0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 8
Printed in U.S.A.

Methoxychlor Stimulates Estrogen-Responsive Messenger Ribonucleic Acids in Mouse Uterus through a Non-Estrogen Receptor (Non-ER)α and Non-ERβ Mechanism*

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ABSTRACT

This study examined the effects of the xenoestrogen methoxychlor (Mxc) on messenger RNA (mRNA) concentrations of two estrogen-responsive uterine genes, lactoferrin (LF) and glucose-6-phosphate dehydrogenase (G6PD). Ovariectomized wild-type (WT) and estrogen receptor (ER)α-knockout (ERαKO) mice were treated with Mxc or estradiol-17β (E_2) to determine whether Mxc acts via pathways that involve ERα. In WT mice, both E_2 and Mxc stimulated increases in uterine LF and G6PD mRNA concentrations in a dose-dependent manner. Competitive pretreatment with the pure antiestrogen ICI 182,780 dramatically reduced E_2-stimulated increases in mRNA concentrations but had no effect on Mxc-induced effects. Competitive pretreatment with E_2 had only a partially inhibitory effect on Mxc-induced responses. In the ERαKO mouse, E_2 had little effect on uterine LF or G6PD mRNA concentrations, whereas Mxc stimulated marked increases in both LF and G6PD mRNAs. The Mxc-induced increases in LF and G6PD mRNAs in the ERαKO mouse were not suppressed by competitive pretreatment with either E_2 or ICI 182,780. Fold increases in mRNA concentrations for both genes induced by Mxc were similar for WT and ERαKO mice. The results surprisingly indicate that a xenoestrogen, Mxc, can increase LF and G6PD mRNA concentrations by a mechanism that is not mediated through ERα or ERβ, and acts through another pathway. (*Endocrinology* **140:** 3526–3533, 1999)

METHOXYCHLOR (Mxc) is a pesticide in current use that was developed as a replacement for dichlorodiphenyltrichloroethane (DDT). Data from several studies in rats indicate that Mxc behaves like a typical estrogen, comparable with estradiol-17β (E_2). Mxc stimulates uterine growth and hypertrophy (1, 2) and increases uterine peroxidase (3) and ornithine decarboxylase activities (4). Mxc can also accelerate vaginal opening and induce persistent vaginal cornification (5), as well as increased uterine estrogen receptor (ER) expression (6, 7). However, in spite of the evidence for estrogenic activity, it is not yet known how, or through which ER, Mxc exerts its effects.

Mxc was long believed to act through the classic ER (ERα) protein, a ligand-activated transcription factor and a member of a large family of evolutionarily conserved nuclear hormone receptors. However, the discovery of an additional ER, ERβ (8, 9), has made it necessary to reevaluate estrogen action. Although the functional importance of ERβ *vs.* ERα is not yet established, the tissue-specific distribution of these two receptor forms (10) may imply tissue-specific agonistic or antagonistic actions of estrogens. For example, it has been shown that Mxc itself acts as an estrogen agonist at the level of uterus and oviduct but as an antagonist in the ovary (11). Although it has been shown that Mxc binds to both ERα and ERβ (10, 12), it is not yet known through which receptor Mxc or its estrogenic metabolite(s) acts. Mxc is converted *in vivo* by the liver to 2,2-bis(p-hydroxyphenyl)-1,1,1,-trichloroethane (HPTE). HPTE is thought to be the principal active metabolite of Mxc because it has a higher affinity for ERα than Mxc (13) and shows potent *in vitro* estrogenic activity (4).

The widespread presence in the environment of chemicals with the capacity to disrupt the functioning of the endocrine system has been extensively studied. These chemicals include pesticides and herbicides such as Mxc, DDT, chlorodecone (kepone), the polychlorinated biphenyls, and phenolic compounds; and they may act via many different mechanisms. One category of endocrine-disrupting chemicals are those that are able to bind to ERs (14) and have effects similar to those of endogenous estrogens. DDT, for example, now banned in the United States for pesticide use, has been shown to advance vaginal opening and increase ovarian and uterine weights in rats (15, 16). Kepone, another pesticide, also induces precocious vaginal opening in immature rats (17), and polychlorinated biphenyls too have been shown to induce precocious puberty and uterine growth in rats (18, 19). These chemicals can act *in vivo* via multiple mechanisms [for example, o,p'DDT, a structural analog of Mxc, binds to ERs, whereas p,p'DDE binds to androgen receptors (20)]. Though the action mechanisms of these estrogenic chemicals are not clear, these compounds have received a great deal of attention, in the past decade, as a possible cause of certain cancers and impaired reproduction in animals (21–23).

Received October 21, 1998.

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* This work was supported by grants from the Environmental Protection Agency (R825295010), the National Institutes of Health (ES-08272), and the U.S. Army (DAMD 17–97-1–7171).

Kuiper *et al.* (10, 12) have shown that some natural estrogens, including various xenobiotics and the estradiol metabolite 4-hydroxy estradiol (a catecholestrogen) may act through binding to both ERα and ERβ. Using the ERα knockout (ERαKO) mice, which show negligible or no classical responses to E_2 (24), Das *et al.* (25, 26) have shown that 4-hydroxy estradiol and the xenoestrogen kepone have estrogenic actions mediated via a non-ERα and non-ERβ pathway.

In this study, we set out to characterize the effects and actions of the widely-used estrogenic pesticide Mxc on the messenger RNA (mRNA) concentrations of estrogen-responsive genes in the mouse uterus. Lactoferrin (LF) and glucose-6-phosphate dehydrogenase (G6PD) are two well-known estrogen-responsive genes (27–30), and this study includes both dose-response and time-course effects of Mxc on these genes. To separate out effects that might be mediated via ERα, from those mediated by ERβ and/or other receptors, we examined the effects of Mxc on estrogen-inducible increases in mRNA concentration in the ERαKO mice and wild-type (WT) controls. Our results demonstrate that Mxc can induce increases in estrogen-sensitive mRNA concentrations in a manner similar to E_2 but through a pathway that does not involve either the classical ERα or the recently discovered ERβ.

Materials and Methods

Chemicals

E_2 (1,3,5 [10]-Estratriene-3, 17β-diol) and Mxc (DMDT; 1,1,1,-Trichloro-2,2,-bis-[p-methoxyphenyl]ethane) were purchased from Sigma Chemical Co. (St. Louis, MO). ICI-182,780 (ICI) was purchased from Tocris (Bristol, UK).

Animals and injection schedule

Animals were maintained and treated in accordance with University of Missouri Animal Care and Use Committee guidelines. Adult WT (+/+) or homozygous ERαKO (−/−) sibling mice of the same mixed genetic background (129/C57BL/6J) were ovariectomized and rested for 2 weeks before treatment. All treatments were given as two dorsal sc 0.1-ml injections, 6 h apart, of olive oil (vehicle control; Sigma Chemical Co.), E_2 (10 or 100 μg/kg BW), ICI (15 mg/kg), or Mxc (1.8, 3.75, 5.7, 7.5, 10.5, 15, 22, 30, 45, and 60 mg/kg BW), and animals were killed 12 h after the final injection. In a separate group of mice, Mxc (15 mg/kg) was injected sc together with E_2 at doses of 10 and 100 μg/kg BW, or with ICI at a dose of 15 mg/kg BW. In these animals, E_2 and ICI were injected 30 min before Mxc injection.

Time-course studies (2, 6, 12, 18, and 24 h for Mxc; and 12, 18, and 24 h for estradiol) were carried with a single injection of Mxc (15 mg/kg) or estradiol (10 μg/kg). All compounds (0.1 ml/mouse) were injected dorsally sc in olive oil vehicle.

Isolation of RNA

Treated animals were euthanized, and the uterus was quickly collected and snap frozen in liquid nitrogen. Total RNA was isolated using Tri-Reagent (Sigma Chemical Co.). After isolation, total RNA concentration was measured in a spectrophotometer. Based on the optical density reading, all RNA samples were brought to a concentration of 1 μg/μl and run out on a 1% agarose gel to confirm the uniformity of the 18S and 28S RNA bands. The integrity and quality of the purified RNA were also monitored by measurement of the A260/280 ratio. Only RNA samples exhibiting a 260/280 ratio greater than 1.6 and showing integrity of RNA by electrophoresis were used in further experiments.

Reverse transcriptase (RT)-PCR

Complementary DNA (cDNA) was prepared for LF, G6PD, and RPL7, using specific antisense primers (0.4 μM) in the presence of 0.25 μl of avian myeloblastosis virus (AMV-RT), 2.5× AMV-RT buffer, 0.25 mM $MgCl_2$, and 1 mM deoxynucleotide triphosphates, in a total reaction vol of 20 μl. RPL7 was used as a housekeeping gene to further countercheck for uniform RNA loading and to monitor the efficiency of RT reaction. One microgram of RNA was used as template in each reaction. The RT reaction was carried out at 48 C for 1 h, and the AMV-RT was then inactivated at 93 C for 3 min and brought to 14 C for 10 min. One microliter of sample cDNA template for each gene, including RPL7, was then amplified by PCR in separate sets of reactions. A negative control (reaction mix but no template) was run in each RT-PCR reaction, both in RT and PCR reactions, to monitor for nonspecific amplification.

For RT and PCR of mouse LF, the primers used were 5′-AGGAAAGCCCCCCTACAAAC-3′ [nucleotide number (nt) 289–308, sense] and 5′-GGAACACAGCTCTTTGAGAAGAAC-3′ (nt 564–541, antisense); GenBank accession no. D88510.

The primers used for mouse G6PD were 5′-CTCCTGCAGATGTTGTGTCT-3′ (nt 842–861, sense) and 5′-TCATTGGGCTGCATACGGA-3′ (nt 1245–1227, antisense); GenBank accession no. Z11911.

The primers for mouse RPL7 were 5′-TCAATGGAGTAAGCCCAAAG-3′ (nt 383–402, sense) and 5′-CAAGAGACCGAGCAATCAAG-3′ (nt 628–609, antisense); GenBank accession no. M29016.

For each gene, PCR was done in the presence of specific sense and antisense primers (0.4 μM), 0.1 mM $MgCl_2$, 0.4 mM deoxynucleotide triphosphates, 0.25 μl Fisher-*Taq* DNA polymerase (Fisher Scientific, St. Louis, MO), and 2× Fisher-*Taq* polymerase buffer in a total reaction vol of 50 μl. The thermal cycling condition for LF and RPL7 was 30 cycles at 94 C for 30 sec, 55 C for 30 sec, 68 C for 50 sec, with a preincubation at 94 C for 3 min and final incubation at 68 C for 7 min. For G6PD, the thermal cycling conditions were slightly different at 30 cycles at 94 C for 30 sec, 55 C for 40 sec, and 68 C for 1 min. Pre- and postincubation temperatures were the same as above. Uniformity of RNA loading for each sample was confirmed by electrophoresis of the RPL7 cDNA, and then LF and G6PD cDNA samples were coamplified with six different concentrations of competitor, as described below.

Competitive RT-PCR of LF

Competitive RT-PCR was the method chosen to quantify the changes of RNA message because of the very limited amount of RNA obtainable from ERαKO mouse uteri. It is essentially the same procedure as that employed by Das *et al.* (25). The competitor template contains the same primer template sequence as the mouse target cDNA. This competitor template was a gift from Drs. S. K. Das and S. K. Dey and was generated by introducing a nonspecific DNA fragment into a mouse target cDNA clone. A 185-bp blunt-ended fragment (*Ssp*I), obtained from a pGEM7Zf(+) vector, was inserted into the LF cDNA at the *Stu*I site. This DNA template was used as the competitor for competitive PCR of LF cDNA templates derived from the RT of uterine RNAs. One tenth of the total RT product was coamplified with 10-fold increasing amounts of the competitive template (1 fg–100 pg) by PCR for 30 cycles, with the mixture of sense and antisense oligonucleotides. The final sizes of the competitor template and target cDNA were 460 bp and 275 bp, respectively. The PCR amplification conditions were the same as for RT-PCR.

Competitive RT-PCR of G6PD

The competitor template contains the same primer-annealing site as mouse target cDNA. A PCR product (350 bp) of the G6PD cDNA, generated by using the mouse G6PD primers described above, was subcloned into the PGEM-T Easy Vector. A 200-bp foreign piece of blunt-ended (*Cla*I) DNA was inserted (blunt-end ligation) within this PCR product in the *Kpn*I restriction site and was used as a competitor template for quantitative PCR. The amplification conditions were the same as for RT-PCR, and the final sizes of the competitor template and target cDNA were 550 bp and 350 bp, respectively. For G6PD, one fifth of the RT product was coamplified with 10-fold increasing amounts of the competitive template (1 fg–100 pg) by PCR for 30 cycles with the mixture of sense and antisense oligonucleotides. One fifth of the RT product was used in PCR amplification of G6PD instead of the one tenth

used for LF, because G6PD mRNA was expressed at lower levels than LF mRNA.

Amplified products were separated on 2% agarose (Agarose Low EEO, Fisher Scientific) gels and stained with ethidium bromide. Gels from different assays were scanned, and optical density units (peak area) for each sample and competitor were determined by using Gptools, version 3.0 (BioPhotonics Corp., Ann Arbor, MI). The ratio of band intensities of the competitor and target cDNA was calculated for each sample and plotted against the amounts of competitor. For each sample, a separate standard curve was prepared to determine the amount of mRNA for each specific gene. The amount of target cDNA was determined from the logarithm plot at the zero equivalence point, which represents 10% of the total (because only one tenth of the total reaction was used) for LF and 20% for G6PD total (because one fifth of the total reaction was used).

Data analysis and statistics

The concentration of mRNA for each gene was calculated as fg/μg total RNA. Statistical analysis of the data was performed by ANOVA, followed by LSM *t* test, using a SAS computer program (SAS system, version V- 6.12, TS 020). Significance was accepted at $P < 0.05$.

Results

To standardize the assay for measurement of LF and G6PD mRNAs stimulated by Mxc, we examined the effect of different cycle numbers and different starting RNA concentrations for each gene, under both untreated and treated (E2-treated) conditions. These studies demonstrated the linearity and validity of the assay; and using these data (not shown), we selected a 30-cycle program and a starting concentration of 1 μg mRNA as optimal for use in further experiments.

After performing the RT reaction, each sample was amplified with its respective competitor template and quantified as described (details in *Materials and Methods*). Fig. 1A shows a representative diagram of coamplification of competitor and target cDNA for LF and G6PD genes in both WT and ERαKO animals. Figure 1B shows an example of the logarithmic plots for control and induced samples (oil- and estradiol-treated WT animals) that are used to calculate mRNA concentrations.

Time course of LF and G6PD mRNA responses to E_2

A short time-course study was carried out with E_2 (10 μg/kg) as a positive control (Fig. 2A). For LF, a slow increase in mRNA concentrations starts from around 3-fold at 12 h, rising to about 6-fold at 18 h. The maximum (14-fold) response within the timeframe studied for LF was seen at 24 h. In contrast, G6PD gave a peak response (17-fold) at 12 h and a decline in response (down to 4.8-fold) at 18 h. A further stimulation (6-fold) started at 24 h, suggesting its stimulation to be bimodal.

Time course of LF and G6PD mRNA responses to Mxc

Ovariectomized mice were injected with Mxc at 15 mg/kg BW, and tissues were collected at 2, 6, 12, 18, and 24 h after treatment. For LF mRNA, no response was seen until 12 h after treatment, when a 2-fold increase was seen in both WT and ERαKO mice (Fig. 2B). A 7-fold increase was found at 18 h, which was the time of maximum response for LF in this timeframe, followed by a slight decline (down to 5-fold) at 24 h. No differences in responses were observed between WT and ERαKO mice in this time-course study.

For G6PD, no response was observed at 2 h, either in WT

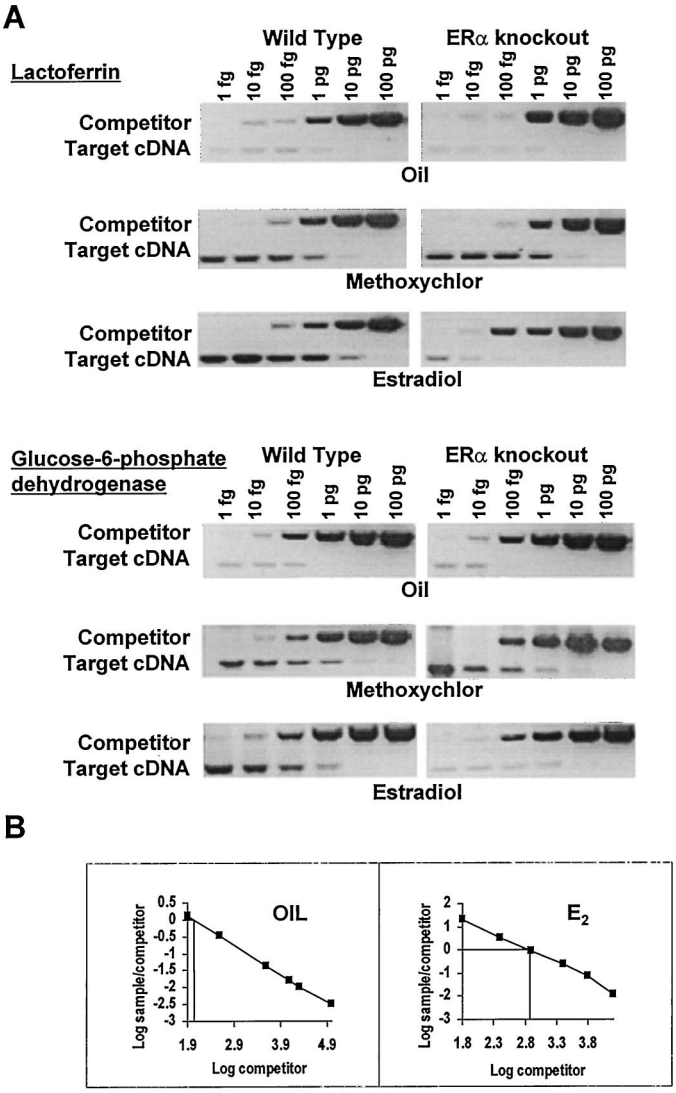

FIG. 1. A, Representative example of competitive PCR, where target cDNA was coamplified with its specific competitor. For both genes, six different concentrations of competitor (1 fg–100 pg) were coamplified with each sample (control and treated) cDNA. B, Representative logarithmic plots of competitor *vs.* sample/competitor for control and E_2-stimulated sample. The zero equivalence point was used to calculate the target cDNA concentration.

or ERαKO mice (Fig. 2C). A slight increase (2-fold) was observed at 6 h in WT mice and also (3.8-fold) in ERαKO animals. At 12 and 18 h, WT mice did not show any further increase beyond that at 6 h (about 3-fold), but a 6-fold increase was found at 24 h. However, ERαKO mice showed a further increase (to about 6-fold) at 12 h and 18 h and gave a peak response (9-fold) at 24 h.

For further studies, 18 h was chosen for the end point, because this appeared optimal for both genes within the timeframe studied.

Effects of different doses of Mxc on uterine LF mRNA concentrations

Mxc (3.75, 5.7, 7.5, 10.5, 15, 22, 30, 45, and 60 mg/kg) induced increases in LF mRNA concentrations in a dose-

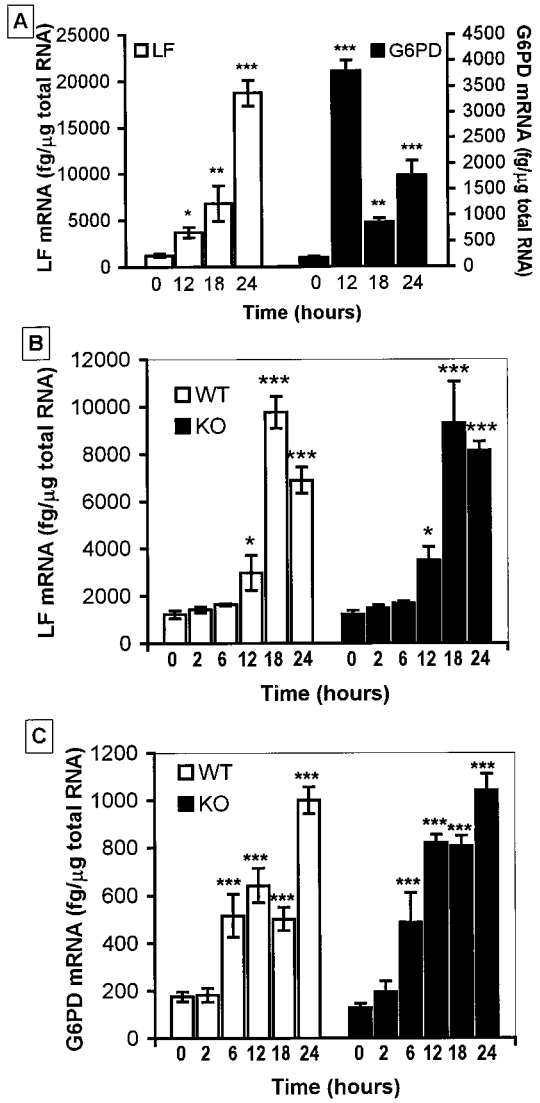

FIG. 2. A, LF and G6PD gene expression in WT mice after a single injection of 10 μg/kg E_2. Animals were killed at different times after injection (12, 18, and 24 h). RNA quantitation was done by quantitative RT-PCR and the result obtained as fg/μg total RNA. n = 2–11 animals per group. B, Time-course study of methoxychlor effects on LF mRNA concentrations in WT and ERαKO mice. Animals were given a single injection of 15 mg/kg Mxc and were killed 2, 6, 12, 18, and 24 h after treatment. n = 3–11 animals per group. C, Time-course study of methoxychlor effects on G6PD mRNA concentrations in WT and ERαKO mice. Animals were given a single injection of 15 mg/kg Mxc and were killed 2, 6, 12, 18, and 24 h after treatment. n = 2–10 animals per group. All data represent mean ± SE. *, $P < 0.05$ *vs.* control treatment; **, $P < 0.01$ *vs.* control treatment; ***, $P < 0.001$ *vs.* control treatment.

dependent manner in WT mice, up to 30 mg/kg (Fig. 3A). Little effect was seen before the 5.7-mg/kg dose, after which message concentrations rose to a peak at 30 mg/kg and then declined. The 30-mg/kg dose seemed to be a maximally effective dose in WT mice. In WT animals, a sharper decline in message was seen, starting from 45 mg/kg; and a gradual fall was seen at the 60-mg/kg dose. The scenario in the ERαKO mouse was quite different. Although the 1.8-mg/kg dose was ineffective, the next lower doses (3.75, 5.7, and 7.5

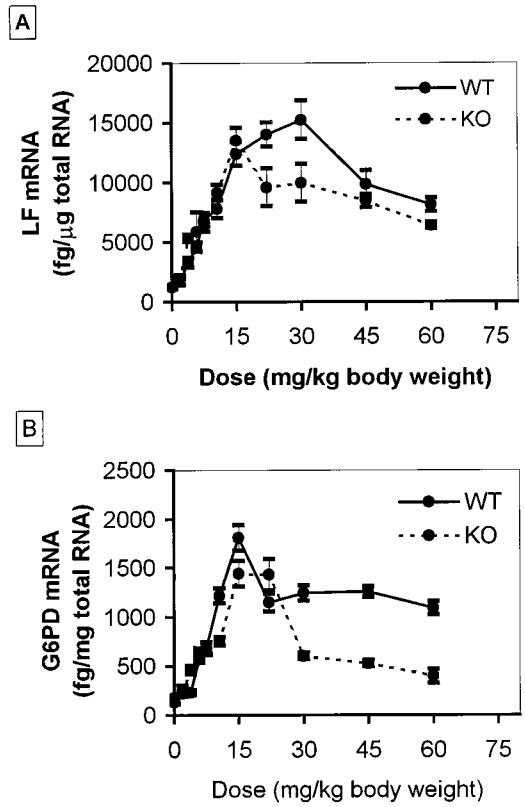

FIG. 3. Dose-response study of methoxychlor effects on LF (A) and G6PD (B) mRNA concentrations in WT and ERαKO mice. Animals were injected with different doses of Mxc in two consecutive injections, 6 h apart, and were killed 12 h after the final injection. Data represent mean ± SE (n = 2–13 animals per group for LF; n = 2–10 animals per group for G6PD).

mg/kg) gave similar magnitude responses (about 4.5-fold increase), forming a short plateau. After this plateau, a further stimulation was seen at the 10-mg/kg dose (7-fold), with a sharper increase (up to 10.5-fold) at 15 mg/kg; message concentrations declined after this dose, down to 4.8-fold at 60 mg/kg.

Effects of different doses of Mxc on uterine G6PD mRNA concentrations

The responsiveness to Mxc of G6PD followed a pattern almost similar to that of LF, in terms of dose response (Fig. 3B). Although the lower (1.8- and 3.75-mg/kg) doses seemed ineffective in WT animals, injection with Mxc at the higher doses induced increases in G6PD mRNA concentrations in WT animals in a dose-dependent manner, rising to a maximum-fold increase at 15 mg/kg. After this point, a decrease in mRNA concentrations was seen, although fold increase did not drop further and remained at around 7-fold at the higher doses (22, 30, and 60 mg/kg).

ERαKO mice again showed a small plateau response at the lower doses (3.5-fold at 3.75 mg/kg, and 5-fold at 5.7 and 7.5 mg/kg). A further increase in message concentration was seen at 10.5 mg/kg (5.9-fold), and a sharp increase at 15 mg (12-fold) was observed that was maintained at 22 mg (11-fold). Nevertheless, as seen for LF, a fall in G6PD mRNA

concentration was seen at 30 mg (4.6-fold), 45 mg (4-fold), and 60 mg (3-fold).

Effect of estradiol 17β and ICI on Mxc-induced increases in LF mRNA concentrations

This study was carried out to check whether competitive pretreatment with estrogen or antiestrogen would inhibit the Mxc-induced stimulation of LF (Fig. 4A) and G6PD (Fig. 4B) mRNAs. Pretreatment with E_2 at 10 μg/kg did not inhibit, but did reduce, the magnitude of the Mxc-induced increases in LF mRNA concentrations in both WT and ERαKO mice (Fig. 4A). The fold increase was reduced from 10- to 7-fold in WT mice and from 10- to 8-fold in ERαKO animals. E_2 alone, at doses of 10 and 100 μg/kg, gave 14.6-fold and 16.4-fold increases, respectively, in WT mice; whereas no responses were observed in ERαKO mice other than a very slight (3-fold) stimulation at 10 μg/kg. Pretreatment with E_2 at 100 μg/kg considerably reduced the Mxc-induced LF response (from 10- to 2.7-fold increase) in WT mice. The ERαKO mice showed magnitudes of response almost similar to those of Mxc, both with and without pretreatment with either dose of E_2, although the lower dose of E_2 seemed to inhibit the Mxc-induced effect (from 10.5- to 8-fold). Treatment with the antiestrogen ICI alone, at a dose of 15 mg/kg, had no effect on LF mRNA concentrations. Pretreatment with ICI had no effect on the Mxc-induced response in WT or ERαKO mice, but the same dose did inhibit the response to estradiol in WT mice.

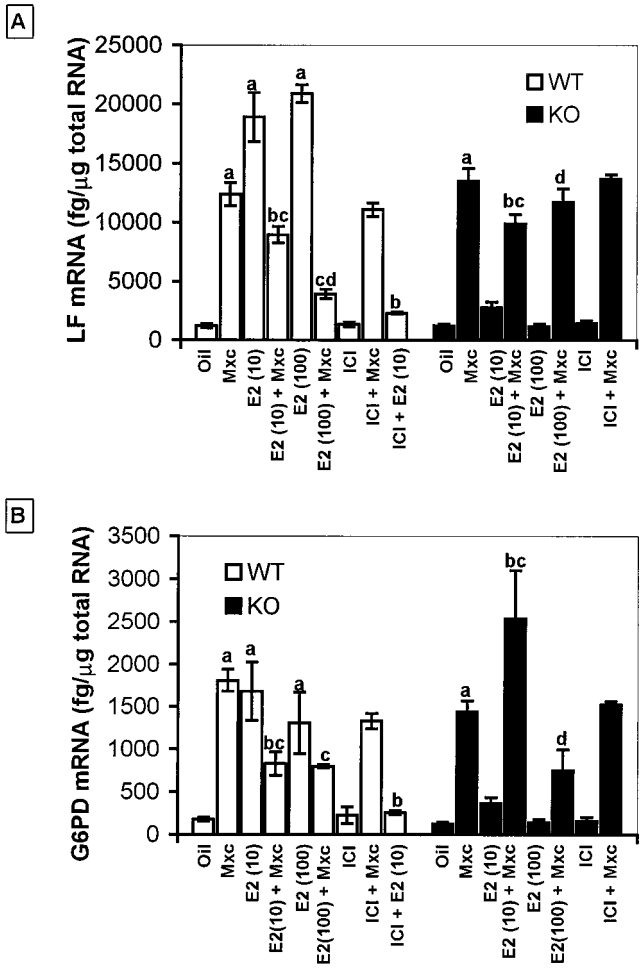

FIG. 4. Effects of E_2 and ICI on Mxc-induced effects on LF (A) and G6PD (B) mRNA concentrations. Animals were injected with oil, Mxc at 15 mg/kg, E_2 at 10 μg/kg ($E_2$10) or 100 μg/kg ($E_2$100), or ICI at 15 mg/kg. For dual treatments, E_2 or ICI was injected 30 min before injecting Mxc. All treatments were given as two series of injections, 6 h apart, and the animals were killed 12 h after the final injection. Data represent mean ± SE (n = 2–13 animals per group for LF; n = 2–13 animals per group for G6PD). Significance (at $P < 0.05$ or greater): a, value significant *vs.* oil; b, value significant *vs.* E_2 (10 μg/kg); c, value significant *vs.* Mxc; d, value significant *vs.* E_2 (100 μg/kg).

Effect of estradiol 17β and ICI on Mxc-induced increases in G6PD mRNA concentrations

Pretreatment with E_2 (10 μg/kg) in WT mice reduced the Mxc-induced increases in G6PD mRNA concentrations from 10- to 4.5-fold, consistent with the LF results (Fig. 4B). In contrast, this dose of E_2 did not inhibit the Mxc-induced increases in G6PD mRNA concentrations in the ERαKO mouse and, instead, had an additive effect. However, the higher, 100-μg/kg E2 dose led to a partial reduction of the Mxc-induced response in both subject groups (from 10- to 5-fold in the WT, and 11- to 6-fold in the ERαKO). The response pattern of G6PD message to ICI was similar to that for LF. ICI did not have any effect when injected alone, inhibited the E_2-induced response seen in WT mice, but did not inhibit Mxc-induced G6PD response in WT or ERαKO mice.

Discussion

For screening of environmental estrogens in the future, it will be important to recognize all the molecular pathways through which these compounds may be working. E_2-induced activation of the LF gene has been shown to be mediated through ERα by an imperfect palindromic ERE in the 5′-flanking region of the LF gene (28); less is known about G6PD activation. To determine whether uterine responses to Mxc were also mediated through ERα, we compared the effects of these agents on uterine LF and G6PD mRNA concentrations in ovariectomized ERαKO and WT mice by using quantitative RT-PCR.

We show here that, in the WT mouse, Mxc (like E_2) stimulated increases in uterine LF and G6PD mRNA in a saturable, dose-dependent manner and that E_2-stimulated increases were dramatically reduced by competitive pretreatment with the pure antiestrogen ICI. In contrast, the increases in mRNA concentrations induced by Mxc were not inhibited by this antiestrogen. Competitive pretreatment with E_2, at 10 μg/kg, had only a partially inhibitory effect on the Mxc-induced responses, and the antiestrogen alone did not influence the concentrations of uterine LF or G6PD mRNA. Collectively, these results indicate that, under normal conditions, the WT uterus responds to both Mxc and E_2, in terms of increased LF and G6PD mRNA concentrations. Moreover, the lack of ICI inhibition of Mxc-induced uterine LF and G6PD mRNA accumulation, and their only-partial inhibition by E_2, suggested that Mxc can act through an additional independent signaling pathway not involving

ERα or ERβ. We confirmed these results by using ovariectomized ERαKO mice.

In the ERαKO mouse, we show that E_2 was ineffective at stimulating marked increases in uterine LF or G6PD mRNA concentrations, agreeing with work by Das *et al.* (25). Mxc, however, did stimulate both LF and G6PD mRNA, in a saturable and dose-dependent manner, indicative of a receptor-mediated mechanism of action. This increase in uterine LF and G6PD mRNA concentrations, induced by Mxc, was not suppressed by competitive pretreatment with E_2 or ICI in the manner seen in WT animals, thus confirming that Mxc can work through a non-ERα and non-ERβ mechanism. It is interesting to note that both LF and G6PD mRNA concentrations were stimulated equally in WT and ERαKO mice, suggesting that the predominant pathway for Mxc action on these mRNAs is not via ERα. Again, the lack of inhibition by ICI or E_2 of Mxc-induced effects indicates that ERα and ERβ are minimally involved in mediating the effects of Mxc on LF and G6PD mRNA concentrations. In support of this, it should be noted that the concentrations of ERβ are remarkably low, in comparison with ERα, in the WT and ERαKO mouse uterus (31).

Taken together, these uterine responses to Mxc in the mouse clearly establish the presence of a pathway that is not mediated via the classical ERα or ERβ but, instead, through an additional signaling pathway. However, although the saturable dose responses are indicative of a receptor-mediated mechanism, this pathway may not necessarily involve an additional ER (such as an ERγ). It is possible, for example, that Mxc effects are mediated via a membrane receptor or another nuclear receptor.

Although the molecular pathway(s) by which Mxc alters these estrogen-sensitive uterine mRNA concentrations has yet to be characterized, several possibilities can be ruled out. First, although ERαKO mice lack full-length ERα, they may still have alternatively spliced forms of ERα. The existence of alternatively spliced forms of ERα that contain the ER ligand-binding domain has recently been documented, either with sequence changes upstream of the exon 5/6 boundary in rat pituitary or lacking exon 5 in rat brain and human smooth muscle cells (32–34). Second, it is possible that effects of estrogenic ligands in ERαKO mice could be mediated by different ER subtypes, such as ERβ or its alternatively spliced forms (35–37). However, it is known that E_2 and antiestrogens bind to both ERα and ERβ ligand-binding domains (12). Our observations demonstrate that E_2 has little or no effect on ERαKO uterine LF and G6PD mRNA concentrations, and that neither E_2 nor ICI markedly inhibits Mxc-induced increases in uterine LF and G6PD mRNAs in ERαKO mice. This lack of inhibition suggests that the effects of this xenoestrogen on the uterine LF and G6PD genes are not mediated via the ligand-binding domains of ERα or ERβ. A third factor to consider is that the LF gene has been shown to have two promoter regions (38), and it is possible that Mxc may exert its effects on LF and G6PD through promoter regions on these genes that differ from those used by E_2.

One important factor to consider is that mRNA concentrations, at any given point in time, represent a balance between synthesis and degradation. Estrogen has been shown to regulate the stability of specific mRNAs (39), and the possibility remains that xenoestrogens may regulate mRNA concentrations through altering expression or via effects on stabilization or destabilization of mRNAs.

The relative pharmacokinetics of Mxc as a pro-drug and E_2 and ICI as drugs might be considered to present technical difficulties for this study, especially in competition experiments. However, we do not believe that this is likely to be a problem, because our dose-response times (12 h after last dose) are short, and the inhibitory effects of ICI on estradiol are evident in the WT controls throughout the time course of our experiments. From the controls, it is clear that ICI is still present and would be capable of inhibiting Mxc if it were working through either an ERα or ERβ ligand-binding domain. In addition, it has been shown that ICI is a comparatively long-acting drug (days longer than tamoxifen), with sustained antiestrogenic effects from a single injection in oil that last at least 3 weeks (39a). Therefore, even if it takes hours, or even a day, for Mxc to be metabolized into an active form, the antiestrogenic effect of ICI would still exist when the active form of Mxc was generated. From this, it is clear that ICI is still present and capable of inhibiting the pro-drug Mxc or its metabolites if it were working through either a ERα or ERβ ligand-binding domain.

It has been suggested (40) that one way xenobiotics disrupt endocrine function is by interfering with the ability of natural ligands to bind receptors and/or binding proteins, perhaps at multiple levels of activation. Mxc is known to be active *in vivo*, acting as a proestrogen, which requires metabolism for estrogenic effectiveness (41). Mxc's metabolites have been less extensively studied than Mxc itself, but Gaido *et al.* (42) have reported that HPTE, thought to be the active *in vivo* metabolite, acts as an agonist for ERα but as an antagonist for ERβ. Katzenellenbogen *et al.* (43) have also reported finding ER ligands that are full agonists via ERα but antagonists via ERβ, as well as one gene, quinone reductase, whose activity is up-regulated by antiestrogens acting through ERα and ERβ. This mixed agonist/antagonist function has also been reported for other compounds. The antiestrogen hydroxytamoxifen has mixed agonist/antagonist activity through ERα, depending on the tissue and gene (44, 45), and can exert synergistic effects when combined with E_2 (46).

We had expected that, because both Mxc and E_2 are thought to act through ERα, their effects would be additive, but this was not seen. It is possible that, in the WT uterus (that is, in the presence of ERα), Mxc may act as a mixed agonist/antagonist to E_2 action by partial binding with ERα, as well as acting through another pathway. These interactions may, in some way, account for the unexpected decrease in Mxc activity we observed in WT animals when E_2 was added. It is also possible that the receptor used by Mxc is an ERα repressor.

The additive effect of Mxc, with low doses of E_2, in the ERαKO mouse, was also unexpected. Although the error bar for these samples was unusually large, the decrease in Mxc-induced increases in G6PD mRNA levels in the presence of E_2 (100 μg/kg) was not statistically different from Mxc alone. Clearly, this *in vivo* system is complex, and it involves potential interactions of ERα, ERβ, and ERα-ERβ heterodimers, as well as interactions between these ERs and the additional receptor used by Mxc. Further, there is likely to be some

degree of cross-talk between ERα/ERβ and the Mxc-signaling pathway. It is also possible that there are activators or repressors that are important in the control of ER function whose normal actions are altered by the lack of ERα in the ERαKO mouse uterus.

One interesting feature was the differing time course of Mxc-induced responses for G6PD and LF mRNA. Mxc stimulated both LF and G6PD mRNA concentrations in a dose-dependent fashion, but the increase in LF message concentrations was slower than that of G6PD (increases in LF mRNA concentrations were not seen until 12 h, whereas increases in G6PD mRNA concentrations were seen at around 6 h). This is similar to the findings of Curtis *et al.* (47), examining the effects of DES and its metabolites, and suggests that Mxc acts on these two genes through two different mechanisms, one more rapid than the other. Another fact that suggests independence of the two pathways is that, for G6PD (but not LF) mRNA concentrations, the lower dose of E_2 (10 μg/kg) seemed to be additive with Mxc, in the ERαKO mouse. Additive or synergistic actions of E_2 are not unknown and have been reported elsewhere, with testosterone in prostate growth (48), with kepone in uterus (49), and with isomers of DDT in MCF-7 cells (50). Finally, in these experiments, there is also a clear difference in how LF and G6PD are regulated in the presence of E2 and Mxc together. Understanding of the molecular cause of these differences awaits a detailed comparative analysis of the LF and G6PD promoter regions in *in vitro* transcriptional reporter assays.

In summary, our findings in ERαKO mice demonstrate that a xenoestrogen can up-regulate the expression of two estrogen-responsive genes in the uterus, via one or more pathways that do not seem to involve ERα or ERβ. Currently, there is much interest in xenoestrogens and other environmental endocrine disrupters because of their potential adverse effects on human and animal health. Further characterization of this novel pathway will enhance our understanding, not only of xenoestrogen action but also of diverse steroid hormone and endocrine disrupter actions in target organs.

Acknowledgments

The authors are thankful to Drs. S. K. Das and S. K. Dey (of the University of Kansas Medical Center, Kansas City) for their kind gift of the LF competitor template. The authors also thank Professor R. Michael Roberts for his earnest cooperation and constant encouragement, Dr. Alan Ealy for helping in statistical analysis, and Dr. Ed Curran for critically reviewing the manuscript.

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