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13. ABSTRACT *(Maximum 200 Words)*

Previous studies suggest the presence of a distinct non-ERα/ERβ estrogen-signaling pathway in the ERαKO mouse. To further characterize the receptor mechanism regu-lating these novel 4OHE2-induced responses, we have synthesized [^{3}H]4OHE2 (4-hydroxy-estradiol) and [^{3}H]4OHE1 (4-hydroxyestrone) using a cytochrome P450-mediated enzymatic procedure. Using ERαKO cellular cytosolic extracts, [^{3}H]4OHE2 specific binding was competed with various unlabeled catechol estrogen compounds but not unlabeled 17βE2 and ICI 182,780. [^{3}H]4OHE2 binding studies indicated significant binding differences among various tissues in WT (wild-type), ERαKO and ArKO (aromatase knockout) female mice which may indicate regulation by either E2 or ER□. In WT & ERαKO mice, the highest concentra-tions of specific [^{3}H]4OHE2 binding were found in the bladder, lung & skeletal muscle. Interestingly, the highest concentrations of specific [^{3}H]4OHE2 binding among ArKO tissues were found in the mammary, uterus and ovary. Scatchard analysis of [^{3}H]4OHE1 binding in ERαKO mice identified a single class of high-affinity ($K_d \cong 1.7$ nM), saturable binding sites in several tissues not competed by E2. Collectively, our results suggest the inter-action of these radiolabeled catechol estrogen compounds with a putative mouse "ERγ" protein. Efforts to clone this "Erγ" are ongoing.

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Introduction:

Importance of Catechol Estrogen Responses in Human Breast Cancer

Catechol estrogens have been studied extensively since enzymes capable of synthesizing them *in vivo* were discovered in the late fifties and early sixties. Their chemistry, biology, and potential functions have been well reviewed over the years (3-7). We will briefly review the importance and significance of catechol estrogens here. Catechol estrogens' structure and metabolism are diagrammed in **Figure 1** below.

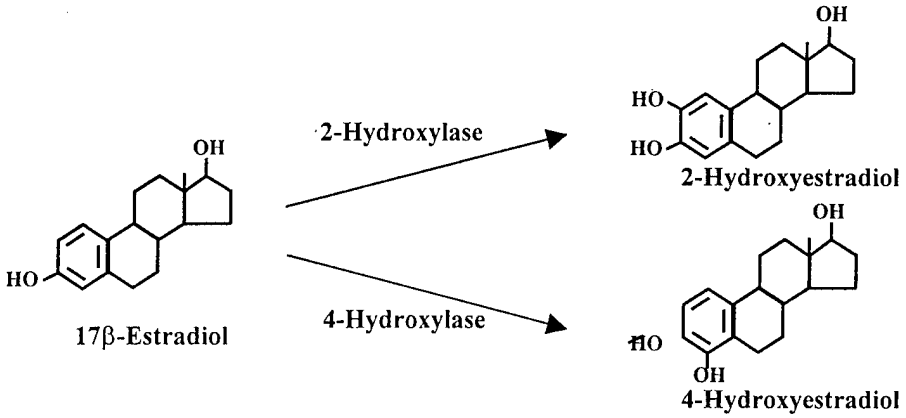


Figure 1. Metabolic conversion of 17β-estradiol to catechol estrogens: 2-hydroxyestradiol and 4-hydroxyestradiol.

Investigating the overall mechanism of estrogens' actions through all their receptors is crucial in understanding breast tumor progression, prognosis, and therapy. Catechol estrogens have been found to be tumor associated in many studies of human breast cancer (Refs. 74-101), human uterine cancer (8), and hamster kidney tumor formation (9,10). Their biological function is thought to be highly localized because of their rapid clearance from plasma (11). Ratios of 2- and 4-hydroxyestradiol concentrations vary between tissues because of differences in rates of synthesis and metabolism to their methoxy derivatives (6-8,12). The tissue specificity data and the data presented in Preliminary Studies (102) strongly support the hypothesis that catechol estrogens are more than simple catabolic products destined for excretion, but have potentially important roles in human physiology and disease as ligands for as yet uncharacterized receptors. We hypothesize that the existence of the putative 4-hydroxyestradiol receptor has been masked, mainly because catechol estrogens will also bind and activate the classic ER protein (13,14), which is present at relatively higher concentrations in humans.

BODY

Candidate Proteins Involved in Non-Classical Estrogen Responses

Candidate proteins fall into one, or possibly more, of the categories of estrogen response proteins listed in **Table 1** on the next page. Proteins #1 - #12 are listed below the Table as candidates for the putative 4-hydroxyestradiol receptor (4OHER), listed briefly for completeness, and/or listed for their potential as confounding factors in characterizing the putative 4-hydroxyestradiol receptor.

Table 1: Response Found with a Biologically Active "Estrogen" that is Mediated by a Particular Type of Potential Estrogen Response Protein

	Types of Estrogen Response Proteins				
"Estrogens"	A1(ER)	A2(ASER)	B1(NCER)	B2(4OHER)	B3(MXCR)
17β-Estradiol	Yes	?	?	No	No
4-Hydroxyestradiol	Yes	?	?	Yes	No?
Methoxychlor (metabolites?)	Yes	?	?	No?	Yes
[Other Natural Estrogens: Estrone, Estriol, Other Catechol estrogens, Flavones, Phytoestrogens. **Synthetic Estrogens:** Diethylstilbestrol, Tamoxifen; Kepone, Some Other Pesticides, Insecticides, Herbicides, etc.]	Yes	?	?	?	?

Five Types of Estrogen Response Proteins

A1. Classical Full Length Estrogen Receptor (ER) Protein

Examples; Nuclear receptor from ER gene, also called ER-alpha membrane (non-genomic) receptor?, Welshons' non-translocatable receptor?,

A2. Non-classical Alternately Spliced Estrogen Receptor (ASER) Proteins

Examples; Alternate splice form of A1, Shupnik's alternately spliced pituitary ER mRNA coding for a novel protein? and potential alternately spliced protein, artifact in ER-α minus mice?

B1. Non-classical Estrogen Response (NCER) Proteins

Examples; Gustafsson's ER-beta, Type II ER, tamoxifen receptor, other catechol estrogen receptors / orphan receptors (G-protein / 7 transmembrane or steroid receptor super families)?, Welshons' non-translocateable receptor?, c-erbB2, membrane (non-genomic) receptor?

B2. Putative 4-Hydroxyestradiol Receptor (4-OH ER) Protein

B3. Putative Methoxychlor Receptor (MXCR) Protein

Includes receptors for possible methoxychlor metabolites that may be mediating responses.

Table 1 Footnote:

Description of potential estrogen response protein candidates (#1-#12)

1) Classic, wild-type, full length ER (or Type A1) protein

In general, non-classical, genomic estrogen response pathways potentially involve at least one of the non-ER proteins (described below). Additional, non-classical, non-genomic estrogen response pathways (15-18) that may be mediated through the classic ER protein or other non-ER proteins are not part of this proposal.

2) Catechol estrogen "receptor(s)" - (Putative 4-hydroxyestradiol receptor?)

Catechol estrogens are produced by the hydroxylation of the 2 and 4 positions of estradiol (see Figure 1, page 20). While catechol estrogens bind to the classic ER with high affinity (14,15), 17b-estradiol does not bind to a partially purified membrane "catechol estrogen" receptor (19). Because of this lack of 17b-estradiol binding, the fascinating literature suggesting novel functions for catechol estrogens (reviewed earlier) and most importantly, the responses seen

with 4-hydroxyestradiol in the ER-α minus mice (see Preliminary Studies section), we also hypothesize that a unique receptor for catechol estrogens may exist. Methoxychlor metabolites are known that contain the catechol structure of two adjacent hydroxyls on an aromatic ring (20). Because of the similarities in chemical structure, it is possible to speculate that a methoxychlor metabolite might also bind to a putative catechol estrogen receptor. Proposed competition studies would allow us to examine this question (see ***Specific Aim #2****).*

3) Jan-Ake Gustafsson's Estrogen receptor - beta

ER-beta, a novel member of the steroid receptor super family, has been cloned by Gustafsson et al. (personal communication, 21,22). Homology of ER-b to the classic estrogen receptor (now termed ER-alpha) is 17% in the N-terminal region, 96% in the DNA-binding domain, 29% in the hinge region, and 55% in the estradiol-binding domain. ER-beta expressed in rabbit reticulocyte lysates binds to estradiol with an affinity of 0.6 nM. It is 485 amino acids long with a molecular weight of 54,000 daltons and can activate transcription through an estrogen response element. ER-b is found in rat prostate from which it was cloned, primarily in the epithelial cells (ER-a is primarily in prostate stromal cells), in the uterus, and in most other tissues but not in mammary gland or in the breast cancer cell lines, MCF-7, ZR75 or T47D. In uterus ER-beta seems to be present at 5-10% of the concentration of classic ER. We are now checking, as is likely, to see if this explains the residual estradiol-binding activity found in the ER-α minus mice. Unique binding characteristics to distinguish ER-alpha and -beta have not yet been released. The lack of inhibition by estradiol and ICI of the 4-hydroxyestradiol response excludes ER-beta as a candidate for the putative 4-hydroxyestradiol receptor (21,22, 102 – see Appendix).

4) Orphan receptors of the Steroid Receptor Superfamily, ERR1 and ERR2

The estrogen-related receptors 1 and 2 (ERR1 and ERR2) have limited homology to the ER gene and are not reported to bind estrogens (catechol estrogens, methoxychlor and kepone are not mentioned) (23). Their native ligands are unknown (hence the name orphan receptors) and, except for their homology to ER, they have no known functional connection to estradiol. Because a response has been observed to 4-hydroxyestradiol, methoxychlor and kepone in ER-α minus mice, we must seriously consider that the putative receptors to which they bind may be orphan members of the steroid receptor superfamily. Homology to a known steroid-binding gene family would allow the screening of cDNA libraries under low stringency conditions and would enable additional candidate genes for the putative methoxychlor receptor, like ERR1 and ERR2, to be cloned, expressed, and analyzed (see ***Specific Aim #4****).*

5) Membrane estrogen receptor or binding protein

Despite nearly 30 years of reports it is only recently that a "membrane estrogen receptor" has gained qualified acceptance (24). Evidence for membrane estrogen receptors come from reports based on biochemical isolation (25-27), immunocytochemistry (28), fluorescent labeled estradiol (29,30) and estrogen immobilization on an inert support (31). It is not clear that these all represent the same protein because of the widely divergent techniques used in the analyses.

6) Type II Estrogen Receptor

This low affinity estrogen binding protein with a binding affinity lower than the classic ER has been the subject of recent successful purification reports (32,33). The type II ER is 73 kd in size with a Kd for estradiol of 24 nM. The ER-α minus residual uterine estradiol-binding activity (see #10) appears not to be Type II ER because of its 0.2 nM K_d for estradiol (1,2).

7) Putative Tamoxifen Receptor

There exists a tamoxifen-binding protein that is distinguished from the classic ER on the basis of binding specificity studies (34-36). Estradiol has little or no affinity for this "tamoxifen" receptor (34). In preliminary experiments we have not found any biological responses to tamoxifen in the ER-α minus mice; unlike methoxychlor, kepone or 4-hydroxyestradiol, tamoxifen does not induce lactoferrin mRNA.

8) Welshons' non-translocatable cytoplast ER

Currently there is a form of estrogen receptor that is not found in the nucleus after estrogen stimulation in estrogen-sensitive breast cancer cells (37). Present evidence suggests a post-translationally modified ER, perhaps positioned to mediate non-classical, non-genomic effects.

9) c-erbB2, also called neu or HER2

There has been a surprising recent report, unconfirmed as far as I know, that the protooncogene, c-erbB2 binds estradiol with a 2.7 nM K_d (38). This protein is a 185 kd transmembrane glycoprotein similar to the EGF receptor. Estradiol is reported to activate the tyrosine kinase activity of c-erbB2 and down regulate this protein.

10) Residual Uterine Estradiol-Binding Activity Found in ER-α Minus Mice (Artifact?)

Recent work from our own lab has demonstrated that Gustafsson's ER-beta is the likely source of most of the 5-10% residual uterine estradiol-binding activity (1,2) and not ER-a, since that is the level that has been reported for ER-

beta in the uterus (21,22). No classic estradiol responses potentially mediated through this residual activity have yet been found (1,24) although differential display suggests they exist (data not shown).

11) Shupnik's translation product (ASER) from alternately-spliced pituitary ER mRNA

This alternately spliced form of ER mRNA is present in high levels in rat pituitary (39,40). We have made the necessary oligonucleotides and will use them to look for an ASER mRNA in the ER-α minus uterus and pituitary. (No resources were requested for these studies in this proposal.)

12) A novel NCER protein

A completely novel NCER protein might be detected in the homozygous ER-α minus mice. A lack of homology to other known receptors when attempting to clone a completely novel, putative 4-hydroxyestradiol receptor ***(See Aim #4)*** *would require isolation and cloning techniques that utilized affinity chromatography or expression cloning.*

Cell-Type Specific Uterine Expression of Lactoferrin (LF) Gene in ER-α minus Mice Exposed to Estradiol-17β, Methoxychlor, Kepone, or 4-Hydroxyestradiol (4-OH-E_2)

Early experiments were performed to examine whether uteri of homozygous ER-α minus mice mutated for the ER gene respond to various estrogenic compounds (**102, 103, 103**). Estrogenic compounds examined were estradiol-17β (primary estrogen), methoxychlor (41-43, 102), kepone (44), and 4-hydroxyestradiol a catechol estrogen. Additional experiments were carried out using tamoxifen and ICI-182,780 (antiestrogens), and ICI-182,780 in conjuction with estradiol-17β or 4-hydroxyestradiol.

Surprising and exciting results were observed. Consistent with previous findings (2), treatment with estradiol-17β failed to induce the LF gene in the uterus of ovariectomized ER-α minus mouse. However, to our surprise, treatments with 4-OH-estradiol-17β, methoxychlor (**103**), or kepone induced the expression of this gene in the uterine epithelium of ovariectomized ER-α minus mice; the expression was most prominent in the luminal epithelium. These results have been verified via quantitative PCR (102,103). Treatment with the ER-specific antagonist, ICI-182,780 alone (45,46), did not influence this uterine gene. However, again to our surprise, ICI-182,780 did not interfere with the induction of the LF gene by 4-OH-estradiol. Because estradiol is non-responsive, it also was used in competition experiments with 4-OH-estradiol-17β, where it was also unable to interfere with LF induction.

Additional control experiments with the COMT (catechol O methyl transferase) inhibitor, U-0521, (47) have shown that it does not stimulate LF mRNA induction. This is important because catechol estrogens are capable of inhibiting COMT, which is necessary for the inactivation of dopamine, a catecholamine. Increased local dopamine levels might induce LF mRNA synthesis through the catecholamine receptors. The lack of effect by a COMT inhibitor indicates that 4-hydroxyestradiol is not acting indirectly to stimulate LF mRNA induction. In addition, since COMT alone does increase active dopamine levels, the likelihood is decreased that the lactoferrin response is mediated through a catecholamine receptor.

These results suggest the presence of a signaling pathway that is not mediated via the conventional ER, but rather by the putative 4-hydroxyestradiol receptor. (The competitive inhibition results of 4-hydroxyestradiol with estradiol or ICI-182,780 are very important because they tend to exclude any confounding problems with the ER-α minus residual uterine estradiol-binding activity / Gustafsson's estradiol-binding ER-beta (21,22).) These results suggest that an acute treatment with 4-hydroxyestradiol induces the LF gene in a cell-type specific manner in the mouse uterus. In recent results, we have confirmed the original results with lactoferrin mRNA and also observed that glucose 6 phosphate dehydrogenase is also induced in ERa-minus mice uterus by methoxychlor but not by estradiol (103).

Hypothesis/Purpose:

4-Hydroxyestradiol and other biologically important estrogens work, not only through the classic ER protein, but also through their own unique non-ER receptors. These receptors can be readily characterized in an ER-α minus mouse background. Specifically, estrogen responses in the uterus and mammary gland are not mediated exclusively by the classical ER protein but in addition by specific types of non-classical estrogen response (NCER) proteins. Characterization of estrogen responses and NCER proteins in ER-α minus mice will lead to a fuller understanding of the physiological and pathological roles of all estrogens in breast cancer.

Original Technical Objectives/Specific Aims:

The first estrogen responses found in ER-α minus mice are to 4-hydroxyestradiol (a catechol estrogen), methoxychlor, and kepone. We are proposing initially to characterize the 4-hydroxyestradiol response and the putative 4-hydroxyestradiol receptor, because of the importance of catechol estrogens in breast cancer.

Specifically, this grant proposes experiments to:

Specific Aim #1. Characterize lactoferrin mRNA responses to 4-hydroxyestradiol in ER-α minus mice.

Specific Aim #2. Characterize the putative 4-hydroxyestradiol receptor in ER-α minus mice.

Specific Aim #3. Compare the specificity of the responses to 4-hydroxyestradiol with those of estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in ER-α minus mice.

Specific Aim #4. Clone the putative 4-hydroxyestradiol receptor (4OHER).

Experimental Design / Methodology:

Hypothesis #1: Lactoferrin will respond to 4-hydroxyestradiol through a receptor-mediated pathway.

Specific Aim #1.

Characterize lactoferrin mRNA responses to 4-hydroxyestradiol in ovariectomized (ovex), ER-α minus mice.

1a. *In vivo* uterine characterization of lactoferrin induction via *in situ* hybridization analysis.

i. Dose response by within-run computer image analysis (Ambion)

ii. Specificity by comparison of 4-hydroxyestradiol responses with these 5 compounds: estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen

1b. *In vivo* tissue specificity and quantitation of lactoferrin mRNA response to 4-hydroxyestradiol via *in situ* hybridization analysis and quantitative PCR. Examine uterus and mammary gland.

1. Rationale:

1a. Characterization of lactoferrin mRNA response to 4-hydroxyestradiol provides potential clues about purification and function of the putative receptor for 4-hydroxyestradiol. A dose response curve will be obtained *in vivo* then *in vitro* to get an estimate of the physiological/pharmacological concentration range of 4-hydroxyestradiol. This will give us a rough idea about the putative receptor binding constant. Two of the six compounds, 4-hydroxyestradiol and methoxychlor, were chosen because they elicit responses in ER-α minus mice. The catechol estrogen, 4-hydroxyestradiol, is made from estradiol by a hydroxylase and methoxychlor is known to be metabolized to a catechol (20). 2-Hydroxyestradiol is usually found in varying rations where 4-hydroxyestradiol is made (See **Figure 1**). 4-Methoxyestradiol is considered to be an inactivated form of 4-hydroxyestradiol. However, this 4-methoxy form is less active only with ER; it is not clear if it will also be inactive with 4OHER. 2-Hydroxyestradiol and 4-methoxyestradiol if unresponsive, as well as estradiol, tamoxifen, and ICI-182,780 will be used in competition studies to roughly determine the specificity of the receptor response.

1b. Determination of lactoferrin mRNA expression in various tissues after 4-hydroxyestradiol exposure will provide information about the receptor's tissue distribution. Tissues to be checked express lactoferrin (48-50). Quantitation will be by quantitative PCR. If possible, a response in a primary uterine culture would rule out an indirect endocrine action of 4-hydroxyestradiol.

1. Experimental Design / Methods / Expected Results / Potential problems:

1a. Steroids are from Steraloids Inc. (Wilton, NH); ultrapure methoxychlor is from Radian (Austin, TX). *In situ* hybridization will be performed to determine the dose response of methoxychlor induction of uterine lactoferrin mRNA. Initial doses will be one oral dose in oil followed by a 24-hour interval of 500 ng, 5 ug, 50 ug, 500 ug, and 5 mg per 30 gram mouse. If it does not appear saturable, we will try to go to 50 mg. A time course, from 2 hours after the last optimal dose from above, will be extended out until values return to background. This will determine the rate of the reversibility of the response. Estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, tamoxifen, and ICI-182,780 will be used individually (doses determined by dose response curve) and in combination (at 100 fold excess) with 4-hydroxyestradiol to determine if they will stimulate or inhibit the response and are working through the same receptor mechanism.

1b. The listed tissues express lactoferrin (48-50) and will be examined by *in situ* hybridization for responses at the optimal dose found in uterus. These optimally responding tissues will be examined for dose response to roughly compare their binding and response parameters with the uterine receptor.

Hypothesis #2: There is a unique receptor for 4-hydroxyestradiol distinct from ER-α and ER-β.

Specific Aim #2.

Characterize the putative 4-hydroxyestradiol receptor in uteri from ER-α minus mice.

2a. Localization by subcellular fractionation

2b. Analyze for saturable binding, binding affinity, and ligand specificity

2. Rationale / Experimental Design / Methods / Expected Results:

A receptor by definition must display low capacity, saturability, and specificity with a binding affinity consistent with its dose response curve. Thus, binding studies with [6,7-^{3}H]-4-hydroxyestradiol will be performed by whole cell uptake in primary uterine culture. Competition studies will be performed with estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, tamoxifen, and ICI 182,780 to demonstrate specificity. 2-Hydroxyestradiol and 4-methoxyestradiol will be used because they are estradiol metabolites and are found wherever 4-hydroxyestradiol is found. Similarly, methoxychlor is also known to have catechol and methoxy metabolites (20). It is possible that catechol estrogens and methoxychlor may share the same non-ER receptor as well as being capable of activating ER. Unique binding specificity will allow us to utilize wild type swine or bovine abattoir sources to purify the 4-hydroxyestradiol receptor away from any unique estradiol-binding protein or wild type ER protein. Subcellular fractionation studies will be done to help determine which type of receptor super family the putative methoxychlor receptor belongs. If the receptor is nuclear or cytoplasmic, and not found in the membrane, it is more likely to be a member of the steroid receptor super family. Binding affinity and specificity (competition) studies will be performed with ^{3}H-catechol estrogens synthesized and purified in our labs.

Hypothesis #3: The 4-hydroxyestradiol receptor will induce unique & specific responses distinct from both estradiol and 4-hydroxyestradiol action through ER-α and ER-β. These are best found by utilizing ovex, ER-α minus mice.

Specific Aim #3.

Messenger RNA responses to 4-hydroxyestradiol will be compared with those potentially observed with estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in the following assays:

3a. Differential display PCR in ER-α minus uteri treated with the 6 listed estrogens/compounds.

3b. Northern & *in situ* hybridization analysis for candidate mRNA responses, like progesterone receptor and glucose-6-phosphate dehydrogenase in uterus, mammary gland, and other selected tissues.

3. Rationale/Experimental Design/Methods:

Time course and dosage regimens will be as described earlier. Select mRNA candidates will be analyzed for their response to the 6 listed compounds. Candidate mRNA responses include lactoferrin, cox-1, progesterone receptor and glucose-6-phosphate dehydrogenase (51); others may be substituted depending upon the results of differential display PCR or subtractive hybridization. These mRNAs have been chosen, because they are known to respond to estradiol in uterus (2) (and therefore are estrogenic responses) and because we have shown that the first two respond to 4-hydroxyestradiol in ER-α minus uterus where estradiol does not invoke a response. Primarily we will examine the uterus and mammary gland for responses, but other tissues will be frozen away and examined, if other funding becomes available.

3. Expected Results/Potential problems:

Responses to 4-hydroxyestradiol and methoxychlor are expected. Estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, and tamoxifen are potentially responsive. From our preliminary data with the ER antagonist, ICI 182,780, it is not expected that estradiol will also work through the putative 4-hydroxyestradiol receptor. However, estradiol, methoxychlor, and tamoxifen are capable of being metabolized to catechols, which could also induce responses. We, of course, expect to see this metabolism but it should alter the same mRNAs

detected by *in situ* hybridization or DDRT-PCR as 4-hydroxyestradiol. Time course experiments and HPLC checks for metabolism of estradiol will be essential (See **Figure 1**).

Hypothesis #4: The 4-hydroxyestradiol receptor protein can be cloned by sequence homology because it is likely to be a member of the steroid receptor super family. Alternatively, the 4-hydroxyestradiol receptor cDNA can be isolated by expression cloning or sequence obtained from receptor protein affinity purification.

Specific Aim #4.

Clone the putative 4-hydroxyestradiol receptor.

4a. Test for 4-hydroxyestradiol binding and responses through Gustaffson's ER-beta.

4b. Screen for receptors that change concentration by differential display PCR analysis in the steroid receptor families using anchored oligonucleotide primers.

4c. Screen uterine ER-α minus mouse cDNA library with probes from conserved sequences of the steroid receptor super family via low stringency hybridization.

4d. Test expressed candidate orphan receptor cDNAs for binding to 4-hydroxyestradiol.

4e. Expression cloning using [^{3}H]- 4-hydroxyestradiol.

4f. Purification by affinity chromatography, then obtain partial peptide sequence for raising epitope specific antibodies or synthesizing oligonucleotide probes for screening of ER-α minus cDNA libraries.

4g. After full-length cDNA clone isolation and sequencing from one of the approaches above (a-f) we will confirm or verify identity of putative receptor by:

i. *in vitro* expression and binding to labeled 4-hydroxyestradiol, or

ii. testing for transcriptional activation of a lactoferrin promoter reporter construct (or other 4-hydroxyestradiol-responsive promoter) with the receptor bound to 4-hydroxyestradiol.

4h. Tissue specific localization of 4-hydroxyestradiol receptor mRNA expression will be ascertained by RT-PCR, Northern blot analysis, or *in situ* hybridization.

4. Rationale / Experimental Design / Methods:

To better elucidate the function of the 4-hydroxyestradiol NCER protein (putative 4-hydroxyestradiol receptor or 4OHER), we must isolate and clone its cDNA. For thoroughness we will need to check the binding of 4-hydroxyestradiol to Gustafsson's expressed ER-beta clone (22) which we have PCR amplified from mouse. 4-Hydroxyestradiol binding ability and/or transcriptional induction characterization of any NCER protein is essential for verification that we have isolated the correct receptor protein. For expression cloning, we will need a way to distinguish the NCER protein from the ER protein, hopefully by their steroid binding characteristics. Thus, detailed specificity, saturability and affinity binding studies will be needed from Specific Aims #1 and #2.

An unpublished, but highly successful method utilized by the PI in the isolation of orphan steroid receptors, is to utilize differential display PCR with anchored oligonucleotides from highly conserved regions from the gene family one is trying to clone, rather than the normal anchored oligos from the poly A tail. The twist here is to utilize DDRT-PCR rather than simple degenerate or low stringency PCR, because then one can screen only for mRNAs that change in concentration after 4-hydroxyestradiol exposure. Because, most steroid receptors down regulate their own mRNA levels, this allows one to distinguish the 4-hydroxyestradiol receptor sequence from other members of its gene family which will have very similar sequences. This is a significant problem because some gene families, like the steroid/nuclear receptor families, contain over a hundred members.

The most widely used procedure with which we have a great deal of experience is the screening of libraries with low stringency probes or PCR amplification under low stringency conditions. The probe used will be from the first zinc finger of the DNA-binding domain of the steroid receptor super family, similar to what the PI utilized in the cloning of the androgen receptor gene and cDNA (52-54).

Candidate cDNA approaches using the proteins discussed in **Footnote Table 1,** (ERR1 and ERR2) also will be tested because of their homology to the estrogen receptor. An alternative procedure to clone the putative 4-hydroxyestradiol receptor would be to prepare ER-α minus uterine cDNA expression libraries in mammalian cells and screen for [^{3}H]- 4-hydroxyestradiol -binding activity (55-57).

While affinity column purification is a potential approach, it is not being proposed as the primary approach, because of both the probable low amounts of activity and the higher efficiency of other approaches. However, an affinity column made from a 4-hydroxyestradiol derivative would likely bind to the ER protein (14). This ER binding to the column could be blocked with estradiol and then an easily obtained abattoir source of receptor from pig or cow uterus would be used. Using this abattoir source to obtain sufficient quantities of purified protein for amino acid sequencing and/or antibody preparation will be difficult, but we do have experience in this type of purification (53). Oligonucleotides generated from protein sequence data or antibodies raised against the NCER protein (or synthetic peptide fragments) will allow cloning of the NCER cDNA from a library for further studies (52,54).

Finally, we will need to express the protein and demonstrate its 4-hydroxyestradiol-binding characteristics or transcriptional activation ability to confirm that we have cloned the correct receptor. Localization by *in situ* hybridization would confirm functional response data from Aim #1 and potentially lead to additional tissues capable of responding to 4-hydroxyestradiol.

4. Expected Results/Potential problems:

A 4-hydroxyestradiol receptor clone will be isolated that is a member of the steroid receptor super family. ER-beta is unlikely to be the 4-hydroxyestradiol receptor because ER-beta can bind and be activated by estradiol and the 4-hydroxyestradiol response was not affected by estradiol competition (preliminary data not shown). Studies of 4-hydroxyestradiol binding to ER-beta and of 4-hydroxyestradiol activation of transcription are needed to determine if ER-beta is mediating the actions of methoxychlor.

Potential problems are legion in the cloning of any novel receptor, but standard biochemical and molecular biology techniques (which the PI is familiar with) are available to surmount them. Subcellular localization will help determine if it is a nuclear receptor. However, if it should localize to the nucleus and/or happen to not be a member of the steroid receptor super family, then approaches 4e and 4f should still be viable. Prioritization of effort will be to try approaches 4a, 4b, 4c, then 4d before going to 4e. Then finally, if necessary, we will develop and utilize an affinity column approach (4f).

General Methods:

Procedures for *In Situ* Hybridization Analysis

(To study estrogen responses, we examined the cell-type specific expression of the LF gene in the mouse uterus by *in situ* hybridization (58,59) in collaboration with SK Dey and SK Das, UKMC (102). In control experiments wild type, ovariectomized mice (C57BL/6) were given a single injection (sc) of oil (0.1 ml/mouse), estradiol-17β (250 ng/mouse), kepone (15 mg/kg), the antiestrogen ICI-182,780 (50 ug/mouse) or the same dose of E_2 30 min after an injection of the same of ICI. Mice were killed 12 h after the last injection and their uteri collected for in situ hybridization.

Labeling and Purification of Catechol Estrogens

Radioactive chemicals are obtained from Dupont NEN (Boston, Massachusetts). Hexa-labeled [6,7-^{3}H]-estradiol is enzymatically treated with human cyp1b1 (4-hydroxylase) with NADPH as a cofactor (60). Since both 2- and 4-hydroxyestradiol (primarily the later) are produced in this system, labeled catechol estrogens are separated and purified by HPLC in Wade Welshons' laboratory (60). Catechol estrogens have stability problems due to oxidation and must be purified by HPLC before use (60). Stability of the labeled catechol estrogens is preserved by addition of 10 mM ascorbic acid to all buffers.

[^{3}H]-Estradiol or [^{3}H]-4-hydroxyestradiol binding assay

Sexually mature mice of each ER genotype are ovariectomized and 7 days later cytosol and nuclear extracts are prepared. Aliquots of each are assayed for [^{3}H]-estradiol or [^{3}H]-4-hydroxyestradiol binding as described previously (61). For normalization of binding data, DNA content of the nuclear fraction is measured using the procedure of Labarca and Paigen (62).

Preparation of mouse uterine cells for primary cell culture.

Immature or ovariectomized female ER-α minus or wild type mice are utilized in the isolation of uterine cells (24). Estrogen-free growth medium is replaced with fresh growth medium the day after plating. Medium is changed again the two days prior to experimentation.

[^{3}H]-Estradiol and [^{3}H]-4-hydroxyestradiol binding - Whole cell uptake assay

This binding assay is performed according to the method of Welshons *et al.* (41,63). Cells will be incubated at 37°C in medium labeled with [^{3}H]estradiol or [^{3}H]-4-hydroxyestradiol in the presence (nonspecific binding) or absence (total binding) of a 100-fold excess of unlabelled ligand. For normalization of binding data, well content of DNA and protein are measured, using the procedures of Labarca and Paigen (62) and Bradford (64) respectively.

Differential display reverse transcriptase PCR (DDRT-PCR) analysis of mRNA

DDRT-PCR is a powerful tool for the analysis of subtle changes in gene expression in tissues and cell lines (65). Early use of this technique has had mixed success in laboratories around the world, but with improvements in methods it has become more reliable (66). This technique allows detection of differentially expressed genes without using specific probes for known gene products and is ideally suited to our purposes of detecting potential subtle 4-hydroxyestradiol, methoxychlor or estradiol, as well as other estrogens, responses in ER-α minus mice. RNA is isolated using a kit (Purescript RNA Isolation Kit) obtained from Gentra Systems, Inc. (Minneapolis, MN). Differential display of mRNA is examined using a kit obtained from GenHunter Corporation (Brookline, MA) (65-67). Steroid-regulated gene expression can ideally be studied by DDRT-PCR because there are relatively few changes in gene expression that occur. The PI has in the past successfully used the technique with "diabetic" tissue culture cell models to isolate novel glucose-responsive orphan steroid receptors from diabetic model cell lines (data not shown, 68). The method is more reproducible in *vitro* culture than *in vivo,* but with three separate uteri, each being run in duplicate, (6 samples per treatment) we reduce our problems with false positives. However, differentially expressed mRNA bands, which are isolated, cloned into plasmids, and expression changes must be confirmed by analysis as probes on Northern gels, by *in situ* hybridization, or by quantitative PCR. After confirmation and sequencing, these probes are used as markers of differential gene expression due to the presence or absence of ER or a particular estrogen treatment.

Quantitation of LF mRNA levels by RT/competitive polymerase chain reaction of the Effects of Estradiol, Kepone or 4-Hydroxyestradiol on Uterine Lactoferrin mRNA levels in Ovariectomized Wild Type or ER-α Minus Mice

Construction of the mutant templates (See Reference 103 in Appendix for details).

To perform a competitive PCR for LF mRNAs, a mutant template (the competitor), containing the same primer template sequences as those of target cDNA competing for primer annealing and amplification, is generated by introducing a non-specific DNA fragment into a mouse LF cDNA clone (71). A 185 bp blunt-ended fragment (SspI) obtained from pGEM7Zf(+) vector, was ligated with the LF cDNA in pGEM4Z at the StuI site. This DNA construct was used to serve as a competitor template to carry out the quantitation of LF mRNA levels in uterine tissues.

RT and competitive PCR.

Total RNA (1 μg) was reverse-transcribed using an antisense oligo (5' GGAACACAGCTCTTTGAGAAGAAC 3') for mouse LF mRNA. The protocol for the RT reaction, for the PCR reaction and the cycle parameters have been described (72). The competitive PCR was performed using the method as described (73). In brief, a fixed amount (1/10th) of the total RT product and increasing amounts (10-fold serial increases) of the mutant template are co-amplified for 30 cycles by PCR, using the mixture of sense (5' AGGAAAGCCCCCCTACAAAC 3') and antisense (as shown above) oligos. The PCR amplified products were analyzed by Southern hybridization using a ^{32}P-end labeled internal oligo (5' CTGCTGTTCTTCACGACTGCTACC 3'). Direct radioimaging of the Southern blot was performed by Ambis

image analysis system to estimate the radioactive intensity of the bands of the target cDNA (276 bp) and competitor cDNA (461 bp). The ratio of band radioactive intensities of the competitor and target cDNAs was calculated for each sample and plotted against the amounts of competitor. The amount of target cDNA is determined from the logarithm plot at zero equivalence point. The efficiency of RT reaction was controlled by measuring the ribosomal protein L-7 (rpl7) mRNAs levels in each sample.

Molecular Biology / Genetic Procedures

Standard procedures will be utilized unless specifically modified as indicated (69,70).

Key Research Accomplishments/Progress

Task/Specific Aim #1. Characterize lactoferrin mRNA responses to 4-hydroxyestradiol in ER-α minus mice. Specificity and dose response.

A paper has been published describing the specificity of lactoferrin mRNA response to 4OHE2 (102). We have had a surprisingly hard time getting RNA from mouse tissues uncontaminated by lactoferrin amplified DNA so that we can do the dose response for 4-hydroxyestradiol. It should be possible because we were able to get a nice saturable dose response curve for methoxychlor which may be acting through a catechol mechanism as well (103). We are likely to replace the in situ approach with a Taq Man quantitation approach because of the ease of analysis and the more quantitative nature of this new technique.

We have had a difficult time obtaining reproducible results from RNA isolated from 4-hydroxyestradiol-treated mouse uterine tissues. This has been a prevalent problem especially among ERαKO mouse uterine tissues. However, we believe this problem to be related to RNA concentration, and not RNA isolation technique, given the extremely small size of the ERαKO mice uteri. Consequently, ERαKO mice uteri will be pooled for a given treatment group (e.g., specific 4-hydroxyestradiol dose, specific 4-hydroxyestradiol treatment time) in order to generate both a dose response curve and time course curve for 4-hydroxyestradiol-induced uterine lactoferrin mRNA response. Although *in situ* hybridization and/or quantitative RT-PCR were originally proposed as methods for lactoferrin mRNA quantitation, we will likely utilize Taq Man quantitation given the ease of analysis and the more sensitive quantitative nature of this new technique.

Task/Specific Aim #2. Characterize the putative 4-hydroxyestradiol receptor in ER-α minus mice.

We have tritium labeled two catechol estrogens and we have characterized their binding to a high affinity protein (proteinase K sensitive, data not shown) in various mouse tissues and cell lines as shown below. This provides strong evidence for an "ER-gamma".

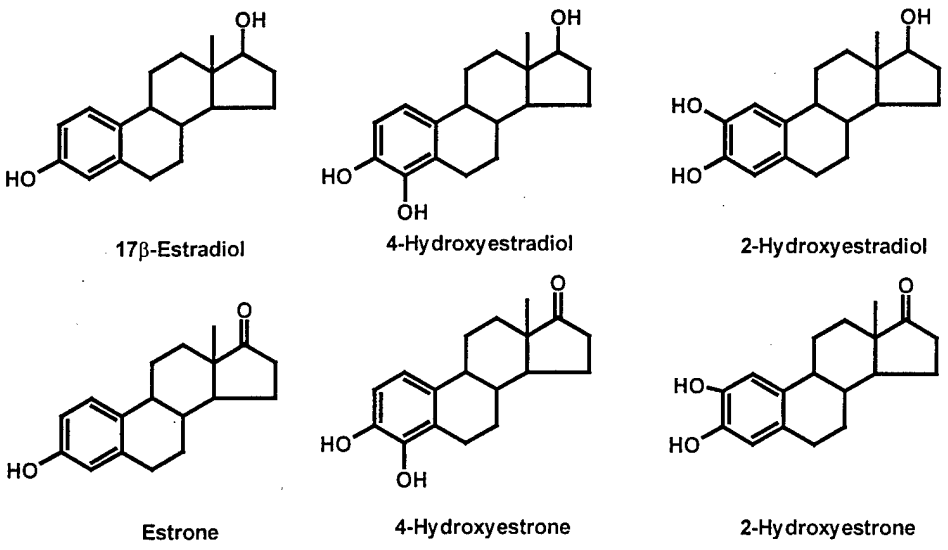


Figure 1.
Structures of 17β-estradiol, 4-hydroxyestradiol, 2-hydroxyestradiol, estrone, 4-hydroxyestrone, and 2-hydroxyestrone.

[^{3}H]-4-Hydroxyestradiol/ [^{3}H]-4-hydroxyestrone Binding.
[^{3}H]-4-hydroxyestrone and [^{3}H]-4-hydroxyestradiol (Figures 1 and 2) were synthesized to be used in various binding studies including tissue distribution and competition studies. Ligand purification and identity were obtained by HPLC (Figures 3A, 3B and 3D). Purified [^{3}H]4-hydroxyestrone was shown to co-elute with an unlabeled 4-hydroxyestrone standard (Figures 3A and 3B). As an additional identity check, the ketone on the 17 position of the steroid was reduced using sodium borohydride to an alcohol, thereby generating 4-hydroxyestradiol. The product of the reaction was shown to co-elute with a 4-hydroxyestradiol standard, thereby confirming the identity of the original labeled ligand as 4-hydroxyestrone (Figure 3C).

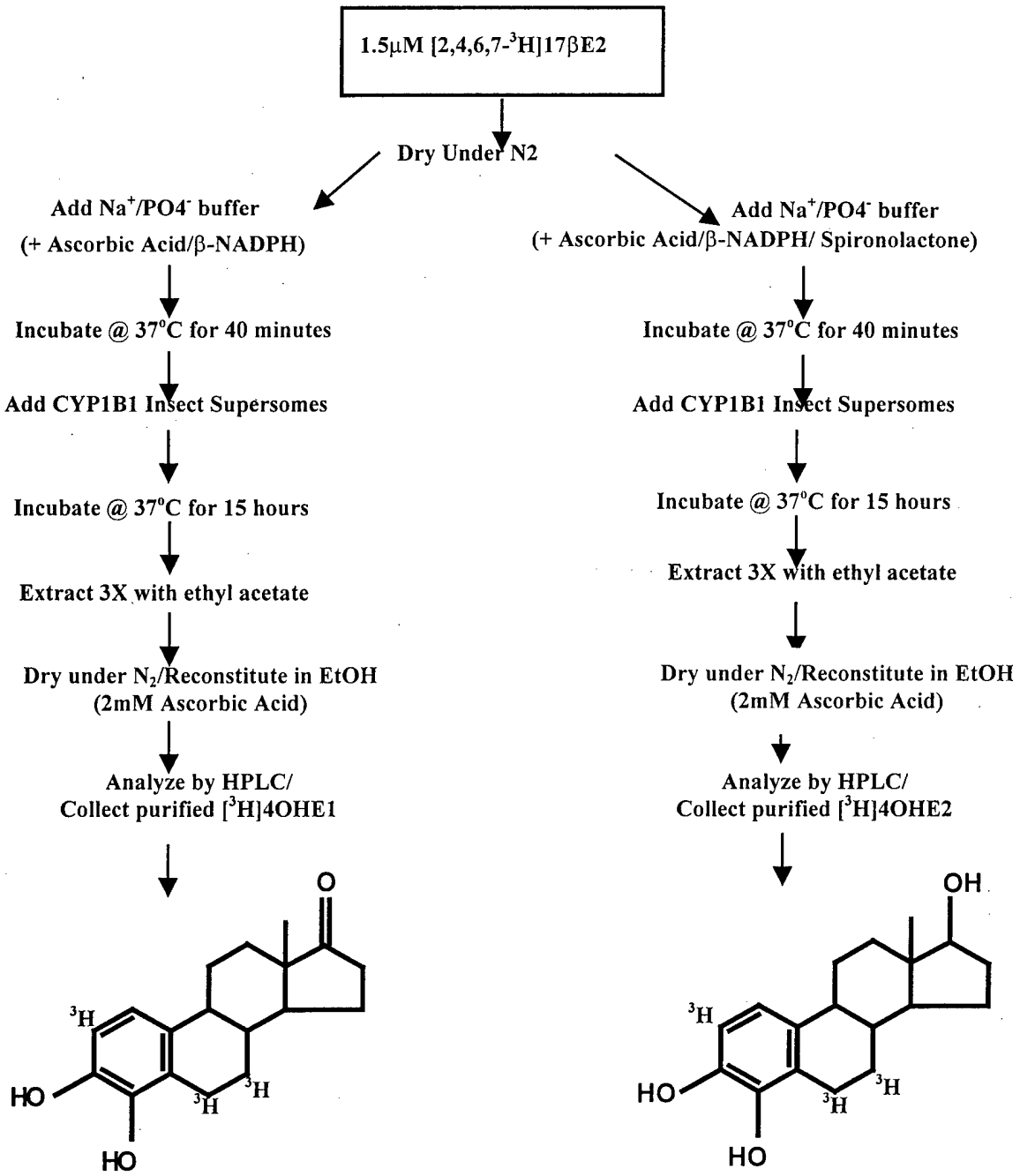
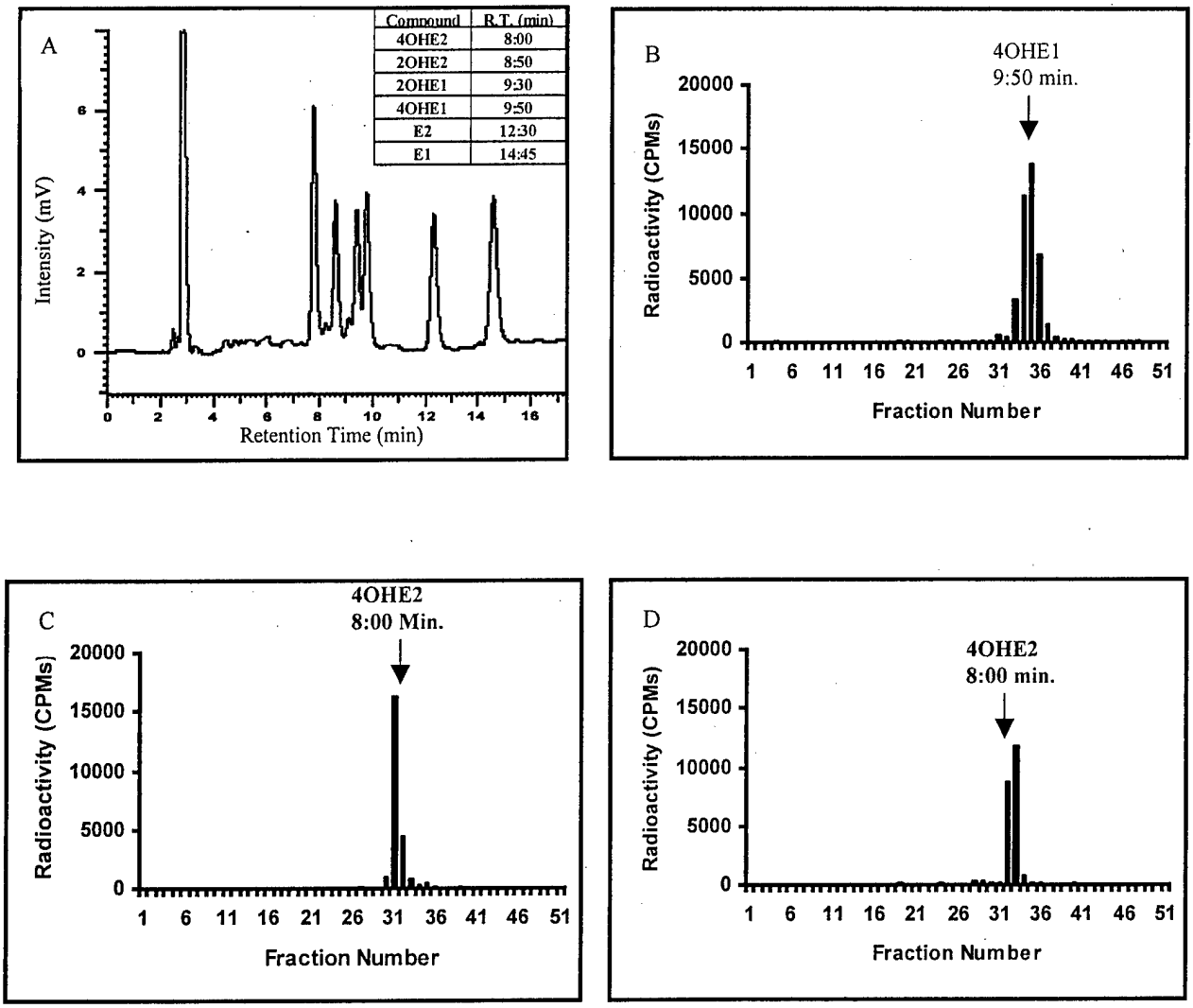


Figure 2. Sequence of steps involved in synthesizing and purifying [^{3}H]4-hydroxyestrone and [^{3}H]4-hydroxyestradiol.

Figure 3. HPLC analysis of various estrogens. The mobile phase consisted of 60:40 A:B (v:v). Component A consisted of 60:40 methanol: acetonitrile, component B consisted of water. A flow rate of 0.8 mL/min and a Phenomenex Luna C-18 250 X 4.5 mm 5 micron column was used. **A.** UV chromatogram of unlabeled 4-hydroxyestradiol (4OHE2), 2-hydroxyestradiol (2OHE2), 2-hydroxyestrone (2OHE1), 4-hydroxyestrone (4OHE1), 17β-estradiol (E2) and estrone (E1) standards. **B.** CPM chromatogram of purified [^{3}H]-4-hydroxyestrone. **C.** CPM chromatogram of Sodium borohydride reduced [^{3}H]-4-hydroxyestrone. Peak elutes at a retention time corresponding to 4-hydroxyestradiol, confirming correct identity of the synthesized ligand as 4-hydroxyestrone. **D.** CPM chromatogram of purified [^{3}H]-4-hydroxyestradiol.



To determine if the binding activity was estrogen regulated, tissue distribution studies were performed in three mouse genotypes, wild type, ERαKO and aromatase knockout (61). All assays were performed in the presence of excess unlabeled 17β-estradiol to saturate any ERα and ERβ present to prevent them from binding to the [^{3}H]-4-hydroxyestradiol. The tissue distribution of [^{3}H]-4-hydroxyestradiol binding for each mouse genotype is listed in Figure 4A. As can be seen, there is a wide distribution of binding across tissues and differences between animal genotype, suggesting estrogen regulation. This binding is seen in the uterus, a tissue shown to elicit an estrogenic response to 4-hydroxyestradiol that is independent of ERα and ERβ (8). Because of this, we are referring to this binding entity as the catechol estrogen receptor (CER).

To further examine the possibility of estrogen regulation, wild type animals were ovariectomized and either treated with a 17β-estradiol implant or not treated. The implant used releases enough estradiol to raise

the serum E2 levels to 300-400 pg/mL Tissues were removed from these animals and tested for [^{3}H]4-hydroxyestradiol binding in the presence of 500 nM 17β-estradiol to saturate any known ER that may be present. There was a difference in specific [^{3}H]4-hydroxyestradiol binding observed in the uterus and lung of these animals suggesting CER regulation by 17β-estradiol (Figure 4B).

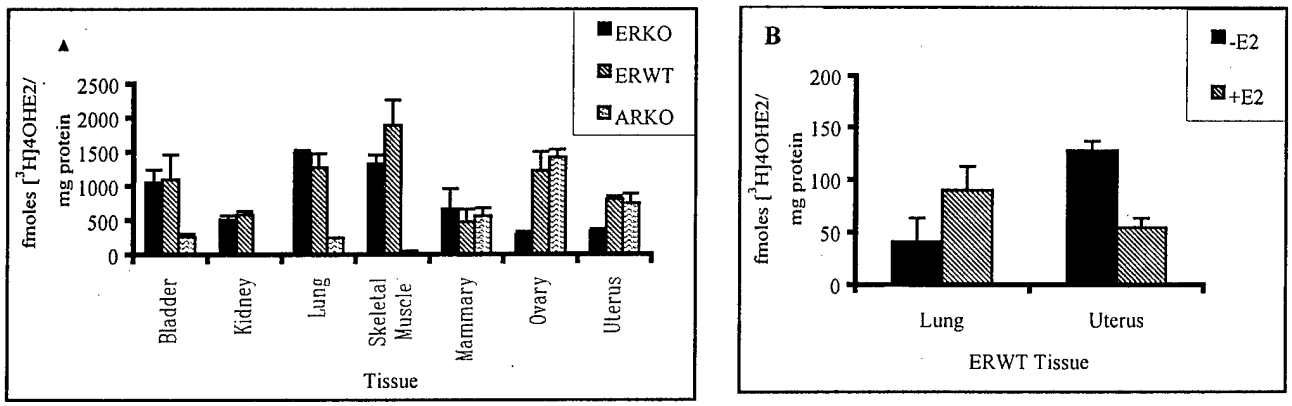


Figure 4. A. Tissue distribution of [^{3}H]-4-hydroxyestradiol binding in wild type, estrogen receptor α knockout and aromatase knockout mice. 1 nM [^{3}H]-4-hydroxyestradiol was added to determine total binding. Nonspecific bound was determined by adding 1 nM [^{3}H]-4-hydroxyestradiol and 500 nM unlabeled 4-hydroxyestradiol. Amount of specific bound [^{3}H]-4-hydroxyestradiol was determined by subtracting nonspecific bound from total bound. Bound [^{3}H]-4-hydroxyestradiol was separated from free ligand by dextran coated-charcoal **B.** To determine possible CER regulation by 17β-estradiol, adult wild type mice were ovariectomized and differentially treated with 17β-estradiol. 1 nM [^{3}H]-4-hydroxyestradiol was added to determine total. Nonspecific bound was determined by adding 1 nM [^{3}H]-4-hydroxyestradiol and 500 nM unlabeled 4-hydroxyestradiol. Amount of specific bound [^{3}H]-4-hydroxyestradiol was determined by subtracting nonspecific bound from total bound. Uteri and lung samples show a marked difference in [^{3}H]-4-hydroxyestradiol binding upon hormone treatment suggesting 17β-estradiol regulation in this tissue.

As an attempt to define additional ligands this receptor binds, various cold competitors were screened to identify compounds that would compete for [^{3}H]-4-hydroxyestradiol binding (Table 1). The most promising competitors to date are 4-hydroxyestradiol, 4-hydroxyestrone, 2-hydroxyestradiol, 2-hydroxyestrone and 2-hydroxyestrone-17-acetate. This suggests that the steroid D ring may be amenable to modification for affinity column synthesis.

Compound	% [^{3}H]4OHE2 Bound ± SE
17β-E2	100 ± 1
4OHE1	8 ± 0
4OHE2	0 ± 1
2OHE1	46 ± 1
2OHE2	23 ± 1
2OHE3	23 ± 1
2-MethoxyE2	70 ± 1
4-MethoxyE2	99 ± 1
2OHE2-17Acetate	39 ± 2
E2-17Acetate	101 ± 2
ICI 182,780	88 ± 2
Epinephrine	88 ± 3
Flutamide	89 ± 2
Genistein	110 ± 3
Spironolactone	119 ± 3
Tamoxifen	99 ± 1

Table 1. [^{3}H]-4-hydroxyestradiol binding competition in ERKO Lung in the Presence of Various Competitors. 1 nM labeled ligand was added along with 500 nm unlabeled competitor. Bound ligand was separated from free ligand by the addition of dextran-coated charcoal. All analysis were conducted in the presence of 500nM cold 17β-estradiol to block binding to estradiol-binding proteins like ERα & ERβ.

To further characterize the putative receptor binding, [^{3}H]-4-hydroxyestrone (Figure 1) was synthesized (Figure2 to be used in various binding studies including tissue distribution and ligand competition studies. Ligand purification and identity were obtained by HPLC (Figure 3). Purified [^{3}H]4-hydroxyestrone was shown to co-elute with an unlabeled 4-hydroxyestrone standard, supporting its identity as 4-hydroxyestrone. Binding affinities and specificities were similar between 4-hydroxyestradiol and 4-hydroxyestrone, suggesting interaction with the same putative receptor.

Because binding specificities and affinities were similar and the non-specific background binding was lower for 4OHE1 than 4OHE2, we used [^{3}H]-4-hydroxyestrone for Scatchard binding analyses. Scatchard analyses using [^{3}H]-4-hydroxyestrone was performed on cell extracts prepared from ERαKO mice. All binding analyses were done in the presence of 500 nM unlabeled estradiol to saturate any known ER that may be present. Ovary, uterus, kidney and liver extracts were tested and all tissues showed high affinity, saturable binding site, possessing a K_d of approximately 1.5 nM in each tissue (Figure 5). Using a homologous competition assay, [^{3}H]-4-hydroxyestradiol gave a similar binding affinity of 5 nm in lung (data not shown).

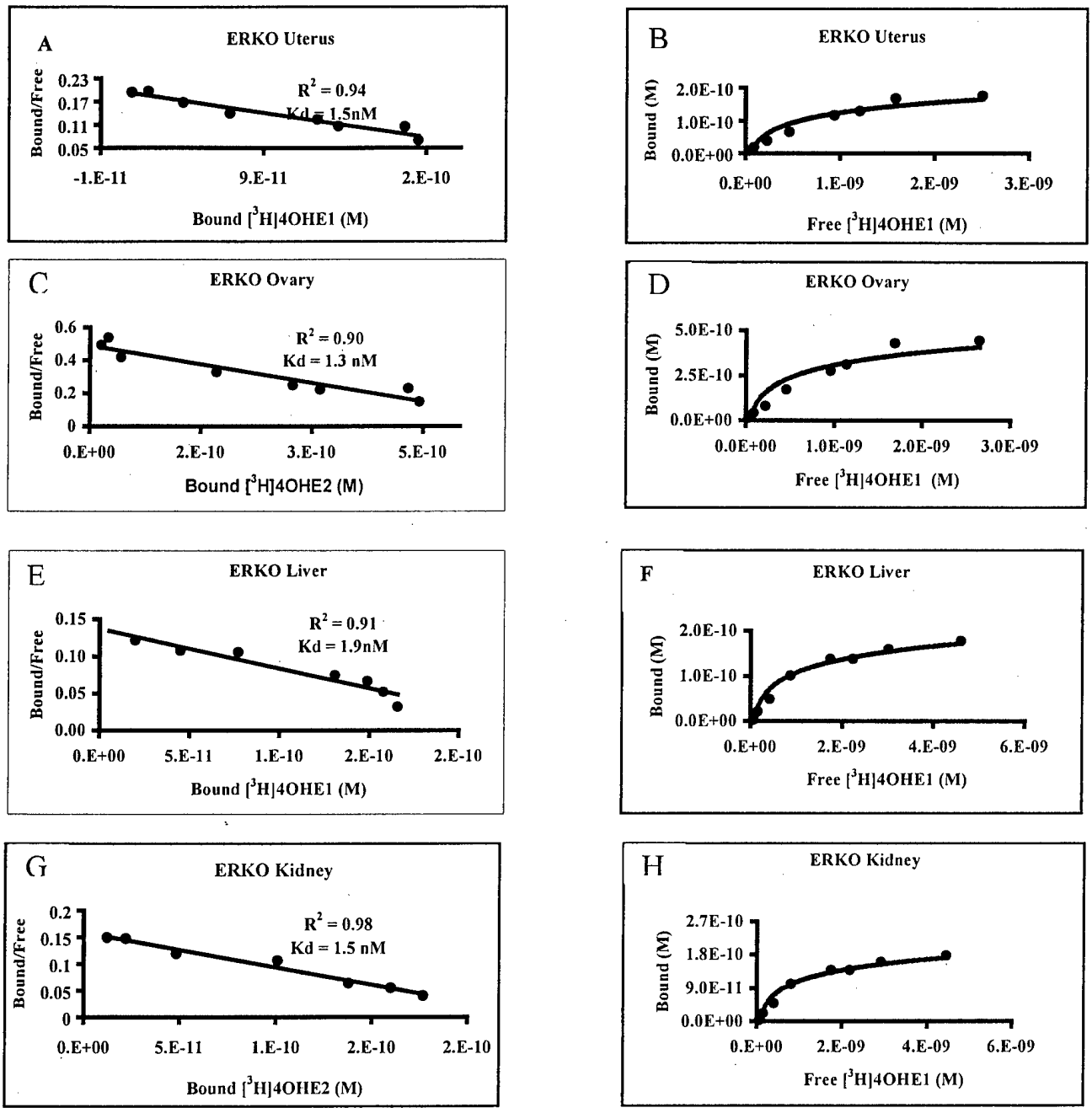


Figure 5. Scatchard plots showing binding affinity and saturation plots showing saturability of [^{3}H]-4-hydroxyestrone in various ERKO tissues. All assays were performed in the presence of 500 nM 17β-estradiol to saturate any ERβ present. Bound ligand was separated from free ligand using dextran-coated charcoal. **A**. Scatchard analysis performed in ERKO uterus with corresponding saturation plot (**B**). **C**. Scatchard analysis performed in ERKO ovary along with the corresponding saturation plot (**D**). **E**. Scatchard analysis performed in ERKO liver along with the corresponding saturation plot (**F**). **G**. Scatchard analysis performed in ERKO kidney along with the corresponding saturation plot (**H**).

ERα/ERβ-Negative Cells.

The MCF-7 breast cancer cell line is the most widely studied *in vitro* model of estrogen action in breast cancer. ERα and certain steroid receptor coactivators are expressed in relatively high concentrations in this cell line (43,44). In addition, numerous genes have been identified to be under transcriptional control by 17β-estradiol in the MCF-7 cell line (45,46). Dr. Edward Curran (University of Missouri) developed two 17β-estradiol nonresponsive cell lines, C4-12-5 and C4-12-6, while he was a graduate student in the laboratory of Dr. Wade Welshons (University of Missouri). These cells were isolated by maintaining the parent cell line in estrogen free media. In our lab we performed whole cell uptake assays to demonstrate the lack of estradiol binding in the C4-12-5 or the C4-12-6 cell lines (data not shown). To determine if the cells were estrogen responsive, we transfected a luciferase reporter containing an ERE (ptkEREluc) into the C4-12-5 cell line and then treated with 17β-estradiol. The C4-12-5 cell line showed no response unless ERα or ERβ were cotransfected into the cells (Figure 6). As a control, this same experiment was performed in COS-1 cells as well, which are known to be ERα/ERβ negative.

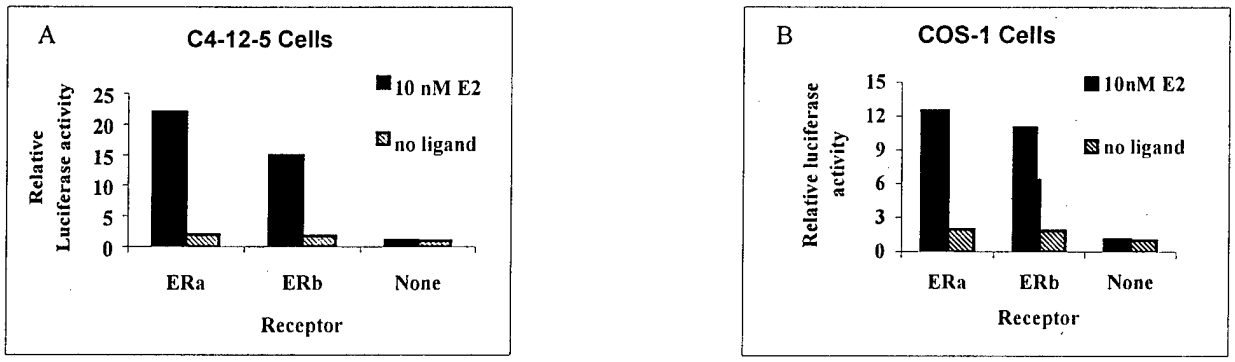


Figure 6. Transcriptional Activation of a Reporter Gene in Estrogen Receptor α/β Negative Cells. **A**. C4-12-5 estrogen receptor negative cells were transiently transfected with either 50 nmoles of ERα, ERβ or empty vector. Cells were then treated with either 10 nm E2 or no hormone and the activation of a reporter gene containing an estrogen response element in the promoter was measured. **B**. COS-1 cells were transiently transfected with either 50 nmoles of ERα, ERβ or no receptor. Cells were then treated with either 10 nm E2 or no hormone and the activation of a reporter gene containing an estrogen response element in the promoter was measured. Neither cell line showed activation in the presence of E2 unless hormone was added, confirming a lack of estrogen receptors α and β. Variation between replicates was less than 5 %.

In addition to binding and transcriptional activation, western blotting with an ERα antibody confirmed the lack of an ERα protein and RNase protection assays showed the lack of ERα mRNA (data not shown). Using RT-PCR, the N-terminal region of ERβ mRNA could be detected at very low levels. When taken together, the lack of 17β-estradiol binding, the inability to activate an estrogen responsive reporter, the negative western blot and RNase protection assays demonstrate that ERα is absent in these cells. These tests also demonstrate that the ERβ protein is either not translated or is expressed at very low concentrations in these cells.

The C4-12-5 and the COS-1 cell line were shown in very recent results to bind [^{3}H]-4-hydroxyestradiol, indicating the presence of CER (Figure 7 below). The C4-12-5 cell line will be the model used to try to characterize a CER response via a reporter gene assay.

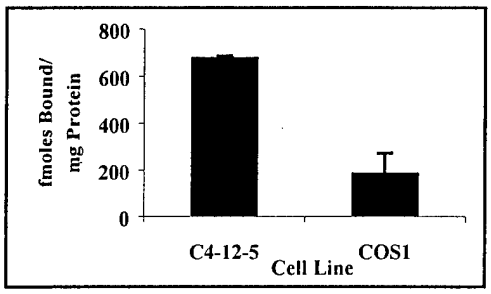


Figure 7. Binding of [^{3}H]-4-hydroxyestradiol in ERα/ERβ negative cell lines. Assays were performed in the presence of 500 nM 17β-estradiol to saturate any estrogen receptors present. 1 nM [^{3}H]-4-hydroxyestradiol was added to determine total binding. Nonspecific bound was determined by adding 1 nM [^{3}H]-4-hydroxyestradiol and 500 nM unlabeled 4-hydroxyestradiol. Bound ligand was separated from free ligand by dextran-coated charcoal. Amount of specific bound [^{3}H]-4-hydroxyestradiol was determined by subtracting nonspecific bound from total bound.

Task/Specific Aim #3. Compare the specificity of the responses to 4-hydroxyestradiol with those of estradiol, 2-hydroxyestradiol, 4-methoxyestradiol, methoxychlor, and tamoxifen in ER-α minus mice. Differential display and candidate responses.

Early results by differential display PCR are promising for a specific mRNA response unique to 4-hydroxyestradiol but not inhibited by ICI or observed with estradiol, thus indicating the 4OHE2 response is not mediated by ER-α or ER-β. Based upon elevated uterine lactoferrin mRNA expression by 4-hydroxyestradiol in ovariectomized ERαKO female mice via a non-ERα, non-ERβ mechanism (102), we employed differential display analysis to identify other estrogen-responsive genes uniquely regulated by 4-hydroxyestradiol in the uteri ERαKO female mice.

Considering the limitations with differential display RT-PCR, microarray technology would allow us to simultaneously analyze thousands of genes at once, some of which we believe are uniquely regulated by 4-hydroxyestradiol via "ERγ". Identification of the genes specifically regulated by ERβ and ERγ are necessary in order to determine their functions. A microarray initiative to aid in determining these functions would be immensely helpful. Our own efforts to explore this microarray approach have quickly run into the obvious problem of cost. Microarray technology is still an expensive approach but it is obviously the technique of choice for gene expression analysis. We hope to utilize microarray technology in the very near future as costs become more affordable, but will continue with differential display analysis until such time. In the mean time a paper has been published that shows in ERaKO uteri there are additional genes induced by both estradiol and catechol estrogen that are not mediated by ERa or ERb (Das S.K., Tan J., Raja, S., Jyotsnabaran H., Paria B.C. and Dey S.K., 2000. Estrogen targets genes involved in protein processing calcium homeostasis and wnt signaling in the mouse uterus independent of estrogen receptor-α and-β. PNAS USA 275:28834-28842.).

Task/Specific Aim #4. Clone the putative 4-hydroxyestradiol receptor (4OHER).

4a. Test for 4-hydroxyestradiol binding and responses through Gustaffson's ER-beta.

4b. Screen for receptors that change concentration by differential display PCR analysis in the steroid receptor families using anchored oligonucleotide primers.

4c. Screen uterine ER-α minus mouse cDNA library with probes from conserved sequences of the steroid receptor super family via low stringency hybridization.

4d. Test expressed candidate orphan receptor cDNAs for binding to 4-hydroxyestradiol.

4e. Expression cloning using [^{3}H]- 4-hydroxyestradiol.

4f. Purification by affinity chromatography, then obtain partial peptide sequence for raising epitope specific antibodies or synthesizing oligonucleotide probes for screening of ER-α minus cDNA libraries.

4g. After full-length cDNA clone isolation and sequencing from one of the approaches above (a-f) we will confirm or verify identity of putative receptor by:

i. *in vitro* expression and binding to labeled 4-hydroxyestradiol, or

ii. testing for transcriptional activation of a lactoferrin promoter reporter construct (or other 4-hydroxyestradiol-responsive promoter) with the receptor bound to 4-hydroxyestradiol.

4h. Tissue specific localization of 4-hydroxyestradiol receptor mRNA expression will be ascertained by RT-PCR, Northern blot analysis, or *in situ* hybridization.

Progress:

1998 Ongoing.

4a. 4-hydroxy estradiol has been shown in a paper by Gustafsson in March 1997 Endocrinology to bind to both ER-α and ER-β. However, this is not the mechanism of the 4-hydroxy estradiol response we have been observing because ER-β is blocked/binds by ICI and estradiol. We are currently testing a new hypothesis that an alternatively spliced form of ER-α or ER-β and/or a heterodimerization of the two may provide unique specificity for 4-hydroxy estradiol.

4b, 4c, 4e, and 4f. Ongoing but very early stages.

4a and 4d. These studies continue. In preliminary data stably transfected ERa and Erb in ER negative MCF-7 cell lines have shown no detectable estrogen binding in the ER-beta lines but do show binding in the ER-a stably transfected lines. Interestingly, when these two types of cell lines are transfected with a construct containing an ERE promoter driving a luciferase reporter and exposed to estradiol, the ER-b line activates the reporter better than the ERa line. Western analyses are ongoing to confirm the quantities of the stably transfected ER-beta. If confirmed, this is the first time that anyone has shown in any cell line that there is a lack of correlation between binding and transcription activation, which may lead to a better understanding of factors that alter the ligand binding specificity, affinity and kinetics of ER. We have also isolated ERR1, ERR3 and will soon have ERR3. We will check to see if it will bind to catechol estrogens and have isolated various ER-beta alternatively spliced forms and will check their responses to catechol estrogens. The MCF-7 cell line in Figure 7 contains catechol estrogen binding activity (ER-gamma?) but does not activate ERE-driven reporters when transfected into them and treated with 4hydroxyestradiol.

4b-4f Ongoing. Many of these are contingent on obtaining labeled catechol estrogens which after much effort we are able to routinely make and purify.

One additional approach that could be used to purify the receptor would be to use an antibody that recognizes the catechol estrogen receptor (CER, also called "ER-gamma" earlier) on an affinity column to pull out any protein that has that motif. A test to determine if the antibody binds to the CER receptor would be to look for a shift in the [^{3}H]4-hydroxyestradiol binding peak on a Sephadex-sizing column or on a sucrose density gradient in the presence of the antibody (104). If a shift were observed, it would indicate the receptor has increased size in the presence of the antibody, meaning that the antibody binds to the receptor. Various ERα or ERβ antibodies could quickly be screened by these methods to check for affinity. Since CER is most likely a homolog with some homology, it is possible that an antibody would cross react. This antibody could then be used to screen an expression library to isolate the cDNA. Cell Extracts have been run over a sephadex G-100

sizing column and the fractions tested for [^{3}H]4-hydroxyestradiol binding (data not shown). The resolution obtained will be adequate to detect a shift due to the binding of an antibody.

We are once again redesigning new primers designed from the host of new fish ERs that have been reported. Recent reports are very encouraging. For example, there are now 3 ERs in goldfish. These primers will be used to amplify mammalian DNAs and cDNAs in hopes of finding a third mammalian estrogen receptor. The original attempt at this with just a few fish ERs was unsuccessful.

Reportable Outcomes:

1) A copy of this paper was attached two years ago as an appendix. (Reference 102. Estrogenic responses in estrogen receptor-alpha deficient mice reveal a novel estrogen signaling pathway. Das SK. Taylor JA. Korach KS. Paria BC. Dey SK. Lubahn DB. Proceedings of the National Academy of Sciences USA 94: 12786-12791, 1997.)

2) A copy of this paper was attached a year as an appendix. (Reference 103. Methoxychlor stimulates estrogen-responsive mRNAs in mouse uterus through a non-estrogen receptor-a and non-estrogen receptor-b mechanism. Ghosh D Taylor JA. Green JA Lubahn DB. Endocrinology. 140(8):3526-33, 1999.)

3. A recent review is in press titled: Estrogen receptor- and aromatase-deficient mice provide insight into the roles of estrogen within the ovary and uterus Rosenfeld CS, Roberts RM, Lubahn DB Molecular Reproduction and Development *in press* 2001

4. Abstract to be presented at the Annual Endocrine Society Meeting in Denver in June 2001 is below:

4-Hydroxylated Catechol Estrogen Binding in Wild-Type, ERαKO and ArKO Mice: Evidence for "ERγ"?

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Estrogens influence diverse physiological processes in target organs via direct interaction with nuclear ligand-activated transcription factors ERα and ERβ. However, despite classical ERα- and β-signaling pathways, there is increasing evidence that gene expression and regulation of cell functions by 17βE2 are mediated by novel (yet uncharacterized) estrogen-response proteins. Estrogenic responses not inhibited by ER antagonists have been well documented in recent years (Das *et al.*, 1998; Ghosh *et al.*, 1999; Singh *et al.*, 2000). In addition to the parent 17βE2 compound, catechol estrogen metabolites are believed to elicit biological responses independent of ERα and ERβ. Although previously considered to be inactive metabolic breakdown products of the circulating primary estrogens, catechol estrogens have been implicated in implantation, parturition and estrogen-induced carcinogenesis and may activate their own unique receptors and/or effectors. Das *et al.* (1997, 2000) have demonstrated recently that 4-hydroxyestradiol (4OHE2) is able to regulate the expression of several estrogen target genes (including lactoferrin) in the uteri of estrogen receptor α-knockout (ERαKO) mice, independent of ICI 182,780 treatment. These findings suggest the presence of a distinct non-ERα/ERβ estrogen-signaling pathway in the ERαKO mouse uterus.

In an attempt to further characterize the receptor mechanism regulating these novel 4OHE2-induced responses, we have synthesized [^{3}H]4OHE2 and [^{3}H]4OHE1 (4-hydroxyestrone) using a cytochrome P450-mediated enzymatic procedure. Using ERαKO cellular cytosolic extracts, [^{3}H]4OHE2 specific binding was competed only with the unlabeled catechol estrogen compounds 2-hydroxyestrone, 2-hydroxyestradiol, 2-hydroxyestriol, 4OHE1 and 4OHE2. Unlabeled 17βE2 and ICI 182,780 failed to compete with this binding. [^{3}H]4OHE2 binding studies indicated significant binding differences among various tissues in WT (wild-type), ERαKO and ArKO (aromatase knockout) female mice. In WT and ERαKO mice, the highest concentrations of specific [^{3}H]4OHE2 binding were found in the bladder, lung and skeletal muscle, whereas reproductive tissues such as the uterus and ovary contained lower but significant concentrations of specific [^{3}H]4OHE2 binding. Compared to ERαKO mice, both WT and ArKO mice possessed elevated specific [^{3}H]4OHE2 binding concentrations in the uterus and ovary. Interestingly, the highest concentrations of specific [^{3}H]4OHE2 binding among ArKO tissues were found in the mammary, uterus and ovary. Scatchard analysis of [^{3}H]4OHE1 binding in ERαKO mice identified a single class of high-affinity ($K_d \cong 1.7$ nM), saturable binding sites in several tissues not competed by unlabeled 17βE2. Collectively, our results suggest the interaction of these radiolabeled catechol estrogen compounds with a putative mouse "ERγ" protein. Characterization of this novel protein is of major importance because of its potential involvement in additional roles of estrogens and their receptors in several human diseases such as osteoporosis, coronary heart disease and breast cancer.

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Conclusions:

Endogenous estrogens can be hydroxylated at multiple sites by NADPH-dependent cytochrome P450 enzymes. The catechol estrogens, 2- and 4-hydroxyestradiol, are a major group of estrogen metabolites formed by the aromatic hydroxylation of 17β-estradiol at the C-2 and C-4 positions, respectively. In mammalian species, catechol estrogen formation from 17β-estradiol is quantitatively the most important metabolic pathway of this endogenous sex hormone. Among the different metabolites of 17β-estradiol, only 2- and 4-hydroxyestradiol have been found to bind to both ERα and ERβ with a relatively high affinity. Although previously believed to be to be benign excretory products, recent evidence suggests that catechol estrogens may be local mediators of estrogen action that possess potent biological and endocrine activities of their own (102,103). In MCF-7 cells, 2- and 4-hydroxyestradiol have been shown to significantly stimulate cell growth and increase progesterone receptor. Effects on embryo implantation, gonadotropin release, partruition and increases in uterine weight also have been reported. More importantly, catechol estrogens have been implicated in hormone-induced carcinogenesis as reviewed earlier (74-101).

To better understand the receptor mechanisms mediating the multitude of estrogenic effects, Lubahn *et al.* (1) generated ERα "knock-out" (ERαKO) mice using homologous recombination techniques. In these transgenic mice, uterine mRNA expression of the estrogen-responsive gene lactoferrin has been shown to be up-regulated by the catechol estrogen 4-hydroxyestradiol and methoxychlor, but not by 17β-estradiol (102, 103). These results suggest the presence of a novel non-ERα, non-ERβ estrogen signaling pathway.

Recent data with labeled catechol estrogens show binding in several tissues that is saturable, specific and of nmolar affinity that is not ER-alpha or ER-beta. This combined with the *in vivo* responses reported earlier strongly suggests a novel ER-gamma" is present in mouse mammary glands and in the MCF-7 breast cancer cell line.

Our new working hypothesis is that the methoxychlor receptor (103) is also the catechol estrogen receptor. This is based primarily on the similar structure of the ligands and the capacity of methoxychlor to be metabolized to a catechol form like the catechol estrogen receptor (20).

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