

Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors α and β

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ABSTRACT

The rat estrogen receptor (ER) exists as two subtypes, ER α and ER β , which differ in the C-terminal ligand binding domain and in the N-terminal transactivation domain. In this study we investigated the messenger RNA expression of both ER subtypes in rat tissues by RT-PCR and compared the ligand binding specificity of the ER subtypes.

Saturation ligand binding analysis of *in vitro* synthesized human ER α and rat ER β protein revealed a single binding component for 16 α -iodo-17 β -estradiol with high affinity [dissociation constant (K_d) = 0.1 nM for ER α protein and 0.4 nM for ER β protein]. Most estrogenic substances or estrogenic antagonists compete with 16 α -[¹²⁵I]iodo-17 β -estradiol for binding to both ER subtypes in a very similar preference and degree; that is, diethylstilbestrol > hexestrol > dienes-
trole > 4-OH-tamoxifen > 17 β -estradiol > coumestrol, ICI-164384 >

estrone, 17 α -estradiol > nafoxidine, moxestrol > clomifene > estriol, 4-OH-estradiol > tamoxifen, 2-OH-estradiol, 5-androstene-3 β ,17 β -diol, genistein for the ER α protein and dienes-
trole > 4-OH-tamoxifen > diethylstilbestrol > hexestrol > coumestrol, ICI-164384 > 17 β -estradiol > estrone, genistein > estriol > nafoxidine, 5-androstene-3 β ,17 β -diol > 17 α -estradiol, clomifene, 2-OH-estradiol > 4-OH-estradiol, tamoxifen, moxestrol for the ER β protein. The rat tissue distribution and/or the relative level of ER α and ER β expression seems to be quite different, *i.e.* moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal for ER α and prostate, ovary, lung, bladder, brain, uterus, and testis for ER β . The described differences between the ER subtypes in relative ligand binding affinity and tissue distribution could contribute to the selective action of ER agonists and antagonists in different tissues. (*Endocrinology* **138**: 863–870, 1997)

ESTROGENS INFLUENCE the growth, differentiation and functioning of many target tissues. These include tissues of the male and female reproductive systems such as mammary gland, uterus, ovary, testis, and prostate. Estrogens also play an important role in bone maintenance and in the cardiovascular system, where estrogens have certain cardioprotective effects (1). Estrogens are mainly produced in the ovaries and testis. They diffuse in and out of all cells, but are retained with high affinity and specificity in target cells by an intranuclear binding protein, termed the estrogen receptor (ER). Once bound by estrogens, the ER undergoes a conformational change, allowing the receptor to bind with high affinity to chromatin and to modulate transcription of target genes (2). Steroid hormone receptors consist of a hypervariable N-terminal domain that contributes to the transactivation function; a highly conserved central domain responsible for specific DNA binding, dimerization, and nuclear localization, and a C-terminal domain involved in ligand binding and ligand-dependent transactivation function (1). The rat ER cDNA was cloned from uterus and found to be highly homologous to the ER complementary DNAs

(cDNAs) cloned from mouse, human, and chicken (3). We recently cloned a novel rat ER cDNA from prostate (4), which we suggested be named rat ER β subtype to distinguish it from the previously cloned ER cDNA (consequently ER α subtype). The rat ER β cDNA encodes a protein of 485 amino acid residues with a calculated mol wt of 54200. Rat ER β protein is highly homologous to rat ER α protein, particularly in the DNA binding domain (> 90% amino acid identity) and in the C-terminal ligand binding domain (LBD) (55%). Saturation ligand binding experiments with *in vitro* synthesized ER β protein revealed a single binding component for 17 β -estradiol (E₂) with high affinity [dissociation constant (K_d) = 0.6 nM]. Expression of ER β was investigated by *in situ* hybridization, and prominent expression was found in rat prostate (secretory epithelial cells) and ovary (granulosa cells). In cotransfection experiments of Chinese hamster ovary (CHO) cells with an ER β expression vector and an estrogen-regulated reporter gene, maximal stimulation of reporter gene activity was found during incubation with 1 nM E₂ (4).

The biological significance of the existence of two ER subtypes is at this moment unclear. Perhaps the existence of two ER subtypes provides, at least in part, an explanation for the selective actions of estrogens in different target tissues (5). In fact, the high degree of interspecies conservation of the individual ER subtypes throughout vertebrate evolution (Ref. 6 and our unpublished observations) could suggest that the basis for the selective effects of estrogens resides in the control of different subsets of estrogen-responsive promoters by

Received September 27, 1996.

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* Supported in part by grants from The Netherlands Organization for Scientific Research (NWO) and from the Karolinska Institute.

† Supported by grants from the Swedish Cancer Society and from the European Union (EU-PL95-1223) Climate and Environment Program.

the two ER subtypes. This would implicate differential expression of the ER subtypes in target tissues.

The overall homology between the rat ER α protein LBD and rat ER β protein LBD is not more than 55% (Fig. 1). Interestingly, the ER β protein LBD encompassing amino acid residues 223–457 has a low homology with the ER α protein LBD between amino acid residues 344–403, whereas outside this stretch the homology is considerably higher (amino acid residues 223–343 and 404–457). The structural core of the LBD of the human ER α protein has recently been mapped by restricted proteolysis, and only one single region within this core was found to be easily accessible to proteases (7). This surface-exposed protease accessible region (human ER α LBD amino acid residues 465–468) is in the center of the stretch showing lowest homology with ER β protein. The amino acid sequence stretches of the ER β LBD between amino acids 223–343 and 404–457 are probably, similarly to the highly homologous stretches in the ER α LBD, part of a compact hydrophobic (non-surface-exposed) entity directly contacting the ligand. Although several parts of these stretches are completely conserved, and the amino acid alterations often are conservative, it is possible that interesting differences in ligand binding affinity and/or specificity exist between the ER subtypes. Chemically quite diverse compounds (estrogens, some androgens, phytoestrogens, antiestrogens, and environmental estrogens) have been shown in the past to have estrogenizing activity and to interact with the ER from rat uterus and human breast tumor cells (Ref. 8 and references therein).

In the present study we investigated the ligand binding specificity of the two ER subtypes and the transcript tissue distribution in the adult rat.

Materials and Methods

Materials

The radioligand 16 α -[¹²⁵I]iodo-E₂ ([¹²⁵I]E₂) was obtained from New England Nuclear (Boston, MA). The unlabeled steroids E₂, 17 α -estradiol, estrone, estriol, dehydroepiandrosterone, 5 α -dihydrotestosterone, testosterone, progesterone, corticosterone, moxestrol (11 β -methoxy-17 α -ethynyl-1,3,5(10)-estratrien-3,17 β -diol), 4-hydroxy-estradiol (1,3,5(10)-estratriene-3,4,17 β -triol), 2-hydroxy-estradiol (1,3,5(10)-estratriene-2,3,17 β -triol), 5-androstenediol (5-androstene-3 β ,17 β -diol), 4-androstenediol (4-androstene-3 β ,17 β -diol), 3 α -androstenediol (5 α -andro-

stane-3 α ,17 β -diol), 3 β -androstenediol (5 α -androstane-3 β ,17 β -diol), 5 α -androstenedione (5 α -androstane-3,17-dione), 5 β -androstenedione (5 β -androstane-3,17-dione), 4-androstenedione (4-androstene-3,17-dione), norethynodrel (17 α -ethynyl-17-hydroxy-5(10)-estren-3-one), norethindrone (19nor-4-androsten-17 α -ethynyl-17 β -ol-3-one), 19-nortestosterone (4-estren-17 β -ol-3-one), β -sitosterol (24 β -ethyl-5-cholesten-3 β -ol), and estrone-3-sulfate (3-hydroxy-1,3,5(10)-estratrien-17-one-3-sulfate) were obtained from Steraloids Inc. (Wilton, NH) except for dehydroepiandrosterone and moxestrol (RU 2858), which were obtained from Ikapharm (Ramat-Gan, Israel) and from Roussel Uclaf (Romainville, France), respectively.

The phytoestrogen coumestrol (2-(2,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Eastman Kodak (Rochester, NY) and genistein (4,5,7-trihydroxyisoflavone) and β -zearalanol (2,4-dihydroxy-6-[6 β ,10-dihydroxyundecyl]benzoic acid μ -lactone) were from Sigma (St. Louis, MO).

The synthetic estrogens diethylstilbestrol (4,4'-(1,2-diethyl-1,2-ethenediyl)bisphenol), hexestrol (4,4'-(1,2-diethyl-1,2-ethane-diyl)bisphenol), and dienestrol (4,4'-(1,2-diethylidene-1,2-ethane-diyl)bisphenol) were obtained from Sigma. The antiestrogens tamoxifen (1-(*p*- β -dimethylamino-ethoxyphenyl)-*trans*-1,2-diphenylbut-1-ene), 4-OH-tamoxifen (1-(*p*-dimethylamino-ethoxyphenyl)-1-(4-hydroxyphenyl)-2-phenylbut-1-ene), clomiphene (1-(*p*- β -diethylaminoethoxyphenyl)-1,2-diphenylchloro-ethylene), nafoxidine 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)-phenoxy]-ethyl)pyrrolidine hydrochloride, and ICI-164384 (*N*-*n*-butyl-11-(3,17 β -dihydroxyestra-1,3,5(10)-trien-7 α -yl)-*N*-methylundecanamide) were obtained from Sigma or synthesized by KaroBio AB (ICI-164384) (Huddinge, Sweden). The environmental estrogens Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) and methoxychlor (1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane) were obtained from Aldrich (Germany). The structural formulas and chemical properties of all the competitors used can be found in the Merck Index or elsewhere (8–10).

Sephadex G25 columns (QS-2A) were obtained from Isolab (Akron, OH). All other chemicals were of the highest purity available.

In vitro transcription and translation

The 2.6 kbp rat ER β cDNA (4) was subcloned into the EcoRI site of pBluescript (Stratagene, La Jolla, CA). The plasmid pT7 β HER (11) containing the wild type (HEGO) human ER α sequence was a kind gift from Dr. B.W. O'Malley and co-workers (Baylor College of Medicine, Houston, TX). Human ER α and rat ER β protein was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega, Madison, WI) with T7-RNA polymerase, during a 90 min reaction at 30 C. Translation reaction mixtures (50- μ l portions) were snap-frozen and stored at -70 C until further use.

Saturation ligand binding analysis

Translation reaction mixtures were diluted in buffer A (20 mM HEPES, pH = 7.9; 150 mM NaCl, 10% wt/vol glycerol, 1 mM EDTA, 6 mM monothioglycerol, and 10 mM Na₂MoO₄) and kept at 4 C. Aliquots equivalent to 0.25 μ l ER α translation mixture or 2 μ l ER β translation mixture were incubated in duplo with 10–800 pM [¹²⁵I]-E₂ in the presence or absence of a 300-fold excess of diethylstilbestrol for 16 h at 4 C. The final incubation volume was 200 μ l, and to the ER α incubation series unprogrammed reticulocyte lysate was added to equalize the total protein concentrations. Free and unbound radioligand was separated by gel filtration over G-25 columns at 4 C as described (12). Bound radioactivity was measured in a Wallac γ -counter (Turku, Finland) with 70% efficiency. Specific binding was determined by subtracting nonspecific binding from total binding, and the free ligand concentration was estimated by subtracting total bound ligand from added ligand. The equilibrium K_d was calculated as the free concentration of radioligand at half-maximal binding by fitting data to the Hill equation (13) and by linear Scatchard transformation (14). Curve fitting was done in Kaleidagraph 2.1.3 (Abelbeck Software, PA).

Ligand competition experiments

Competitors were dissolved in dimethylsulfoxide at a concentration of 1 mM, except for coumestrol, genistein, and β -zearalanol, which were dissolved in ethanol. Translation reaction mixtures were diluted with

223	LVLITLLEAEPFNVLVS-RPSMPFTEASMMSLIKLADKELVHMIGWAKCI	rat ER β
320	M.SA..D...LIYSEYD..R..S....GL..N...R....N...RV	rat ER α
272	PGFVELSLIDQVRLLESCWMEVLMGLMWRSDHPGKLIFAPDLVLRDE	rat ER β
370	...GD.N.H...H...CA.L.I...I..V...ME....L...N.L...NQ	rat ER α
322	GKCVBGLILEIFDMLLATTSRFFELKLOHKEYLCVKAMITLINSMPY-LAS	rat ER β
420	MV.....S.....MN..GE.FV.L.SI.....GV.TF.S.	rat ER α
371	ANQFAESSRKLTILHNAVITDALWWTAKSGISSQQSVRLANLMLLSHV	rat ER β
470	ILKSL..EKDITHRV.DKIN.T.IHLM..A.LITL...HR...Q..LI...I	rat ER α
421	RHISNKGMEHLISMCKCNVVPYDILLEMNAHILRG	rat ER β
520	..M.....YN.....L.....D..R.HAPA	rat ER α
	558	

FIG. 1. Alignment of amino acid sequences of rat ER α protein (GenBank database Y00102) LBD (amino acid residues 320–558), and rat ER β protein (GenBank database U57439) LBD (amino acid residues 223–457). For alignment, Clustal analysis using MEGALIGN/DNASTAR software was used.

buffer A and kept at 4 C. Aliquots equivalent to 0.25 μ l ER α translation mixture or 2 μ l ER β translation mixture were added to dilutions containing [125 I]-E $_2$ and the respective competitors. The final concentration of radioligand was 125–150 pM, and the incubation time was 16 h at 4 C. Unprogrammed reticulocyte lysate was added to the ER α series to equalize protein concentrations. Competitors were present at concentrations between 10^{-4} M and 10^{-10} M; each competition curve consisting of eight concentrations in duplicates. Free and bound ligand were separated by gel filtration over Sephadex G-25 columns as described (12). The data were evaluated by a nonlinear four-parameter logistic model (15) to estimate the IC $_{50}$ value (the concentration of competitor at half-maximal specific binding). Relative binding affinity (RBA) of each competitor was calculated as the ratio of concentrations of E $_2$ and competitor required to reduce the specific radioligand binding by 50% (= ratio of IC $_{50}$ values). The RBA value for E $_2$ was arbitrarily set at 100. The Cheng-Prusoff equation (13, 14) was used to calculate the K $_i$ of the various competitors.

PCR analysis of rat tissue total RNA

Male and female rats (6–8 weeks old) were killed by cervical dislocation, and tissues were collected. Tissue samples were immediately processed for total RNA isolation according to the acid guanidinium thiocyanate-phenol-chloroform single-step extraction protocol (16). The integrity and quality of the purified RNA was controlled by formaldehyde denaturing agarose gel electrophoresis and by measurement of the A260/A280 nm ratio. Only RNA samples exhibiting an A260/A280 ratio >1.6 and showing integrity of the RNA by electrophoresis were used in further experiments. The RNA isolated from spleen and brain cortex appeared degraded and was discarded.

Random hexamer-primer cDNA synthesis was performed as described (17, 18). For the PCR amplification, 5% of the synthesized cDNA was added to a PCR reaction mixture as described (17) and amplified for 30 cycles by incubation at 95 C for 30 sec, 57 C for 15 sec, 72 C for 60 sec, and a final incubation at 72 C for 3 min, all in a PCR 9600 thermocycler (Perkin-Elmer, Norwalk, CT). The oligonucleotides erbkg1: 5'TCCCCG-CAGCACCAGTAACC (+38 relative to ATG) and erbkg2: 5'TC-CCTCTTTGCGTTTGACTA (+279 relative to ATG) were used for amplification of a 262-bp fragment of the ER β messenger RNA (mRNA). The oligonucleotides kgb5: 5'AATTCTGACAATCGACGCCAG (+472 relative to ATG) and kgb6: 5'GTGCTTCAACATTCTCCCTCCTC (+794 relative to ATG) were used for amplification of a 344-bp fragment of the rat ER α mRNA. The oligonucleotides used for the amplification of actin mRNA are previously described (17). After agarose gel electrophoresis and blotting to nitrocellulose filters, the PCR products were hybridized to the internal oligonucleotides: ERUR 4: 5'GGGACTCTTTGAGGT-TCTGC (+163-182 relative to ATG) for ER β , KG50: 5'GCAGC-GAGAAGGGAAACATGA (+518-538 relative to ATG) for ER α , and actin primer: 5'GATGACCCAGATCATGTTGA (+434-454 relative to ATG) for actin according to a previously described protocol (17).

Results

Saturation ligand binding analysis of ER protein

The ER can be isolated from the cytosol of target cell extracts as a large nontransformed (*i.e.* non-DNA binding) 7–8S oligomeric complex, which contains hsp90 and hsp70 (2). It is believed that heat-shock proteins function to help fold the ER protein properly and to protect the hydrophobic hormone binding domain from inappropriate interactions (2). Rabbit reticulocyte lysates contain large amounts of several heat-shock proteins as hsp90 and hsp70, and have been used extensively for the study of ER complex formation with hsp, as well as for the study of requirements for steroid binding and interactions with DNA (2, 6, 11). When ER β protein was synthesized *in vitro* and labeled with a saturating dose of [3 H]-E $_2$ and analyzed on sucrose density gradients, a single peak of specifically bound radioactivity was observed. The sedimentation coefficient of this complex was

about 7S, and it shifted to 4S in the presence of 0.4 M NaCl (not shown). It was therefore decided to use human ER α and rat ER β protein synthesized in reticulocyte lysates for the ligand binding experiments.

To obtain optimal conditions for the determination of equilibrium K $_d$ s and RBAs of various ligands, the ER concentration in the binding assay was lowered to 10–20 pM. At these low ER concentrations radioligand and/or competitor depletion can be excluded while maintaining high receptor recovery during separation of bound and unbound ligand by the use of a gel filtration assay instead of the traditional charcoal adsorption assay (12). The low ER concentration made it necessary to employ radioiodinated estradiol as a probe, because the specific radioactivity of tritiated estradiol was too low to maintain sufficient accuracy. Radioiodinated E $_2$ (16 α -[125 I]iodo-E $_2$) binds to the ER with high affinity and specificity as shown by its use in dry-mount autoradiographic techniques and various ligand binding assays (19, 20).

In Fig. 2 the result of a saturation ligand binding assay with [125 I]-E $_2$ is shown. Single point assays (not shown) were used to equalize the amount of ER α and ER β protein used (10–15 pM). The nonspecific binding was $\leq 8\%$ of total binding over the whole radioligand concentration range used. The K $_d$ values calculated from the saturation curves (Fig. 2) were 0.06 nM for ER α protein and 0.24 nM for ER β protein. Linear transformation of saturation data (Scatchard plots in Fig. 2) revealed a single population of binding sites for 16 α -iodo-E $_2$ with a K $_d$ of 0.1 nM for the ER α protein and 0.4 nM for the ER β protein. The measured K $_d$ values are in agreement with the finding that almost maximal stimulation of reporter gene activity by ER α and ER β protein was previously found during incubation with 1 nM E $_2$ (4). Although the ER β protein has a four times lower affinity for 16 α -iodo-E $_2$ in this system compared with the ER α protein, both K $_d$ values are within

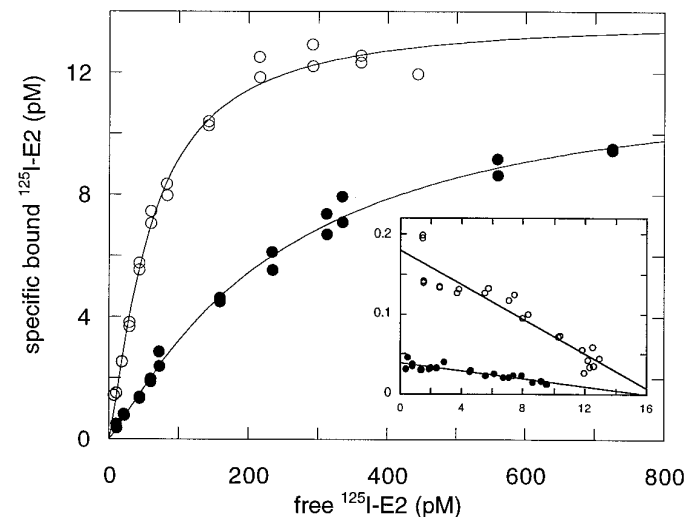


FIG. 2. Binding of 16 α -[125 I]iodo-E $_2$ to *in vitro* synthesized ER α and ER β protein in presence or absence of a 300-fold excess of diethylstilbestrol for 16 h at 4 C. Unbound radioactivity was removed as described, and specific bound counts (ER α = $\circ$; ER β = $\bullet$) were calculated by subtracting nonspecific bound counts from total bound counts. Inset, Scatchard analysis of specific binding giving a K $_d$ of 0.1 nM for ER α protein and a K $_d$ of 0.4 nM for ER β protein.

the range (0.1–1 nM) generally reported for estradiol binding to ERs in various systems (1).

Ligand binding specificity of ER α and ER β protein

Measurements of the equilibrium binding of the radioligand in the presence of different concentrations of unlabeled competitors provides readily interpretable information about the affinities of the latter, provided that radioligand and/or competitor depletion are avoided. Competition ex-

periments were performed using ER α and ER β protein concentrations of 10–15 pM and a [125 I]-E $_2$ concentration of about 150 pM, so that for both ERs the total receptor concentration was $\leq 0.1 K_d$ and the radioligand concentration was ≥ 10 times the ER concentration. Under such experimental conditions radioligand or competitor depletion can be excluded (14).

In total 37 substances were tested for both ER subtypes (Fig. 3 and Table 1). In Fig. 3 several examples of typical

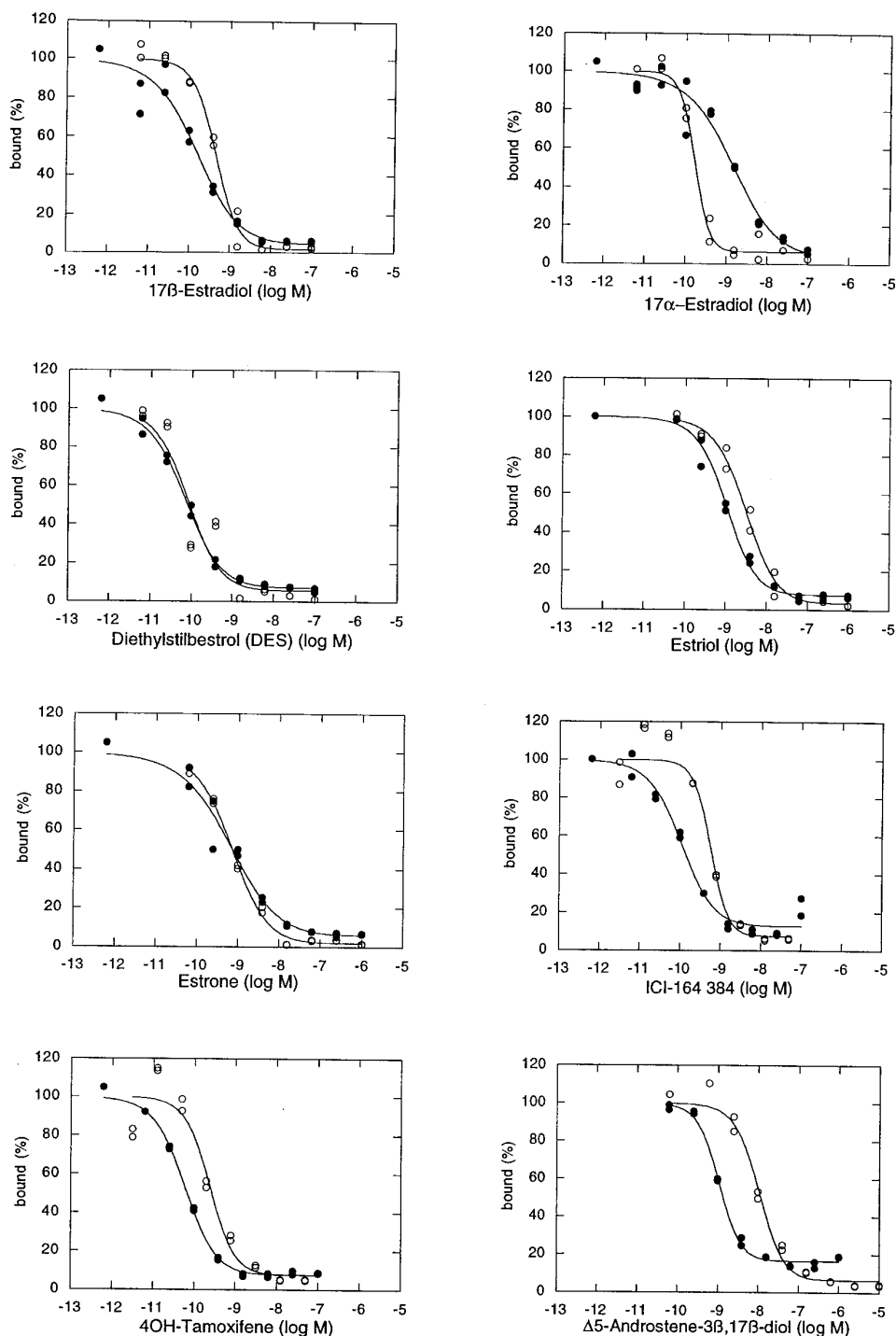


FIG. 3. Competition by several nonradioactive estrogenic substances and antiestrogens for 16 α -[125 I]iodo-E $_2$ binding to *in vitro* synthesized ER α protein (○) and ER β protein (●). Incubation was for 16 h at 4 C, and bound and unbound radioligand were separated as described.

TABLE 1. Binding affinity of various compounds for ER α and ER β

Compound	RBA ^a		K _i (nM) ^b	
	ER α	ER β	ER α	ER β
E ₂	100	100	0.13	0.12
Diethylstilbestrol	468	295	0.04	0.05
Hexestrol	302	234	0.06	0.06
Dienestrol	223	404	0.05	0.03
Estrone	60	37	0.3	0.4
17 α -Estradiol	58	11	0.2	1.2
Moxestrol	43	5	0.5	2.6
Estriol	14	21	1.4	0.7
4-OH-Estradiol	13	7	1.0	1.9
2-OH-Estradiol	7	11	2.5	1.3
Estrone-3-sulfate	<1	<1		
4-OH-Tamoxifen	178	339	0.1	0.04
ICI-164384	85	166	0.2	0.08
Nafoxidine	44	16	0.3	0.8
Clomifene	25	12	0.9	1.2
Tamoxifen	7	6	3.4	2.5
5-Androstenediol	6	17	3.6	0.9
3 β -Androstenediol	3	7	6	2
4-Androstenediol	0.5	0.6	23	19
3 α -Androstenediol	0.07	0.3	260	48
5 α -Dihydrotestosterone	0.05	0.17	221	73
Dehydroepiandrosterone	0.04	0.07	245	163
19-Nortestosterone	0.01	0.23	765	53
5 α -Androstanedione	<0.01	<0.01		
Testosterone	<0.01	<0.01		
5 β -Androstanedione	<0.01	<0.01		
4-Androstenedione	<0.01	<0.01		
Coumestrol	94	185	0.14	0.07
Genistein	5	36	2.6	0.3
β -Zearalanol	16	14	0.8	0.9
Bisphenol A	0.05	0.33	195	35
Methoxychlor	0.01	0.13	1774	90
Norethindrone	0.07	0.01	152	1084
Norethynodrel	0.7	0.22	14	53
Progesterone	<0.001	<0.001		
Corticosterone	<0.001	<0.001		
β -Sitosterol	<0.001	<0.001		

^a RBA of each competitor was calculated as ratio of concentrations of E₂ and competitor required to reduce the specific radioligand binding by 50% (= ratio of IC₅₀ values). RBA value for E₂ was arbitrarily set at 100. The IC₅₀ of E₂ was 0.21 nM for ER α protein and 0.13 nM for ER β protein.

^b The Cheng-Prusoff formula (13, 14) was used to calculate the K_i of the various competitors.

competitor curves obtained are shown. In all cases monophasic curves were obtained for compounds with significant affinity. The slopes of the curves were almost similar, enabling the use of IC₅₀ values to calculate RBA values (Table 1), with an RBA value of 100 for E₂ for each receptor. For the ER α as well as ER β protein, the estradiol binding was stereospecific because 17 α -estradiol showed a two times and 10 times lower affinity, respectively (Table 1), compared with E₂, which is in agreement with previous findings on stereospecific binding of estradiol by the ER (21). However, in making such comparisons, it should be kept in mind that most, if not all, ER ligand binding studies done in the past 30 yr actually involved mixtures of ER α and ER β protein. This is certainly the case for many studies in which rat uterus cytosol was used (see following). The present study is the first in which the ligand binding properties of both ER subtypes are measured separately, and caution is needed when

comparing RBAs from this study with the previous studies involving mixtures of ER subtypes.

For the physiological estrogens, the order of competition was E₂ > estrone, 17 α -estradiol (ER α) > estriol > catecholestrogens, 17 α -estradiol (ER β) > estrone-3-sulfate. Overall the ER α and ER β proteins show the binding characteristics and relative affinity for the physiological estrogens found to be characteristic for an ER protein (1, 8, 22). The stilbene estrogens, which consist of a composite diphenolic ring structure, bind with high affinity to both ER subtypes. However, different orders of competition were found: diethylstilbestrol > hexestrol > dienestrol > (E₂) for ER α and dienestrol > diethylstilbestrol > hexestrol > (E₂) for ER β .

The extra methoxy group at C11 and the ethynyl group at C17 of moxestrol (RU 2858) lowered the affinity compared with E₂ for the ER α protein by only a factor of 2 but for the ER β protein by a factor of 20. Moxestrol is in use as a radioligand in ER assays, and is known to have a lower binding affinity than E₂ under certain assay conditions (23).

The triphenylethylene (anti)estrogens were developed by successive chemical modifications of the triphenylethylene nucleus, formed by the addition of an extra phenyl ring to the stilbene nucleus as present in for instance diethylstilbestrol (9, 10). Interestingly, the measured order of affinity for the tested triphenylethylene (anti)estrogens was the same for both ER subtypes: 4OH-tamoxifen >> nafoxidine > clomifene > tamoxifen. The steroidal antiestrogen ICI 164384 had a high affinity for ER α as well as for ER β , confirming that extensions at C7 do not preclude ligand-ER interactions (9, 24).

It has been known for a long time that a number of compounds classified as androgens (C19 steroids) can evoke estrogen-like effects in the female genital tract and in the mammary glands (25). Of all the androgens tested only those with a hydroxyl group at C3 and C17 had significant affinity for both ER subtypes (Table 1). The relative flatness of the A-ring with respect to the B-ring is also important, given the clear difference in affinity for both ER subtypes between 5-androstenediol and 4-androstenediol. The binding affinity of 3 β -androstenediol and 5-androstenediol for both ER subtypes is in agreement with previous studies showing specific binding to the rat uterus ER and estrogenic responses in rat uterus and mammary tumors for both steroids (26, 27).

Norethynodrel and norethindrone, progestins derived from 19-nor-testosterone, and 19-nor-testosterone itself have an intrinsic estrogenic potential as shown by the induction of alkaline phosphatase activity in ER-positive human endometrial cancer Ishikawa cells (28). The apparent binding affinity of norethynodrel and norethindrone for both ER subtypes was however, only about 1/500th of that for E₂ (Table 1), and the need for a conversion into more active metabolites by aromatization or hydroxylation at C-3 has been suggested (28).

Several plant-derived nonsteroidal compounds such as genistein and coumestrol have estrogenic activity (8). These compounds increase rat uterine weight and stimulate growth of breast tumor cells and compete with E₂ for binding to ER protein as well as stimulate the activity of reporter genes in the presence of ER protein (Ref. 29 and references therein). Both coumestrol and genistein had a significantly higher

affinity for ER β protein (Table 1), which is interesting in the light of the high expression of ER β mRNA in the secretory epithelial cells of the prostate, and the prostate cancer protective properties that have been associated with these compounds (30). Zearalanols are fungal metabolites or derivatives thereof that have been associated with estrogenizing syndromes in cattle fed with mold-infected grain (8). Despite the fact that zearalanols are structurally very different to known steroidal and nonsteroidal estrogens, they interact with the rat uterus cytosolic ER (31). Also, in our competition assays β -zearalanol interacted with both ER subtypes with a similar affinity (Table 1), as was reported previously for the rat uterus ER protein (31).

Abnormal sexual development in reptiles as well as the increasing incidence of certain human reproductive tract abnormalities (such as hypospadias) has been associated with increased exposure to and body burdens of so-called estrogenic environmental chemicals (32, 33). These effects from estrogenic chemicals as, for instance, the pesticide methoxychlor and the plastics ingredient bisphenol A, are postulated to be mediated via the ER because these compounds have estrogenic effects (increase of uterine weight) in female rats (8, 32, 33). Bisphenol A and methoxychlor both inhibited the binding of [125 I]-E $_2$ by the ER α and ER β protein, and the inhibition seemed to be stronger for the ER β protein (Table 1). However, it was clearly a very low affinity interaction, and the fact cannot be excluded that it involved different sites on the ER than those involved in the binding of E $_2$.

Expression of ER α and ER β mRNA in rat tissues

To determine the relative distribution of ER α and ER β mRNA, total RNA was isolated from rat tissues and used for RT-PCR using primers specific for each ER subtype. All tissues were taken from 6- to 8-week-old male rats, except uterus and ovary, which were taken from 8-week-old female rats. Although this assay was only semiquantitative, it is clear that the relative distribution of both ER subtypes was quite different (Fig. 4). Highest expression of ER β mRNA was found in the ovary and prostate, which is in agreement with our previous *in situ* hybridization experiments using male and female rats of similar ages (4). In addition, testis, uterus, bladder, and lung showed moderate expression, whereas pituitary, epididymis, thymus, various brain sections, and spinal cord reveal low expression of ER β mRNA. The ER α mRNA was highly expressed in epididymis, testis, pituitary, uterus, kidney, and adrenal, which all showed moderate or no expression of ER β mRNA. Aside from weak expression in thalamus/hypothalamus, the brain sections tested were negative for ER α mRNA. Ovary and uterus, which are known to contain high amounts of ER protein (1), clearly expressed both ER subtypes. All organs from male rats previously described to display specific binding of [3 H]-E $_2$ to an 8S cytosolic protein, *i.e.* liver, lung, adrenal, pituitary, prostate, epididymis, and testis, showed clear expression of either ER subtype mRNA or both (34, 35).

Discussion

The ligand binding affinity of ER α and ER β protein is overall quite similar for the physiological ligands, certainly

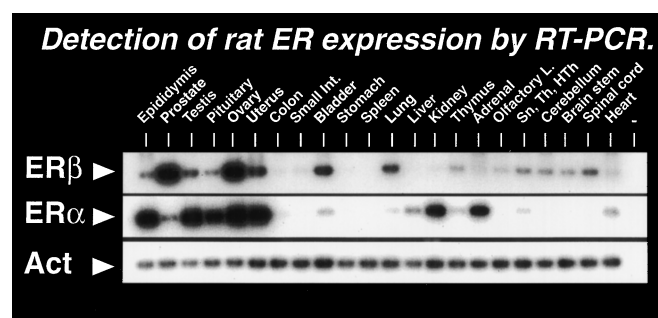


FIG. 4. Rat tissue distribution of ER α mRNA and ER β mRNA determined by RT-PCR (see *Materials and Methods*). Autoradiograms are shown of blots after hybridization with oligonucleotide probes specific for ER β (top), ER α (middle), and actin (bottom). Sn, Substantia nigra, preopticus; Th, thalamus; Hth, hypothalamus; Olfactory L., olfactory lobes; Small Int., small intestine.

when only the order of competition is compared. The most interesting difference was found for 17 α -estradiol, which has a five times higher affinity for ER α protein. The physiological action of 17 α -estradiol is quite different from that of E $_2$, because 17 α -estradiol is a short-acting estrogen and actually a time-dependent mixed agonist-antagonist in the rat/mouse uterus (1). Short-acting estrogens (estrone, estriol, and 17 α -estradiol) do cause nuclear binding of the hormone-receptor complex but only for a short period of time (1). It would be interesting to see whether the difference found for 17 α -estradiol in our ligand binding assays is also present in a transactivation assay system. For the other types of ligands tested, *i.e.* antiestrogens, androgenic steroids, and phytoestrogens, there are some interesting differences in the RBAs (Table 1), but it remains to be seen whether these differences are also reflected in a transactivation assay system using different cellular backgrounds. The ER β protein clearly displays all the ligand binding characteristics of a classical ER protein (1, 8–10, 21, 22, 26–29, 31, 36), and therefore it seems unlikely that a unique physiological ligand for the ER β protein exists. The relative order of ligand binding for various estrogens (diethylstilbestrol, estrone, and estriol) is slightly different in this study than in our previous study (4). Our previous rather preliminary assay (4) was hampered by relatively high levels of nonspecific radioligand binding (30–40% compared with about 5% in this report), which might explain the difference.

A question of considerable interest is why, despite the numerous ligand binding assays performed for the ER protein, an indication for the existence of two ER subtypes was never published. Of course, distinguishing between a mixed population of receptor subtypes and a homogeneous receptor population by saturation or homologous/heterologous competition assays is generally difficult. This is only possible with certainty when the two subtypes differ sufficiently in affinity (10- to 100-fold), and the range of ligand concentrations examined is wide. Furthermore, the proportions of the two subtypes must be appropriate (37). Of all the radioligands used in ER assays (E $_2$, DES, hexestrol, moxestrol, 16 α -iodo-estradiol), the difference in affinity for moxestrol between both ER subtypes is the greatest (8-fold) in our experiments. In this regard, it should be realized that to detect the existence of receptor subtypes the higher affinity

subtype should be less abundant than the lower affinity subtype (37). Most ER ligand binding assays have been done with uterus extracts and breast tumor extracts or cell lines, and it could be that the right conditions for the detection of receptor subtypes are not fulfilled in these cases. We have been unable to detect the ER β mRNA in various breast tumor cell lines (MCF-7, T47D, ZR75-1) by RT-PCR (our unpublished observations), whereas both subtypes are expressed in rat (Fig. 4) and human uterus (not shown).

In prostate and ovary, the two tissues that express high levels of ER β mRNA, it has been difficult to demonstrate the presence of ERs by immunostaining with the available ER antibodies, although specific binding of E₂ could be measured (1, 8, 34). In human and rat prostate at best only weak staining in stromal smooth muscle cells was found (38, 39), which is in contrast with our results showing high expression of ER β mRNA in the prostate secretory epithelial cells (4). In the rat and human ovary, specific binding of estradiol was found in intact follicles and granulosa cells (40–41), but no ER could be detected with available ER antibodies (41). These discrepancies could be explained by the fact that the most frequently used ER protein antibodies, H-222 and H-226 (42), do not cross-react with rat ER β protein on immunoblots (our unpublished observations). The above findings and our results could indicate that the ER β protein is the predominant if not the only ER subtype present in rat prostate and ovary. Of course this remains to be proven when specific ER β protein antibodies become available. The fact that disruption of the ER α gene *in vivo* did not eliminate the ability of small follicles to grow, as is evident from the presence of secondary follicles and antral follicles in the ER α knockout mouse (43), also argues for the presence of alternative ER (ER β ?) molecules. In fact, rat uterus, ovary, testis, epididymis, and pituitary clearly express both ER subtypes mRNAs. Although we have no data on ER β mRNA expression or protein concentration in tissues of the ER α knock-out mouse (43), the possible presence of ER β protein should be kept in mind when interpreting experiments using the ER α knock-out mouse. Furthermore, in the uterus of the ER α knock-out mouse, residual E₂ binding could be measured (43), which is likely caused by the presence of ER β protein. In the brain of the ER α knock-out mouse, specific binding of E₂ and modulation of progesterone receptor gene expression by E₂ was observed (44). Again this is most likely caused by the presence of ER β protein, because the ER β mRNA is broadly expressed in the rat brain and probably also in the mouse brain at a low level. Detailed mapping of ER α and ER β expression in rat/mouse brain by *in situ* hybridization or using specific antibodies for each subtype is of clear interest given the fact that for the localization of ERs in the brain of various species antibodies that do not recognize ER β have been used (45).

Acknowledgments

The authors thank Drs. Margaret Warner and Albert Brinkmann for advice and suggestions and Dr. Geoffrey Greene for providing ER antibodies.

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