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NUCLEAR CONCENTRATION OF ESTRIOL IN SUPERFUSED HUMAN ENDOMETRIUM; COMPETITION WITH ESTRADIOL

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SUMMARY

Human endometrial slices were superfused with tritiated estriol (E_3). No metabolism of E_3 was noted. The ratio of concentrations of the hormone in tissue and superfusion medium $(T/M)_{E_3}$ was found to be approximately 4. When the concentrations of [^{3}H]-E_3 in the medium were increased from 18 to 700 ng/ml. the intracellular concentrations of E_3 were proportionally elevated. However, the level of E_3 tightly bound to nuclei reached a maximum value. This value was equivalent to the saturation level of nuclear bound estradiol (E_2) obtained when another portion of the same tissue was superfused with excess E_2. Competition of E_3 and E_2 for nuclear binding was observed when tissue slices were superfused with mixtures of the two tritiated steroids. The nuclear E_2 displaced was stoichiometrically replaced by E_3, likely as a result of competition for the same binding sites of the receptor in the nucleus. A 50% displacement of E_2 from the receptor in the nucleus was achieved when the ratio of intracellular concentrations of E_3 and E_2 was about 2. Calculations are presented to show that this ratio corresponds to the relative association constants of the E_2- and E_3-receptor complexes in the nucleus.

INTRODUCTION

The characteristics of the binding of estriol (E_3) to a receptor in rat uterus have been studied by Geynet *et al.* [1]. Using the cytosol fraction, these authors found that the dissociation constant of the E_3-receptor complex was three to six times larger than the K_d of the estradiol–receptor complex. The E_3-receptor complex extracted from nuclei had approximately the same sedimentation constant that the nuclear estradiol–receptor complex on sucrose density gradient ultracentrifugation. Brecker *et al.* [2] found that E_3 was an effective inhibitor of estradiol (E_2) binding to rat uterus by injecting a mixture of E_3 and tritiated E_2 to female rats. Wotiz and Scublinsky have related this observation to the effectiveness of E_3 administration in preventing pregnancy in the rat [3].

Although E_3 appears to have high affinity for the E_2 receptor, its estrogenic actions on the rat uterus are limited. Only the early effects produced by E_2, such as water imbibition [4, 5] and glucose uptake [5] were observed after a single injection of E_3. A single large dose of E_3 was not capable of mimicking the long term uterotrophic actions of E_2 [4, 5, 6] presumably because the E_3-receptor complex does not remain in the nucleus long enough to elicit the nuclear events that provoke these effects[5].

The purpose of our studies was to investigate the metabolism of E_3, the saturation level of E_3 tightly bound to nuclei and the competition of E_3 and E_2 for nuclear binding in human endometrium. A tracer superfusion technique, described elsewhere [7], was used to measure the nuclear uptake of estrogens. Superfusion of mixtures of [^{3}H]-E_2 and [^{3}H]-E_3 was possible because no conversions between these two steroids occur in human endometrium.

MATERIAL AND METHODS

Tissue

Human endometrium specimens were obtained either by curettage or after hysterectomy. The tissue was immediately cut into thin slices and divided into two or three portions which were superfused at 37°C with Earl's balanced salt solution, 1 mg/ml of glucose (GIBCO), containing the tritiated estrogen. The superfusions were conducted at a rate of 20 ml/h, using an apparatus described in detail elsewhere [8, 9]. Dating of the specimens of endometrium by histologic examination was kindly performed by Dr. L. Deppish, Department of Pathology, Mount Sinai School of Medicine.

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Tracers

[6,7-^{3}H]-E_2 (S.A. = 48 Ci/mmol), [6,7-^{3}H]-E_3 (S.A. = 53·1 Ci/mmol), [4-^{14}C]-E_1 (S.A. = 51 mCi/mmol) and [4-^{14}C]-E_2 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts, and purified by paper or thin layer chromatography before use.

Description of experiments

The metabolism of E_3 in human endometrium was studied by incubating tissue slices with [^{3}H]-E_3 at 37°C for two hours, either in a batchwise manner (Table 1, Exps. 10 and 11) or by superfusion (Table 1, Exps. 6a and 6b). The labeled steroids were extracted from the medium from the total tissue homogenate and from an 800 *g* nuclear fraction, and purified following procedures described in the next section.

In order to determine the maximum attainable levels of tightly bound nuclear E_3, tissue slices were superfused with different concentrations of [^{3}H]-E_3. Concentrations of labeled hormone were then measured in tissue homogenates and in thoroughly washed nuclear pellets. Tissue slices were also superfused with mixtures of [^{3}H]-E_2 (20–40 ng/ml) and [^{3}H]-E_3 to study the competition of E_3 and E_2 for nuclear binding. The relative concentrations of superfused [^{3}H]-E_3 to [^{3}H]-E_2 was varied from 1 to 100.

Isolation and purification of steroids

Superfused tissue slices were washed with cold isotonic saline and homogenized at 0–4°C in Tris (0·01 M) and EDTA (0·0015 M) buffer, pH 7·4, in a glass tissue grinder. Aliquots of the homogenate were taken to measure the steroid concentrations in tissue, the rest of the homogenate was centrifuged at 800 *g*, at 0–4°C. The nuclear pellet was washed several times by repeated suspension during a 2 to 3 min interval, in 3 ml of fresh buffer, followed by 10 min centrifugation. Labeled steroids were measured in each wash and in the final nuclear pellet.

Methanolic solutions of carriers for estrone (E_1), E_2 and E_3 (500 μg each) and suitable amounts of [^{14}C]-E_1 and [^{14}C]-E_2 indicators were added to samples of tissue homogenate or nuclear pellet. The amounts of precipitated protein or DNA were determined by the methods of Lowry [10] and Burton [11], respectively. Fractions of the extracts were taken to measure total radioactivity in each sample. The rest of the extract was chromatographed on Silica Gel GF (Analtech, Inc., Newark, Delaware) thin layer plates, using the system chloroform–methanol (92:8 v/v). In this system, E_1, E_2 and E_3 were separated (R_F values: 0·88 for E_1, 0·62 for E_2, 0·25 for E_3). The ultraviolet absorbing zones corresponding to these compounds on the developed plates were eluted with methanol. Estrone and E_2 were rechromatographed in other systems, described in a previous publication [9]. These purification steps are sufficient to obtain radiochemically pure E_1 and E_2 [9]. E_3 was further purified by thin layer chromatography, using the system chloroform–methanol, 4:1 v/v (R_F = 0·7). In one experiment, the purity of the isolated [^{3}H]-E_3 was verified by addition of 10 mg of E_3 and crystallization of the mixture from methanol.

A liquid scintillation spectrometer (Packard Tri-Carb, Model 3951) was used for the measurements of radioactivity. Samples of labeled E_1 and E_2 were counted in 10 ml of Toluene Spectrafluor (Amersham Searle Corp., Chicago, Ill.) and samples of [^{3}H]-E_3 were counted in 0·5 ml of water plus 10 ml of Insta-Gel (Packard Co., Inc., Downers Grove, Ill.). The loss of [^{3}H]-E_1 or [^{3}H]-E_2 in each sample was estimated from the loss of a known amount of [^{14}C]-E_1 or [^{14}C]-E_2 added to the sample before purification. The loss of [^{3}H]-E_3 was estimated by measuring spectrophotometrically the amount of carrier E_3 recovered. Intracellular or nuclear concentrations of the steroids (ng per gram tissue or ng per mg DNA) were determined by dividing the concentrations of labeled estrogens (c.p.m./g tissue or mg DNA) by the specific activity of superfused [^{3}H]-E_2 or [^{3}H]-E_3. A detailed description of the calculations was presented elsewhere [9, 12].

RESULTS

Stability of labeled E_3 in human endometrium

Table 1 shows the results obtained from analysis of endometrial slices superfused with [^{3}H]-E_3. In each sample, the total radioactivity extracted from the tissue corresponded to [^{3}H]-E_3, as shown by t.l.c. or crystallization. These results indicate that E_3 is not significantly metabolized in superfused human endometrium.

Intracellular concentration of E_3

Table 2 presents values of concentrations of labeled E_2 and E_3 in superfusion medium, tissue homogenate and washed nuclei obtained during superfusion of tissue slices with these compounds. The intracellular concentration of E_3 increased in proportion to the concentration of E_3 in the medium. The ratio of intracellular to superfused concentrations of E_3, denoted by $(T/M)_{E_3}$, was found to be about 4, with a range of 2·2–7·7.

The ratio of intracellular concentrations of labeled E_1 and E_2 obtained during superfusion of [^{3}H]-E_2, ([^{3}H]-E_1/[^{3}H]-E_2)$_{E_2}$ in Table 2, does not change significantly by the addition of E_3 (up to 4 μg/ml) to the

Table 1. Stability of $[^3H]$-E_3 in human endometrium

Exp. no.	S.A. of $[^3H]$ E_3 incubated (c.p.m./ng)	Samples	Total radioactivity extracted (c.p.m.)	Radioactivity as E_3 after last t.l.c.* (c.p.m.)	Radioactivity as E_3 after crystallization†
10	448,000	Medium	3,500,000	3340,000	
		Tissue homogenate	43,700	43,000	
		Washed nuclei	2650	2640	
6a	2930	Tissue homogenate	550	550	
		Washed nuclei	640	670	
6b	2930	Tissue homogenate	1990	1880	
		Washed nuclei	510	500	
11	448,000	Tissue homogenate	306,000	306,000	298,000

* Values corrected for losses of E_3 added to the samples.
† Value calculated from the specific activity of the crystals.

superfusion medium. Since this ratio is related to the level of 17β-E_2 dehydrogenase activity in human endometrium [12, 13], it may be concluded that E_3 does not have a direct effect on the activity of the enzyme under our experimental conditions. In contrast, addition of 17α-ethinyl estradiol to the medium (1–4 μg/ml) reduced the ratio of intracellular concentrations of labeled E_1 and E_2 (unpublished results).

Removal of labeled steroids from the isolated nuclei

Figure 1 shows nuclear concentrations of E_3, E_2 and E_1 during successive washings of labeled nuclear pel-

Table 2. Intracellular and nuclear concentrations of labeled E_1, E_2 and E_3

		Perfusion medium				Intracellular		Washed nuclei				
Exp. no.	Type of tissue	E_2 (c.p.m./ml $\times 10^{-3}$)	(ng/ml)	E_3 (c.p.m./ml $\times 10^{-3}$)	(ng/ml)	(c.p.m./g tissue $\times 10^{-3}$) E_2	E_3	(c.p.m./mg DNA) E_2	E_3	T/M* E_2	T/M* E_3	($[^3H]$-E_1/$[^3H]$-E_2)†
1a	Prolif.	215	28	—	—	1130	—	8390	—	5·3	—	2·0
1b		215	28	—	4000	758	—	830	—	3·5	—	2·4
2a	Prolif.	160	21	—	—	1780	—	9240	—	11	—	0·8
2b		160	21	—	53	1500	—	5880	—	9·4	—	0·95
2c		160	21	—	375	1470	—	2630	—	9·2	—	0·9
3a	Early secret.	260	35	—	—	185	—	1860	—	0·7	—	12
3b‡	(19–21)	260	35	298	641	153	488	930	120	—	—	—
3c		260	35	298	641	226	716	196	160	0·9	2·4	13
4a	Prolif.	140	26	—	—	—	—	3600	—	—	—	3·9
4b		140	26	59	22	416	284	2210	780	3·0	4·8	3·2
4c		—	—	59	22	—	306	—	2130	—	5·2	—
5a	Prolif.	280	24	—	—	1100	—	5220	—	4·0	—	2·6
5b		280	24	243	25	1240	650	4270	810	4·4	2·7	2·3
5c		280	24	2100	217	1230	6430	1550	2370	4·4	3·1	2·6
6a	Prolif.	—	—	52	18	—	402	—	2380	—	7·7	—
6b		—	—	221	75	—	1460	—	2100	—	6·6	—
7a	Prolif.	399	36	—	—	2750	—	5290	—	7·0	—	0·9
7b		399	36	869	118	2750	2170	3850	1530	7·0	2·7	1·0
8a	Prolif.	179	35	—	—	659	—	1600	—	3·7	—	2·3
8b		179	35	579	198	555	1220	600	890	3·1	2·1	2·8
9a	Prolif.	308	28	—	—	1340	—	6160	—	4·3	—	1·5
9b		308	28	2110	274	2560	4200	2290	2280	8·0	2·0	1·9

* T/M denotes the ratio between the concentrations of a superfused labeled estrogen $[^3H]$-E_2 or $[^3H]$-E_3) in the tissue (c.p.m./g) and in the superfusion medium (c.p.m./ml).
† $[^3H]$-E_1/$[^3H]$-E_2 denotes the ratio between the intracellular concentrations of labeled E_1 and E_2 at the steady state during superfusion of labeled E_2.
‡ Only $[^3H]$-E_2 was superfused during the 1st h; after this period, the tissue slices were transferred to another superfusion chamber and superfused for another hour with the mixture of $[^3H]$-E_2 and $[^3H]$-E_3.

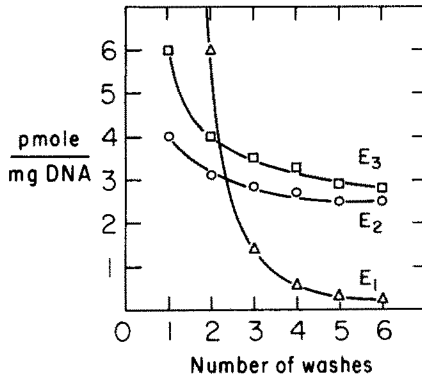

Fig. 1. Concentrations of estrogens in nuclear pellets during successive washes after superfusion of slices of human endometrium with either [^{3}H]-E_2 or [^{3}H]-E_3 (Experiments 4a and 4c, Table 2).

lets originated from one tissue specimen. Slices of endometrium were divided into two portions, one of which was superfused with [^{3}H]-E_2 and the other with [^{3}H]-E_3 (Table 2, Exps. 4a and 4c). As can be seen in Fig. 1, the fractions of E_3 in the nucleus which are recovered by each washing were similar to those of E_2 and were much smaller than the fractions corresponding to E_1. The nuclear concentration of E_1 after six washes was less than one-tenth the concentration of E_2 or E_3. These results indicate that E_3 is bound to endometrial nuclei almost as tightly as E_2 while E_1 can be easily removed from the nuclear pellet.

Saturation level of E_3 in nuclei

The saturation level of E_3 in the nucleus was studied by dividing a specimen of proliferative endometrium into two portions which were superfused at two different concentrations of [^{3}H]-E_3, 18 ng/ml and 75 ng/ml (Table 2, Exps. 6a and 6b). The concentrations of E_3 in washed nuclei were identical in both experiments even though the intracellular concentrations of E_3 increased proportionally to the concentration of the steroid in the medium. This result indicates the saturability of tight binding sites for E_3. In another experiment, 4, two portions of the same specimen were superfused with either [^{3}H]-E_2 or [^{3}H]-E_3. Equivalent saturation levels for E_3 and E_2 were found.

Competition of E_2 and E_3 for nuclear binding

The concentration of E_2 tightly bound to the nuclei was effectively reduced by addition of E_3 to the superfusion medium. Figure 2 presents a plot of nuclear concentrations of E_2 vs the ratio of the intracellular concentrations of E_3 and E_2. Relative nuclear concentrations of E_2 were used in this graph since the

Table 3. Time studies of intracellular and nuclear concentrations of E_2 during superfusion of a secretory endometrium

	Time, h		
	0·7	1	2
Medium (ng/ml)	37	37	37
Intracellular (ng/mg DNA)	3·5	3·9	4·2
Washed nuclei (ng/mg DNA)	0·2	0·2	0·2

levels of E_2 in nuclei vary from one specimen to another. The E_2 levels achieved in the absence of E_3 in the medium were assigned the value of 100.

The competition between E_2 and E_3 for receptor sites may occur either in the cytoplasma, prior to the translocation of the receptor to the nucleus, in the nucleus or in both of these two compartments. Experiment 3b was designed to study the possibility that E_3 displaces E_2 from an E_2-receptor complex already localized in the nucleus. In this experiment, tissue slices were superfused first with [^{3}H]-E_2 for 1 h, a time which is sufficient to reach saturation levels in the nucleus, as is evident from data shown in Table 3. After the 1st h of superfusion with [^{3}H]-E_2 alone, the slices were superfused for another hour with a mixture of [^{3}H]-E_2 and [^{3}H]-E_3. The results obtained from this experiment (3b) are listed in Tables 2 and 4. Replacement of nuclear E_2 by E_3 is evident. The nuclear concentrations of these hormones would depend upon their relative intracellular concentrations and the association constants of these two steroids to the receptor in the nucleus.

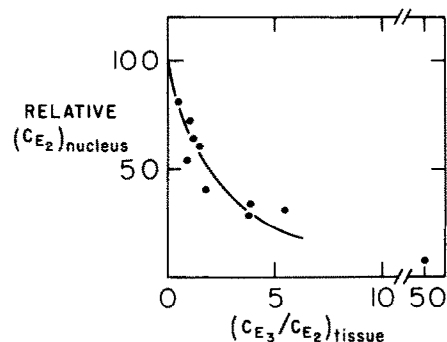

Fig. 2. Concentrations of E_2 in nuclear pellets at various intracellular ratios of E_3 and E_2 measured at the steady state during superfusion of slices of human endometrium with mixtures of these steroids. The concentrations of E_2 superfused (20–40 ng/ml) were sufficient to yield maximum levels of receptors in the nucleus. Nuclear concentrations of E_2 are expressed as percentages of the values determined in the absence of E_3.

Table 4. Concentration of E_2 and E_3 in nuclei

Exp. no.	Steroids superfused	Intracellular concentration pmol/mg DNA E_2	E_3	Concentration in nuclei pmol/mg DNA E_2	E_3	$E_2 + E_3$
3a	E_2	13	—	0·9	—	0·9
3b	$E_2 + E_3$	12	500	0·5	0·8	1·3
3c	$E_2 + E_3$	14	710	0·1	1·2	1·3
4a	E_2	37	—	2·5	—	2·5
4b	$E_2 + E_3$	40	60	1·5	1·0	2·5
4c	E_3	—	61	—	2·7	2·7
5a	E_2	69	—	1·6	—	1·6
5b	$E_2 + E_3$	79	42	1·3	0·3	1·6
5c	$E_2 + E_3$	77	410	0·5	0·8	1·3
7a	E_2	190	—	1·8	—	1·8
7b	$E_2 + E_3$	170	170	1·3	0·7	2·0
8a	E_2	83	—	1·2	—	1·2
8b	$E_2 + E_3$	81	313	0·4	1·0	1·4
9a	E_2	129	—	2·0	—	2·0
9b	$E_2 + E_3$	154	278	0·8	1·0	1·8

Table 4 shows the intracellular and nuclear concentrations of E_2 and E_3 obtained during superfusion of endometrium with mixtures of E_2 and E_3. In each parallel run, a stoichiometric replacement of tightly bound E_2 by E_3 was observed.

DISCUSSION

The results shown in Table 4 indicate that E_2 and E_3 are competing for the same binding sites. Therefore, the relative constants of association of E_2 and E_3 to the receptor in the nucleus can be estimated. The calculations are based on the assumption of fast intracellular mixing of the estrogen not bound to the receptor (i.e. nuclear or cytoplasmic E_2 or E_3 unbound or loosely bound to other proteins and macromolecules). The following equations can then be considered:

$$K_{aE_2} = \frac{[E_2R]}{[E_2] \times [R]} \qquad (1)$$

and

$$K_{aE_3} = \frac{[E_3R]}{[E_3] \times [R]} \qquad (2)$$

where K_{aE_2} and K_{aE_3} are the association constant of E_2 and E_3 to the receptor, $[R]$ is the concentration of unbound receptor, and $[E_2R]$ and $[E_3R]$ are the concentrations of steroid-receptor complexes. $[E_2]$ and $[E_3]$ denote the concentrations of the unbound compounds.

Since the concentrations of E_2 and E_3 in the superfusion medium were large enough to saturate the receptors. The binding of practically all of the intracellular estrogen is of the non-specific, high capacity, low affinity type. Note that less than 5% of the total amount of steroids in the tissue in the experiments in which a mixture of E_2 and E_3 was superfused is present in a tightly bound form in the nucleus. If we now assume that E_2 and E_3 have the same affinity constants of association to the non-saturable binders, then the concentrations of unbound estrogens, $[E_2]$ and $[E_3]$, are proportional to their total intracellular concentrations, listed in Table 4. On the basis of this assumption, equations 1 and 2 can be rearranged and simplified to the following form:

$$\frac{K_{aE_2}}{K_{aE_3}} = \frac{[E_2R] \times [E_3] \text{ intracellular}}{[E_3R] \times [E_2] \text{ intracellular}}. \qquad (3)$$

From Fig. 2, it follows that 50% replacement of E_2 by E_3 (i.e. $[E_2R] = [E_3R]$) is achieved when the ratio $([E_3]/[E_2])$ is about 2. Therefore, from Eq. 3, K_{aE_2}/K_{aE_3} is also about 2. Similar ratios of association constants were estimated in rat cytosol [1].

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