Specific binding of 4-hydroxyestradiol to mouse uterine protein: evidence of a physiological role for 4-hydroxyestradiol

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Abstract

There are several indications of a possible physiological role for 4-hydroxyestradiol (4-OHE_2) in hormone-responsive tissues. To examine a hormonal activity of 4-OHE_2, we have studied the binding of ^{3}H-labeled 4-OHE_2 to mouse uterine cytosolic protein. In uteri of 3-week-old mice, total binding was 319·4 ± 13·9 fmol/mg protein. Binding in the presence of excess unlabeled 4-OHE_2 dropped to 82·1 ± 1·7 fmol/mg protein, whereas 214·6 ± 9·4 fmol/mg protein bound while incubating in an excess of unlabeled 17β-estradiol (E_2). The difference between the two binding values in the presence of excess steroid (132·5 ± 11·1 fmol/mg protein) is taken as selective binding of 4-OHE_2 to a specific protein. In mice older than 4 weeks, the specific 4-OHE_2 binding declined: 32·0 ± 4·0 fmol/mg protein at 8 weeks, 54·8 ± 6·3 fmol/mg protein at 12 weeks and 54·6 ± 5·2 fmol/mg protein at 9 months. Of other organs tested (liver, kidney, lung and whole brain) only lung showed significant selective binding of 4-OHE_2. When E_2-binding sites are blocked, 4-OHE_2 binding follows first-order kinetics, yielding a dissociation constant (K_d) value of 11·8 ± 2·1 nM. The specific binding of 4-OHE_2 was not inhibited by any other steroids or estrogen metabolites that were tested, except for 2-hydroxyestradiol (2-OHE_2), which displayed competitive inhibition of 4-OHE_2 binding with an inhibition constant (K_i) value of 98·2 ± 12·6 nM. These results lead us to conclude that 4-OHE_2 binds to a specific binding protein, distinct and different from binding to estrogen receptors (ERα and ERβ). The physiological role of this binding remains to be elucidated.

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Introduction

The pathophysiological role of 17β-estradiol (E_2) and its metabolite 4-hydroxyestradiol (4-OHE_2) in hormonal carcinogenesis has been investigated at great length (reviewed in Liehr 2001). Evidence is accumulating, however, to support the hypothesis that 4-OHE_2 is not merely a metabolite formed to facilitate the excretion of E_2, but may have a physiological role of its own.

The major pathway for oxidative metabolism of E_2 in the liver is 2-hydroxylation. Formation of 4-OHE_2 occurs as a quantitatively minor pathway, typically less than 15% of 2-hydroxylation (Zhu & Conney 1998). The enzymes responsible for catalyzing this reaction belong to the cytochrome P450 family and include CYP1A2 and the CYP3A families (Kerlan *et al.* 1992, Lee *et al.* 2003). 4-OHE_2 is formed through a lack of specificity of these enzymes. The most likely function of hepatic estrogen metabolism is to prepare the steroids for excretion.

In contrast to this metabolic profile seen in the liver, 4-hydroxylation is a dominant pathway of catechol estrogen formation in several extrahepatic tissues in both animal models and humans. Selective 4-hydroxylation of E_2, with little or no 2-hydroxylation activity, has been observed in human uterine myoma, where 4-OHE_2 is the predominant catechol estrogen, formed at rates 5-fold higher than those in the surrounding myometrium (Liehr *et al.* 1995). CYP1B1 has been identified as a major enzyme catalyzing the 4-hydroxylation of E_2. In fact, E_2 may be the physiological substrate for human CYP1B1, principally because of its low K_m value (0·71 μM) for E_2 (Hayes *et al.* 1996). This specific 4-hydroxylase has been identified in many of those organs of rodents in which chronic estrogen exposure induces malignant or benign tumors: hamster kidney (Kirkman 1959), mouse uterus (Newbold *et al.* 1990, Newbold & Liehr 1999) or rat pituitary gland (Bui & Weisz 1988). As mentioned above, specific 4-hydroxylation of E_2 also occurs in normal or neoplastic human tissues, such as myometrium (Liehr *et al.* 1995) and breast (Liehr & Ricci 1996).

Selective expression of estrogen 4-hydroxylase activity in target tissues does not inactivate the parent estrogen but may be a mechanism for maintaining hormonal activity in these tissues (Zhu & Conney 1998). Tissue-specific metabolism of E_2 is likely a form of differential regulation of estrogenic action and may point to a distinct

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physiological role for 4-OHE_2. For example, 4-OHE_2 has been shown to upregulate the uterine expression of lactoferrin in estrogen-receptor (ER)-knockout (ERKO) mice 60-fold over vehicle control, while E_2 produced only a doubling of lactoferrin mRNA (Das *et al.* 1997). This upregulation of lactoferrin by 4-OHE_2 was not inhibited by ICI 182,780, an estrogen-receptor antagonist, indicating a pathway independent of both ERα and ERβ. Also in ERKO mice, 4-OHE_2 has been implicated in mammary growth and development (Weisz *et al.* 1993). Paria *et al.* (1990) showed that 4-OHE_2 plays a definitive role during blastocyst implantation. More-recent data suggest that both E_2 and 4-OHE_2 are essential for implantation. E_2 prepares the progesterone-primed uterus to the receptive state via interaction with the classical estrogen receptor, while 4-OHE_2 makes the blastocyst 'implantation-competent' via the generation of prostaglandins (Paria *et al.* 1998). The 4-hydroxylase activity in the uterus of the pregnant mouse changes drastically as the pregnancy progresses. E_2 and progesterone elevate the levels of the specific 4-hydroxylase activity on day 4 of the pregnancy, precisely the time at which implantation occurs (Paria *et al.* 1990). Also, a surge in the 4-hydroxylase activity was noted in the pig blastocyst on days 12 and 13 of pregnancy, a time that corresponds to blastocyst implantation (Mondschein *et al.* 1985).

2-Hydroxyestradiol (2-OHE_2) and 4-OHE_2 possess different physiologic potencies and functions. For example, the uterotrophic potency of 4-OHE_2 is close to that of E_2, while that of 2-OHE_2 is considerably weaker (Barnea *et al.* 1983, Paria *et al.* 1990). This is true even though both catechol estrogens have similar binding affinities to ERα and ERβ, albeit 5–10-fold weaker than that of E_2 (Schütze *et al.* 1994). 4-Methylestradiol, which is incapable of being metabolized to 4-OHE_2, is an estrogen agonist with about 25% the hormonal activity of E_2 based on relative binding to the ER and induction of the progesterone receptor in MCF-7 cells (Vollmer *et al.* 1991). Despite this, 4-methylestradiol is incapable of inducing uterine weight gain (Qian & Abul-Hajj 1990, Ball *et al.* 1983). This indicates that 4-hydroxylation of E_2 is necessary for the expression of at least some of the estrogenic effects of E_2.

4-OHE_2 induces the expression of vascular endothelial growth factor-A (VEGF-A) through a phosphoinositide 3-kinase-mediated pathway (Gao *et al.* 2004). Also through a phosphoinositide 3-kinase pathway, independent of described estrogen receptors, 4-OHE_2 has been shown to activate the antioxidant-responsive element, which plays a role in gene expression of phase II metabolism enzymes (Lee *et al.* 2003). It has also been shown to be capable of stimulating the proliferation of human female osteoblastic cells, independently of the two known estrogen receptors (Seeger *et al.* 2003).

All the above-mentioned data indicate a role for 4-OHE_2 other than that of an estrogenic metabolite binding to estrogen receptor(s) (ERα and/or ERβ), formed solely for facilitating excretion. In an attempt to understand this potential physiological role for 4-OHE_2, we have investigated the selective binding of 4-OHE_2 to binding proteins other than the described estrogen receptors in wild-type mice.

Materials and Methods

Materials

[6,7-^{3}H]E_2 (specific activity, 50 Ci/mmol) was obtained from American Radiolabeled Chemicals (St Louis, MO, USA). E_2, 4-OHE_2 and 2-OHE_2 were obtained from Steraloids (Newport, RI, USA). Potassium nitrosodisulfonate was obtained from Aldrich Chemicals (Milwaukee, WI, USA). Hydroxyapatite (HAP) was obtained from Bio-Rad (Hercules, CA, USA). Tris base, EDTA, dithiothreitol, Na_2MoO_4, KCl and glycerol were all obtained from Sigma Chemicals (St Louis, MO, USA).

Animals

All mice used were non-inbred Swiss NIH high-fertility strain. The source of the animals was the nude mouse colony at the Stehlin Foundation for Cancer Research. Animals used for the study were female mice of various ages, ranging from newborn to 10 months old. They were selected from non-nude animals. In addition, in order to select for animals of precise ages, animals were bred specifically for this study. For this purpose, 10 homozygous dominant females were bred, at any one time, with five homozygous dominant males. Their offspring were killed at 1, 2, 3, 4, 8 or 16 weeks of age. Experimental groups consisted of tissue collected and pooled from several animals. Six tissue pools were used to accumulate the data from uterus and three from lung, while all other data were collected in duplicate. Animals did not undergo any treatments and were not administered any drugs at any point before being killed. All animal handling and euthanasia was conducted in accordance with Institutional Animal Care and Use Committee-approved procedures.

Synthesis of radiolabeled 4-OHE_2

[6,7-^{3}H]4-OHE_2 was synthesized from [6,7-^{3}H]E_2 according to a modified procedure detailed by Gelbke *et al.* (1973). The resulting radiolabeled catechol estrogens were purified by reversed-phase HPLC using a C_{18} column. The solvent gradient of aqueous buffer (75 mM citric acid/25 mM ammonium acetate)/acetonitrile/methanol from 80:15:5 to 30:50:20 (by vol.) at 1 ml/min over 50 min resolved the products as follows: 4-OHE_2 at 31·2 min, 2-OHE_2 at 32·3 min and E_2 at 38·4 min. Solutions of the pure ^{3}H-labeled 4-OHE_2 were stored in the presence of 10% acetic acid at −80 °C until used.

Preparation of the extract

Animals were killed and their uteri removed. The uteri were washed in ice-cold 1 × PBS (4% NaCl, 1% KCl, 0·47% Na_2HPO_4, 0·1% KH_2PO_4, pH 7·3) and subsequently homogenized in about 3 vol. of ice-cold KTEDMG buffer (300 mM KCl, 1 mM sodium EDTA, 10 mM Tris base, 1 mM dithothreitol and 10 mM Na_2MoO_4, pH 7·3; made up in a 10% glycerol solution) containing protease inhibitors. Gentle homogenization was carried out in glass homogenizers to prevent denaturation of the proteins. Cytosolic fractions of the uterine homogenate were prepared by ultracentrifugation (40 000 r.p.m. (165 000 ***g***) at 4 °C for 1 h) in a Beckman L7–65 ultracentifuge. Total protein concentrations of the fractions were determined using the Bradford protein assay.

Binding assay

For total binding, the total cytosolic fraction resulting from each experimental group was normalized to 1 mg total protein/ml, divided into six aliquots (200 µl) and incubated with 2·0 nM radiolabeled [6,7-^{3}H]4-OHE_2. For the determination of non-specific binding, another set of tubes received a 400-fold molar excess (800 nM) of unlabeled 4-OHE_2, E_2 or 4-OHE_2 plus E_2 in addition to the radiolabeled steroid. After incubation at 4 °C for 18–20 h (overnight), bound and unbound radioligand were separated by using HAP: 400 µl of a 1:1 suspension of HAP in TED buffer (10 mM Tris base, 10 mM sodium EDTA and 1 mM dithiothreitol, pH 7·3) were added to the incubation volume (200 µl) and the mixture incubated on ice for about 15 min, vortexing every 5 min. The HAP was then spun down to a tight pellet (3000 r.p.m. for 10 min at 4 °C). The pellet was washed three times with 2 ml aliquots of ice-cold TED containing 1% Tween 80. Bound steroid was extracted by resuspending the HAP in 2 ml ethanol (100%) for 15 min at room temperature, vortexing occasionally. The HAP was spun down and an aliquot (1 ml) of the supernatant was counted in a liquid scintillation counter.

Unlabeled saturation assays

These experiments were conducted in a similar fashion to the binding assay except that instead of the 400-fold molar excess of E_2 or 4-OHE_2, increasing concentrations of unlabeled 4-OHE_2 or 2-OHE_2 were used to generate a saturation curve with unlabeled reactants.

Hormonal specificity studies

Hormonal specificity studies were conducted in a similar manner as above, using a 400-fold molar excess of the selected steroid in the presence or absence of a 400-fold molar excess of E_2, to rule out cross-binding to estrogen-binding sites. Since ethanol is used to denature the proteins and extract the radioligand, binding observed is reversible binding and not the result of a covalent bonding of the catechol to the protein, as described previously (Abul-Hajj & Cisek 1988). Specific binding is expressed as (total binding − non-specific binding) and is expressed as fmol of [6,7-^{3}H]4-OHE_2 bound per mg cytosolic protein.

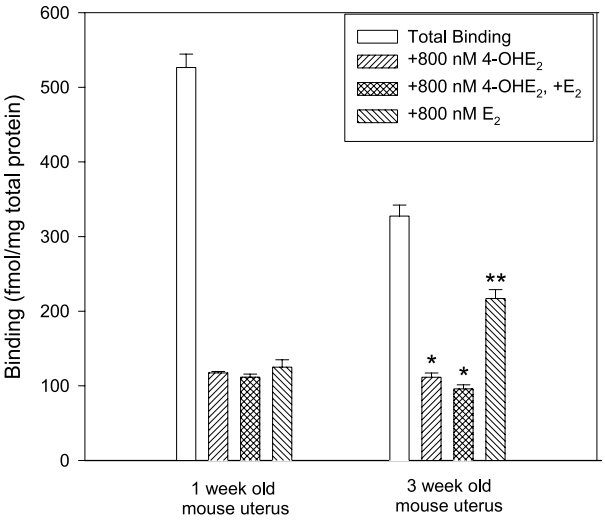

Figure 1 Binding of 2 nM [6,7-^{3}H]4-OHE_2 to 1- and 3-week-old mouse uterine protein alone and in the presence of 800 nM unlabeled 4-OHE_2, 800 nM 4-OHE_2 + 800 nM E_2 or 800 nM E_2. *denotes significant difference versus total binding; ** denotes significant difference versus both total binding and binding in the presence of excess 4-OHE_2 ($P<0·0001$, $n=6$ experimental groups).

Results

Uterine protein collected from 3-week-old animals showed a total 4-OHE_2 binding of 319·4 ± 13·9 fmol/mg protein (Fig. 1). In the presence of excess unlabeled 4-OHE_2 the binding recorded was 82·1 ± 1·7 fmol/mg protein, but in the presence of excess unlabeled E_2 the binding decreased only to 214·6 ± 9·4 fmol/mg protein. The difference between displacements of ^{3}H-labeled 4-OHE_2 by unlabeled E_2 and unlabeled 4-OHE_2 was taken as evidence of specific binding to a 4-OHE_2-binding protein. This binding to uterine protein of 3-week-old animals served as a positive control in more than a dozen independent experiments and consistently yielded the specific 4-OHE_2 binding shown in Fig. 1. There was little or no difference between binding in the presence of excess unlabeled 4-OHE_2 and 4-OHE_2 plus E_2, indicating binding of the catechol estrogen to a specific binding protein in addition to any ERα and/or ERβ binding. 4-OHE_2 binds to the ER with a dissociation

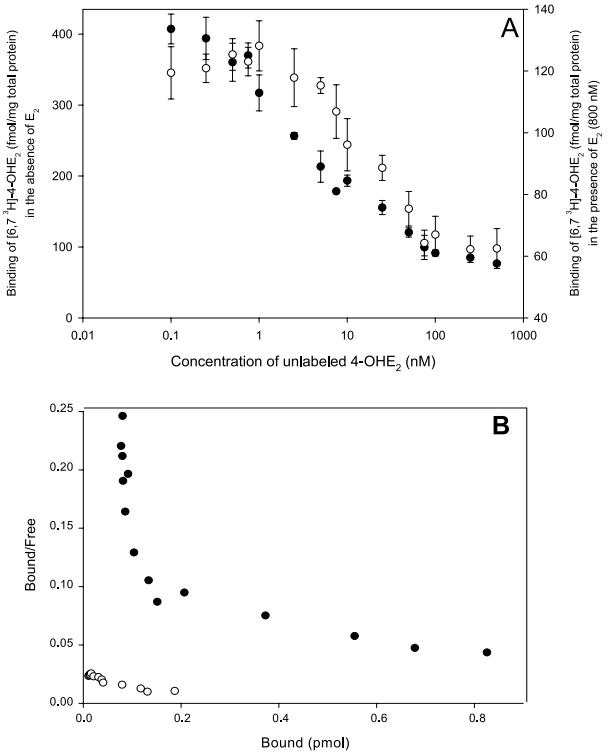

Figure 2 (A) Displacement of [6,7-^{3}H]4-OHE$_2$ from 3-week-old mouse uterine protein by increasing concentrations of unlabeled 4-OHE$_2$ in the presence of 800 nM E$_2$ (○, right-hand *y* axis) and in the absence of E$_2$ (●, left-hand *y* axis). (B) The same data presented as a Scatchard plot.

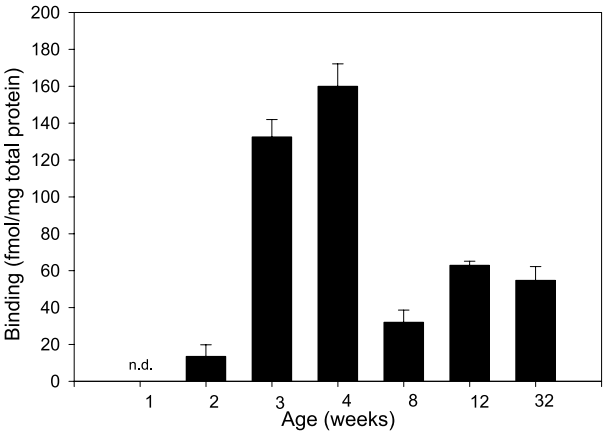

Figure 3 Specific binding of [6,7-^{3}H]4-OHE$_2$, i.e. the difference between binding in the presence of unlabeled E$_2$ and binding in the presence of unlabeled 4-OHE$_2$ at various ages in the mouse uterus. Data shown for 1- and 3-week-old animals are derived from data shown in Fig. 1. n.d., specific binding not detected.

constant (K_d) of 0·21 nM (Barnea *et al.* 1983, Schütze *et al.* 1994) and so a portion of the total binding of the radioligand would have to account for such binding. The data derived from 3-week-old animals were used as a gold standard, as the results were verified in more than a dozen independent experiments.

In contrast to the 3-week-old animals, uterine tissue collected from 1-week-old animals was markedly different. Although high levels of total 4-OHE$_2$ binding to uterine protein were detected in 1-week-old mice (Fig. 1), this binding was entirely displaced by E$_2$. Binding likely includes the relatively low levels of ER identified previously (Shigeta *et al.* 1996) by Northern analysis in this tissue and possibly other binding proteins.

When increasing concentrations of unlabeled 4-OHE$_2$ (0·1–500 nM) were incubated with a fixed concentration of 4-OHE$_2$ (2 nM), a sigmoidal binding-inhibition curve was observed as the radioligand was gradually displaced from its specific binding sites by the increasing concentrations of the unlabeled ligand, as seen in Fig. 2A. If viewed as a Scatchard plot, two distinct binding components can be observed (Fig. 2B). In order to distinguish the binding of 4-OHE$_2$ to the ER(s) from its specific binding to its own putative receptor, the unlabeled saturation study was also conducted in the presence of 800 nM E$_2$. In the presence of this saturating concentration of E$_2$ the displacement curve of 4-OHE$_2$ was much steeper, as can be seen in Fig. 2A. If viewed as a Scatchard plot, only a single binding component can be seen (Fig. 2B). The K_d value for the specific binding of 4-OHE$_2$ was calculated from these and other similar experiments to be 11·8 ± 2·1 nM. The K_d calculated from the high-affinity component of the Scatchard plot was 0·29 ± 0·03 nM. This corresponds to previously reported K_d values of 4-OHE$_2$ for the ER in MCF-7 cells (Schütze *et al.* 1994).

As shown in Fig. 3, specific 4-OHE$_2$ binding – the difference in displacement of the radioligand by E$_2$ and 4-OHE$_2$ – as described above, is age-dependent. Levels were low, barely detectable, during the first weeks of life, and then reach a peak of 159·9 ± 12·2 fmol/mg protein at 4 weeks (puberty occurs in the mouse around 3–4 weeks of age), before returning to relatively low levels at later ages.

Specific 4-OHE$_2$ binding was also observed in the lung (n=3, P<0·015), where concentrations of estrogen receptors are low (Stabile *et al.* 2002; Fig. 4). Liver, kidney and whole brain of 4-week-old mice were also examined, but showed no appreciable specific binding of 4-OHE$_2$.

Hormonal specificity of the binding of 4-OHE$_2$ was examined by performing competition assays with various estrogens, catechols and other steroidal compounds. The results are shown in Fig. 5. As described above, 800 nM E$_2$ partially displaced radiolabeled 4-OHE$_2$ from its binding sites, while unlabeled 4-OHE$_2$ was much more efficient in this respect (Fig. 5A). Of the compounds tested, only 2-OHE$_2$, 4-hydroxytamoxifen, 6α-hydroxyestradiol and 11β-hydroxyestradiol showed any signs of being able to displace radiolabeled 4-OHE$_2$ from its binding sites.

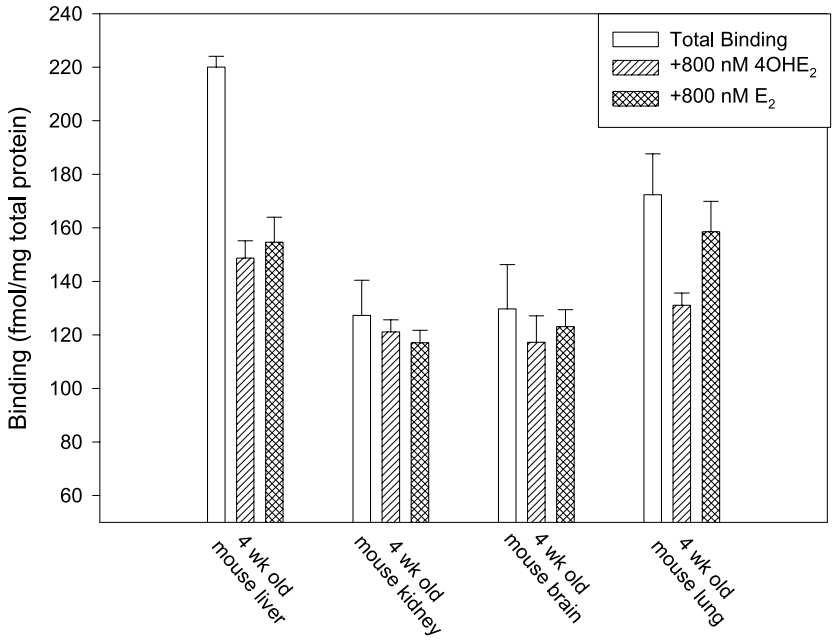

Figure 4 Binding of [6,7-^{3}H]4-OHE$_2$ in various mouse organs, alone (white bars; Total binding) or in the presence of 800 nM unlabeled 4-OHE$_2$ or 800 nM unlabeled E$_2$. Specific binding of [6,7-^{3}H]4-OHE$_2$ in the lung was significantly different from binding of E$_2$ (P<0·015).

The affinity of 2-OHE$_2$ for the estrogen receptor has been established previously (Barnea *et al.* 1983, Schütze *et al.* 1994). 4-Hydroxytamoxifen has been shown previously to have significant affinity for the estrogen receptor (Foster *et al.* 1985) and 6α- and 11 β-hydroxyestradiol have been shown to exert estrogenic effects or to be significant estrogenic metabolites (Dehennin *et al.* 1984, Segaloff & Gabbard 1984). Our data are consistent with binding of these compounds to E$_2$-binding sites, but not to 4-OHE$_2$-binding sites.

The displacement of radioligand caused by each steroid increased in the presence of excess (800 nM) E$_2$, to approximately the displacement observed in the presence of E$_2$ as the only antagonist but not significantly more (Fig. 5B). This indicates that the displacements caused by the steroids tested were from estrogen-specific binding sites, not 4-OHE$_2$-specific sites. The only exception to this was 2-OHE$_2$. At the concentration tested (800 nM), 2-OHE$_2$ could displace 4-OHE$_2$ from its specific binding site as well as from its binding to E$_2$-binding sites. To further examine the binding of 2-OHE$_2$ to the specific 4-OHE$_2$-binding site, a parallel study was conducted using the same amounts of radioligand and increasing concentrations of either unlabeled 4-OHE$_2$ or unlabeled 2-OHE$_2$. The results can be seen in Fig. 6, which shows 2-OHE$_2$ to be a competitive inhibitor of 4-OHE$_2$ binding, albeit with an inhibition constant (K_i) 10-fold greater than the K_d value of 4-OHE$_2$ (98·2 ± 12·6 nM).

The binding assay has been designed to exclude covalent binding of the radioligand to proteins, as described in the Materials and Methods section. The ligand–protein complex was denatured with 100% ethanol and the radioligand taken up into the solvent. The amount of radioligand dissolved in the ethanol was assayed and used to calculate protein binding. Moreover, binding experiments were carried out in the presence of 1% dithiothreitol, a reducing agent used to prevent oxidation of 4-OHE$_2$ to the semiquinone or quinone forms, the reactive molecules in covalent binding of catechol estrogens to cellular macromolecules (Abul-Hajj & Cisek 1988).

Discussion

Our data clearly demonstrate specific binding of 4-OHE$_2$ to a selective binding protein. Although 4-OHE$_2$ binding to ERα is known (Barnea *et al.* 1983), our data demonstrate protein binding distinct and different from binding to ER. Total binding of 4-OHE$_2$ likely includes binding to ERα, ERβ, a putative 4-OHE$_2$ receptor and possibly other binding proteins. The difference in displacement of ^{3}H-labeled 4-OHE$_2$ by unlabeled 4-OHE$_2$ and unlabeled E$_2$, however, is clear evidence of the existence of a specific binding protein, which may stimulate a hormonal signaling pathway distinct and different from that of E$_2$.

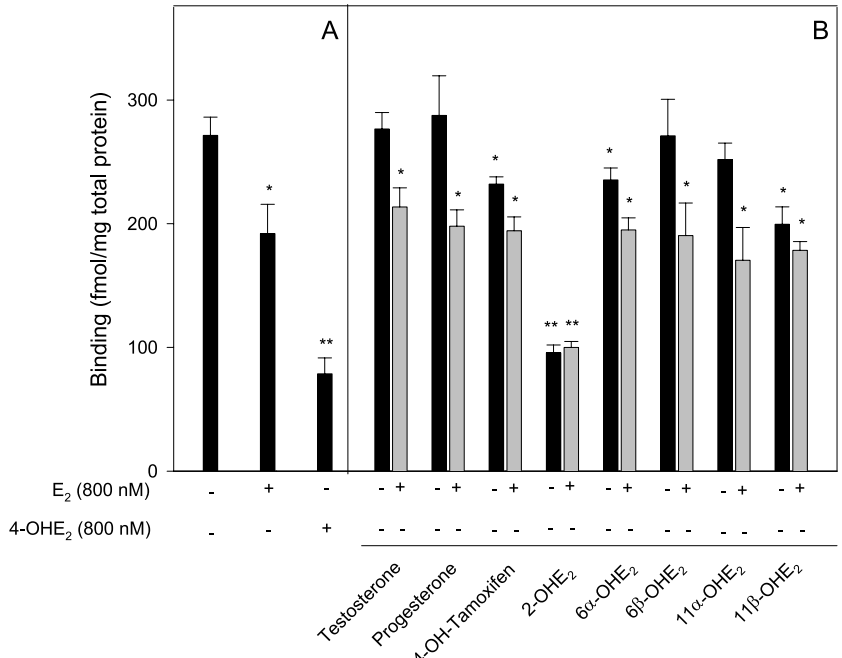

Figure 5 (A) Binding of 2 nM [6,7-^{3}H]4-OHE_2 to uterine protein from 3-week-old mice in the presence or absence of 800 nM E_2 or 800 nM 4-OHE_2. (B) Binding of 2 nM [6,7-^{3}H]4-OHE_2 to uterine protein from 3-week-old mice in the presence of 800 nM of each of the indicated steroids. Binding was carried out in the absence (black bars) or presence (gray bars) of 800 nM unlabeled E_2. Each bar represents the mean ± S.D. from five replicates. *Significant difference (P<0·05) from binding in the absence of any unlabeled steroids; **significant difference (P<0·05) from total binding (2 nM [6,7-^{3}H]4-OHE_2 only) as well as from binding in the presence of unlabeled E_2. 4-OH-Tamoxifen, 4-hydroxytamoxifen; OHE_2, hydroxyestradiol.

Binding of 4-OHE_2 to the ER(s) has been measured in the past (Schütze *et al.* 1994) during investigation of the physiological role of catechol metabolites of E_2. That study corroborated previous results (Barnea *et al.* 1983) that 4-OHE_2 binds to the ER, but dissociates more slowly than E_2. Therefore hydroxylation of E_2 may be a mechanism for prolonging estrogenic action in certain tissues (Zhu & Conney 1998). Schütze *et al.* (1994), however, observed higher 'non-specific binding' when using radiolabeled 4-OHE_2 rather than E_2. Since they were using diethylstilbestrol to displace the radioligand, this higher value for non-specific binding could be attributed to binding of the radioligand to a 4-OHE_2-specific binding site, such as the one described in this study, from which it could not be displaced by diethylstilbestrol.

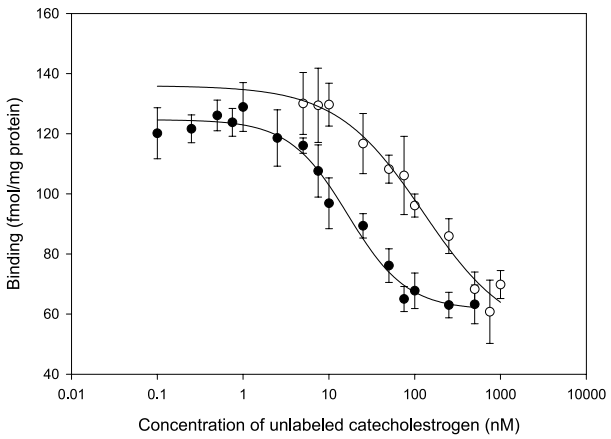

Figure 6 Comparison of displacement of 2 nM [6,7-^{3}H]4-OHE_2 by increasing concentrations of unlabeled 4-OHE_2 (●) and unlabeled 2-OHE_2 (○). Both unlabeled saturation studies were conducted in the presence of 800 nM unlabeled E_2. Studies were conducted in 3-week-old mouse uterine protein preparations.

In fact, as described in this text, none of the steroids tested except 2-OHE_2 were capable of displacing radiolabeled 4-OHE_2 from its specific binding site, even when using a 400-fold molar excess. The 10-fold lower affinity of 2-OHE_2 for the 4-OHE_2-specific binding site indicates that the binding of 2-OHE_2 is not a hormonal effect. Also, even though the K_m values of both catechol estrogens for catechol-O-methyl transferase are similar, 2-OHE_2 is much more rapidly methylated than 4-OHE_2 (Roy *et al.* 1990). This results in 2-OHE_2 levels that are universally low (Emons *et al.* 1987), nowhere close to 98 nM, which

we have determined as its K_d for the 4-OHE_2-specific binding site.

Philips *et al.* (2004) recently described specific 4-OHE_2 binding in ERKO mice. The K_d they report however, are a full order of magnitude lower than the one reported in this paper in 3-week-old wild-type mice. This difference could indicate a different binding dynamic in the presence of ER or even the existence of a totally different binding protein. The decline in the level of specific 4-OHE_2 binding in mice aged more than 4 weeks old (Fig. 3) could also point to a different protein being expressed during puberty.

Catechol estrogens are easily oxidizable to the semi-quinone and quinone forms, which may bind covalently to proteins. The protein binding described by us, however, is different from covalent binding, as evident from the reversal of binding by the ethanol wash. Moreover, the lack of binding in mouse uterus at 1 week of age or in other organ sites indicates specific reversible binding and not non-specific, covalent binding, which would be expected to occur in any tissue.

The existence of the specific binding indicates that 4-OHE_2 has physiological activity, as indicated previously by its role in blastocyst implantation (Paria *et al.* 1990, 1998) and lactoferrin gene expression (Das *et al.* 1997). Both these events take place in the uterus, an organ where we detect binding to a specific protein. The specific binding of 4-OHE_2 described in this text could account for this behavior of 4-OHE_2. Circulating levels of 4-OHE_2 are undetectable. Within hormonally active tissues, however, such as human breast cancer, high levels in the nanomolar range have been detected (Yue *et al.* 2003). This is due to the fact that in the blood serum enzymes, such as catechol-O-methyl transferase, rapidly metabolize 4-OHE_2, while these enzymes are not necessarily present in tissues where 4-OHE_2 is formed *in situ*. The distribution of CYP1B1, the isozyme responsible for selective 4-hydroxylation of E_2, is known (Shimada *et al.* 1996) and could be a good indicator of tissues that express 4-OHE_2-specific binding.

Our data indicate that 4-OHE_2 may be formed in the uterus by CYP1B1 (Shimada *et al.* 1996) as a signaling molecule. The existence of this estrogen 4-hydroxylase in organs other than uterus, such as lung (Shimada *et al.* 1996), point towards an as-yet unknown role of this steroid in other organ sites. The exact nature of the binding protein as well as the more-specific role of 4-OHE_2 in the lung and other organs remain to be elucidated.

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References

Abul-Hajj YJ & Cisek PL 1988 Catechol estrogen adducts. *Journal of Steroid Biochemistry* **31** 107–110.

Ball P, Emons G, Haupt O & Knuppen R 1983 Pharmacological effects of 2- and 4-methyloestradiol as a probe to test the biological important of 2- 4-hydroxylation of oestrogens (catecholestrogen formation). *Acta Endocrinologica* **102** 150–152.

Barnea ER, MacLusky NJ & Naftolin F 1983 Kinetics of catecholestrogen-estrogen receptor dissociation: a possible factor underlying differences in catecholestrogen biological activity. *Steroids* **41** 643–656.

Bui QD & Weisz J 1988 Monooxygenase mediating catecholestrogen formation by rat anterior pituitary is an estrogen-4-hydroxylase. *Endocrinology* **124** 1085–1087.

Das SK, Taylor JA, Korach KS, Paria BC, Dey SK & Lubhan DB 1997 Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct signaling pathway. *PNAS* **94** 12786–12791.

Dehennin L, Blacker C, Reiffsteck A & Scholler R 1984 Estrogen 2-, 4-, 6- or 16-hydroxylation by human follicles shown by gas chromatography-mass spectrometry associated with stable isotope dilution. *Journal of Steroid Biochemistry* **20** 465–471.

Emons G, Merriam GR, Pfeiffer D, Loriaux DL, Ball P & Knuppen R 1987 Metabolism of exogenous 4- and 2-hydroxyestradiol in the human male. *Journal of Steroid Biochemistry* **28** 499–504.

Foster AB, Jarman M, Leung OT, McCague R, Leclercq G & Devleeschouwer N 1985 Hydroxy derivatives of tamoxifen. *Journal of Medicinal Chemistry* **28** 1491–1497.

Gao N, Nester RA & Sarkar MA 2004 4-Hydroxy estradiol but not 2-hydroxy estradiol induces expression of hypoxia-inducible factor 1 alpha and vascular endothelial growth factor A through phosphatidylinositol 3-kinase/Akt/FRAP pathway in OVCAR-3 and A2780-CP70 human ovarian carcinoma cells. *Toxicology and Applied Pharmacology* **196** 124–135.

Gelbke HP, Haupt O & Knuppen R 1973 A simple chemical method for the synthesis of catechol estrogens. *Steroids* **21** 205–218.

Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ & Sutter TR 1996 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *PNAS* **93** 9776–9781.

Kerlan V, Dreano Y, Bercovici JP, Beaune PH, Floch HH & Berthou F 1992 Nature of cytochrome P450 involved in the 2-/4-hydroxylations of estradiol in human liver microsomes. *Biochemical Pharmacology* **44** 1745–1756.

Kirkman H 1959 Estrogen-induced tumors of the kidney. III. Growth characteristics in the Syrian hamster. *National Cancer Institute Monographs* **1** 1–57.

Lee JM, Anderson PC, Padgitt JK, Hanson JM, Waters CM & Johnson JA 2003 Nrf2, not the estrogen receptor, mediates catechol estrogen-induced activation of the antioxidant responsive element. *Biochimica et Biophysica Acta* **1629** 92–101.

Liehr JG 2001 Genotoxicity of the steroidal oestrogens oestrone and oestradiol: possible mechanism of uterine and mammary cancer development. *Human Reproduction Update* **7** 273–281.

Liehr JG & Ricci MJ 1996 4-Hydroxylation of estrogens as marker of human mammary tumors. *PNAS* **93** 3294–3296.

Liehr JG, Ricci MJ, Jefcoate CR, Hannigan EV, Hokanson JA & Zhu BT 1995 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. *PNAS* **92** 9220–9224.

Mondschein JS, Hersey RM, Dey SK, Davis DL & Weisz J 1985 Catecholestrogen formation by pig blastocysts during the preimplantation period: biochemical characterization of estrogen-2/4-hydroxylase and correlation with aromatase activity. *Endocrinology* **117** 2339–2346.

Newbold RR & Liehr JG 1999 Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Research* **60** 235–237.

Newbold RR, Bullock BC & McLachlan AJ 1990 Uterine adenocarcinoma in mice following developmental treatment with estrogen. *Cancer Research* **50** 7677–7681.

Paria BC, Chakraborty C & Dey SK 1990 Catechol estrogen formation in the mouse uterus and its role in implantation. *Molecular and Cellular Endocrinology* **69** 25–32.

Paria BC, Lim H, Wang X-N, Liehr JG, Das SK & Dey SK 1998 Coordination of differential effect of primary estrogen and catecholestrogen on two distinct targets mediates embryo implantation in the mouse. *Endocrinology* **139** 5235–5246.

Philips BJ, Ansell PJ, Newton LG, Harada N, Honda S-I, Ganjam VK, Rottinghaus GE, Welshons WV & Lubahn DB 2004 Estrogen receptor-independent catechol estrogen binding activity: protein binding studies in wild-type, estrogen receptor-α KO, and aromatase KO mice tissues. *Biochemistry* **43** 6698–6708.

Qian X-D & Abul-Hajj YJ 1990 Synthesis and biological activity of 4-methylestradiol. *Journal of Steroid Biochemistry* **35** 745–747.

Roy D, Weisz J & Liehr JG 1990 The O-methylation of 4-hydroxyestradiol is inhibited by 2-hydroxyestradiol: implications for estrogen-induced carcinogenesis. *Carcinogenesis* **11** 459–462.

Schütze N, Vollmer G, Wunsche W, Grote A, Feit B & Knuppen R 1994 Binding of 2-hydroxyestradiol and 4-hydroxyestradiol to the estrogen receptor of MCF-7 cells in cytosolic extracts and in nuclei of intact cells. *Experimental and Clinical Endocrinology* **102** 399–408.

Segaloff A & Gabbard RB 1984 Structure-activity relationships of estrogens: effects of esterification of the 11 beta-hydroxyl group. *Steroids* **43** 111–123.

Seeger H, Hadji P & Mueck AO 2003 Endogenous estradiol metabolites stimulate the *in vitro* proliferation of human osteoblastic cells. *International Journal of Clinical Pharmacology and Therapeutics* **41** 148–152.

Shigeta H, Newbold RR, McLachlan JA & Teng C 1996 Estrogenic effect on the expression of estrogen receptor, COUP-TF, and lactoferrin mRNA in developing mouse tissues. *Molecular Reproduction and Development* **45** 21–30.

Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP & Sutter TR 1996 Activation of chemically diverse procarcinogens by human cytochrome P450 1B1. *Cancer Research* **56** 2979–2984.

Stabile LP, Davis AL, Gubish CT, Hopkins TM, Luketich JD, Christie N, Finkelstein S & Siegfried JM 2002 Human non-small cell lung cancer tumors and cell derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen. *Cancer Research* **62** 2141–2150.

Vollmer G, Wunsche W, Schutze N, Feit B & Knuppen R 1991 Methyl and bromo derivatives of estradiol are ligands for the estrogen receptor of MCF-7 breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **39** 359–366.

Weisz J, Bui QD, Roy D & Liehr JG 1993 Elevated 4-hydroxylation of estradiol by hamster kidney microsomes: a potential pathway of metabolic activation of estrogens. *Endocrinology* **131** 655–661.

Yue W, Santen RJ, Wang JP, Li Y, Verderame MF, Bocchinfuso WP, Korach KS, Devanesan P, Todorovic R, Rogan EG & Cavalieri EL 2003 Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. *Journal of Steroid Biochemistry* **86** 477–486.

Zhu BT & Conney AH 1998 Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* **19** 1–27.

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