice have previously been shown to be an accurate predictor of developmental effects of DES in humans (29). Nulliparous reproductive-age female CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were housed in a temperature-controlled room (22 C) with a 14-h light, 10-h dark cycle. Animals were housed in standard polycarbonate mouse cages. Food (Purina Chow, Purina Mills, Richmond, IN) and water were provided ad libitum. All mice weighed 20–25 g. The Yale University Animal Use and Care Committee approved all animal experiments.

For the uterotrophic assay, on d 0 (1 week after arrival), bilateral ovariectomy (O VX) was performed via a single mid-dorsal incision. On d 14 (2 wk after bilateral ovariectomy), the mice were randomly divided into four groups. The groups received MXC (1 mg/d) dissolved in DMSO; EA 2.4 µg/kg-d; DES 40 µg/kg; or vehicle only. All injections were intraperitoneal. For histological analysis, sections of the miduterus were fixed in formalin; 5-µm tissue sections were cut and stained with hematoxylin and eosin. Tissue sections were evaluated for epithelial cell height using a microscope (Olympus Corp., New Hyde Park, NY). Cell height was determined by obtaining three measurements from five areas from five mice.

For neonatal exposure CD-1 mice were treated with MXC (2 or 0.2 mg/kg-d) or vehicle control by ip injection from d 1 until d 14. Analysis was performed on 2-wk-old and 8-wk exposed female offspring.

Animals were killed by cervical dislocation under anesthesia using ketamine/xylazine (75:25 mg/kg). Uteri were removed, blotted, and weighed. Uteri were then fixed in 4% formalin and embedded in paraffin for histological and immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical analysis of Hoxa10 expression was performed as previously described (30). Slides were deparaffinized and dehydrated through a series of xylene and ethanol washes, followed by permeabilization in 95% cold ethanol. After a 5-min rinse in distilled water, slides were steamed in 0.01 M sodium citrate buffer for 20 min and cooled for 20 min. Slides were rinsed for 5 min in PBS with 0.1% Tween 20 (PBST), and sections were circumscribed with a hydrophobic pen. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min followed by a 5-min PBST wash. Nonspecific binding was blocked with 1.5% normal horse serum in PBST for 1 h at room temperature. The primary Hoxa10 antibody (sc-17159) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Slides were incubated in the primary antibody overnight at 4 C. Normal goat IgG (Santa Cruz Biotechnology) was used as a negative control. Horse α-goat biotinylated secondary antibody was purchased from Vector Laboratories (Burlingame, CA) and applied for 1 h at 4 C. Slides were washed in 1× PBST, incubated in ABC Elite (Vector) for 15 min at room temperature, washed in 1× PBST, and incubated for 5 min in diaminobenzidine (Vector). A 20-sec exposure to hematoxylin was used as a counterstain. All slides were processed simultaneously. Slides were rehydrated through 3-min ethanol and xylene washes and mounted with Permount. Each group consisted of at least four mice.

Fluorescence microscopy

Uteri were fixed with 4% paraformaldehyde for 10 min at room temperature. After three washes with PBS cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and washed again. For Hoxa10 staining, sections were incubated with primary antibody dilutions of 1:200 in PBS/1% BSA for 30 min. Samples were then washed three times with PBS followed by staining with the secondary fluorescein isothiocyanate-linked antibody diluted in PBS/1% BSA. After three final washes with PBS, cells were analyzed by using a microscope (Zeiss, New York, NY).

Cell culture

Ishikawa cells are a well-differentiated endometrial adenocarcinoma cell line that has previously been used to model human endometrial epithelial cells. Ishikawa cells express ERs, progesterone receptors, and other markers of endometrial function (31–36). HOX gene expression has been previously well characterized in these cells (37–39).

Ishikawa cells were maintained in 25-cm² flasks using DMEM supplemented with 10% (vol/vol) fetal bovine serum. Cells were maintained at 37 C in a humidified atmosphere (5% CO² in air) and allowed to reach confluency. Ishikawa cells were passaged by standard methods of trypsinization and plated in 96-well culture dishes until they reached 80% confluence. The cells were then treated with serum-free, phenol red-free media for 24 h before MXC treatment. After a 24-h incubation to serum-free, phenol-red-free media, the media were removed from the confluent Ishikawa cells and the cells treated with DMEM containing MXC at concentrations ranging from 10⁻⁵ to 10⁻² m for 24 h. Control cells were treated with an equivalent concentration of diluent (DMSO).

Western analysis

Ishikawa cells were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM NaSO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), centrifuged at 12,000 x g for 2 min at 4 C and supernatant collected. The protein content was quantified by the Bradford method using a protein assay kit (Bio-Rad Laboratories, Richmond, CA). Sixty-microgram aliquots were loaded onto a 6% sodium dodecyl sulfate polyacrylamide gel, size fractioned, and transferred to a nitrocellulose membrane using a transblot apparatus (Bio-Rad Laboratories) at 100 V for 2 h at 4 C. The membrane was immersed in a 3% gelatin-Tris-buffered saline (TBST: 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 8.0]) blocking solution for 30 min at room temperature, washed for 10 min in TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween 20 [pH 7.5]), and then incubated overnight at 4 C with a 1 µg/ml dilution of HOXA10 polyclonal antibody (Santa Cruz Biotechnology). The membrane was washed with TBST for 5 min at room temperature and incubated for 1 h with a 6 µg/ml dilution of goat antimouse IgG-horseradish peroxidase (Bio-Rad Laboratories). The membrane was then washed twice in TBS for 5 min at room temperature and immersed in a horseradish peroxidase color developer buffer (Bio-Rad Laboratories) for 30 min. Photographs were taken immediately after color development.

Real-time RT-PCR

Quantitative real-time RT-PCR was performed using the LightCycler SYBR Green RT-PCR kit from Roche (Stockholm, Sweden). One microgram of total RNA was reverse transcribed in 20 µl of reaction mixture containing 10 mM each of dATP, dCTP, dGTP, and deoxythymidine triphosphate; 20 pmol oligo(dT); 40 U/µl ribonuclease inhibitor, 10 U/µl avian myeloblastosis virus-reverse transcriptase, and 10 × AMV-RT buffer for 30 min at 61 C. PCR for Hoxa10 was performed for 45 cycles of 95 C for 2 sec; 65 C for 5 sec; and 72 C for 18 sec. The Hoxa10 intron-spanning primers were selected using the primer selection program Primer3 developed by the Whitehead Institute.
Cycler and adjusted to the quantitative expression of created. Quantitation of samples were determined with the Roche Light-products during amplification. A quantitative standard curve was then labeled DNA in a final volume of 25 μl containing 25 mM Tris-HCl, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5 mM MgCl₂, 10 μg/ml salmon sperm DNA, and 10% glycerol. All samples were fractionated for 3 h at 200 V in a 4% nondenaturing polyacrylamide gel containing 1 × TBE at 4 °C. The gel was dried under a vacuum at 80 °C for 45 min and exposed overnight on X-OMAT film (Kodak, Rochester, NY) and subsequently developed.

EMSAs were performed as previously described (40–42). Complementary single-stranded oligodeoxynucleotides were synthesized and annealed to incorporate the ER binding site and flanking sequences located 5′ of the transcription site of the HOXA10 gene. Sequence of HOXA10 ERE oligonucleotide is as described previously (15). Cells treated with E2 (10⁻⁸ M) alone, MXC (25 or 50 μM), or both were used as a source of nuclear extracts. Nuclei were isolated and nuclear extracts prepared from Ishikawa cells growing at log phase by the method described by Dignam et al. (43). Binding reactions were performed on ice for 30 min using 2 μg Ishikawa cell nuclear extract and 80,000 cpm of labeled DNA in a final volume of 25 μl containing 25 mM HEPES (pH 7.6), 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5 mM MgCl₂, 10 μg/ml salmon sperm DNA, and 10% glycerol. All samples were fractionated for 3 h at 200 V in a 4% nondenaturing polyacrylamide gel containing 1 × TBE at 4 °C. The gel was dried under a vacuum at 80 °C for 45 min and exposed overnight on X-OMAT film (Kodak, Rochester, NY) and subsequently developed.

Results

Uterotrophic response to MXC

The uterotrophic response to ip administration of MXC (1 mg/kg), E2, DES, or vehicle control was assessed after 3 d. The wet uterine weight was evaluated in three OVX animals from each group. Figure 1A shows the uterine weight after administration of E2, DES, MXC, or control (OVX). Intraperitoneal injection of E2, DES, or MXC resulted in a rapid increase in uterine wet weight of approximately 1.25- (MXC) to 5 (DES)-fold, consistent with previous findings. Results from the treated animals were compared with control using Student’s t test. All treatments were significantly different from control. E2 or DES treatment resulted in a significantly greater increase in uterine weight than treatment with MXC.

Similarly uterine endometrial epithelial height was measured histologically after administration of the same compounds. Each compound induces a change in epithelial height, compared with the OVX control mice uterine epithelium. E2 or DES each induced a greater increase in height than did MXC (Fig. 1B).

Effects of MXC on HOXA10 expression in vitro

To develop a cell model of adult uterine exposure, the effect of MXC on HOXA10 expression was evaluated in Ishikawa cells. Ishikawa cells are a well-differentiated human uterine adenocarcinoma line in which HOX gene expression has been previously well characterized (37–39). mRNA and protein expression was determined by real-time RT-PCR and Western analysis, respectively, after treatment with MXC, E2, or DES. Ishikawa cells were treated with either 10⁻⁸ M E2 or DES or with MXC at concentration ranging from 1 to 50 μM. Dimethylsulfoxide (DMSO) was used as a diluent and served as the diluent control. HOXA10 mRNA expression was measured using both real-time RT-PCR normalized to actin expression and presented as fold induction compared with diluent, or Northern analysis normalized to glyceraldehyde-3-phosphate dehydrogenase expression.

HOXA10 mRNA levels, as measured by real-time RT-PCR after MXC treatment, are demonstrated in Fig. 2A. A dose-responsive increase in HOXA10 expression is noted with increasing concentrations of MXC. Ishikawa cell HOXA10 mRNA levels were increased 6- to 8-fold after treatment with 25 or 50 μM MXC. A small, but significant, increase was detected after treatment with 1 μM MXC.

Compared with the control diluent-treated cells, as previously described, E2 increased HOXA10 mRNA expression and DES minimally increased mRNA expression in this cell line (Fig. 2B). MXC at 25 × 10⁻³ m increased HOXA10 mRNA expression nearly to the level seen with 10⁻⁸ M DES treatment.
Induction of HOXA10 protein expression in response to MXC treatment was measured in Ishikawa cells using Western analysis as demonstrated in Fig. 2C. Positive control cells were treated with a vector that constitutively expressed HOXA10, as previously described (40, 44). The negative control consisted of BT20 cells known not to express HOXA10 (40). Treatment with diluent control resulted in low but detectable HOXA10 protein expression. One to 50 μM MXC treatment results in a dose-responsive increase in HOXA10 protein expression. The dose-responsive increase was verified by densitometry.

Effects of neonatal MXC exposure on Hoxa10 expression

Neonatal exposure occurred from d 1 to 14 post partum with examination of the 2-wk-old exposed offspring. Seven mice were treated with MXC and three with vehicle control (DMSO). The 2-wk-old exposed female mice were killed and the uteri examined for gross and histologic anomalies. Immunohistochemistry was performed for Hoxa10 expression. No gross or histologic changes were seen in the MXC-treated uteri, compared with control. To identify both spatial and quantitative alterations in Hoxa10 expression, immunohistochemistry was performed on the exposed uteri. Hoxa10 spatial boundaries of expression were identical in the control and MXC-treated mice. Figure 3 demonstrates the expression of Hoxa10 in the uterus of control and MXC-treated mice. Neonatal exposure results in decreased expression of this gene. Whereas a small number of cells still demonstrated a signal representing Hoxa10 expression, most cells did not express Hoxa10 at levels above background.

Effects of neonatal MXC on subsequent adult Hoxa10 expression

To evaluate the effect of neonatal MXC exposure on adult Hoxa10 expression, mice were treated with MXC or vehicle control as described above. Four mice were treated in each group. Animals were killed 8 wk after termination of exposure. Figure 4 demonstrates Hoxa10 protein expression. Whereas uteri from vehicle control-treated animals express...
abundant Hoxa10, MXC-treated animals demonstrate significantly reduced expression. Neonatal MXC treatment produces lasting changes in Hoxa10 expression.

Effects of MXC on ER binding to the HOXA10 estrogen response element (ERE)

We have previously shown that ERα and ERβ bind to the HOXA10 ERE and that the affinity of binding is not altered by DES (15). Here we investigated the effect of methoxychlor on ER binding to the HOXA10 ERE. EMSA was used to assess ER binding to the HOXA10 ERE in the absence and presence of methoxychlor. ERα and ERβ from Ishikawa cell nuclear extract have previously been shown to bind and retard electrophoretic mobility of this element (15). Labeled HOXA10 ERE was used to bind Ishikawa cell nuclear extract, previously shown to contain both ERα and ERβ (15). As demonstrated in Fig. 5, migration of the HOXA10 ERE through polyacrylamide was retarded by nuclear extract from Ishikawa cultured in the presence of 10^{-8} M E2. The addition of 25 μM MXC to the culture media with E2 results in a significantly decreased amount of shifted probe, compared with that shifted in the presence of E2 alone. MXC alone induces limited ER binding to the HOXA10 ERE; this degree of binding was greater than seen using nuclear extract from un-

treated cells (not shown). MXC disrupts E2-induced binding of ER; however, MXC alone will permit limited ER binding to the ERE in vitro.

Discussion

MXC is a widely used pesticide. It was developed as an alternative to dichlorodiphenyl-trichloroethane; however; it
may also have the originally unanticipated property of interfering with the endocrine action of estradiol. Here we examined the effect of MXC on an estrogen-responsive gene necessary for uterine development and adult function. Targeted disruption of Hoxa10 or blocking maternal Hoxa10 with antisense results in a failure of uterine function, namely the ability to support embryo implantation (16, 17). Hoxa10 expression is regulated by estrogens in both humans and mice, and an ERE mediating this effect has recently been characterized (14, 15, 39). Exposure to the well-known non-steroidal estrogen DES results in uterine anomalies, in part by disrupting the normal pattern of Hox gene expression (8). Here we hypothesized that MXC would also result in altered Hoxa10 expression and would disrupt ER binding to the Hoxa10 ERE.

In vitro MXC induced HOXA10 expression in Ishikawa cells. At high concentration MXC induced HOXA10 expression nearly as well as a low dose of DES but not to the level seen after E2 treatment. This finding was at first surprising, considering the in vitro findings of reduced Hoxa10 expression after MXC treatment. Furthermore, MXC is thought to derive estrogenic activity after conversion to its hydroxy and bis-hydroxy metabolites (18, 45, 46). However, commercial preparations of MXC are known to contain small amounts of these metabolites and may account for the estrogenic activity at high doses (47). We have also not excluded the possibility of Ishikawa cells metabolizing MXC, resulting in the active compounds.

In vivo, neonatal MXC exposure decreases Hoxa10 expression. The neonatal reproductive tract is undergoing significant morphogenesis and development for approximately 14 d after birth. Normally Hoxa10 is expressed in most uterine stromal cells; MXC exposure resulted in a dramatic and rapid restriction of HOXA10 expression to a small number of cells. Although there was no obvious morphological effect of this altered Hox gene expression, there is similarly no obvious histological difference between wild-type mice and mice lacking Hoxa10. Hoxa10 (+/−) mice are unable to support embryo implantation (17). Diminished Hoxa10 expression in mice treated with MXC may similarly affect subsequent reproductive function. One mechanism by which lasting effects on the reproductive tract occur after exposure to endocrine disrupting chemicals is by permanently altering developmental gene expression.

The apparent contrast in the in vitro and in vivo effects is likely due in part to more efficient metabolism of MXC in the mouse than in vitro. As discussed above, hydroxy and bis-hydroxy metabolites of MXC are more potent endocrine disruptors. Additionally, in vitro (in the absence of E2) MXC functioned as a weak E2 agonist, whereas in the presence of E2, in vivo MXC functioned as an E2 antagonist. As demonstrated by the EMSA results, MXC allowed ER binding to the HOXA10 ERE in the absence of E2; however, MXC disrupted E2-induced ER binding. In vivo, in the presence of E2, MXC likely functions by preventing E2-driven ER binding and activation of transcription from the HOXA10 ERE. Additional effects of MXC may be mediated by non-ER-dependent mechanisms as previously reported (48).

The effect of neonatal exposure persists in the adult; subsequent Hox gene expression is diffusely diminished. The resulting reproductive competence of these animals was not tested here because other investigators have documented a deleterious effect on fertility (49). Exposure in early pregnancy produces a dose-dependent decline in the number of implantation sites (20). MXC exposure blocks the decidual cell response, inhibiting implantation and support of the early embryo (28). Similarly, Hoxa10 is necessary for decidualization; targeted disruption of Hoxa10 results in defective decidualization and lack of implantation (50). The diminished Hoxa10 expression seen here after MXC exposure may be the molecular mechanism for the defective decidual response and failed implantation seen with exposure to this agent.

In utero exposure to endocrine disruptors may produce biochemical and functional defects in offspring that are not as immediately apparent as structural anomalies. Many phenotypes may become relevant only at particular junctures, such as reproduction, or only when combined with other defects or environmental challenges. For example, the effects of DES exposure in humans were often subtle, delayed, dose dependent, and varied with timing of exposure. Only a subset develops cancer or experience adverse reproductive outcomes. MXC has a lasting effect on the expression of a gene required for reproductive tract development and function. The effect of MXC exposure in humans is not well characterized and, like the effects of DES exposure, may be subtle. Because MXC and DES have similar effects on developmental gene expression in exposed animals, the effect of human exposure to either of these two endocrine disruptors may be similar.

Acknowledgments

Received March 22, 2005. Accepted May 3, 2005.

Address all correspondence and requests for reprints to: Hugh S. Taylor, Division of Reproductive Endocrinology, Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520. E-mail: hugh.taylor@yale.edu.

This work was supported by National Institutes of Health Grants ES10610 and HD36887.

References

46. Eroschenko VP, Johnson TJ, Rouke AW 2000 Estradiol and pesticide methoxychlor do not exhibit additivity or synergism in the reproductive tract of adult ovariectomized mice. J Toxicol Envir Health A 60:407–421
57. Cermik D, Selam B, Taylor HS 2003 Regulation of HOXA10 expression by testosterone in vitro and in the endometrium of patients with PCOS. J Clin Endocrinol Metab 88:238–243
68. Ghosh D, Taylor JA, Green JA, Lubahn DB 1999 Methoxychlor stimulates estrogen-responsive messenger ribonucleic acids in mouse uterus through a non-estrogen receptor (non-ER) α and non-ER β mechanism. Endocrinology 140:3526–3535
70. Lim H, Ma L, Ma WG, Maas RL, Dey SK 1999 Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidu- alization in the mouse. Mol Endocrinol 13:1005–1017