Estrogen Receptor-Independent Catechol Estrogen Binding Activity: Protein Binding Studies in Wild-Type, Estrogen Receptor-α KO, and Aromatase KO Mice Tissues†

Brian J. Philips,[‡,¶] Pete J. Ansell,[‡] Leslie G. Newton,[‡] Nobuhiro Harada,[§] Shin-Ichiro Honda,[§] Venkataseshu K. Ganjam,[||,+] George E. Rottinghaus,[⊥,+] Wade V. Welshons,[||,+] and Dennis B. Lubahn*[,‡,#,△,+]

Departments of Biochemistry, Veterinary Biomedical Sciences, Child Health, and Animal Sciences, Veterinary Medical Diagnostic Laboratory, and MU Center for Phytonutrient and Phytochemical Studies, University of Missouri, Columbia, Missouri 65212, and Division of Molecular Genetics, Fujita Health University, Toyoake, Aichi, Japan

Received December 1, 2003; Revised Manuscript Received April 2, 2004

ABSTRACT: Primary evidence for novel estrogen signaling pathways is based upon well-documented estrogenic responses not inhibited by estrogen receptor antagonists. In addition to 17β-E2, the catechol estrogen 4-hydroxyestradiol (4OHE2) has been shown to elicit biological responses independent of classical estrogen receptors in estrogen receptor-α knockout (ERαKO) mice. Consequently, our research was designed to biochemically characterize the protein(s) that could be mediating the biological effects of catechol estrogens using enzymatically synthesized, radiolabeled 4-hydroxyestrone (4OHE1) and 4OHE2. Scatchard analyses identified a single class of high-affinity ($K_d \approx 1.6$ nM), saturable cytosolic binding sites in several ERαKO estrogen-responsive tissues. Specific catechol estrogen binding was competitively inhibited by unlabeled catechol estrogens, but not by 17β-E2 or the estrogen receptor antagonist ICI 182,780. Tissue distribution studies indicated significant binding differences both within and among various tissues in wild-type, ERαKO, and aromatase knockout female mice. Ligand metabolism experiments revealed extensive metabolism of labeled catechol estrogen, suggesting that catechol estrogen metabolites were responsible for the specific binding. Collectively, our data provide compelling evidence for the interaction of catechol estrogen metabolites with a novel binding protein that exhibits high affinity, specificity, and selective tissue distribution. The extensive biochemical characterization of this binding protein indicates that this protein may be a receptor, and thus may mediate ERα/β-independent effects of catechol estrogens and their metabolites.

Catechol estrogens are estrogen metabolites formed by the aromatic hydroxylation of 17β-estradiol (17β-E2[1]) and estrone (E1) at either the C-2 or C-4 position (*1*). They are generated by the activity of different intracellular hydroxylases, all of which are cytochrome P450 (CYP450) monooxygenase-associated enzymes. Catechol estrogens are formed in a variety of organs and cell systems of the rat (*2*, *3*) and mouse (*4*, *5*) and in human tissues (*6*, *7*). In mammalian species, catechol estrogen formation from 17β-E2 is quantitatively the most important metabolic pathway of this endogenous sex hormone (*8*), rivaling the parent estrogens in concentration (*9*).

Although the exact physiological role of catechol estrogens remains uncertain, a number of their biological effects have been described. Hormonal activities of these estrogen metabolites have been demonstrated in MCF-7 cells via stimulation of cell growth (*7*, *10*) and increased expression of the progesterone receptor (*7*). Effects on embryo implantation (*5*, *11*, *12*), gonadotropin release (*13*), parturition (*14*),

† This study was supported in part by NIH grants ES08272 and PO1-ES-10535 to D.B.L. and a Breast Cancer grant from the Department of the Army (DAMD-17-97-1-7171) to D.B.L.

* Address correspondence to this author at Room 110A Animal Sciences Research Center, 920 E. Campus Dr., University of Missouri, Columbia, MO 65211. Tel.: (573) 884-6781. Fax: (573) 882-6827. E-mail: LubahnD@missouri.edu.

‡ Department of Biochemistry, University of Missouri.

|| Department of Veterinary Biomedical Sciences, University of Missouri.

⊥ Veterinary Medical Diagnostic Laboratory, University of Missouri.

Department of Child Health, University of Missouri.

△ Department of Animal Sciences, University of Missouri.

+ MU Center for Phytonutrient and Phytochemical Studies, University of Missouri.

§ Fujita Health University.

¶ Current address: Department of Molecular Pharmacology and Cancer Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263.

[1] Abbreviations: AR, androgen receptor; ArKO, aromatase knockout; BPA, bisphenol A; CYP450, cytochrome P450; CYP1B1, cytochrome P450 1B1; DES, diethylstilbestrol; DHEA, dehydroisoandrosterone; DHT, dihydrotestosterone; E1, estrone; E2-17-acetate, estradiol 17-acetate; E3, estriol; EDTA, ethylenediaminetetraacetic acid; ERα, estrogen receptor-α; ERβ, estrogen receptor-β; ERKO, estrogen receptor knockout; ERαKO, estrogen receptor-α knockout; ERR, estrogen-receptor related; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; HPTE, 2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane; $NaBH_4$, sodium borohydride; PCR, polymerase chain reaction; PK, proteinase K; TEG, Tris buffer supplemented with EDTA and glycerol (10% v/v); WT, wild-type; 2OHE1, 2-hydroxyestrone; 2OHE2, 2-hydroxyestradiol; 2OHE2-17-acetate, 2-hydroxyestradiol 17-acetate; 2OHE3, 2-hydroxyestriol; 2-methoxyE1, 2-methoxyestrone; 2-methoxyE2, 2-methoxyestradiol; 4OHE1, 4-hydroxyestrone; 4OHE2, 4-hydroxyestradiol; 4OH-tamoxifen, 4-hydroxytamoxifen; 4-methoxyE2, 4-methoxyestradiol; 16α-E1, 16α-estrone; 17α-E2, 17α-estradiol; 17β-E2, 17β-estradiol; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

10.1021/bi036154j CCC: $27.50
Published on Web 05/07/2004

angiogenesis (*15*), and increases in uterine dry and wet weight (*1*, *16*) also have been reported. Furthermore, catechol estrogens have been implicated in estrogen-induced carcinogenesis (reviewed in ref *17*) in both humans (*18*) and animals (*19*, *20*) through redox cycling mechanisms.

Despite classical ERα- and ERβ-signaling pathways, several lines of evidence suggest that the actions of catechol estrogens could be mediated through alternative mechanisms via activation of their own unique receptors and/or effectors. Such mechanisms of action include putative catechol estrogen binding sites located in cytosol and cellular membranes (*21*−*24*). Additional evidence suggests that these binding sites may function as intracellular transcription factors, potentially related to both ERα and ERβ. With the use of estrogen receptor-α knockout (ERαKO) female mice (*25*) and an ER antagonist (ICI 182,780), work done in collaboration with our laboratory (*26*) demonstrated that 4-hydroxyestradiol (4OHE2) was able to induce the mRNA expression of lactoferrin in the uteri of these ERα-deficient mice. 17β-Estradiol also failed to inhibit this uterine response. Furthermore, 4OHE2 was able to induce the mRNA expression of multiple uterine genes in ERαKO mice, independent of ICI 182,780 treatment (*27*). Taken together, these results strongly suggest the presence of a distinct catechol estrogen-signaling pathway in the ERαKO mouse uterus, independent of both ERα and ERβ.

To characterize the putative receptor mechanism regulating this novel 4OHE2-induced response in ERαKO female mice, we have synthesized radiolabeled 4-hydroxyestrone (4OHE1) and 4OHE2 via a unique CYP450-mediated enzymatic procedure, and then extensively characterized their interaction with a novel non-ERα/non-ERβ binding protein in various tissues in wild-type (WT), ERαKO, and aromatase knockout (ArKO) female mice. Although putative catechol estrogen binding sites have been identified previously in several cell and tissue systems (*21*−*24*), we believe that the murine binding protein identified here is both unique and distinct on the basis of its ligand specificity, tissue localization, and estrogen response characteristics. Also, our findings provide additional and significant information regarding the potential role of these distinct catechol estrogen binding proteins in estrogen-mediated responses.

MATERIALS AND METHODS

Animals. Adult WT and ERαKO male/female mice of the same genetic background (C57BL/6J) were bred and maintained in accordance with the University of Missouri Animal Care and Use Committee guidelines. The mothers were fed Purina Laboratory mouse chow soy-based formulation 5008 (Ralston-Purina Co., St. Louis, MO) until weaning, and then fed soy-based formulation 5001 (Ralston-Purina Co.). Mice were provided water ad libitum and exposed to a daily 12 h light−12 h dark cycle. ArKO male/female mice (bred from mice kindly provided by Shin-Ichiro Honda) were maintained under the same conditions. Genotypes of the mice were ascertained by PCR analysis, as described previously (*25*, *28*).

Cell Culture. All cells were maintained as previously described (*29*).

Chemicals. The radioligand [2,4,6,7-^{3}H]17β-E2 (85−99 Ci/mmol) was purchased from Amersham Pharmacia Biotech Corp. (Arlington Heights, IL). The unlabeled steroids 17β-E2, E2-17-acetate, E1, estriol (E3), 16α-estrone (16α-E1), 16-epiestriol, progesterone, diethylstilbestrol (DES), dexamethasone, and spironolactone were obtained from Sigma Chemical Co. (St. Louis, MO). The unlabeled steroids 17α-estradiol (17α-E2), 2-hydroxyestrone (2OHE1), 2-hydroxyestradiol (2OHE2), 2-hydroxyestradiol 17-acetate (2OHE2-17-acetate), 2-hydroxyestriol (2OHE3), 4-hydroxyestrone (4OHE1), 4-hydroxyestradiol (4OHE2), 2-methoxyestrone (2-methoxyE1), 2-methoxyestradiol (2-methoxyE2), 4-methoxyestradiol (4-methoxyE2), dehydroisoandrosterone (DHEA), and 5-androstenediol were purchased from Steraloids Inc. (Wilton, NH). The catecholamines epinephrine and norepinephrine, as well as tamoxifen (TAM), 4-hydroxytamoxifen (4OH-TAM), (+)-catechin, (−)-catechin, (+)-epicatechin, quercetin, and kepone were purchased from Sigma Chemical Co. The phytoestrogen genistein was obtained from Indofine Chemical Co. (Belle Mead, NJ), and the synthetic antiestrogen ICI-182,780 was obtained from Tocris (Bristol, UK).

[^{3}H]4OHE1 Synthesis. Approximately 1.5 μM [2,4,6,7-^{3}H]17β-E2 (130 μCi) was dried under nitrogen and then incubated with 500 μL of reaction buffer (3.3 mM magnesium chloride, 100 mM sodium phosphate buffer, pH 7.4 (at 23 °C)) containing 2 mM β-NADPH (Sigma Chemical Co.) for 40 min at 37 °C. The reaction was initiated by the addition of 600 μL of cytochrome P450 1B1 (CYP1B1) Insect Supersomes (Gentest Corp., Woburn, MA), which possessed high 17β-hydroxysteroid dehydrogenase (17β-HSD) type II activity. This enzymatic activity, in turn, facilitated the formation of radiolabeled 4OHE1 from [2,4,6,7-^{3}H]E1. The reaction mixture was incubated at 37 °C for 7 h, after which time 450 μL of Insect Control Supersomes (Gentest Corp.) were added. The reaction was allowed to proceed for 14 h at 37 °C and then terminated by the addition of ethyl acetate. The reaction samples were extracted with ethyl acetate, and the extracts were pooled and dried under nitrogen. The resulting residue was dissolved in EtOH containing 2−3 mM ascorbic acid (Sigma Chemical Co.) and analyzed for metabolite composition by HPLC. Ascorbic acid was utilized as a reducing agent to prevent spontaneous oxidation of the radiolabeled product (*30*).

[^{3}H]4OHE2 Synthesis. The procedure was similar to [^{3}H]-4OHE1 synthesis with some minor variations. Spironolactone (0.6 mM; Sigma Chemical Co.) was added to the reaction mixture as a 17β-HSD type II inhibitor (*31*). The reaction mixture, containing 250 μL of CYP1B1 Insect Supersomes, was allowed to proceed for 5 h at 37 °C and then terminated by the addition of ethyl acetate. The radiolabeled products were extracted, reconstituted, and analyzed as above.

HPLC Analysis. Steroids were analyzed and purified by HPLC using a 5 μm Luna reversed-phase C_{18} column (250 × 4.6 mm) (Phenomenex, Torrance, CA) and a mobile phase consisting of 36% methanol:24% acetonitrile:40% double-distilled water. All separations were performed at room temperature at a flow rate of 0.8 mL/min. Unlabeled steroids were detected by UV absorbance, while labeled steroids were detected by scintillation counting of collected fractions. All solvents were of HPLC grade and were purchased from Fisher Scientific Corp. (Pittsburgh, PA).

Biochemical Verification of [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2. To confirm the molecular identity of the synthesized labeled catechol estrogens, two reactions were

conducted: nonenzymatic sodium borohydride ($NaBH_4$; Sigma Chemical Co.) reduction and enzymatic 17β-HSD oxidation (via Insect Control Supersomes). The purity and stability of both radiolabeled products were verified by HPLC analysis approximately every month, with no degradation noted.

(a) [2,6,7-^{3}H]4OHE2. Following HPLC purification, 1.6 nM [2,6,7-^{3}H]4OHE2 was dried under nitrogen and then incubated with 0.8 mL of reaction buffer for 30 min at 37 °C. The reaction was initiated by the addition of 200 μL of CYP1B1 Insect Control Supersomes and allowed to proceed for 17 h at 37 °C. The reaction was then terminated by the addition of ethyl acetate. The resulting products were extracted and reconstituted, and an aliquot was analyzed for [2,6,7-^{3}H]4OHE1 catechol metabolite composition by HPLC.

After HPLC confirmation of the enzymatic conversion of radiolabeled 4OHE2 to radiolabeled 4OHE1, the remaining [2,6,7-^{3}H]4OHE1 sample was dried under nitrogen and then incubated in 1 mL of double-distilled water containing 0.05% acetic acid (Sigma Chemical Co.) and 50 μM $NaBH_4$ for approximately 1 h. The reaction was terminated as above, and an aliquot was analyzed for [2,6,7-^{3}H]4OHE2 catechol metabolite composition by HPLC.

(b) [2,6,7-^{3}H]4OHE1. For this labeled metabolite, only the nonenzymatic $NaBH_4$ reduction reaction was utilized for catechol metabolite biochemical confirmation as described above.

Tissue Cytosol Preparation. Animals were sacrificed and their tissues immediately removed, stripped of adhering fat, wrapped in aluminum foil, and quickly immersed in liquid nitrogen. The tissues were stored at −80 °C until use. Tissues were homogenized in TEG buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10% glycerol, 3 mM sodium azide, pH 7.4 (at 23 °C)) on ice, employing a Tissue Tearor (Biospec Products Inc., Racine, WI). The homogenized solution was centrifuged at 10000*g* for 15 min at 4 °C, and the supernatant then was centrifuged at 300000*g* for 2 h at 4 °C. The resulting cytosol samples were stored at −80 °C until use. Protein concentrations of tissue cytosol extracts were determined using the Total Protein diagnostic kit with bovine serum albumin as the standard (Sigma Chemical Co.).

Saturation Binding Analysis. ERαKO tissue cytosol samples (∼100−900 μg of protein) were incubated first with 0.5 μM unlabeled 17β-E2 for approximately 1.5 h at 4 °C to prevent the binding of labeled and unlabeled 4OHE1/4OHE2 to ERα and ERβ receptors. Radiolabeled 4OHE1 (∼1 Ci/mmol), in the presence or absence of approximately 1000-fold excess unlabeled 4OHE2, was added to the cytosol samples and allowed to incubate overnight (∼16 h) at 4 °C to achieve equilibrium binding. Bound and free ligand were separated by dextran-coated charcoal, and an aliquot of bound radioactivity was measured by scintillation counting (*32*). Scatchard analysis (*33*) was used for linear transformation of the data to determine the dissociation constant (K_d).

Ligand Specificity/Tissue Distribution Experiments. Tissue cytosol (∼20−600 μg of protein) was incubated first with 0.5 μM unlabeled 17β-E2 for 1.5 h at 4 °C. Approximately 1 nM [2,6,7-^{3}H]4OHE1 or [2,6,7-^{3}H]4OHE2 (∼1 Ci/mmol), in the presence or absence of 500 nM unlabeled competitor, was added to the cytosol samples. Binding assays were performed as described above.

[2,6,7-^{3}H]4OHE1 Metabolism Analysis. Binding assays were performed as described above, but in the presence or absence of 3 mM ascorbic acid. The reaction samples were extracted with ethyl acetate, and the extracts were pooled and dried under nitrogen. The resulting samples were reconstituted in HPLC mobile phase (see above), and aliquots were analyzed for metabolite composition by HPLC.

RESULTS

Biochemical Synthesis/Purification of [^{3}H]4OHE1 and [^{3}H]4OHE2. In efforts to biochemically characterize a putative catechol estrogen binding protein, we have enzymatically synthesized and purified two radiolabeled catechol estrogen compounds, [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2, using commercially available [2,4,6,7-^{3}H]17β-E2 and the CYP450 enzyme CYP1B1. This enzyme has been characterized as a specific 4-hydroxylation enzyme (*34*−*38*), contributing to the predominant formation of 4-hydroxylated catechol estrogens from the parent compounds 17β-E2 and E1 (Figure 1).

Prior to HPLC purification of radiolabeled catechol estrogens, unlabeled corresponding standards were chromatographed and retention times accurately determined (Figure 2). Typical HPLC CPM profiles of [2,4,6,7-^{3}H]17β-E2 metabolism incubated with CYP1B1 enzyme, in the presence and in the absence of spironolactone (17β-HSD inhibitor), are illustrated in parts A and C of Figure 3, respectively. On the basis of these radioactive profiles, sufficient percentage yields of both [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 (approximately 30−35% final yield) were obtained under these reaction conditions. Aliquots of representative final purified extracts of [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 ligands are depicted in parts B and D of Figure 3, respectively. Both labeled ligands were ∼95% pure on the basis of HPLC CPM analysis. To prevent spontaneous oxidation, both radiolabeled catechol estrogens were stored in 100% EtOH containing 2 mM or 3 mM ascorbic acid (*30*).

Enzymatic and nonenzymatic biochemical means were used for verification of the molecular identities of the radiolabeled compounds (data not shown). For [2,6,7-^{3}H]-4OHE2, an aliquot was reacted first with Insect Control CYP1B1 enzyme extract, resulting in almost complete conversion to [2,6,7-^{3}H]4OHE1 via 17β-HSD type II activity. Upon subsequent reaction with the reducing agent $NaBH_4$, [2,6,7-^{3}H]4OHE1 was converted back to the original radiolabeled ligand [2,6,7-^{3}H]4OHE2 (likely a racemic mixture). In a similar manner, nonenzymatic reaction of [2,6,7-^{3}H]-4OHE1 with $NaBH_4$ resulted in the predominant formation of [2,6,7-^{3}H]4OHE2 (racemic mixture), confirming the identity of [2,6,7-^{3}H]4OHE1 as substrate (data not shown).

Saturation Binding Analysis. Saturation curves illustrating the binding of [2,6,7-^{3}H]4OHE1 metabolite (see [2,6,7-^{3}H]-4OHE1 Metabolism Analysis section of Results) to ERαKO kidney (A), liver (B), ovarian (C), and uterine (D) tissue cytosols are displayed in Figures 4 and 5 (liver and ovarian tissues only). At apparent saturation, ERαKO kidney, liver, and uterine cytosols displayed specific binding concentrations of approximately 0.2 nM, while ERαKO ovarian cytosol revealed specific binding concentrations of nearly 0.4 nM. Accordingly, the percentages of specific binding for the four tissue samples were approximately 4% for kidney and liver,

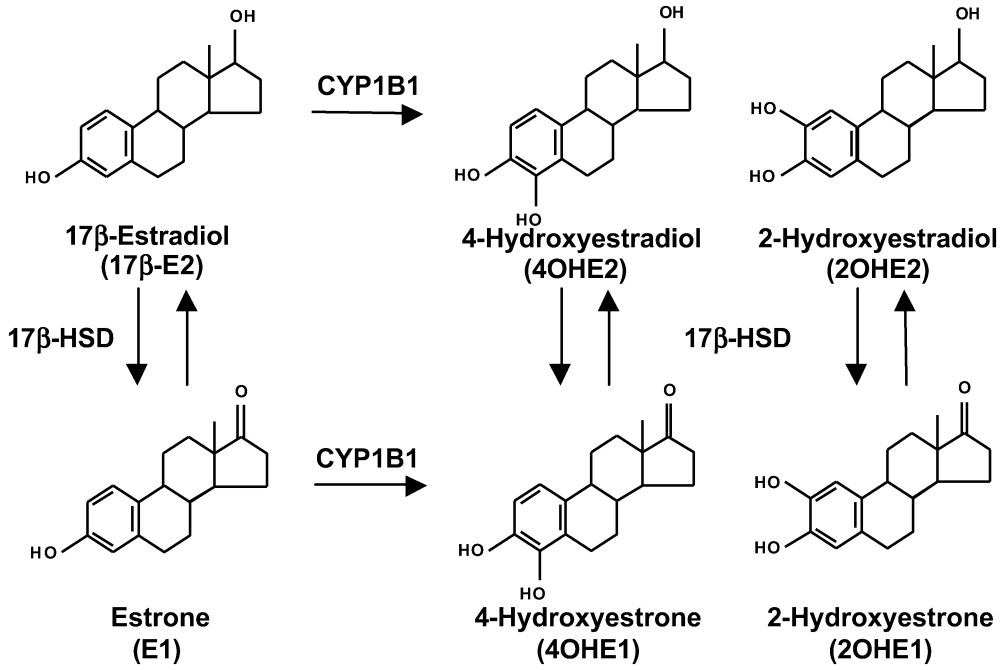

FIGURE 1: Chemical structures of 17β-estradiol (E2), estrone (E1), and related catechol estrogen metabolites 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2), 2-hydroxyestrone (2OHE1), and 4-hydroxyestrone (4OHE1). 17β-Hydroxysteroid dehydrogenase (17β-HSD) enzymatically interconverts E1 and E2. Cytochrome P450 1B1 (CYP 1B1) specifically metabolizes E1 and E2 to the 4-OH (~80%) and 2-OH (~20%) estrogen derivatives. Catechol estrogens possess two vicinal hydroxyl groups in their A ring, reminiscent of the catecholamines.

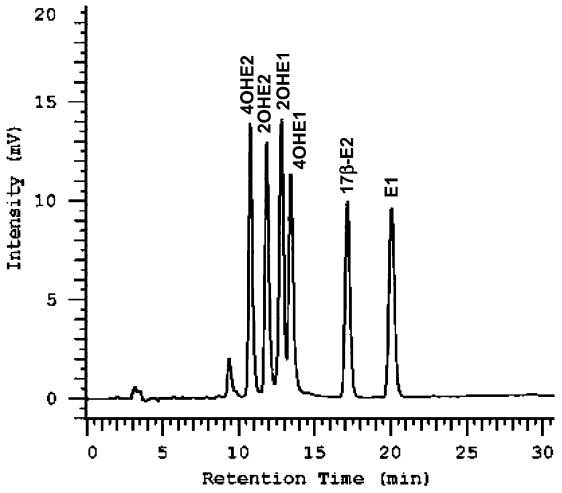

FIGURE 2: HPLC chromatogram of 17β-estradiol (17β-E2), estrone (E1), and related catechol estrogens. Unlabeled steroidal compounds were separated and corresponding UV profiles determined by HPLC analysis as described in Materials and Methods.

7% for uterus, and 13% for ovary. Also, for all tissue cytosol binding curves, nonspecific binding demonstrated linearity with increasing radiolabeled ligand concentrations (Figures 4 and 5). Linear transformation of the saturation data (inset Scatchard plots in Figure 4) indicated a single population of binding sites for the 4OHE1 metabolite with an average K_d of 1.6 nM. Specifically, K_d values of 1.5, 1.9, 1.3, and 1.5 nM were demonstrated with kidney, liver, ovarian, and uterine ERαKO tissue cytosols, respectively, for specific [2,6,7-^{3}H]4OHE1 metabolite binding. On the basis of Scatchard plot calculations, these measured K_d values are similar to those K_d values obtained for catechol estrogen binding (0.1−0.5 nM) (*8*, *39*) and 17β-E2 binding (0.1−1 nM) (*32*, *40*) to estrogen receptor proteins. Among the four ERαKO tissues analyzed, specific [2,6,7-^{3}H]4OHE1 metabolite binding determined under equilibrium conditions indicated that kidney cytosol possessed the highest concentration (B_{max}) of [2,6,7-^{3}H]4OHE1 metabolite binding sites (Figure 4A).

Ligand Specificity. The specificity of [2,6,7-^{3}H]catechol estrogen metabolite binding was ascertained by measuring the ability of various steroidogenic compounds to compete for binding with both [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]-4OHE2 in ERαKO lung tissue cytosol. The lung tissue was utilized because of both its abundance and its high specific [2,6,7-^{3}H]catechol estrogen metabolite binding concentration.

Numerous estrogenic and steroidogenic compounds were tested (Table 1) and were grouped into three categories for purposes of clarity and convenience: catechol estrogen competitors, steroid/hormone competitors, and phytoestrogen/catecholamine competitors. For both specific [2,6,7-^{3}H]-4OHE1 and [2,6,7-^{3}H]4OHE2 metabolite binding concentrations, the unlabeled catechol estrogens tested exhibited very similar inhibition profiles (Table 1A). The catechol compounds 2OHE1, 2OHE2, 2OHE2-17-acetate, 2OHE3, 4OHE1, and 4OHE2 all competitively inhibited specific [2,6,7-^{3}H]catechol estrogen metabolite binding very effectively. The methoxy derivatives 2-methoxyE1, 2-methoxyE2, and 4-methoxyE2 were much weaker competitive inhibitors.

The majority of estrogenic/steroidogenic ligands analyzed did not competitively inhibit specific [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 metabolite binding (Table 1B). For example, in addition to 17β-E2, other estrogenic (natural or synthetic) compounds such as BPA, DES, 17α-E2, E2-17-acetate, E3, and kepone did not compete for specific [2,6,7-^{3}H]catechol estrogen metabolite binding. Other steroid hormones (e.g., androstenediol, dexamethasone, and progesterone) and several well-known ER/AR antagonists (e.g., flutamide, ICI 182,780, tamoxifen, and 4OH-tamoxifen) also

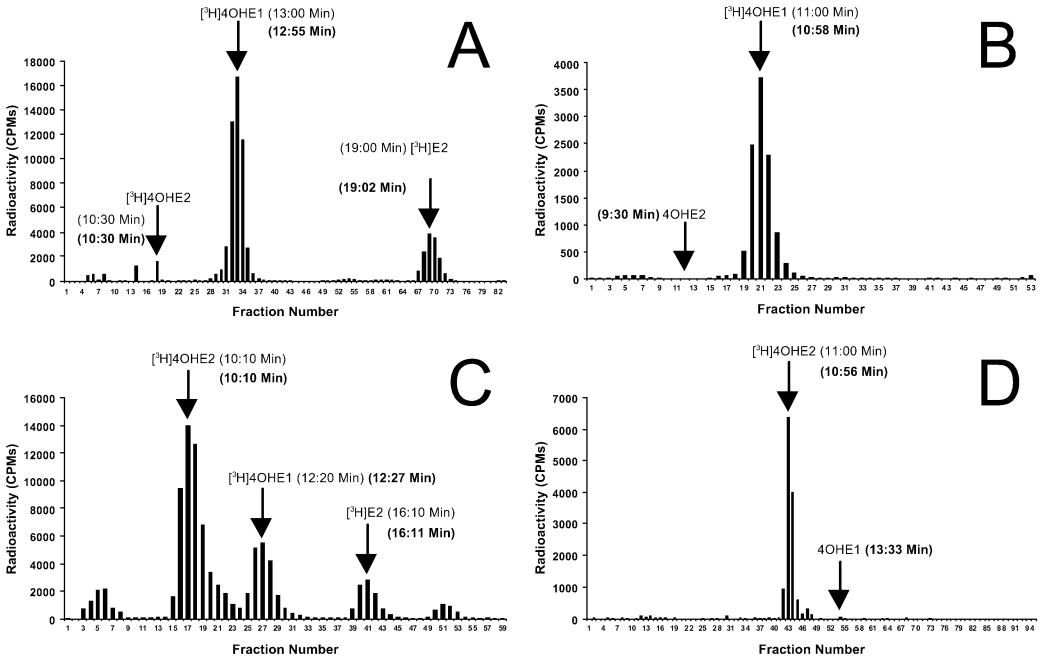

FIGURE 3: HPLC CPM profiles of [2,4,6,7-^{3}H]17β-E2 metabolism via cytochrome P450 1B1: synthesis of catechol estrogens [2,6,7-^{3}H]-4OHE1 and [2,6,7-^{3}H]4OHE2. Approximately 1.5 μM [2,4,6,7-^{3}H]17β-E2 was incubated with CYP1B1 Enzyme Supersomes without (A) or with (C) 0.6 mM spironolactone. In [2,6,7-^{3}H]4OHE2 synthesis reactions, spironolactone was utilized as a 17β-HSD type II enzyme inhibitor. For determination of [2,6,7-^{3}H]catechol estrogen synthesis, aliquots from each reaction mix were analyzed by HPLC chromatography. HPLC profiles of reaction mix extracts were determined using a LUNA reversed-phase C_{18} column with an isocratic mobile phase (36:24:40) of methanol:acetonitrile:double-distilled water. The flow rate was 0.8 mL/min, and the absorbance was monitored at 260 nm. CPM peaks of labeled estrogens are indicated by arrows with corresponding retention times. Retention times of unlabeled compounds are shown in bold type. Profiles of purified [2,6,7-^{3}H]4OHE1 (B) and [2,6,7-^{3}H]4OHE2 (D) products from syntheses reactions are shown. Both radiolabeled ligands were ~95% pure on the basis of HPLC CPM analyses.

demonstrated weak competitive profiles. These data strongly suggest that other nuclear receptor superfamily members, including the androgen receptor, the glucocorticoid receptor, the mineralocorticoid receptor, and the progesterone receptor, are not involved in specific binding of radiolabeled catechol estrogens or their metabolites under these conditions. Of the catecholamines tested in the competitive binding assays (see Figure 6), norepinephrine was a surprisingly stronger competitive inhibitor than epinephrine (Table 1C), although both were much weaker competitive inhibitors than the catechol estrogens.

Several plant-derived compounds with estrogenic activity (i.e., phytoestrogens) also were assayed for their ability to competitively inhibit specific [2,6,7-^{3}H]catechol estrogen metabolite binding (Table 1C). Genistein, a flavonoid with reported increased affinity for ERβ compared to ERα (*32*), exhibited a weak competition profile. Conversely, catechin/epicatechin compounds and quercetin, all with structural resemblance to catechol estrogens (see Figure 6), exhibited strong inhibition profiles. Specifically, catechin and epicatechin compounds competitively inhibited approximately 50% of specific [2,6,7-^{3}H]catechol estrogen metabolite binding, whereas quercetin competitively inhibited 100% of specific binding.

Tissue Distribution (WT, ERαKO, and ArKO Mice). To determine the relative tissue distribution of a specific catechol estrogen binding protein, competition binding studies were conducted in a wide range of female WT, ERαKO, and ArKO murine tissues. The three genotypes of mice allowed relative concentrations of catechol estrogen binding sites to be assessed among tissues under varied concentrations of systemic 17β-E2 (high concentrations, ERαKO; moderate concentrations, WT; low concentrations, ArKO). Serum concentrations of 17β-E2 are nearly 10 times higher in ERαKO female mice compared to WT mice (*41*), while ArKO animals possess very low serum concentrations of 17β-E2 compared to their WT counterparts (*42*).

Using radiolabeled 4OHE2 ligand, significant binding concentration differences both within and among tissues of these varied genotypic mice were quite evident (Figure 7). Relative concentrations of specific [2,6,7-^{3}H]4OHE1 metabolite binding displayed a similar pattern among various murine tissues, although this is not shown. In WT female animals, the highest concentrations of specific [2,6,7-^{3}H]-4OHE2 metabolite binding were found in bladder, lung, ovary, and skeletal muscle. Moderate concentrations of specific [2,6,7-^{3}H]4OHE2 metabolite binding were evident in kidney, mammary, and uterine tissues, while the lowest concentrations were demonstrated in the brain, heart, and liver (Figure 7).

Among ERαKO female tissues, bladder, lung, and skeletal muscle displayed the highest specific [2,6,7-^{3}H]4OHE2 metabolite binding concentrations. Lower but significant binding concentrations were found in the kidney, liver, mammary, ovary, and uterus. Analysis of specific [2,6,7-^{3}H]4OHE2 metabolite binding concentrations in brain and heart tissues revealed very weak binding (Figure 7). To validate the proteinaceous nature of this putative catechol estrogen binding site, pretreatment of ERαKO lung tissue with proteinase K prior to overnight incubation completely eliminated specific binding by both [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 metabolites (data not shown), suggesting stable interactions of radiolabeled catechol estrogen metabolites with a cytosolic protein.

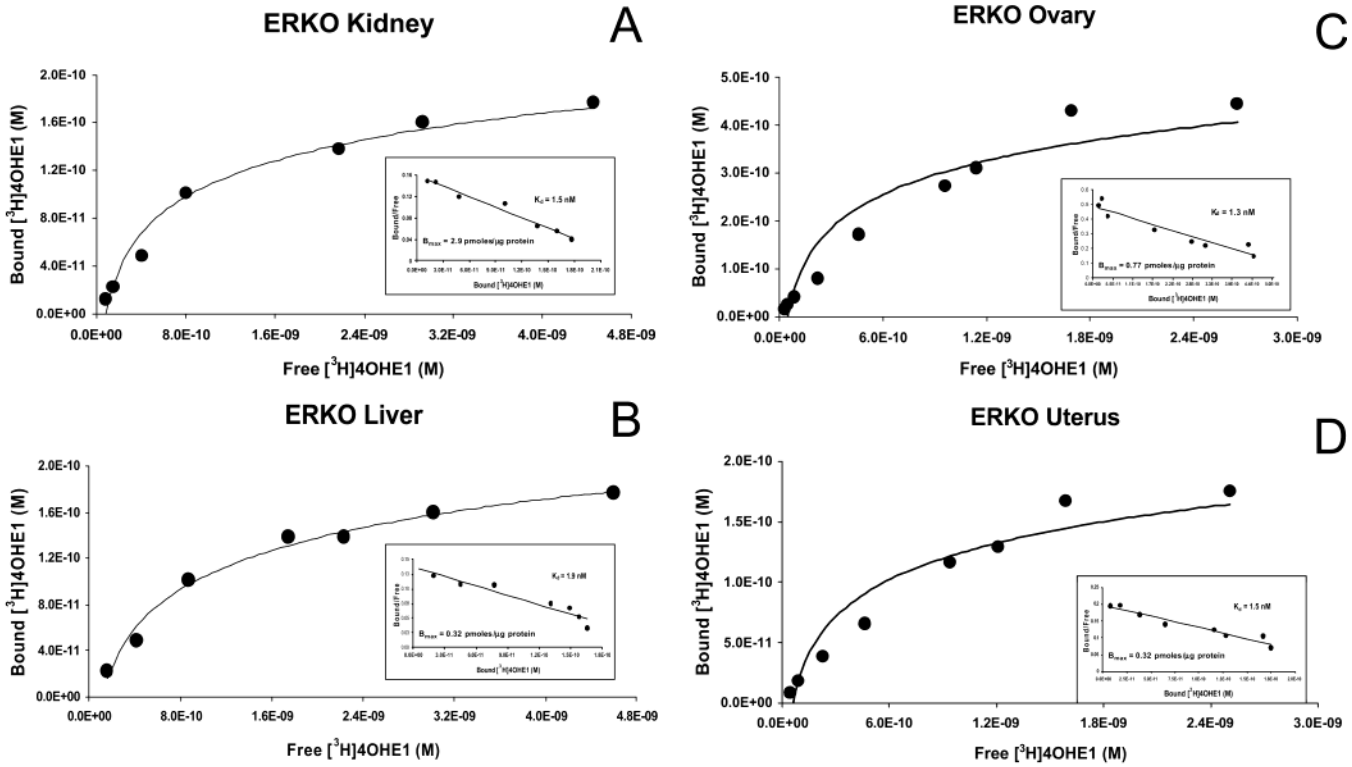

FIGURE 4: Saturation and Scatchard (insets) analyses of [2,6,7-^{3}H]4OHE1 metabolite binding in ERαKO mouse tissue cytosol. Female ERαKO mouse cytosol samples (from kidney (A), liver (B), ovarian (C), and uterine (D) tissues) were labeled with [2,6,7-^{3}H]4OHE1 in the presence of 1000-fold excess unlabeled competitor as detailed in Materials and Methods. Unbound radioactivity was removed as described, and specific binding was calculated by subtracting nonspecific binding from total binding. Saturation curves were fitted using logarithmic regression analysis. Scatchard analyses of specific [2,6,7-^{3}H]4OHE1 metabolite binding concentrations indicated an average $K_d = 1.6$ nM. B_{max} values are adjusted for total micrograms of cytosolic protein.

In comparison to WT and ERαKO female mice, ArKO females possessed much lower specific [2,6,7-^{3}H]4OHE2 metabolite binding concentrations (Figure 7). Among tissues of ArKO female mice, the highest concentrations of specific [2,6,7-^{3}H]4OHE2 metabolite binding were found in mammary, ovary, and uterus. Moderate concentrations of specific metabolite binding were evident in the bladder, heart, and lung, while several tissues, including the brain, kidney, liver, and skeletal muscle, possessed low specific binding concentrations.

In addition to female mice, select tissues (brain and prostate) from male WT, ERαKO, and ArKO mice were analyzed for specific [2,6,7-^{3}H]catechol estrogen metabolite binding concentrations (data not shown). These two tissues were assessed for both specific [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 metabolite binding concentrations because both ERα and ERβ mRNA are abundant and widespread in male and female rodent brains (*32*, *43*) and because ERβ was first cloned from rat prostate tissue (*40*). In prostate tissues of adult male WT, ERαKO, and ArKO mice, no significant differences in specific [2,6,7-^{3}H]catechol estrogen metabolite binding concentrations were evident (data not shown). Concentrations of specific [2,6,7-^{3}H]catechol estrogen metabolite binding for this tissue among all three mouse genotypes were low (i.e., <150 fmol/mg of total cytosol protein) compared to other female tissues. Within brain tissue, comparison of specific [2,6,7-^{3}H]catechol estrogen metabolite binding concentrations among male mice demonstrated no significant differences either, with specific [2,6,7-^{3}H]catechol estrogen metabolite binding concentrations <100 fmol/mg of total cytosol protein (data not shown). Consequently, no significant gender differences between male and female WT, ERαKO, or ArKO brain tissues for specific [2,6,7-^{3}H]catechol estrogen metabolite binding were apparent.

[2,6,7-^{3}H]4OHE1 Metabolism Analysis. The concern about metabolic breakdown of estrogenic compounds, especially catechol estrogens (*30*), prompted us to investigate the stability of catechol estrogens in our sample preparations. In all cases, metabolism of [2,6,7-^{3}H]4OHE1 was assessed by ethyl acetate extraction following normal binding assay conditions in the presence or absence of 3 mM ascorbic acid (reducing agent). From these experiments, incubation of ERαKO mouse lysate with varying concentrations of [2,6,7-^{3}H]4OHE1 resulted in nearly complete metabolism of the radiolabeled substrate (data not shown). The addition of ascorbic acid to the reaction mixture was unable to inhibit the enzymatic oxidative metabolism of the radiolabeled ligand, and completely eliminated specific [2,6,7-^{3}H]4OHE1 metabolite binding in each of the lysate samples. Furthermore, the inability to extract nearly 100% of radioactivity with ethyl acetate following incubation suggested covalent/irreversible binding with lysate proteins. In contrast to [2,6,7-^{3}H]4OHE1, in control experiments, no significant metabolism was observed with radiolabeled 17β-E2 (data not shown).

DISCUSSION

Although enigmatic in terms of function, recent evidence has suggested important physiological roles for catechol estrogens in gonadotropin release (*13*), embryo implantation

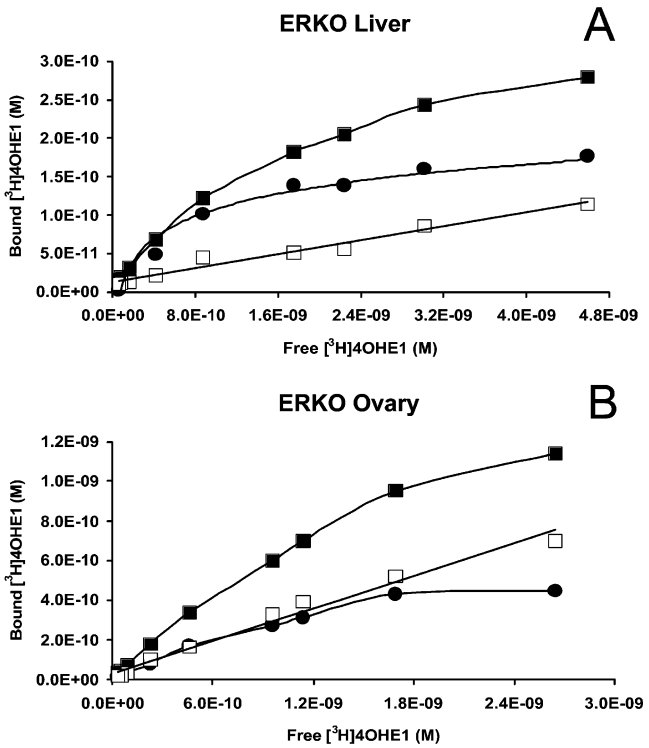

FIGURE 5: Saturation binding of [2,6,7-^{3}H]4OHE1 metabolite to cytosol extracts from ERαKO mouse liver and ovarian tissues. Female ERαKO mouse cytosol samples from liver (A) and ovarian (B) tissues (same as Figure 4) were labeled with [2,6,7-^{3}H]4OHE1 in the presence of 1000-fold excess unlabeled competitor as detailed in Materials and Methods. Total (■), nonspecific (□), and specific (●) binding were calculated as described.

(*5*, *11*, *12*), parturition (*14*), and angiogenesis (*15*). In addition to ERα and ERβ, these estrogenic metabolites are believed to interact with a potential third estrogen-type receptor (*26*). The objective of this study was to biochemically characterize this putative catechol estrogen binding protein in WT, ERαKO, and ArKO mice. Our results demonstrate the existence of such a binding protein (cytosolic) that exhibits characteristics of a typical high-affinity receptor protein. However, the binding described herein is mediated by metabolites of catechol estrogens, rather than the parent compounds. Specific [2,6,7-^{3}H]4OHE1 metabolite binding was reversible and saturable, and demonstrated low capacity (for catechol estrogens) with an approximate K_d of 1.6 nM. We believe that this protein is functionally distinct from other previously described catechol estrogen binding sites (*21*−*24*) on the basis of its ligand selectivity, tissue distribution, and estrogen responsiveness. Accordingly, the present results warrant the examination of this binding protein as a potential mediator of estrogenic action.

Analysis of the Scatchard plots for ERαKO mouse tissues (Figure 4) indicated a K_d range of 1.3−1.9 nM for [2,6,7-^{3}H]4OHE1 metabolite binding. These values are similar to those binding affinities of catechol estrogens for nuclear estrogen receptors (*8*, *39*) and for putative catechol binding proteins (*22*, *24*). However, the B_{max} values reported here (Figure 4) are greater than those reported for high-affinity catechol estrogen binding sites in rat anterior pituitary membranes (13 fmol/mg protein) (*21*) and guinea pig hypothalamic membranes (38 fmol/mg protein) (*23*). It is possible that these differences are attributable to cellular distribution and animal tissue/model variation. Also, as indicated, we believe this cytosolic protein is different from previously described catechol estrogen binding sites demonstrated in cytosol and membrane preparations. Whether the binding sites characterized herein are also found in membrane fractions would need to be verified by further studies.

On the basis of tissue distribution studies (Figure 7), however, the protein binding concentrations were similar to those previously reported for catechol estrogen binding sites (*21*, *23*) and nuclear estrogen receptors (*40*). Disparities in ligand binding methodologies could account for this dis-

Table 1: Inhibition Profiles of Various Estrogenic/Non-estrogenic Compounds for Specific [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 Metabolite Protein Binding[a]

compound	% [^{3}H]4OHE1 bound ± SE	% [^{3}H]4OHE2 bound ± SE
(A) Catechol Estrogen Competitors		
no competitor	100.0 ± 5.7	100.0 ± 1.3
17β-E2	100.0 ± 9.4	100.0 ± 7.3
2OHE1	5.4 ± 3.4	17.5 ± 6.8
2OHE2	20.7 ± 7.0	22.6 ± 2.6
2OHE2-17-acetate	15.9 ± 1.8	18.7 ± 2.6
2OHE3	7.9 ± 13.4	22.9 ± 2.0
4OHE1	0.0 ± 10.9	10.5 ± 7.4
4OHE2	0.0 ± 8.2	0.0 ± 9.8
2-methoxyE1	82.6 ± 17.9	100.6 ± 14.8
2-methoxyE2	73.1 ± 11.5	70.6 ± 6.7
4-methoxyE2	77.1 ± 12.5	85.9 ± 9.2
(B) Steroid/Hormone Competitors		
17β-E2	100.0 ± 9.4	100.0 ± 7.3
adrostenediol	112.4 ± 8.9	75.6 ± 0.5
bisphenol A	102.0 ± 11.6	97.6 ± 3.4
DES	98.2 ± 10.6	63.5 ± 0.6
dexamethasone	113.1 ± 4.0	84.4 ± 2.4
DHEA	107.1 ± 14.0	104.6 ± 11.8
DHT	116.8 ± 3.3	110.4 ± 2.5
16-epiestriol	90.2 ± 1.6	95.4 ± 3.2
E1	93.4 ± 4.7	74.9 ± 0.7
16α-E1	72.6 ± 9.5	77.7 ± 2.7
17α-E2	83.0 ± 13.6	82.6 ± 1.1
E3	100.0 ± 4.5	87.3 ± 5.1
E2-17-acetate	82.9 ± 3.0	86.8 ± 6.6
flutamide	74.7 ± 3.3	89.2 ± 1.6
HPTE	103.7 ± 17.7	73.0 ± 0.6
ICI 182,780	85.3 ± 7.4	87.9 ± 1.1
kepone	88.9 ± 7.6	91.3 ± 9.6
methoxychlor	116.4 ± 1.7	131.5 ± 3.9
progesterone	83.1 ± 3.3	90.4 ± 0.7
spironolactone	98.5 ± 3.8	110.7 ± 3.9
tamoxifen	71.8 ± 3.0	92.8 ± 10.4
4OH-tamoxifen	72.5 ± 4.5	85.3 ± 4.8
(C) Phytoestrogen/Catecholamine Competitors		
17β-E2	100.0 ± 9.4	100.0 ± 7.3
(+)-catechin (2*R*,3*S*)	42.6 ± 4.8	54.6 ± 7.9
(−)-catechin (2*S*,3*R*)	63.5 ± 8.1	61.7 ± 6.6
(+)-epicatechin (2*S*,3*S*)	42.4 ± 4.8	53.9 ± 9.0
epinephrine	80.0 ± 0.9	88.1 ± 2.6
genistein	86.8 ± 2.7	109.5 ± 5.3
norepinephrine	49.7 ± 6.8	62.0 ± 3.8
quercetin	<0	<0

[a] Following preincubation with 500 nM 17β-E2, female ERαKO lung cytosol (from pooled tissue samples) was incubated with 1 nM [2,6,7-^{3}H]4OHE1 or [2,6,7-^{3}H]4OHE2 in the presence of 500 nM unlabeled competitor. Percent specific [2,6,7-^{3}H]catechol estrogen metabolite bound for each compound is relative to either 4OHE1 or 4OHE2 (set at 100% inhibition = 0% specific [2,6,7-^{3}H]catechol estrogen metabolite bound) ± SE (standard error). Data are representative of two/three independent binding experiments or multiple replicates from the same experiment.

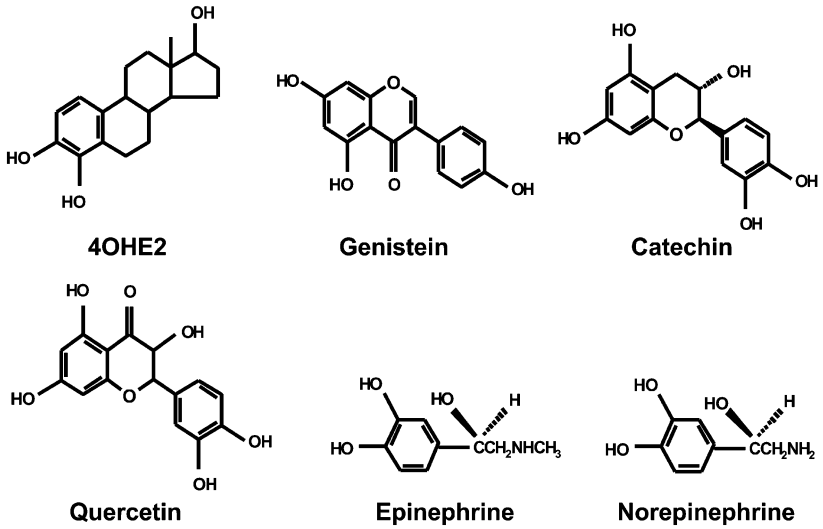

FIGURE 6: Molecular structures of select phytoestrogens and catecholamines. The phystoestrogens quercetin and catechin exhibit structural resemblance to the catechol estrogens (4OHE2) and catecholamines (epinephrine and norepinephrine). Genistein, catechins, and quercetin are believed to possess anti-cancer and/or anti-oxidant properties.

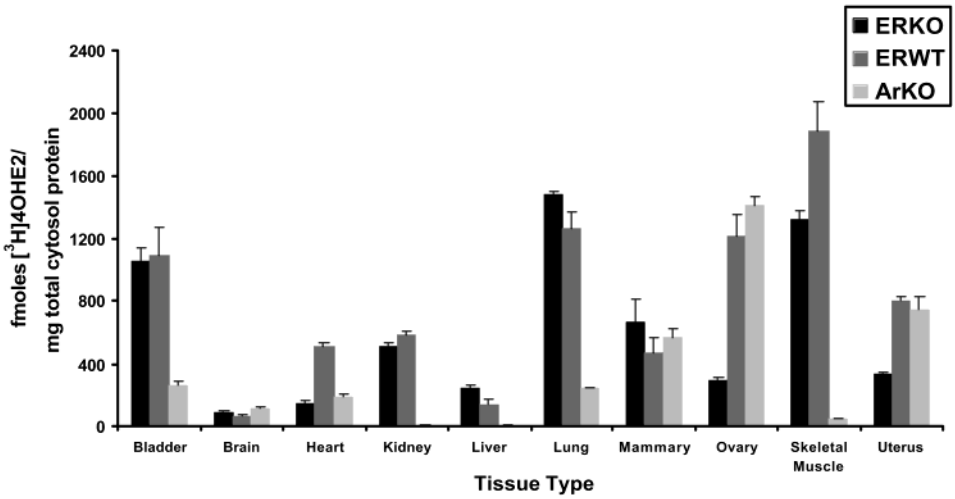

FIGURE 7: Specific [2,6,7-^{3}H]4OHE2 metabolite binding concentrations among tissues in ERWT (wild-type), ERαKO (estrogen receptor-α knockout), and ArKO (aromatase knockout) female mice. Tissue cytosol was incubated with 1 nM [2,6,7-^{3}H]4OHE2 in the presence or absence of 500-fold excess of unlabeled 4OHE2 competitor for 16 h at 4 °C. Specific [2,6,7-^{3}H]4OHE2 metabolite binding was calculated by subtracting nonspecific binding from total binding. Concentrations of specific [2,6,7-^{3}H]4OHE2 metabolite protein binding represent mean values ± SE (standard error) and are adjusted for total milligrams of cytosolic protein. Data are representative of two independent binding experiments or multiple replicates from the same experiment.

crepancy. For example, while saturation binding (Scatchard plots) utilized radiolabeled concentrations approaching receptor saturation, competition binding (tissue distribution plots) utilized sub-saturating concentrations of radiolabeled ligand (to conserve limited ligand resources). Alternative explanations for disparity in the B_{max} values between binding studies include radiolabeled ligand differences and/or the interaction of [2,6,7-^{3}H]4OHE1 and [2,6,7-^{3}H]4OHE2 metabolites with separate binding proteins. However, given the similar inhibitory profiles for both radiolabeled ligands (see Table 1), we believe that these ligands are binding to the same protein.

Assuming a molecular weight of approximately 50 000 g/mol for a putative catechol estrogen binding protein, our binding data (Figures 4 and 5) indicate a total cellular protein binding percentage of approximately 1.6% for ERαKO liver and uterus, 3.9% for ERαKO ovary, and 14.5% for ERαKO kidney. Although high-affinity receptor proteins, like steroid receptors, are normally present at much lower concentrations (*40*), a precedent for a high-affinity/high-capacity estrogen binding protein has been demonstrated. In studies of rat brain, Ramirez et al. (*44*) recently identified that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), present at 10−20% of total cellular protein, binds to 17β-E2 with high affinity (IC_{50} = 50 nM). Because GAPDH competitively interacts in a high-affinity fashion with 17β-E2, it does not represent a potential candidate for our catechol estrogen binding protein; however, these findings (*44*) demonstrate that such a binding protein could exist at elevated concentrations in tissues and may explain the varied tissue distribution of our putative catechol estrogen binding protein among WT, ERαKO, and ArKO female mice (see Figure 7).

To date, speculation on the existence of a novel (catechol) estrogen receptor has been based upon estrogen responsiveness in an ERαKO mouse background (*26*). The 4OHE2-induced lactoferrin response identified by Das et al. (*26*) could be mediated by several mechanisms, including protein

isoforms of ERα and/or ERβ, orphan receptor members of the nuclear receptor superfamily, or cross-talk between growth factors and estrogen receptors (i.e., membrane and/or nuclear ERβ). However, we believe that the most simplistic explanation for such a response in the ERαKO mouse is the existence of a novel estrogen-type receptor.

We are designating this binding protein as a putative estrogen-type receptor protein for several reasons: (1) the inability of both 17β-E2 and ICI 182,780 to inhibit this response suggests this effect is not mediated by the ligand binding domains of either ERα or ERβ; (2) the concentration of 4OHE2 (10 μg/kg) utilized to induce ERαKO uterine lactoferrin mRNA expression is consistent with concentrations ($10^{-8}-10^{-12}$ M) at which hormones are biologically active, strongly implying a receptor-mediated mechanism; (3) the inability of 17β-E2 to induce this lactoferrin response indicates that this parent estrogen compound is not interacting with the ligand binding domains of either ERα or ERβ isoforms; and (4) the lactoferrin response is elicited by an estrogen metabolite (4OHE2) that exhibits structural resemblance to the parent compound 17β-E2.

Our working model is that this putative catechol estrogen binding protein may represent a novel estrogen-type receptor or perhaps a putative orphan receptor. To date, there is no evidence of a specific binding protein for catechol estrogen metabolites (i.e., quinones), which bind to available sulfhydryl and amino groups in proteins in general. Regardless, a receptor is defined by several criteria, including saturability, specificity, and reversibility. Furthermore, it is assumed that the integrity of the ligand is maintained during ligand−receptor interaction. However, in the case of catechol estrogens, this assumption is not valid given their susceptibility to oxidative metabolism and redox cycling (*17*). Consequently, we utilized HPLC analysis to investigate the metabolism of [2,6,7-^{3}H]4OHE1 when incubated with both WT and ERαKO mouse cellular lysate in the presence and in the absence of ascorbic acid, a reported protective antioxidant (*30*).

Results from our studies demonstrated significant oxidative metabolism of this radiolabeled compound under normal binding assay conditions, even in the presence of 3 mM ascorbic acid. A large percentage of radioactivity was not extracted by ethyl acetate, implying potential covalent/irreversible binding to lysate proteins (data not shown). The ability of ascorbic acid to eliminate specific [2,6,7-^{3}H]4OHE1 metabolite binding, but failure to prevent enzymatic oxidative metabolism of the radiolabeled ligand, strongly suggests that the observed specific binding is attributable to oxidized product(s). It remains possible that the specific, reversible binding observed is mediated by unmetabolized, parent catechol estrogens, though the metabolism data indicate otherwise. Although unknown mechanistically, the binding inhibition by ascorbic acid may reflect direct ligand competition or the formation of a nonbinding catechol estrogen intermediary product (via oxidation).

To further characterize the apparent two types of binding revealed from our studies, kinetic analyses were conducted (data not shown). Results from these studies demonstrated binding interactions that were not consistent with traditional ligand−receptor interactions and revealed a K_d value (∼22 nM) that conflicted with the K_d value (∼1.6 nM) obtained from Scatchard analyses (see Figure 4). The conflicting data may be resolved by two potential explanations. First, if the specific binding were covalent, then the calculated binding affinity should be higher (i.e., K_d should be lower). Second, because the parent catechol estrogen ligand is metabolized to both binding and nonbinding ligands (capable of covalent interactions), the concentration of the "true" binding ligand is actually lower than anticipated. Accordingly, this generates an apparent elevated K_d value (22 nM). The increased nonspecific, irreversible binding over time (>16 h) observed for both association and dissociation kinetics (data not shown) supports this assertion. While not discussed here, the identification of the catechol estrogen metabolite, the protein(s) it binds, and the enzymes mediating the metabolism will allow a much clearer interpretation of these data.

In summary, our findings demonstrate the interaction of catechol estrogen metabolites with a novel, and functionally distinct, cytosolic murine binding protein that exhibits high affinity, specificity, and selective tissue distribution. To our knowledge, the present study represents the most extensive characterization of a putative catechol estrogen binding protein in any animal or cell model. The fact that this binding protein shares tissue distribution patterns with both ERs (*32*, *45*, *46*) and estrogen-related receptors (ERRs) (*47*−*49*) could help elucidate its biological role in alternative estrogen-signaling pathways. Evidence that this protein is a receptor must await protein purification, subsequent sequence comparisons, and characterization of receptor responses at differing ligand concentrations. Indeed, if this catechol estrogen binding protein does represent a novel estrogen-type receptor, then this protein, in addition to the known ERα and ERβ, would potentially have major therapeutic implications for cancer, fertility, and cardiovascular disease.

ACKNOWLEDGMENT

We are thankful to Dr. Edward Curran and Dr. Xiaohui Yuan for use of the C4-12-5 (ER-negative), MCF-7 (ER-positive), and COS-1 (ER-positive) cell lines. We also are grateful to Dr. Julia Taylor for assistance in HPLC analysis and animal surgeries/treatments. Additional thanks are due to Dr. Wade Welshons for providing bisphenol A (BPA), Dr. Frederick vom Saal for use of flutamide and dihydrotestosterone (DHT), and Dr. William Kelce for providing the environmental estrogens methoxychlor and 2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE).

REFERENCES

1. Barnea, E. R., MacLusky, N. J., and Naftolin, F. (1983) Kinetics of catechol estrogen-estrogen receptor dissociation: A possible factor underlying differences in catechol estrogen biological activity, *Steroids 41*, 643−656.
2. Fishman, J., and Norton, B. (1975) Catechol estrogen formation in the central nervous system of the rat, *Endocrinology 96*, 1054−1059.
3. Lane, K. E., Ricci, M. J., and Ho, S.-M. (1997) Effect of combined testosterone and estradiol-17β treatment on the metabolism of E_2 in the prostate and liver of noble rats, *Prostate 30*, 256−262.
4. Bunyagidj, C., and McLachlan, J. A. (1988) Catechol estrogen formation in mouse uterus, *J. Steroid Biochem. 31*, 795−801.
5. Paria, B. C., Chakraborty, C., and Dey, S. K. (1990) Catechol estrogen formation in the mouse uterus and its role in implantation, *Mol. Cell. Endocrinol. 69*, 25−32.
6. Ball, P., and Knuppen, R. (1978) Formation of 2- and 4-hydroxyestrogens by brain, pituitary, and liver of the human fetus, *J. Clin. Endocrinol. Metab. 47*, 732−737.

7. Schütze, N., Vollmer, G., and Knuppen, R. (1993) Catechol estrogens are MCF-7 cell estrogen receptor agonists, *J. Steroid Biochem. Mol. Biol. 46*, 781−789.
8. Van Aswegen, C. H., Purdy, R. H., and Wittliff, J. L. (1989) Binding of 2-hydroxyestradiol and 4-hydroxyestradiol to estrogen receptors from human breast cancers, *J. Steroid Biochem. 32*, 485−492.
9. Merriam, G. R. (1979) Catechol estrogens: A brief review, J. Am. Med. Women's Assoc. *34*, 363−370.
10. Lottering, M.-L., Haag, M., and Seegers, J. C. (1992) Effects of 17β-estradiol metabolites on cell cycle events in MCF-7 cells, *Cancer Res. 52*, 5926−5932.
11. Hoversland, R. C., Dey, S. K., and Johnson, D. C. (1982) Catechol estrogen induced implantation in the mouse, *Life Sci. 30*, 1801−1804.
12. Dey, S. K., and Johnson, D. C. (1986) Embryonic signals in pregnancy, *Ann. N.Y. Acad. Sci. 476*, 49−62.
13. Merriam, G. R., Pfeiffer, D. G., and Loriaux, D. L. (1983) Catechol estrogens and the control of gonadotropin and prolactin secretion in man, *J. Steroid Biochem. 19*, 619−625.
14. Biswas, A., Chaudhury, A., Chattoraj, S. C., and Dale, S. L. (1991) Do catechol estrogens participate in the initiation of labor? *Am. J. Obstet. Gynecol. 165*, 984−987.
15. Fotsis, T., Zhang, Y., Pepper, M. S., Adlercreutz, H., Montesano, R., Newroth, P. P., and Schweigerer, L. (1994) The endogenous oestrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumour growth, *Nature 368*, 237−239.
16. Martucci, C. P., and Fishman, J. (1979) Impact of continuously administered catechol estrogens on uterine growth and luteinizing hormone secretion, *Endocrinology 105*, 1288−1292.
17. Yager, J. D., and Liehr, J. G. (1996) Molecular mechanisms of estrogen carcinogenesis, *Annu. Rev. Pharmacol. Toxicol. 36*, 203−232.
18. Liehr, J. G., Ricci, M. J., Jefcoate, C. R., Hannigan, E. V., Hokanson, J. A., and Zhu, B. T. (1995) 4-hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis, *Proc. Natl. Acad. Sci. U.S.A. 92*, 9220−9224.
19. Roy, D., and Liehr, J. G. (1990) Inhibition of estrogen-induced kidney carcinogenesis in Syrian hamsters by modulators of estrogen metabolism, *Carcinogenesis 11*, 567−570.
20. Devanesan, P., Todorovic, R., Zhao, J., Gross, M. L., Rogan, E. G., and Cavalieri, E. L. (2001) Catechol estrogen conjugates and DNA adducts in the kidney of male Syrian golden hamsters treated with 4-hydroxyestradiol: potential biomarkers for estrogen-initiated cancer, *Carcinogenesis 22*, 489−497.
21. Schaeffer, J. M., Stevens, S., Smith, R. G., and Hsueh, A. J. W. (1980) Binding of 2-hydroxyestradiol to rat anterior pituitary cell membranes, *J. Biol. Chem. 255*, 9838−9843.
22. Vandewalle, B., Peyrat, J.-P., Bonneterre, J., and Lefebvre, J. (1985) Catecholestrogen binding sites in breast cancer, *J. Steroid Biochem. 23*, 603−610.
23. Etchegoyen, G. S., Cardinali, D. P., Pérez, A. E., Tamayo, J., and Pérez-Palcios, G. (1986) Binding and effects of catecholestrogens on adenylate cyclase activity, and adrenoreceptors, benodiazepine and GABA receptors in guinea-pig hypothalamic membranes, *Eur. J. Pharmacol. 129*, 1−10.
24. Vandewalle, B., Hornez, L., and Lefebvre, J. (1988) Characterization of catecholestrogen membrane binding sites in estrogen receptor positive and negative human breast cancer cell-lines, *J. Receptor Res. 8*, 699−712.
25. Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., and Smithies, O. (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene, *Proc. Natl. Acad. Sci. U.S.A. 90*, 11162−11166.
26. Das, S. K., Taylor, J. T., Korach, K. S., Paria, B. C., Dey, S. K., and Lubahn, D. B. (1997) Estrogenic responses in estrogen receptor-α deficient mice reveal a novel estrogen signaling pathway, *Proc. Natl. Acad. Sci. U.S.A. 94*, 12786−12791.
27. Das, S. K., Tan, J., Raja, S., Halder, J., 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 estroge receptor-α and -β, *J. Biol. Chem. 275*, 28834−28842.
28. Honda, S., Harada, N., Ito, S., Takagi, Y., and Maeda, S. (1998) Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the *cyp19 gene*, *Biochem. Biophys. Res. Commun. 252*, 445−449.
29. Oesterreich, S., Zhang, P., Guler, R. L., Sun, X., Curran, E. M., Welshons, W. V., Osborne, C. K., and Lee, A. V. (2001) Re-expression of estrogen receptor alpha in estrogen receptor alpha-negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth, *Cancer Res. 61*, 5771−5777.
30. Gelbke, H. P., and Knuppen, R. (1972) A new method for preventing oxidative decomposition of catechol estrogens during chromatography, *J. Chromatogr. 71*, 465−471.
31. Tremblay, M. R., Luu-The, V., Leblanc, G., Noel, P., Breton, E., Labrie, F., and Poireir, D. (1999) Spironolactone-related inhibitors of type II 17beta-hydroxysteroid dehydrogenase: chemical synthesis, receptor binding affinities, and proliferative/antiproliferative activities, *Bioorg. Med. Chem. 7*, 1013−1023.
32. Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., and Gustafsson, J.-Å. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β, *Endocrinology 138*, 863−870.
33. Scatchard, G. (1949) The attraction of proteins for small molecules and ions, *Ann. N.Y. Acad. Sci. 51*, 660−672.
34. Savas, Ü., Bhattacharyya, K. K., Christou, M., Alexander, D. L., and Jefcoate, C. R. (1994) Mouse cytochrome P-450, representative of a new 1B superfamily of cytochrome P-450s: cloning, sequence determination, and tissue expression, *J. Biol. Chem. 269*, 14905−14911.
35. Alexander, D. L., Eltom, S. E., and Jefcoate, C. R. (1997) Ah receptor regulation of CYP1B1 expression in primary mouse embryo-derived cells, *Cancer Res. 57*, 4498−4506.
36. Zhang, L., Savas, Ü., Alexander, D. L., and Jefcoate, C. R. (1998) Characterization of the mouse *Cyp1B1* gene: Identification of an enhancer region that directs aryl hydrocarbon receptor-mediated constitutive and induced expression, *J. Biol. Chem. 273*, 5174−5183.
37. Sutter, T. R., Tang, Y. M., Hayes, C. L., Wo, Y.-Y. P., Jabs, E. W., Li, X., Yin, H., Cody, C. W., and Greenlee, W. F. (1994) Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2, *J. Biol. Chem. 269*, 13092−13099.
38. Tang, Y. M., Wo, Y.-Y. P., Stewart, J., Hawkins, A. L., Griffin, C. A., Sutter, T. R., and Greenlee, W. F. (1996) Isolation and characterization of the human cytochrome P450 CYP1B1 gene, *J. Biol. Chem. 271*, 28324−28330.
39. Merriam, G. R., MacLusky, N. J., Picard, M. K., and Naftolin, F. (1980) Comparative properties of the catechol estrogens, I: Methylation by catechol-O-methyltransferase and binding to cytosol estrogen receptors, *Steroids 36*, 1−11.
40. Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nillson, S., and Gustafsson, J.-Å. (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary, *Proc. Natl. Acad. Sci. U.S.A. 93*, 5925−5930.
41. Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Golding, T. S., Lubahn, D. B., Smithies, O., and Korach, K. S. (1995) Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene, *Mol. Endocrinol. 9*, 1441−1454.
42. Fisher, C. R., Graves, K. H., Parlow, A. F., and Simpson, E. R. (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene, *Proc. Natl. Acad. Sci. U.S.A. 95*, 6965−6970.
43. Shughrue, P. J., Komm, B., and Merchenthaler, I. (1996) The disruption of estrogen receptor-β mRNA in the rat hypothalamus, *Steroids 61*, 678−681.
44. Ramirez, V. D., Kipp, J. L., and Joe, I. (2001) Estradiol, in the CNS, targets several physiologically relevant membrane-associated proteins, *Brain Res. Brain Res. Rev. 37*, 141−152.
45. Couse, J. F., Lindzey, J., Grandien, K., Gustafsson, J.-Å., and Korach K. S. (1997) Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse, *Endocrinology 138*, 4613−4621.
46. Dechering, K., Boersma, C., and Mosselman, S. (2000) Estrogen receptors α and β: Two receptors of a kind? *Curr. Med. Chem. 7*, 561−576.

47. Sladek, R., Bader, J.-A., and Giguère, V. (1997) The orphan nuclear receptor estrogen-related receptor α is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene, *Mol. Cell. Biol. 17*, 5400−5408.
48. Hong, H., Yang, L., and Stallcup, M. R. (1999) Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3, *J. Biol. Chem. 32*, 22618−22626.
49. Heard, D. J., Norby, P. L., Holloway, J., and Vissing, H. (2000) Human ERRγ, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: Tissue-specific isoforms are expressed during development and in the adult, *Mol. Endocrinol. 14*, 382−392.

BI036154J