Journal of Steroid Biochemistry, Vol. 9, pp. 1145–1148

0022-4731/78/1201-1145$02.00/0

HETEROGENEITY OF SATURABLE ESTRADIOL BINDING SITES IN NUCLEI OF HUMAN ENDOMETRIUM. ESTETROL STUDIES

LINDA TSENG and ERLIO GURPIDE
Departments of Obstetrics and Gynecology, State University of New York at Stony Brook, Stony Brook, New York, U.S.A., and Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

(*Received* 20 *March* 1978)

SUMMARY

Competition of [^{3}H]-estradiol (E_2), estetrol (E_4) and ethynyl estradiol (EE) for binding to estrogen receptors in human endometrial cytosol was studied at various competitor/estradiol (C/E_2) ratios. The fraction of E_2 that remains bound to the receptor in the presence of competitor (f) was measured at different concentrations of the competitor. Straight lines were obtained by plotting $(1-f)/f$ vs C/E_2, a result that would be expected if E_2, E_4 and EE compete for the same set of binding sites in endometrial cytosol. The relative values of association constants of E_2, EE and E_4, estimated from the slopes of the regression lines, were 100:70:1.5. Scatchard analysis of binding of each of these labeled compounds yielded values for relative association constants which were in agreement with those from competition studies. Similar concentrations of specific binding sites were obtained using labeled E_2, E_4, and EE.

These results indicate that previously published data on competition of E_4 and E_2 for nuclear binding in human endometrium, interpreted to reveal heterogeneity in estrogen binding sites in the nucleus, are not due to heterogeneity in cytosolic E_2 receptors.

INTRODUCTION

Results of *in vitro* studies on the competition of estradiol (E_2) and estetrol (1,3,5-10 estratriene, 3,15α, 16α, 17β-tetrol, E_4) for saturable binding in human endometrium have been previously reported [1]. The published report described the observation that incubation of tissue slices with mixtures of E_2 and E_4 in various proportions yielded the same amount of receptor-bound estrogen ($E_2 + E_4$) in the nuclei. The proportion of E_2 and E_4 bound to receptors in the nucleus depended on their relative concentration in the medium. An unexpected result from those studies was that about 35% of the estradiol binding sites in the nucleus was clearly more resistant to competition by E_4 than the other 65%. In contrast, ethynylestradiol tested in similar experiments displaced all nuclear bound E_2, following kinetics characteristic of competition for a single class of binding sites.

It was then suggested that the observed differences in competitiveness of E_4 and E_2 at various E_4/E_2 ratios were due to a heterogeneity in the nuclear binding of E_2. Great interest in such a possibility derives from the observation that the amounts of E_2 needed to obtain maximal binding to receptors in rat uterus *in vivo* are higher than those needed for maximal biological effect [2]. It is possible that disproportion in binding and biological action may correlate with heterogeneity in nuclear estradiol binding sites.

The main purpose of the present study was to examine the competition of E_2 and E_4 for binding to receptors in endometrial cytosol, in order to determine whether the heterogeneity in binding sites, evident after interaction of the estrogen–receptor complex with nuclear acceptors, might already exist at the cytoplasmic receptor level.

MATERIALS AND METHODS

Tissue. Human endometrium specimens, obtained after dilatation and curettage or from excised uteri, were immediately transported to the laboratory, cleaned under cold saline, and frozen at −80°C for the preparation of cytosol. All endometria studied were proliferative and histologically normal.

Labeled and unlabeled steroids. Labeled steroids, [6,7-^{3}H] E_2 (S.A.: 48 Ci/mmol), [6,7-^{3}H] EE (S.A.: 40.9 Ci/mmol), and [2,4-^{3}H] E_4 (S.A.: 52 Ci/mmol), were purchased from New England Nuclear Corp. Their radiochemical purity was ascertained by mixing aliquots with authentic standards and measuring specific activities before and after t.l.c. or crystallization.

Crystalline E_4 was purchased from the Lamar Research Group, E_2 was supplied by Steraloids, EE and diethylstilbestrol (DES) by Sigma Chemical Corp. High pressure liquid chromatography of 50 μg of E_4 carried out on a microporasil column (Waters Associates) using chloroform, indicated that this compound was free (<0.1%) of estrone or estradiol.

Preparation of cytosol. The frozen endometrium was pulverized with a Thermavac apparatus, and

homogenized at 4°C in an all-glass tissue grinder in 4 volumes of 10 mM Tris, 1.5 mM EDTA, 0.5 mM dithiothreitol buffer (TED), adjusted to pH 7.4 at 25°C. The homogenate was centrifuged at 100,000 *g* for 45 min. The supernatant was separated and treated with dextran-coated charcoal (5 mg/ml cytosol) for 15 min at 0–4°C, in order to reduce the concentration of endogenous estrogens. After centrifugation at 2000 *g* for 10 min, the cytosol was diluted with TED buffer to obtain a protein concentration of about 5–10 mg/ml. Protein levels were measured by the method of Lowry [3].

Competition of E_2, E_4, and EE for binding in cytosol. Solutions of [^{3}H]-E_2 in TED buffer (1.92×10^6 d.p.m./ml; 18.4 pmol/ml) were used in the competition experiments. Unlabeled competitors (E_4 or EE) were added to obtain the ratios indicated in Table 1. Cytosol (0.2 ml) and TED buffer solution (0.2 ml), containing either [^{3}H]-E_2 or [^{3}H]-E_2 plus competitor, were incubated for 3 h at 23°C. Time studies have indicated that maximum labeling is achieved under these conditions. At the end of this period, 0.6 ml of the dextran-coated charcoal suspension (2.5 mg/ml Norit A, 0.25 mg/ml dextran T-70 in TED buffer) was added, and the mixture was occasionally shaken for 15 min at 0–4°C. The charcoal was separated by centrifugation at 2000 *g* for 10 min. An aliquot of the supernatant (0.5 ml) was transferred to a counting vial and 10 ml of toluene-based scintillation fluid was added. The mixture was shaken in a Vortex mixer to extract the steroid into the organic phase.

Each incubation was carried out in parallel with another in which unlabeled E_2 was present at a 100-fold higher concentration than [^{3}H]-E_2. The amount of radioactivity bound, corresponding to non-specific binding, was subtracted from the bound radioactivity in the tube to which only [^{3}H]-E_2 was added. This difference was considered to correspond to saturable (specific) binding of E_2 to cytoplasmic receptors.

Each assay was conducted in duplicate.

Binding of [^{3}H]-E_4 (S.A.: 115,000 d.p.m./pmol) or [^{3}H]-EE (84,800 d.p.m./pmol) was determined by the same procedures, using about 364,000 d.p.m. per tube. Samples containing [^{3}H]-E_4 were counted in Scintiverse (Fisher Scientific Co.).

Evaluation of competition data; relative values of association constants. Since the amounts of E_2 used in these experiments were in large excess in relation to the available estrogen receptor, the sum of the concentration of receptor bound to E_2 ([E_2R]) and to the competitor ([CR]) was considered to equal the concentration of total receptor available, i.e.

$$[E_2R] + [CR] = [R_T]$$

where $[R_T]$ is the total concentration of receptor determined by incubations with [^{3}H]-E_2 in the absence of competitor.

As previously described [1], competition for a single set of binding sites implies that

$$\frac{K_{aC}}{K_{aE_2}} = \frac{[CR]}{[E_2R]} \frac{[E_2]}{[C]}$$

where K_{aC} and K_{aE_2} are the constants of association of C and E_2 to the receptor.

Under the experimental conditions used, in which the receptor is saturated by the ligands,

$$\frac{[CR]}{[E_2R]} = \frac{[R_T] - [E_2R]}{[E_2R]}$$

$$= \frac{1 - \text{fraction of } E_2 \text{ retained}}{\text{fraction of } E_2 \text{ retained}} = \frac{1 - f}{f}$$

or

$$\frac{\text{Fraction of } E_2 \text{ displaced}}{\text{Fraction of } E_2 \text{ retained}} = \frac{1 - f}{f}$$

$$= \frac{K_{aC}}{K_{aE_2}} \frac{[C]}{[E_2]} \simeq \frac{K_{aC}}{K_{aE_2}} \left(\frac{C}{E_2}\right)$$

where (C/E_2) is the ratio of concentrations of competitor and E_2 in the assay mixture.

A plot of $(1 - f)/f$ vs (C/E_2) served to test the assumption of competition of E_4 or EE with E_2 for a single set of binding sites, which would require that a straight line were obtained. The slope of such a regression line would indicate the relative value of the constants of association of E_2 and the competitor to the receptor.

Cytosol binding saturation analysis, Scatchard plot. In order to determine association constants of E_2, E_4 and EE to estrogen receptors in cytosol and the maximum number of binding sites available, aliquots of cytosol were incubated separately with various amounts of each of the tritiated compounds. The procedure described above was used for the incubations, separation of bound and unbound ("free") steroid, and correction for non-specific binding. The amount of high specific activity labeled estrogens used ranged from 42,400 to 424,000 d.p.m. (0.40–4.0 pmol) for E_2, 39,400 to 394,000 d.p.m. (0.34–3.4 pmol) for E_4, and 42,000 to 424,000 d.p.m. (0.37–3.7 pmol) for EE. The ratios of specifically bound to unbound labeled estrogen (B/F) and the corresponding concentrations of receptor-bound ligand (B, as pmol/ml of assay solution) were plotted in linear coordinates. Binding of the estrogen to a set of sites characterized by a constant of association K_a, would result in a linear function $B/F = -K_aB + nK_a$ where n is the concentration of specific binding sites. The slope of this line corresponds to $-K_a$ and the intercept with the abscissa (B/F = 0) corresponds to n. This value can be referred to the protein content in the assay solution and is usually expressed as fmol of ligand/mg cytosol protein.

RESULTS

Table 1 presents the results of experiments of competition of E_4 and E_2, or EE and E_2, for saturable

Table 1. Competition of estetrol (E_4) and ethynylestradiol (EE) with estradiol (E_2) for binding to receptors in cytosol of proliferative human endometrium

Exp No.	Competition data							Total receptor concentration (fmol/ml protein)	Regression line $\frac{1-f}{f} = a\frac{C}{E_2} + b$		Correlation coefficient	$\frac{K_{aE_2}}{K_{aE}}$
									a	b		
	E_4/E_2 (mol/mol)	4.5	9	13	18	45	89					
1	E_2 displaced (%)	4	10	14	20	31	49	190	0.011	0.016	0.99	95
	$(1-f)/f$	0.04	0.11	0.16	0.25	0.45	0.96					
	E_4/E_2 (mol/mol)	9	18	45	89	180						
2	E_2 displaced (%)	20	27	51	65	77		230	0.018	0.130	0.99	55
	$(1-f)/f$	0.25	0.37	1.0	1.9	3.3						
	E_4/E_2 (mol/mol)	9	18	36	67	89	180					
3	E_2 displaced (%)	5	18	34	48	55	61	81	0.020	0.048	0.99	50
	$(1-f)/f$	0.05	0.22	0.52	0.92	1.2	2.1					
	E_4/E_2 (mol/mol)	9	18	45	89							
4a	E_2 displaced (%)	18	23	46	61			80	0.016	0.350	0.97	60
	$(1-f)/f$	0.22	0.3	0.85	1.6							
	EE/E_2 (mol/mol)	0.18	0.46	0.92	1.8							
4b	E_2 displaced (%)	10	29	38	62			80	0.902	−0.078	0.98	1.1
	$(1-f)/f$	0.11	0.40	0.61	1.6							
	EE/E_2 (mol/mol)	0.23	0.46	0.92	1.8							
5	E_2 displaced (%)	12	16	34	49			55	0.542	−0.010	0.99	1.8
	$(1-f)/f$	0.14	0.19	0.52	0.96							

binding in endometrial cytosol. Each experiment involved assays conducted at various relative ratios of unlabeled competitor and [^{3}H]-E_2 using aliquots of cytosol from a single specimen of proliferative tissue. The concentrations of [^{3}H]-E_2 were maintained constant and above the levels needed for saturation of the receptor present in the sample. The total receptor concentration in each cytosol preparation, expressed as fmol/mg protein and shown in the table, was determined by measuring the binding of [^{3}H]-E_2 in the absence of competitor. This value was taken as unity and the amount of specifically bound [^{3}H]-E_2 in the presence of competitor was expressed as "fraction of E_2 retained"(f). As described above, $(1-f)/f$ should be proportional to the ratio of concentrations of competitor and E_2 if these compounds compete for a single set of binding sites. The table shows the values of slopes and intercepts of the regression lines best fitting the experimental points by the least-square method. The correlation coefficients indicate linearity and, consequently, competition of E_2, EE and E_4 for a single set of binding sites. The relative values of association constants of E_2 and E_4 to the receptor were in the range of 50–95 with an average of 65. In contrast, K_{aE_2} was only 1.1–1.8 times greater than K_{aEE}.

The regression lines corresponding to experiments 3 and 4b are shown in Fig. 1. This figure includes, for purposes of comparison, data previously reported [1], obtained by incubations of endometrial

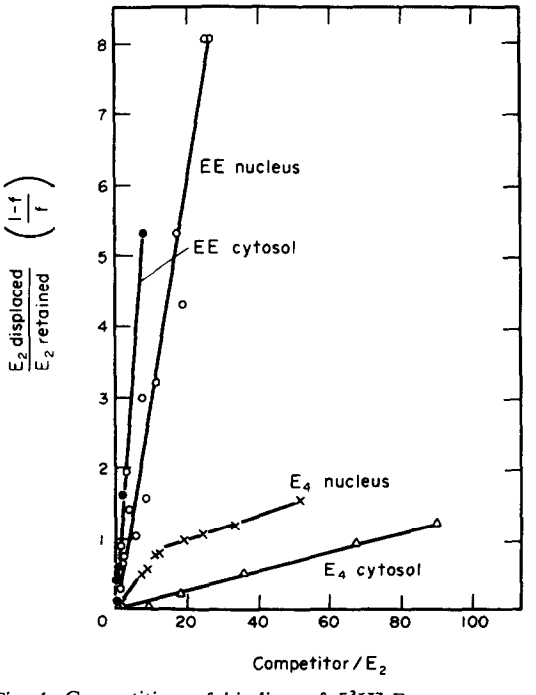

Fig. 1. Competition of binding of [^{3}H]-E_2 to estrogen receptors in nuclei and cytosol of human proliferative endometrium by estetrol (E_4) and ethynylestradiol (EE).

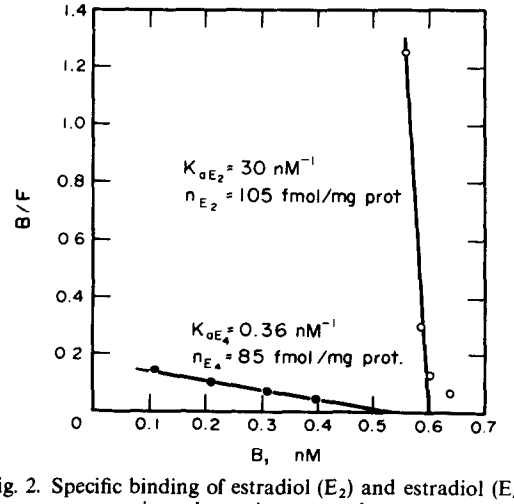

Fig. 2. Specific binding of estradiol (E_2) and estradiol (E_4) in endometrium cytosol.

slices with mixtures of [^{3}H]-E_2 and E_4 or [^{3}H]-E_2 and EE and measurement of nuclear concentrations of specifically bound [^{3}H]-E_2. Lack of linearity is noted only in the nuclear data corresponding to the competition between E_2 and E_4.

The results presented in Table 1 and Fig. 1 can be interpreted to indicate that E_2, EE and E_4 bind to the same set of sites in endometrial cytosol with relative association constants of 100:70:1.5. Specific binding of E_2 and EE in the nucleus also appears to reflect competition for a single set of sites. In contrast, competition of E_2 and E_4 for nuclear binding appears to reflect heterogeneity in nuclear sites, viz. the relative association constants of E_2 and E_4 are 100:16 for about 65% of the sites and 100:1.5 for the other 35%.

The association constants of cytosol receptors, determined by analyses of Scatchard plots from several endometrial specimens, varied from 2 to 30 nM^{-1}. Figure 2 presents the results of one experiment in which binding of [^{3}H]-E_2 and [^{3}H]-E_4 was studied in different aliquots of cytosol of the same endometrial specimen. The ratio of association constants of cytosol receptor for E_2 and E_4 in this example was 100:1.2 ($K_{aE_2} = 30\,nM^{-1}$, $K_{aE_4} = 0.36\,nM^{-1}$). The concentration of binding sites in cytosol were about 100 fmol/mg protein, 20% lower for E_4 than for E_2. Similar agreement was found in the other three experiments. Scatchard plots of data on binding of E_2 and EE to receptors in aliquots of cytosol samples used in experiment 5 (Table 1) indicated a ratio $K_{aE_2}/K_{aEE} = 1.3$ and total concentrations of binding sites of 55 and 63 fmol/mg protein for E_2 and EE, respectively.

DISCUSSION

The heterogeneity of estradiol binding in the nuclei of uterine tissue has been previously suggested on the basis of results from experiments in which the labeled steroid was extracted from nuclear preparations with salt solutions of various concentrations [2] or in the presence of intercalating agents [4], although the significance of the results from differential extraction experiments has been questioned [5]. Differences in the extractability of E_2 and nafoxidine were also interpreted to reflect heterogeneity in nuclear binding sites [4].

The results from the series of experiments reported here are consistent with binding of E_2, E_4, and EE to a single set of sites in human endometrial cytosol. Therefore, the heterogeneity we have observed during competition of E_2 and E_4 for nuclear binding does not seem to be due to heterogeneity in cytosol receptors.

It is of interest to note that the competition of E_4 for binding of E_2 appears to be 10 times more effective in nuclei than in cytosol when measured at intracellular E_4/E_2 concentration ratios below 15 (relative association constants 100:16 from nuclear data and 100:1.5 from cytosol data). This finding may reflect a difference in binding properties of the estrogens to the receptor in cytosol and to the receptor on chromatin acceptor sites. In contrast, the slope of the competition curve for E_2 and E_4 in cytosol is not different from the slope of the competition curve for nuclear binding at intracellular E_4/E_2 ratios greater than 15. The relative values of the association constants of E_2 and EE are 1.5 in cytosol and 3 in nuclei; this difference, however, may not be statistically significant.

The two slopes in the curve of competition of E_2 and E_4 for nuclear binding differ by a factor of 10 (Fig. 1). The existence of these two types of E_2 binding sites in the nucleus may reflect different receptor–acceptor configurations or different location of the complexes on the chromatin.

Martucci and Fishman [6] reported lack of uterotropic activity of E_4, a finding that would tend to support the possibility that the nuclear estradiol receptors which are hardly exchangeable with E_4 may be responsible for biological action. Chang *et al.* (unpublished) failed to see estrogenic or antiestrogenic actions of E_4 injected in rats at levels of 5 μg/day for 4 days, as evaluated by the rate of ovum tubal transport. On the other hand, Holinka and Gurpide (Biol. Reprod., in press) could detect effects of E_4 on uterine weight and alkaline phosphatase activity when high doses of E_4 (10–50 μg/day) were injected into immature rats for 1 or 2 days.

Acknowledgements—This investigation was supported by grant HD 07197, awarded by the National Institutes of Health.

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