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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, ERα and ERβ, 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 ERα and rat ERβ protein revealed a single binding component for 16α-iodo-17β-estradiol with high affinity [dissociation constant (K_d) = 0.1 nM for ERα protein and 0.4 nM for ERβ protein]. Most estrogenic substances or estrogenic antagonists compete with 16α-[^{125}I]iodo-17β-estradiol for binding to both ER subtypes in a very similar preference and degree; that is, diethylstilbestrol > hexestrol > dienestrol > 4-OH-tamoxifen > *17β-estradiol* > coumestrol, ICI-164384 > estrone, 17α-estradiol > nafoxidine, moxestrol > clomifene > estriol, 4-OH-estradiol > tamoxifen, 2-OH-estradiol, 5-androstene-3β,17β-diol, genistein for the ERα protein and dienestrol > 4-OH-tamoxifen > diethylstilbestrol > hexestrol > coumestrol, ICI-164384 > *17β-estradiol* > estrone, genistein > estriol > nafoxidine, 5-androstene-3β,17β-diol > 17α-estradiol, clomifene, 2-OH-estradiol > 4-OH-estradiol, tamoxifen, moxestrol for the ERβ protein. The rat tissue distribution and/or the relative level of ERα and ERβ expression seems to be quite different, *i.e.* moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal for ERα and prostate, ovary, lung, bladder, brain, uterus, and testis for ERβ. 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 ERβ subtype to distinguish it from the previously cloned ER cDNA (consequently ERα subtype). The rat ERβ cDNA encodes a protein of 485 amino acid residues with a calculated mol wt of 54200. Rat ERβ protein is highly homologous to rat ERα 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 ERβ protein revealed a single binding component for 17β-estradiol (E_2) with high affinity [dissociation constant (K_d) = 0.6 nM]. Expression of ERβ 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 ERβ expression vector and an estrogen-regulated reporter gene, maximal stimulation of reporter gene activity was found during incubation with 1 nM E_2 (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

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

The overall homology between the rat ERα protein LBD and rat ERβ protein LBD is not more than 55% (Fig. 1). Interestingly, the ERβ protein LBD encompassing amino acid residues 223–457 has a low homolgy with the ERα 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 ERα 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 ERα LBD amino acid residues 465–468) is in the center of the stretch showing lowest homology with ERβ protein. The amino acid sequence stretches of the ERβ LBD between amino acids 223–343 and 404–457 are probably, similarly to the highly homologous stretches in the ERα 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 16α-[^{125}I]iodo-E_2 ([^{125}I]E_2) was obtained from New England Nuclear (Boston, MA). The unlabeled steroids E_2, 17α-estradiol, estrone, estriol, dehydroepiandrosterone, 5α-dihydrotestosterone, testosterone, progesterone, corticosterone, moxestrol (11β-methoxy-17α-ethynyl-1,3,5(10)-estratrien-3,17β-diol), 4-hydroxy-estradiol (1,3,5(10)-estratriene-3,4,17β-triol), 2-hydroxy-estradiol (1,3,5(10)-estratriene-2,3,17β-triol), 5-androstenediol (5-androstene-3β,17β-diol), 4-androstenediol (4-androstene-3β,17β-diol), 3α-androstanediol (5α-androstane-3α,17β-diol), 3β-androstanediol (5α-androstane-3β,17β-diol), 5α-androstanedione (5α-androstane-3,17-dione), 5β-androstanedione (5β-androstane-3,17-dione), 4-androstenedione (4-androstene-3,17-dione), norethynodrel (17α-ethynyl-17-hydroxy-5(10)-estren-3-one), norethindrone (19nor-4-androsten-17α-ethynyl-17β-ol-3-one), 19-nortestosterone (4-estren-17β-ol-3-one), β-sitosterol (24β-ethyl-5-cholesten-3β-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-[6β,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*-dimethylaminoethoxyphenyl)1-(4-hydroxyphenyl)-2-phenyl-but-1-ene), clomiphene (1-(*p*-(β-diethylaminoethoxy)phenyl)-1,2-diphenylchloro-ethylene), nafoxidine 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphtyl)-phenoxy]-ethyl)pyrrolidine hydrochloride, and ICI-164384 (*N*-*n*-butyl-11-(3,17β-dihydroxyestra-1,3,5(10)trien-7α-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*-methoxy-phenyl)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 ERβ cDNA (4) was subcloned into the *Eco*RI site of pBluescript (Stratagene, La Jolla, CA). The plasmid pT7βhER (11) containing the wild type (HEGO) human ERα sequence was a kind gift from Dr. B.W. O'Malley and co-workers (Baylor College of Medicine, Houston, TX). Human ERα and rat ERβ 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-μl 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 Na_2 MoO_4) and kept at 4 C. Aliquots equivalent to 0.25 μl ERα translation mixture or 2 μl ERβ translation mixture were incubated in duplo with 10–800 pM [^{125}I]-E_2 in the presence or absence of a 300-fold excess of diethylstilbestrol for 16 h at 4 C. The final incubation volume was 200 μl, and to the ERα incubation series unprogrammed reticulocyte lysate was added to equalize the total protein concentrations. Free and unbound radioligand was separated by gelfiltration 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

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223 LVLTLLEAEPPNVLVS-RPSMPFTEASMMMSLTKLADKELVHMIGWAKKI   rat ERß
320 M.SA..D....LIYSEYD..R..S.....GL..N...R......N...RV   rat ERα

272 PGFVELSLLDQVRLLESCWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRDE   rat ERß
370 ...GD.N.H...H...CA.L.I..I..V...ME.....L...N.L...NQ   rat ERα

322 GKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAMILLNSSMYP-LAS   rat ERß
420 ......MV.........S....MMN..GE.FV.L.SI.....GV.TF.S.   rat ERα

371 ANQFAESSRKLTHLLNAVTDALVWVIAKSGISSQQQSVRLANLLMLLSHV   rat ERß
470 TLKSL.EKDHIHRV.DKIN.T.IHLM..A.LTL...HR...Q..LI...I   rat ERα

421 RHISNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRG 457           rat ERß
520 ..M........YN........L........D..R.HAPA 558          rat ERα
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FIG. 1. Alignment of amino acid sequences of rat ERα protein (GenBank database Y00102) LBD (amino acid residues 320–558), and rat ERβ 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 μl ERα translation mixture or 2 μl ERβ 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 ERα 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 gelfiltration 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′TTCCCGGCAGCACCAGTAACC (+38 relative to ATG) and erbkg2: 5′TCCCTCTTTGCGTTTGGACTA (+279 relative to ATG) were used for amplification of a 262-bp fragment of the ERβ 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 ERα 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′GGGACTCTTTTGAGGTTCTGC (+163-182 relative to ATG) for ERβ, KG50: 5′GCAGCGAGAAGGGAAACATGA (+518-538 relative to ATG) for ERα, and actin primer: 5′GATGACCCAGATCATGTTTGA (+434-454 relative 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 hsps, as well as for the study of requirements for steroid binding and interactions with DNA (2, 6, 11). When ERβ 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 ERα and rat ERβ protein synthesized in reticulocyte lysates for the ligand binding experiments.

To obtain optimal conditions for the determination of equilibrium K_ds 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 (16α-[^{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 ERα and ERβ protein used (10–15 pM). The nonspecific binding was ≤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 ERα protein and 0.24 nM for ERβ protein. Linear transformation of saturation data (Scatchard plots in Fig. 2) revealed a single population of binding sites for 16α-iodo-E_2 with a K_d of 0.1 nM for the ERα protein and 0.4 nM for the ERβ protein. The measured K_d values are in agreement with the finding that almost maximal stimulation of reporter gene activity by ERα and ERβ protein was previously found during incubation with 1 nM E_2 (4). Although the ERβ protein has a four times lower affinity for 16α-iodo-E_2 in this system compared with the ERα protein, both K_d values are within

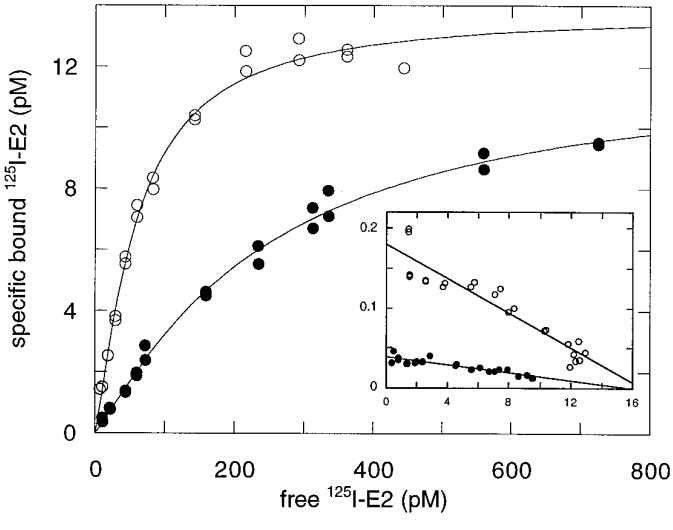

FIG. 2. Binding of 16α-[^{125}I]iodo-E_2 to *in vitro* synthesized ERα and ERβ protein in presence or absence of a 300-fold excess of diethylstilbestrol for 16 h at 4 C. Unbound radioactivity was removed as described, and specific bound counts (ERα = ○; ERβ = ●) were calculated by subtracting nonspecific bound counts from total bound counts. *Inset*, Scatchard analysis of specific binding giving a K_d of 0.1 nM for ERα protein and a K_d of 0.4 nM for ERβ protein.

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

Ligand binding specificity of ERα and ERβ 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 experiments were performed using ERα and ERβ 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 ≤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

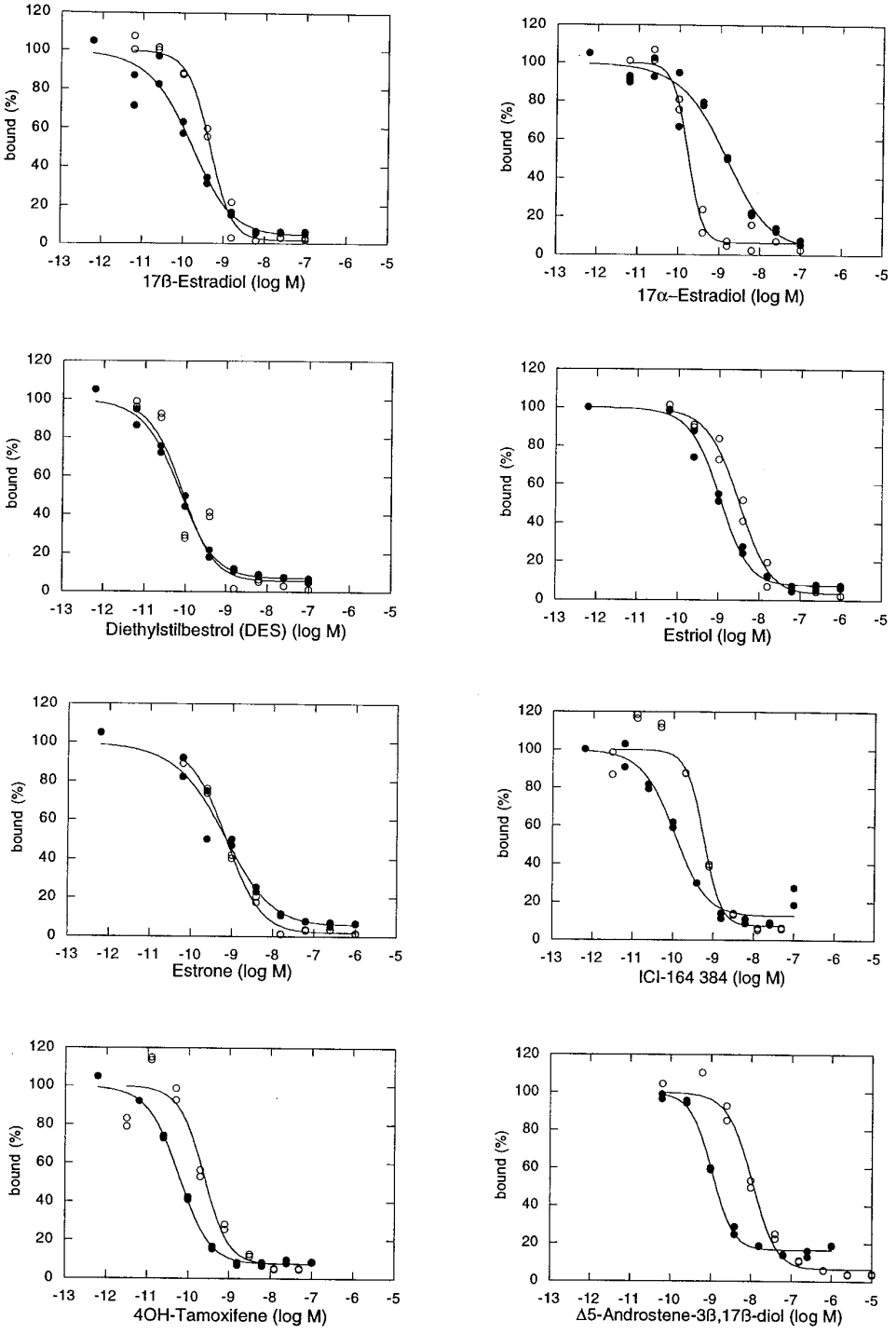

FIG. 3. Competition by several nonradioactive estrogenic substances and antiestrogens for 16α-[^{125}I]iodo-E_2 binding to *in vitro* synthesized ERα protein (○) and ERβ protein (●). Incubation was for 16 h at 4 C, and bound and unbound radioligand were separated as described.

TABLE 1. Binding affinity of various compounds for ERα and ERβ

Compound	RBA[a]		K_i (nM)[b]	
	ERα	ERβ	ERα	ERβ
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
17α-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
3β-Androstanediol	3	7	6	2
4-Androstenediol	0.5	0.6	23	19
3α-Androstanediol	0.07	0.3	260	48
5α-Dihydrotestosterone	0.05	0.17	221	73
Dehydroepiandrosterone	0.04	0.07	245	163
19-Nortestosterone	0.01	0.23	765	53
5α-Androstanedione	<0.01	<0.01		
Testosterone	<0.01	<0.01		
5β-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 E_2 and competitor required to reduce the specific radioligand binding by 50% (= ratio of IC_{50} values). RBA value for E_2 was arbitrarily set at 100. The IC_{50} of E_2 was 0.21 nM for ERα protein and 0.13 nM for ERβ 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 IC_{50} values to calculate RBA values (Table 1), with an RBA value of 100 for E_2 for each receptor. For the ERα as well as ERβ protein, the estradiol binding was stereospecific because 17α-estradiol showed a two times and 10 times lower affinity, respectively (Table 1), compared with E_2, 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 ERα and ERβ 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 E_2 > estrone, 17α-estradiol (ERα) > estriol > catecholestrogens, 17α-estradiol (ERβ) > estrone-3-sulfate. Overall the ERα and ERβ 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 > (E_2) for ERα and dienestrol > diethylstilbestrol > hexestrol > (E_2) for ERβ.

The extra methoxy group at C11 and the ethynyl group at C17 of moxestrol (RU 2858) lowered the affinity compared with E_2 for the ERα protein by only a factor of 2 but for the ERβ 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 E_2 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 ERα as well as for ERβ, 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 3β-androstanediol 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 E_2 (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 E_2 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 ERβ protein (Table 1), which is interesting in the light of the high expression of ERβ 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 ERα and ERβ protein, and the inhibition seemed to be stronger for the ERβ 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 ERα and ERβ mRNA in rat tissues

To determine the relative distribution of ERα and ERβ 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 ERβ 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 ERβ mRNA. The ERα mRNA was highly expressed in epididymis, testis, pituitary, uterus, kidney, and adrenal, which all showed moderate or no expression of ERβ mRNA. Aside from weak expression in thalamus/hypothalamus, the brain sections tested were negative for ERα 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 ERα and ERβ protein is overall quite similar for the physiological ligands, certainly when only the order of competition is compared. The most interesting difference was found for 17α-estradiol, which has a five times higher affinity for ERα protein. The physiological action of 17α-estradiol is quite different from that of E_2, because 17α-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 17α-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 17α-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 ERβ 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 ERβ 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, 16α-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

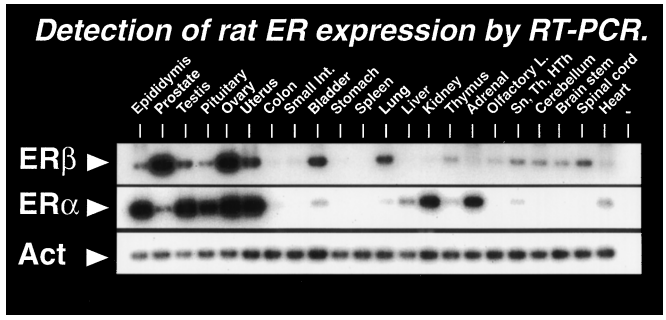

FIG. 4. Rat tissue distribution of ERα mRNA and ERβ mRNA determined by RT-PCR (see *Materials and Methods*). Autoradiograms are shown of blots after hybridization with oligonucleotide probes specific for ERβ (*top*), ERα (*middle*), and actin (*bottom*). Sn, Substantia nigra, preopticus; Th, thalamus;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

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 ERβ 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 ERβ mRNA, it has been difficult to demonstrate the presence of ERs by immunostaining with the available ER antibodies, although specific binding of E_2 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 ERβ 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 ERβ protein on immunoblots (our unpublished observations). The above findings and our results could indicate that the ERβ 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 ERβ protein antibodies become available. The fact that disruption of the ERα 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 ERα knockout mouse (43), also argues for the presence of alternative ER (ERβ?) molecules. In fact, rat uterus, ovary, testis, epididymis, and pituitary clearly express both ER subtypes mRNAs. Although we have no data on ERβ mRNA expression or protein concentration in tissues of the ERα knock-out mouse (43), the possible presence of ERβ protein should be kept in mind when interpreting experiments using the ERα knock-out mouse. Furthermore, in the uterus of the ERα knock-out mouse, residual E_2 binding could be measured (43), which is likely caused by the presence of ERβ protein. In the brain of the ERα knock-out mouse, specific binding of E_2 and modulation of progesterone receptor gene expression by E_2 was observed (44). Again this is most likely caused by the presence of ERβ protein, because the ERβ mRNA is broadly expressed in the rat brain and probably also in the mouse brain at a low level. Detailed mapping of ERα and ERβ 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 ERβ have been used (45).

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