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Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct estrogen signaling pathway

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ABSTRACT **Estrogens are thought to regulate female reproductive functions by altering gene transcription in target organs primarily via the nuclear estrogen receptor-α (ER-α). By using ER-α "knock-out" (ERKO) mice, we demonstrate herein that a catecholestrogen, 4-hydroxyestradiol-17β (4-OH-E_2), and an environmental estrogen, chlordecone (kepone), up-regulate the uterine expression of an estrogen-responsive gene, lactoferrin (LF), independent of ER-α. A primary estrogen, estradiol-17β (E_2), did not induce this LF response. An estrogen receptor antagonist, ICI-182,780, or E_2 failed to inhibit uterine LF gene expression induced by 4-OH-E_2 or kepone in ERKO mice, which suggests that this estrogen signaling pathway is independent of both ER-α and the recently cloned ER-β. 4-OH-E_2, but not E_2, also stimulated increases in uterine water imbibition and macromolecule uptake in ovariectomized ERKO mice. The results strongly imply the presence of a distinct estrogen-signaling pathway in the mouse uterus that mediates the effects of both physiological and environmental estrogens. This estrogen response pathway will have profound implications for our understanding of the physiology and pathophysiology of female sex steroid hormone actions in target organs.**

The precise mechanism(s) of action of a number of estrogenic compounds are as yet poorly understood. Traditionally, the consensus has been that all estrogens act via the classical estrogen receptor-α (ER-α), but the recent identification of another form of ER, ER-β, in mammalian tissues including uterus and ovary (1–5) has necessitated a reevaluation of the actions of estrogenic compounds in target organs. The relative distribution and levels of ER-α and ER-β vary considerably, but the relative biological significance of these two receptor forms is not yet known.

It was recently reported that natural estrogens, catecholestrogens and xenoestrogens (traditionally thought to act via ER-α) bind to both ER-α and ER-β (6). These compounds have estrogenic activities, although their specific action mechanisms remain poorly understood. Catecholestrogens, 2-hydroxyestradiol-17β (2-OH-E_2) and 4-hydroxyestradiol-17β (4-OH-E_2), are produced from primary estrogens via hydroxylation at the C2 or C4 position respectively by catecholestrogen synthases (7). These two estrogens possess differential physiologic potencies and functions: for example, the uterotrophic potency of 4-OH-E_2 is close to that of estradiol-17β (E_2), whereas 2-OH-E_2 is considerably weaker (7, 8). Although catecholestrogens have similar binding affinities for ER-α and ER-β, these binding affinities are about 7- to 14-fold lower than that of the primary estrogen, E_2 (6). Xenoestrogens are environmental estrogens, drawing increasing attention today for their potential adverse effects on both human and animal reproduction (9–11). Their reproductive toxicity is widely believed to be mediated by their binding to ER-α and mimicking certain effects of primary estrogens (12), but again, the specific mechanisms are ill-defined.

The ER-α knockout (ERKO) mouse (13) does not respond to E_2 with respect to uterine expression of lactoferrin (LF), progesterone receptor or glucose-6-phosphate dehydrogenase genes, all of which are E_2-responsive in the wild-type uterus (14, 15). This lack of responsiveness to E_2 is observed even though uteri of ovariectomized ERKO mice still show ≈5–10% of the E_2 binding exhibited in wild-type uteri (13). In wild-type mice, E_2 can modulate the expression of a subset of uterine genes that are likely to alter cellular responses (15), but whether catecholestrogens or xenoestrogens can alter the expression of these same genes in a manner similar to that of E_2 is not known. In this study we explore the ER-α vs. non-ER-α-mediated actions of 4-OH-E_2 and kepone (a xenoestrogen), and examine estrogen-induced gene responses in the ERKO mouse.

MATERIALS AND METHODS

Animals and Injections Schedule. Adult wild-type (+/+) or homozygous (−/−) ERKO sibling mice of the same genetic background (129/J/C57BL/6J) were ovariectomized and rested for 2 weeks before treatment. All treatments were given as two injections at 6-h intervals of oil (control), kepone (15 mg/kg body weight), E_2 (10 μg/kg), 4-OH-E_2 (10 μg/kg), or ICI-182,780 (ICI, 1 or 20 mg/kg). In addition, the following combinations of treatments (at the same doses) were administered: ICI 30 min before the injections of E_2, 4-OH-E_2 or kepone, or E_2 30 min before the injections of 4-OH-E_2. Mice were killed 6 h after the last injection. All of the test agents were dissolved in corn oil and injected (0.1 ml/mouse) subcutaneously.

***In Situ* Hybridization.** *In situ* hybridization was performed as described (15). Each uterine horn was excised, cut into halves, and flash-frozen in freon. Frozen sections (10 μm) were mounted onto poly-L-lysine coated slides and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. Following prehybridization, uterine sections were hybridized with ^{35}S-labeled LF sense or antisense cRNA probes for 4 h at 45°C. After hybridization and washing, the sections were incubated with RNase-A (20 μg/ml) at 37°C for 15 min. RNase-A resistant hybrids were detected by autoradiography after 3–5 days of exposure using Kodak NTB-2 liquid emulsion. The slides were poststained with hematoxylin and eosin.

Northern Blot Hybridization. For Northern blot hybridization, total uterine RNA (6.0 μg) was denatured and separated

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Abbreviations: ER, estrogen receptor; ERKO, ER-α knock out; 4-OH-E_2, 4-hydroxyestradiol-17β; LF, lactoferrin; rpL7, ribosomal protein L-7; ICI, ICI-182,780; E_2, estradiol-17β.

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by formaldehyde/agarose gel electrophoresis, transferred to nylon membranes, and UV cross-linked. Northern blots were prehybridized, hybridized, and washed as described (15). Hybridization was performed in sequence with ^{32}P-labeled cRNA probes (specific activity, $\approx 2 \times 10^9$ dpm/μg) for mouse LF and ribosomal protein L-7 (rpL7). Transcripts were detected by autoradiography (2-h exposure time). Quantitation of radioactivity in hybridized bands was achieved by radioimage analysis (Ambis Systems, San Diego).

Competitive PCR. The method of quantitation of mRNAs by competitive PCR is described elsewhere (16) and was chosen because of the limited amount of RNA obtainable from ERKO mouse uteri. In brief, the competitor template containing the same primer template sequences as those of the target cDNA competing for the primer annealing and amplification was generated by introducing a nonspecific DNA fragment into a mouse target cDNA clone. Specifically, a 185-bp blunt-ended fragment (*Ssp*I), obtained from pGEM7Zf(+) vector, was inserted into the LF cDNA at the *Stu*I site or into the rpL7 cDNA at *Bgl*II site. These DNA templates were used as competitors to carry out the competitive PCR for LF and rpL7 cDNA templates derived by reverse transcription (RT) reaction in uterine RNAs. For RT-PCR the following primers were used: 5′-AGGAAAGCCCCCCTACAAAC-3′ (nt 258–277, sense), 5′-GGAACACAGCTCTTTGAGAAGAAC-3′ (nt 510–533, antisense) for LF mRNA (17), and 5′-TCAATGGAGTAAGCCCAAAG-3′ (nt 359–378, sense) and 5′-CAAGAGACCGAGCAATCAAG-3′ (nt 585–604, antisense) for rpL7 RNA (18). The internal oligonucleotides, 5′-CTGCTGTTCTTCACGACTGCTACC-3′ (nt 339–362, antisense) and 5′-GATTGCCTTGACAGATAATTC-3′ (nt 564–584, sense) were used for Southern blot hybridization of RT-PCR amplified products for LF and rpL7, respectively. Protocols for the RT reaction and PCR amplification were as described (19). In brief, one-tenth of total RT reaction product was coamplified with 10-fold increasing amounts of the competitive template (1–100,000 fg for wild-type or 1–10,000 fg for ERKO samples) by PCR for 30 cycles, with the mixture of sense and antisense oligonucleotides. Amplified products were separated by 1% agarose gel electrophoresis and detected by Southern blot hybridization by using a ^{32}P-labeled internal oligonucleotide. Quantitation of radioactivity in hybridized bands was achieved by radioimage analysis (Ambis Systems). The ratio of radioactivities of the competitor and target cDNA was calculated for each sample and plotted against the amounts of competitor. The amount of target cDNA was determined from the logarithm plot at zero equivalence point, which represents 10% of the total; i.e., only one-tenth of total reaction was used.

Effects of E_2 or 4-OH-E_2 on Uterine Phase I Estrogenic Responses in ERKO Mice. Phase I responses were evaluated

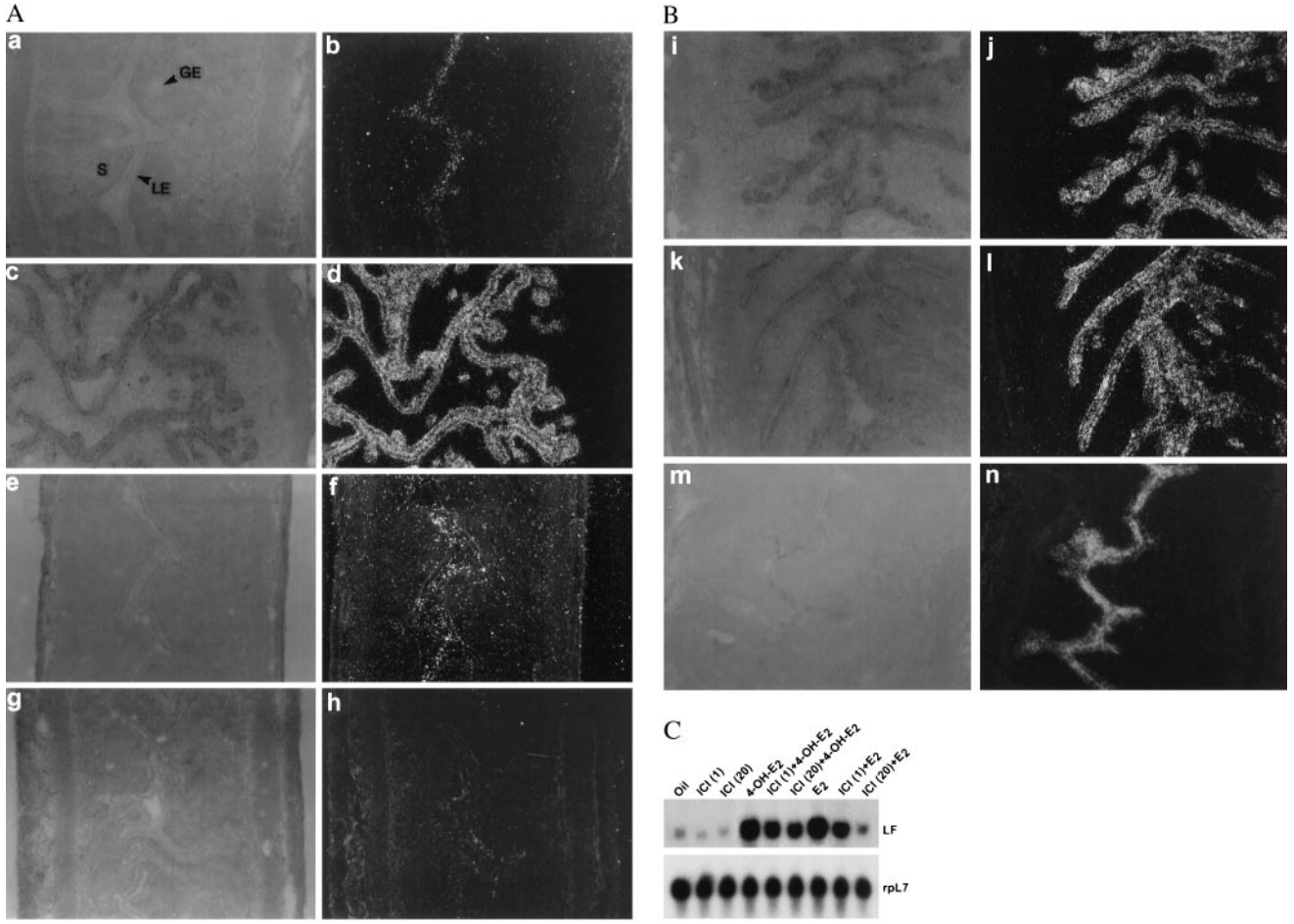

FIG. 1. *In situ* hybridization of LF mRNA in the ovariectomized wild-type mouse uterus (*A*) after exposure to oil (control), E_2 (10 μg/kg), and/or ICI (20 mg/kg), and (*B*) after exposure to 4-OH-E_2 (10 μg/kg) and 4-OH-E_2 plus ICI or kepone (15 mg/kg). Bright- and dark-field photomicrographs of uterine sections are shown. (×100.) (*A a* and *b*) Oil (vehicle). (*c* and *d*) E_2. (*e* and *f*) ICI + E_2. (*g* and *h*) ICI. (*B i* and *j*) 4-OH-E_2. (*k* and *l*) ICI + 4-OH-E_2. (*m* and *n*) Kepone. No positive signals were observed when sections were hybridized with the sense probe (data not shown). LE, luminal epithelium; GE, glandular epithelium; S, stroma. These experiments were repeated three times with three mice in each group, and similar results were obtained. (*C*) Northern blot analysis of LF and rpL7 mRNAs. Effects of ICI {1 mg/kg [ICI (1)] or 20 mg/kg [ICI (20)]} on E_2- or 4-OH-E_2-induced uterine LF mRNA levels in wild-type mice are shown. The pooled total RNA (6 μg) obtained from three to four mice in each group was analyzed. These experiments were repeated twice with independent RNA samples and similar results were obtained.

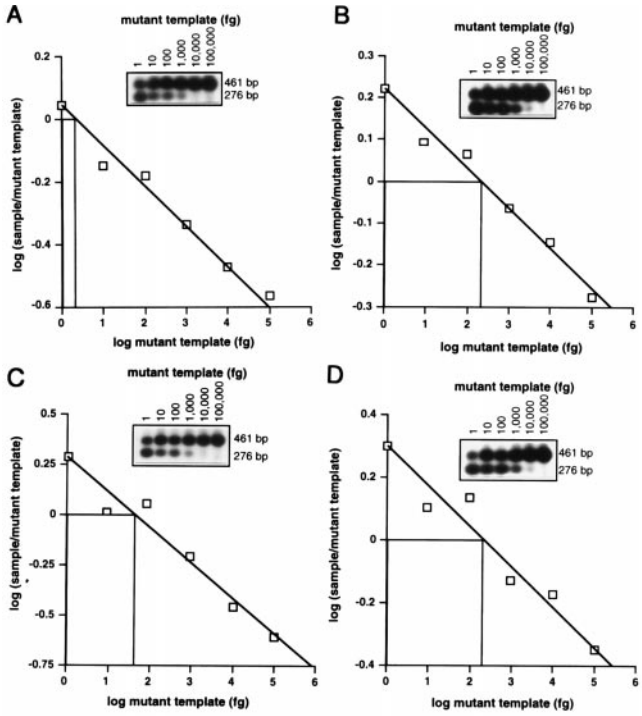

FIG. 2. Competitive RT-PCR for LF mRNA in ovariectomized wild-type mouse uterus. Adult ovariectomized wild-type mice were injected with oil (*A*), E_2 (*B*), kepone (*C*), or 4-OH-E_2 (*D*). The pooled uterine total RNA (1 μg) obtained from three to four mice in each group was reverse-transcribed by using LF or rpL7 mRNA specific antisense oligonucleotides as described. Two products of 461 and 276 bp for the competitor and sample DNA templates, respectively, were detected for LF mRNA. The ratios of radioactivities of the sample bands to those of the mutant are plotted against the amounts of the competitor template. The efficiency of the RT reaction was controlled by measuring the level of rpL7 mRNA in each sample, which was similar in all samples (4.0×10^7 copies/μg of total RNA). These experiments were repeated twice with two independent sets of RNA samples, and similar results were obtained.

by measuring uterine water imbibition and uterine uptake of ^{125}I-labeled BSA as described (20). Ovariectomized mice were given two injections at 6-h intervals of oil (control), E_2 (10 μg/kg), or 4-OH-E_2 (10 μg/kg). At 6 h after the second injection, they were given an intravenous injection of ^{125}I-labeled BSA (0.5 μCi, specific activity 10 μCi/μg; 1 Ci = 37 GBq). After 15 min the mice were perfused with saline and killed. Uterine wet weights were recorded, followed by counting of uterine radioactivity in a γ-counter. After recording uterine radioactivity, the uterine dry weights were determined, and water imbibition was calculated (wet weight minus dry weight). The specificity of uterine uptake of ^{125}I-labeled BSA was determined by comparison with uptakes in other tissues, such as skeletal muscle, liver, and heart.

Table 1. Effects of E_2, 4-OH-E_2, or kepone on uterine LF mRNA levels in ovariectomized wild-type mice

Treatments	Levels of mRNA, fg/μg total RNA	mRNA copies, molecules/μg total RNA	Fold increase
Oil	21.8	4,800	1.0
E_2	2126.4	470,000	97.6
Kepone	426.8	94,000	19.6
4-OH-E_2	1857.4	400,000	85.2

Mice were given two injections at 6-h intervals of oil (control, 0.1 ml/mouse), E_2 (10 μg/kg), 4-OH-E_2 (10 μg/kg), or kepone (15 mg/kg), and were killed 6 h after the last injection. In each treatment group the pooled uterine total RNA from three to four mice was subjected to RT-PCR as described. Values were derived from the zero equivalence point of the logarithmic plot as shown in Fig. 2, and fold increases were calculated with respect to oil value. Values represent the average of two independent experiments.

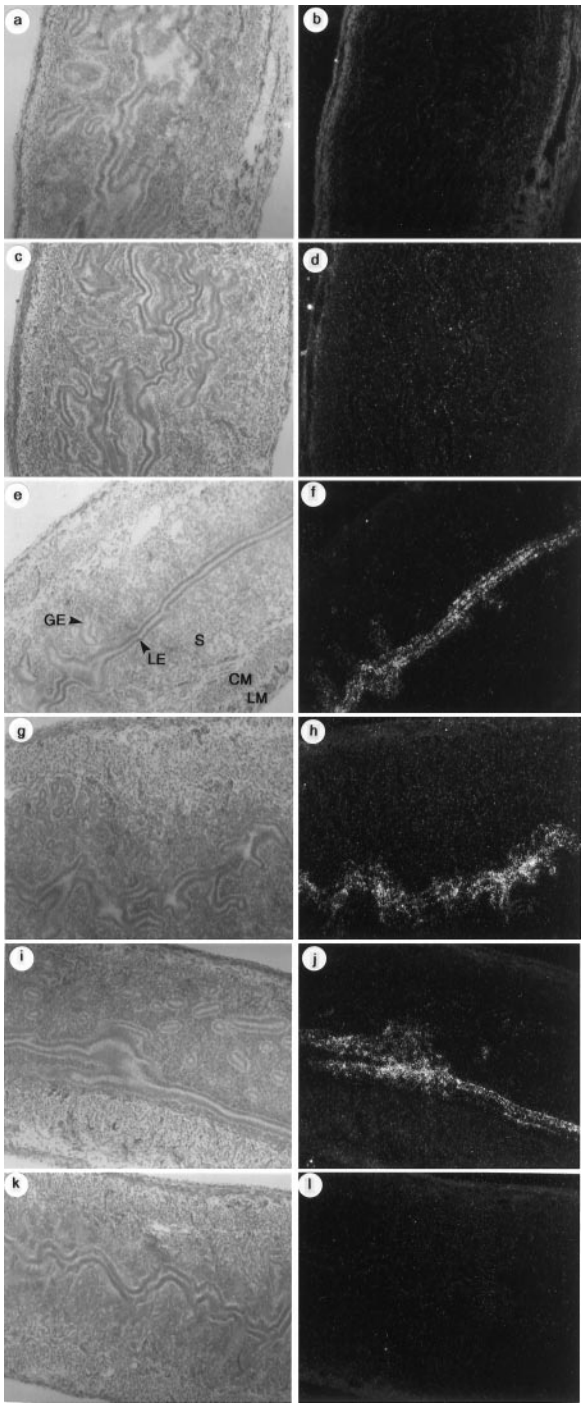

FIG. 3. *In situ* hybridization of LF mRNA in uteri of ovariectomized ERKO mice after injection of oil, E_2 (10 μg/kg), kepone (15 mg/kg), 4-OH-E_2 (10 μg/kg), 4-OH-E_2 plus ICI (20 mg/kg), or ICI alone. Bright- and dark-field photomicrographs of uterine sections are shown. (×100.) (*a* and *b*) Oil. (*c* and *d*) E_2. (*e* and *f*) Kepone. (*g* and *h*) 4-OH-E_2. (*i* and *j*) ICI + 4-OH-E_2. (*k* and *l*) ICI. Sections hybridized with the sense probe did not exhibit any positive signals (data not shown). LE, luminal epithelium, GE, glandular epithelium; S, stroma, CM, circular muscle, LM, longitudinal muscle. These experiments were repeated three times with three mice in each group and similar results were obtained.

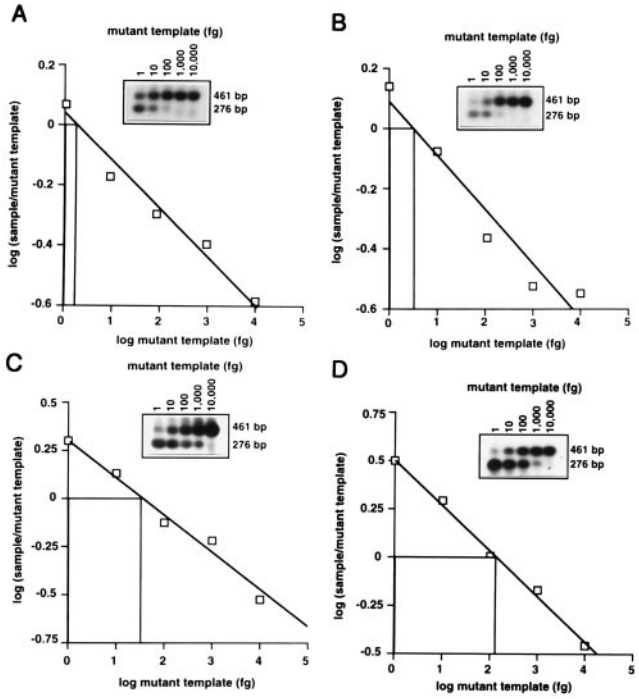

FIG. 4. Competitive RT-PCR for LF mRNA in the ovariectomized ERKO mouse uterus. Adult ovariectomized ERKO mice were injected with oil (*A*), E_2 (*B*), kepone (*C*), or 4-OH-E_2 (*D*) as described in Fig. 2. The pooled uterine total RNA (1 μg) obtained from three to four mice in each group was reverse-transcribed by using LF or rpL7 mRNA specific antisense oligonucleotides as described. These experiments were repeated at least four times with independent RNA pools and similar results were obtained.

Statistics. Statistical analyses were performed by using one-way ANOVA followed by Newman–Keul's multiple range test and by Student's *t* test.

RESULTS AND DISCUSSION

LF is a major estrogen-induced secretory iron-binding glycoprotein of the mouse uterine epithelium, and is thought to have bactericidal and growth-promoting properties (17). E_2-induced activation of the LF gene has been shown to be mediated through ER-α by an imperfect palindromic estrogen response element in the 5′-flanking region of the LF gene (17). To examine whether uterine responses to 4-OH-E_2 or kepone differed from those to E_2, we compared the effects of these agents on uterine LF mRNA levels in ovariectomized ERKO and wild-type mice by using *in situ* hybridization, Northern blot analysis and quantitative PCR.

The results of *in situ* hybridization showed that 4-OH-E_2 and kepone, like E_2, up-regulated the LF mRNA accumulation in wild-type uterine epithelial cells (Fig. 1*A* and *B*). The up-regulation by E_2 was dramatically reduced by pretreatment with the pure antiestrogen ICI at 20 mg/kg (Fig. 1*A*). In contrast, the up-regulation by 4-OH-E_2 was only partially inhibited by this antiestrogen (Fig. 1*B*). These results were further confirmed by Northern blot hybridization (Fig. 1*C*). Thus, 16-fold induction of uterine LF mRNA levels by E_2 was remarkably reduced to only 1-fold by pretreatment with ICI at 20 mg/kg. Quantitation by radioimage analysis revealed that the inhibitory effects of ICI were dose-dependent. In contrast, 15-fold induction by 4-OH-E_2 was only reduced to 11-fold by pretreatment with ICI at 20 mg/kg. The antiestrogen alone did not influence the expression of uterine LF mRNA (Fig. 1*A* and *C*). By using a different methodology, quantitative RT-PCR demonstrated ≈98-, 85-, and 20-fold induction of LF mRNA in the wild-type uterus by E_2, 4-OH-E_2, and kepone, respectively (Fig. 2 and Table 1). Collectively, these results indicate that under normal conditions the uterus responds to these three estrogenic compounds with respect to LF gene expression. Moreover, only partial inhibition of 4-OH-E_2-induced uterine LF mRNA accumulation by ICI suggested that catecholestrogens and presumably xenoestrogens are coupled to an additional and independent signaling pathway not involving ER-α. This speculation was tested by using ovariectomized ERKO mice.

As reported previously (14), E_2 was ineffective at inducing LF mRNA in uterine epithelial cells of ERKO mice (Fig. 3). Surprisingly, both 4-OH-E_2 and kepone stimulated LF mRNA levels in these cells (Fig. 3) with induction levels of ≈60- and 19-fold for 4-OH-E_2 and kepone, respectively (Fig. 4 and Table 2). This induction of uterine LF mRNA by 4-OH-E_2 or kepone was not suppressed by pretreatment with E_2 or ICI (Table 2). It is also interesting to note that 4-OH-E_2 showed 25-fold greater induction of LF mRNA levels in wild-type mice than in ERKO mice (Table 1 vs. Table 2), suggesting that in wild-type mice, 4-OH-E_2 can affect the expression of the LF gene via both ER-α and non-ER-α mediated pathways.

Several primary estrogens are known to exert both early (phase I) and late (phase II) responses in the uterus (20). As shown in Fig. 5, the treatment of ERKO mice with 4-OH-E_2 significantly increased two phase I estrogenic responses, uterine water imbibition, and ^{125}I-labeled BSA uptake. In contrast, E_2 failed to elicit any significant changes in these phase I responses (Fig. 5). No significant changes in ^{125}I-labeled BSA

Table 2. Levels of uterine LF mRNA in ovariectomized ERKO mice after treatment with various agents

Treatments	Levels of mRNA, fg/μg total RNA	mRNA copies, molecules/μg total RNA	Fold increase
Oil (n = 4)	18.9 ± 1.0[a]	4,100 ± 200[a]	1.0
E_2 (n = 4)	27.0 ± 2.7[a]	5,900 ± 200[a]	1.4
4-OH-E_2 (n = 5)	1126.5 ± 40.2[b]	240,000 ± 10,000[b]	59.6
4-OH-E_2 + ICI (n = 3)	1249.7 ± 40.5[b]	280,000 ± 10,000[b]	66.0
4-OH-E_2 + E_2 (n = 3)	1152.0 ± 65.9[b]	250,000 ± 10,000[b]	60.9
Kepone (n = 4)	360.3 ± 24.0[c]	84,000 ± 3,000[c]	19.0
Kepone + ICI (n = 3)	369.7 ± 22.8[c]	82,000 ± 2,000[c]	19.5

Mice were given two injections at 6-h intervals of oil (control, 0.1 ml/mouse), E_2 (10 μg/kg), 4-OH-E_2 (10 μg/kg), kepone (15 mg/kg), 4-OH-E_2 + ICI (20 mg/kg), 4-OH-E_2 + E_2, or kepone + ICI. Mice were killed 6 h after the last injection. "n" denotes the number of replicates. For each replicate the pooled uterine total RNA from three to four mice was subjected to RT-PCR as described. Values were derived from the zero equivalence point of the logarithmic plot as shown in Fig. 4, and fold increases were calculated with respect to oil value. Values with different superscript letters are statistically different ($p < 0.05$, ANOVA followed by Newman–Keul's multiple range test).

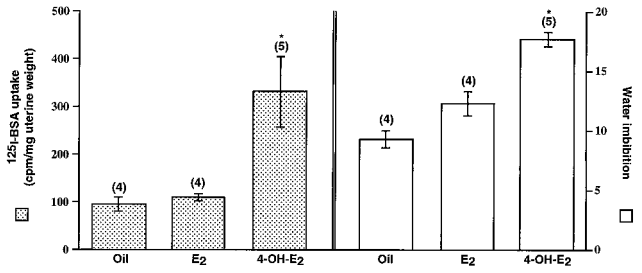

FIG. 5. Effects of E_2 or 4-OH-E_2 on uterine water imbibition (mg) and ^{125}I-labeled BSA uptake in ERKO mice treated with oil (control), E_2 (10 μg/kg), or 4-OH-E_2 (10 μg/kg). Results shown are mean ± SEM. Numbers within parentheses indicate the number of mice used for each treatment group. Values are statistically different ($P < 0.05$, Student's t test) from those treated with oil or E_2.

accumulation were noted in skeletal muscle, liver, and heart by either 4-OH-E_2 or E_2 treatment (data not shown).

Taken together, these uterine responses to estrogens in ERKO mice clearly establish the presence of a pathway that is not mediated via the classical ER-α, but could be mediated by non-ER-α estrogen signaling pathways. Interestingly, several E_2 responses have been observed in mammals lacking ER-α and are proposed to be mediated via ER-β. These mammalian E_2 responses include a small increase in ERKO mouse uterine wet weight after 10 days of high dose E_2 treatment (21), E_2 inhibition of a vascular injury response in ERKO mice (22), and sublingual E_2-induced brachial artery dilation in an ER-α negative man (23).

Although the molecular mechanism(s) by which 4-OH-E_2 or kepone alter uterine functions has yet to be definitively elucidated, several possibilities can be ruled out. First, ERKO mice may have alternatively spliced forms of ER-α (14). The existence of alternatively spliced forms of ER-α that contain the ER 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 (24–26). Second, it is possible that differential effects of the various "estrogenic" ligands in ERKO mice could be mediated by different ER subtypes, such as ER-β or its alternatively spliced forms.¶ However, it is known that E_2 and antiestrogens bind to both ER-α and ER-β ligand-binding domains (6). Our observations demonstrate that E_2 is unable to influence ERKO uterine LF expression, and that neither E_2 nor ICI inhibits 4-OH-E_2- or kepone-induced uterine LF expression in ERKO mice. This lack of inhibition suggests that the effects of this catecholestrogen and xenoestrogen on the uterine LF gene are not mediated via the ligand binding domains of ER-α or ER-β. Further, the level of ER-β is remarkably low in comparison to ER-α in the wild-type mouse uterus (data not shown), and is very low to absent in ERKO mouse uterus.‖

Alternative mechanisms for the mediation of estrogenic responses that cannot be described in terms of either ER-α or ER-β could be via membrane estrogen receptors (27, 28) or other response proteins, such as a novel orphan receptor member of the nuclear receptor superfamily (29). It should also be noted that response to an "estrogen" in a target tissue is not necessarily related to its affinity for or its occupancy of the receptor (30–32), and estrogen-responsive genes with no recognizable estrogen response element have been identified (33–35). In addition, it is now apparent that at least 10 coactivator proteins or basal factors form combinatorial complexes that modulate receptor function (36–39), and ER-α and ER-β themselves form a heterodimeric complex (40, 41). All of these ER complexes have the potential to differentially affect the regulation of gene expression by estrogens or even the receptor's ability to bind specific ligands, and it is possible that kepone and 4-OH-E_2 actions are mediated via the formation of such complexes.

Cross-talk between different intracellular signaling systems may also generate effects that mimic those induced by natural ligands (36–43). Thus the dogma that steroids or their mimics function only by interacting with a specific nuclear steroid receptor that serves as a transcription activating factor may not be totally reflective of *in vivo* mechanisms.

In summary, our findings in ERKO mice demonstrate that a catecholestrogen or a xenoestrogen can up-regulate the expression of an estrogen-responsive gene in the uterus via a pathway that does not appear to involve nuclear ER-α or ER-β. Because xenoestrogens and catecholestrogens are implicated in both embryo implantation (8, 44) and breast tumorigenesis (45), further characterization of this pathway will enhance our understanding of diverse steroid hormone actions in target organs.

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