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COMPETITION OF ESTETROL AND ETHYNYLESTRADIOL WITH ESTRADIOL FOR NUCLEAR BINDING IN HUMAN ENDOMETRIUM

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SUMMARY

Competition of tritiated estetrol (E_4), ethynylestradiol (EE), and estradiol (E_2) for binding to estrogen receptors was studied *in vitro* in human proliferative endometrium. The binding affinities of these compounds, relative to E_2, were estimated from the displacement of [^{3}H]-E_2 specifically bound in the nucleus, measured at various intracellular ratios of concentrations of E_2 and competitor. Ethynylestradiol was found to compete with E_2 for the same set of saturable binding sites with a relative affinity constant of 1/3. Similar calculations applied to the data corresponding to 50% displacement of E_2 by E_4 indicated a relative affinity constant of E_4 of 1/16. However, in contrast with EE, only about 65% of bound E_2 could be exchanged by E_4. This finding reveals a heterogeneity in the E_2-receptor complex bound to nuclei. After correction for this unexchangeable fraction of bound E_2, the relative affinity constant of E_4 to sites for which both E_2 and E_4 can compete was found to be about 1/6.

Of various phenolic steroids tested, only those possessing a 17β hydroxy group competed with E_2 for binding; norethynodrel also reduced the binding of E_2 to human endometrium, *in vitro*.

INTRODUCTION

Studies on competition of steroidal and non-steroidal compounds with estradiol† (E_2) for binding to receptor sites in target tissues serve to evaluate their potential estrogenic (or antiestrogenic) action. Following a procedure previously applied to the study of estriol [1], we have now examined in human proliferative endometrium the competition of tritiated ethynylestradiol (EE), tritiated estetrol (E_4), and several unlabeled compounds for nuclear binding of [^{3}H]-E_2. The method involves incubating the tissue with labeled E_2 and competitor and measuring the displacement of specifically bound nuclear [^{3}H]-E_2 at various intracellular concentration ratios of E_2 and the competing compound, at the steady state. Relative affinity constants and stoichiometry of the replacement of nuclear E_2 by the competitor are calculated from data on concentrations of the labeled compounds in incubation medium, whole tissue homogenates, and thoroughly washed nuclei.

The labeled compounds tested included E_4, a quantitatively important metabolite in the human fetus [2–4] which might have a role in fetal and uterine physiology, and EE, a potent estrogen widely used in oral contraceptive preparations. The displacement of nuclear bound [^{3}H]-E_2 by other unlabeled compounds were measured in order to evaluate structural requirements for competition.

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† Abbreviations and trivial names: E_2, estradiol 17β; E_1, estrone; EE, 17α ethynylestradiol; E_4, 1,3,5-10, estratriene 3,15α,16α,17β-tetrol; DES, diethylstilbestrol; norgestrel, 17α-ethynyl-17β-hydroxy-13β-ethyl-4-gonen-3-one; medroxyprogesterone, 6α-methyl, 17α-hydroxy-4-pregnene-3,20 dione; norethynodrel; 17α-ethynyl, 17β-hydroxy, 5-10 estren-3-one; mestranol, ethynylestradiol 3 methylether.

MATERIALS AND METHODS

Tissue

Specimens of human endometrium were obtained by curettage or after hysterectomy. Proliferative, rather than secretory endometrium, was chosen for these studies since the tissue levels of E_2 receptors are higher [5] and the activities of estradiol 17β dehydrogenase are lower [6] during the follicular phase of the menstrual cycle. This enzyme accelerates the removal of E_2 from the tissue.

The specimens were immediately cut into thin slices, thoroughly washed, divided into various portions and either superfused or batch-incubated, using medium containing [^{3}H]-E_2 and labeled or unlabeled test compounds.

Labeled and unlabeled steroids

[6,7-^{3}H]-E_2 (S.A. = 48 Ci/mmol), [6,7-^{3}H]-EE (S.A. = 40.9 Ci/mmol), [2,4-^{3}H]-E_4 (S.A. = 40 Ci/mmol) were purchased from New England Nuclear Corp. The purity of each tracer was tested by mixing with the authentic steroid and measuring specific activities after t.l.c. or crystallization. Occasionally, radioactive E_2 and E_4 were purified by t.l.c.

Crystalline E_4 was purchased from the Lamar Research Group, Westport, Conn.; E_2, estriol, 16-epiestriol, 17α estradiol, 17-deoxyestradiol, estrone,

testosterone and progesterone were supplied by Steraloids, Pawling, N.Y.; EE and DES were obtained from Sigma Chem. Co., norgestrel was a gift of Wyeth Lab; medroxyprogesterone, a gift from the Upjohn Co.; norethynodrel, a gift from Searle Labs.

Incubations

(a) *Superfusions.* Tissue slices were divided randomly into two 100–200 mg portions which were superfused in parallel, at 37C, for 2 h at 20 ml/h, with Earl's balanced salt solution (GIBCO) containing glucose (1 mg/ml) and [^{3}H]-E_2 either alone or mixed with the compound to be tested for competition. The superfusion set-up has been previously described [7]. The concentration of [^{3}H]-E_2 in the superfused medium (approx. 10^{-7} M) produced intracellular steady state concentrations greater than 20 pmol/mg DNA, sufficient to replace the endogenous hormone and saturate estradiol receptors with the exogenous labeled hormone. Tritiated E_2 and [^{3}H]-EE could be superfused as a mixture because there is no isotope exchange between these compounds. Samples of superfusate collected during the last 40 min of superfusion were used to verify achievement of the steady state.

(b) *Batch incubations.* In experiments designed to evaluate E_4 as a competitor, batch-incubations were performed; the low affinity of E_4 for E_2 receptors would have required too large amounts of [^{3}H]-E_4 to conduct superfusions. Tissue slices were incubated in glass vials with 2 ml of the same medium described above, for 2 h in a water bath at 37C, with shaking. Parallel incubations of different portions of the same tissue with [^{3}H]-E_2 alone and mixtures of [^{3}H]-E_2 and [^{3}H]-E_4 were conducted. The initial concentrations of E_2 (approx. 10^{-6}M) were reduced by no more than 50% during the incubations with proliferative endometrium. The intracellular concentrations of [^{3}H]-E_2 at the end of the incubation were always higher than 20 pmol/mg DNA.

At the end of the superfusion or batch-incubation, the tissue was rapidly washed with cold isotonic saline and homogenized at 0–4C in Tris (10 mM) and EDTA (1.5 mM) buffer, pH 7.4, in a glass tissue grinder. Aliquots of the homogenates were taken and the rest was centrifuged at 800 × *g* at 0–4C. Following procedures already described [1], the nuclear pellet was washed several times by resuspension in fresh buffer. The final pellet was transferred to another tube containing the carriers in methanolic solution. Labeled steroids were measured in each wash and in the final nuclear pellet, as well as in the samples of whole homogenate and incubation medium.

Isolation and measurement of labeled steroids. Methanolic solutions of [^{14}C]-E_2 and carriers for E_2, estrone, and either E_4 or EE (500 μg each) were added to the samples. The amounts of precipitated protein and DNA were determined by the methods of Lowry [8] and Burton [9], respectively. The extracts were chromatographed on Silica Gel GF (Analtech) thin layer plates using the systems chloroform–ethyl acetate, 4-1, to separate E_2 from EE and E_1 (R_F: 0.36, 0.51, and 0.75, respectively), and chloroform–methanol, 92 : 8, to separate E_2 from E_4 and E_1, (R_F: 0.65, 0.22, and 0.85, respectively).

Radioactivity was measured with a liquid scintillation spectrometer (Isocap 300, Nuclear Chicago) after dissolving the samples in Econofluor (New England Nuclear) or in Insta-Gel (Packard Instr. Co.) containing 5% of water. Losses of E_2 were estimated from the ^{3}H/^{14}C ratio in the isolated E_2 and losses of E_4 and EE were determined by measuring spectrophotometrically (280 nm) the recovered amounts of added carriers. Concentrations of labeled estrogens were calculated on the basis of the concentrations of radioactivity and the specific activities of the incubated compounds [1]. Results were expressed as pmol/ml (medium), pmol/mg protein or pmol/mg DNA (homogenate and washed nuclear pellet).

The extent of metabolism of E_2, EE, and E_4 was estimated in separate incubations of each of these labeled compounds with proliferative endometrium. Aliquots of the medium and the tissue homogenate were either counted directly or chromatographed on t.l.c. after addition of authentic steroid to determine the amount of the compound remaining unchanged after 1.5 h of incubation.

Calculations. The ratio of constants of association of E_2 and the competitor to saturable binders in the tissue can be estimated from the ratio of their intracellular concentrations and the fraction of nuclear E_2 displaced by the competitor. For instance, if EE and E_2 compete for the same saturable binding sites and have equal affinity constants to non-saturable binders then, as previously described [1],

$$\frac{K_{aE_2}}{K_{aEE}} = \frac{[E_2R]}{[EER]}\left(\frac{[EE]}{[E_2]}\right)_{\text{intracellular}}$$

where $[E_2R]$ and $[EER]$ are the concentrations of E_2 and EE bound to receptors, as measured in the nuclear fraction, and $([EE]/[E_2])$ intracellular is the ratio of total concentrations of EE and E_2 in tissue (whole homogenate).

If f denotes the fraction of $[E_2R]$ that remains bound to nuclei in the presence of the competitor, it follows from the above equation that

$$\frac{1-f}{f} = \frac{K_{aEE}}{K_{aE_2}}\left(\frac{[EE]}{[E_2]}\right)_{\text{intracellular}} \quad (1)$$

A straight line would then be expected from a plot of $(1-f)/f$ vs the ratio of intracellular concentrations of EE and E_2. The ratio of association constants could be obtained from the slope of the regression line or from the intracellular ratio corresponding to 50% reduction in the nuclear binding of E_2 $[(1-f)/f = 1]$.

RESULTS

About 95% of the labeled EE or E_4 was recovered unchanged, both from tissue and medium, after the

Table 1. Effect of various compounds on nuclear levels of receptor-bound estradiol (E_2)

Compound	Compound: E_2 molarity ratio in medium	Displacement of nuclear receptorbound E_2 (%)
Steroids		
Phenolic, with 17β HO group		
Ethynylestradiol	131	94
Estriol	135	90
16-Epiestriol	91	90
Estetrol	115	62
Mestranol	77	38
Phenolic, without 17β HO group		
17α estradiol	125	NS
17 deoxy estradiol	128	NS
Estrone	30	see legend
Non-phenolic		
Norethynodrel	145	20
Norgestrel	84	NS
Testosterone	85	NS
Progesterone	35	NS
Medroxyprogesterone	67	NS
Non-steroids		
Diethylstilbestrol	84	98

Although addition of estrone (E_1) to the incubation medium results in a reduction of receptor bound [^{3}H]-E_2 in the nucleus, it has been shown that the displacement is effected by E_2 derived from E_1 rather than by direct competition of E_1 for binding sites [10].

incubations, whereas about 50% of E_2 was metabolized, almost entirely to E_1.

Table 1 shows the effects of various compounds on the levels of E_2 tightly bound to nuclei at the steady state during superfusion of [^{3}H]-E_2. Among the phenolic steroids tested, only those possessing a 17β hydroxy group competed with E_2 for nuclear binding. Estrone (E_1) presents a special situation: in spite of reducing the nuclear levels of bound [^{3}H]-E_2, the actual competitor is not E_1 itself but E_2 derived from E_1 in the tissue [10]. Therefore, E_1 could not be considered a direct competitor under these experimental conditions in which E_1 can be converted to E_2. Norethynodrel, which according to the analytical data from Searle Laboratories contained less than 0.2% of mestranol, competed to some extent with E_2, in agreement with previous *in vivo* findings in rat uterus [11]. Diethylstilbestrol, at a concentration in the medium 100 times higher than that of E_2, completely prevented the nuclear binding of [^{3}H]-E_2.

Table 2 shows the data obtained during incubations of mixtures of [^{3}H]-E_2 and [^{3}H]-EE. It can be seen that EE replaces E_2 in the nucleus and that the total molar concentration of specifically bound estrogen remains constant. The extent of displacement of E_2 from nuclear binding is related to the ratio of the intracellular concentrations of EE and E_2, as shown in Fig. 1.

Application of equation 1 to calculate relative affinity constants requires proportionality between the ratio $(1-f)/f$ (fraction of E_2 displaced/fraction of E_2 retained) and the ratio of total intracellular concentrations of EE and E_2. Figure 2 shows that the experimental data corresponding to the displacement of E_2

Table 2. Competition of ethynylestradiol and estradiol for nuclear binding

Exp. No.	Concentrations in Incubation medium (nM) EE	E_2	EE/E_2	Concentrations in Tissue (pmol/mg DNA) EE	E_2	EE/E_2	Concentrations in Nuclei, receptor bound (pmol/mg DNA) E_2 Control	% Displacement	E_2 + EE
VI	14	107	0.13	86	84	1.0	3.5	23	4.4
	25	107	0.24	145	81	1.8	3.5	40	4.2
VII	33	99	0.34	192	99	1.9	2.8	43	2.9
	74	99	0.75	343	88	3.9	2.8	65	2.8
VIII	14	107	0.13	37	31	1.2	1.6	47	1.9
	37	107	0.34	98	37	2.6	1.6	66	1.9
IX	37	81	0.46	128	23	5.6	1.1	51	—
	57	81	0.70	246	22	11	1.1	76	—
X	150	95	1.6	236	28	8.4	3.0	61	—
	660	95	6.9	976	37	26	3.0	89	—
XI	170	170	1.0	860	120	7.1	3.6	75	—
	330	170	1.9	1650	88	19	3.6	81	—
	930	170	5.4	3710	150	25	3.6	89	—
XII	—	—	—	2180	130	17	3.6	84	—

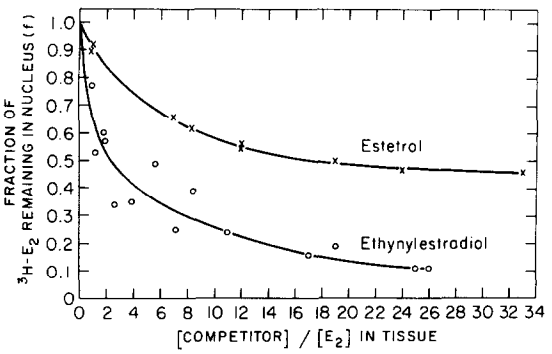

Fig. 1. Displacement of nuclear bound [^{3}H]-estradiol by ethynylestradiol (EE) and estetrol (E_4) as a function of the ratios of intracellular concentrations of EE and E_2 and of E_4 and E_2.

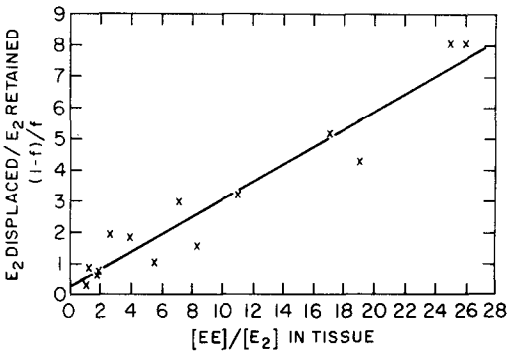

Fig. 2. Displacement of nuclear bound [^{3}H]-estradiol by ethynylestradiol (EE), expressed as the quotient of E_2 displaced/E_2 retained, vs EE/E_2 intracellular concentration ratios.

by EE satisfy this requirement. The slope of the regression line fitting the experimental points is 0.3, i.e., K_{aE_2} is about 3 times greater than K_{aEE}. A similar conclusion could be obtained from the EE/E_2 intracellular ratio corresponding to 50% displacement of nuclear E_2, in Fig. 1.

Table 3 presents the data corresponding to the competition between E_4 and E_2. When tested, the replacement by E_4 of E_2 bound in the nucleus was equimolar. The competition curve corresponding to these data is also shown in Fig. 1. the E_4/E_2 intracellular concentration ratio at which the binding of E_2 to receptors in the nucleus is reduced by 50% is about 16. However, an obvious flattening of the curve is noted at this level of replacement, suggesting that there is a fraction of nuclear E_2 (about 40%) which cannot be displaced by E_4. This suggestion is supported by a comparison of the results of the 2 experiments in which the nuclear saturation levels obtained with [^{3}H]-E_4 and with [^{3}H]-E_2 during parallel incubations with two portions of the same specimen of proliferative endometrium, were measured. In both cases (Table 4), the nuclear concentration achieved with E_4 was only about 65% of the E_2 level. It should be noted that endogenous E_2 in the tissue may account for the occupancy of receptors in the nucleus by E_2 which is not displaceable by [^{3}H]-E_4. According to the values reported by Guerrero *et al.* [12], the expected concentration of E_2 in proliferative endometrium is about 1.2 ng/g or 0.5 pmol/mg DNA. Therefore, incomplete displacement was observed at intracellular concentration ratios of exogenous E_4 and endogenous E_2 as high as 500, or higher.

The values shown in Table 5 were obtained by recalculating the data on competition of E_4 and E_2 on the basis of the exchangeable portion of nuclear E_2 (65% of the saturation value obtained with E_2 alone). Figure 3 presents a plot of the corrected $(1-f)/f$ values versus E_4/E_2 intracellular concentration ratios. The regression line now indicates that

Table 3. Competition of estetrol (E_4) and estradiol (E_2) for nuclear binding

	Concentrations in Incubation medium (nM)			Concentrations in Tissue (pmol/mg DNA)			Concentrations in Nuclei, receptor bound (pmol/mg DNA)		
Exp. No.	E_4	E_2	E_4/E_2	E_4	E_2	E_4/E_2	E_2 Control	% Displacement	$E_2 + E_4$
I	141	59	2.4	46	51	0.9	1.6	10	1.5
II	1610	438	3.7	330	330	1.0	1.6	8	1.6
III	2240	103	22	1300	136	8.3	2.4	38	2.0
	4640	132	35	2340	184	12	2.4	44	2.3
IV	4490	246	18	855	121	7	3.2	34	2.9
	6380	206	31	1170	96	12	3.2	45	—
	11600	217	54	2300	96	24	3.2	53	—
V	2690	51	52	707	37	19	2.1	50	—
	9200	107	86	2400	73	33	2.1	54	—
	6780	59	115	1410	27	52	2.1	62	—

Table 4. Nuclear binding of [^{3}H]-estradiol and [^{3}H]-estetrol in parallel incubations

		Concentrations			
Exp No.	Compound	Medium (nM)	Tissue (pmol/mg DNA)	Nuclei, receptor bound (pmol/mg DNA)	E_4 / E_2
I	E_2	423	254	1.6	0.63
	E_4	342	82	1.0	
II	E_2	493	478	1.6	0.68
	E_4	505	312	1.1	

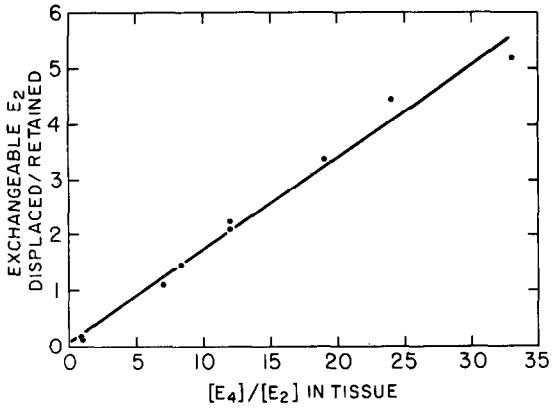

Fig. 3. Displacement of nuclear bound [^{3}H]-estradiol replaceable by estetrol (E_4) at various E_4/E_2 intracellular concentration ratios.

the constant of association of E_4 to the exchangeable receptor sites in the nucleus is only 6 times lower than the constant of association of E_2 to the same sites. It is then apparent that two different types of saturable nuclear binding sites need be considered to interpret the competition between E_4 and E_2, one binding E_2 about 6 times more strongly than E_4 and the other binding E_2 so tightly that competition by E_4 is prevented.

DISCUSSION

Although quantitative studies on competition for binding to receptors in cytosol fractions have yielded valuable results, measurements of competition for nuclear binding in whole cells have the merit of evaluating the overall effect of the competitor on the binding of the hormone. A feature of the method used for this work is the measurement of intracellular concentrations of the competitors, greatly facilitated by the availability of labeled compounds. Data on concentration of the competitors in the incubation medium (or in plasma during *in vivo* experiments) are not fully adequate for quantitative studies of competition, due to the marked differences in the tissue uptake of various steroids. For instance, EE would appear to be a much better competitor than estriol on the basis of the concentrations in medium necessary to produce the same displacement of E_2, even though they show similar association constants relative to E_2 [1]. This discrepancy results from the fact that the ratio of concentrations of EE in tissue and medium, at the steady state, is about 40, whereas the corresponding ratio for estriol is about 4.

It is also of methodologic importance to point out that the practically complete displacement of bound E_2 by EE or DES confirms the adequacy of nuclear washing to remove non-specifically bound [^{3}H]-E_2 in human endometrium. Saturability of binding is indicated by the agreement of the levels of E_2 receptor estimated in different portions of the same specimen from the nuclear concentration of E_2, when only [^{3}H]-E_2 was used, or of either E_2 + EE, E_2 + E_4, or E_2 + estriol [1], when mixtures of these labeled compounds were incubated.

The data obtained from the studies with [^{3}H]-EE show that the competition of EE with E_2 can be analyzed on the basis of a single set of binding sites for both compounds. In contrast, the results obtained with E_4 reveal that about 35% of the E_2 bound to receptors in the nucleus under saturation conditions is not amenable to exchange with the weaker competitor. This finding puts in evidence a heterogeneity in the E_2-receptor complex bound to nuclei. Clark *et al.* [13] have shown a disproportion between the concentration of nuclear binding sites and the biologic response to E_2 in rat uterus, where the maximal rate of glucose oxidation is reached when the concentration of [^{3}H]-E_2 specifically bound in the nucleus is about 60% of the saturation value. Similarly, Katzenellenbogen and Ferguson [14] observed the presence of [^{3}H]-E_2-receptor complex in rat uterus even after treatment with amounts of antiestrogens that completely blocked the E_2-stimulated synthesis of IP (induced protein). Although further studies with E_4 need be performed in the rat uterus, where a very weak competition of E_4 and E_2 for binding to cytosol receptors has been noted by Martucci and Fishman [15], it is of interest to consider the possibility that measurements of nuclear binding of [^{3}H]-E_4 or of competition of E_2 and E_4 in target tissues may allow the distinction between E_2 nuclear binding sites of different relevance to hormonal action.

Table 5. E_4-exchangeable and E_4-nonexchangeable nuclear binding of E_2

Exp. No.	$[E_4]/[E_2]$ intracellular	Receptor bound E_2 in nuclei (pmol/mg DNA) Total Control	Total Remaining	Total Displaced/Remaining	Exchangeable Control	Exchangeable Remaining	Exchangeable Displaced/Remaining
I	0.9	1.6	1.44	0.11	1.04	0.88	0.18
II	1.0	1.6	1.47	0.09	1.04	0.91	0.14
III	8.3	2.4	1.48	0.62	1.56	0.64	1.44
	12	2.4	1.34	0.79	1.56	0.50	2.12
IV	7	3.2	2.11	0.52	2.08	0.99	1.10
	12	3.2	1.76	0.82	2.08	0.64	2.25
	24	3.2	1.50	1.13	2.08	0.38	4.47
V	19	2.1	1.05	1.00	1.36	0.31	3.39
	33	2.1	0.96	1.19	1.36	0.22	5.18
	52	2.1	0.79	1.65	1.36	—	—

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