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MODULATION OF ESTROGEN RECEPTORS IN FOUR DIFFERENT TARGET TISSUES: DIFFERENTIAL EFFECTS OF ESTROGEN vs PROGESTERONE*

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SUMMARY

The effect of pretreatment with estradiol-17β with or without progesterone on estradiol cytosolic receptors was investigated in 4 different target tissues from long term castrate Sprague Dawley rats. Receptor assay was performed by hydroxylapatite adsorption and Scatchard analysis. Treatment with estradiol (0.1 μg/rat) for 48 h *in vivo* increased absolute cytosolic receptor concentrations per mg DNA 3–5-fold for uterine and vaginal tissue, but no increase was observed in pituitary or hypothalamic tissue. These increases in estradiol receptor concentration after 48 h of estrogen exposure should be distinguished from "replenishment" phenomenon after shorter time periods (15–20 h) which demonstrate recovery to baseline of "available" estrogen receptors.

The increase in cytosolic estrogen receptors in uterine and vaginal tissue in response to estrogen is opposed by *in vivo* treatment with estrogen plus progesterone. This opposition cannot be explained by competition from progesterone or negative cooperative interaction with progesterone when analyzed by Scatchard plots and Hill coefficients. Deviations from linearity in Scatchard plots are not explainable in terms of positive or negative cooperativity between estrogen and progesterone. Neither estrogen stimulation of E_2–R_c nor its opposition by P_4 were observed in pituitary or hypothalamic target tissues. These results support the concept of a single, non-interacting binding site for estrogen on the estrogen receptor with similar K_a values for E_2–R_c in all four target tissues studied. However, the uterine and vagina "response" to E_2 stimulation, as measured by changes in E_2–R_c binding sites, was statistically different from the response of pituitary or hypothalamic target tissue.

INTRODUCTION

Estrogens are concentrated by specific target tissues (uterus, vagina, pituitary, hypothalmus) which contain a macromolecular binding protein called the estrogen receptor [1–5]. Estrogen treated uteri respond by undergoing hypertrophy and mitosis. However, the progestational uterus, under the influence of both estrogen and progesterone, becomes distinct from the estrogen dominated uterus showing a decreased epithelial cell division and an increased or "differentiated" secretory function [1, 6, 7]. Since the steroid-receptor interaction is one of the very early events which must occur in each target tissue in order for the tissue to respond, it is possible that the response is limited or potentiated by the availability of the cytosolic estrogen receptor. The term "replenishment" refers to restoration of available estrogen receptor concentration in the first 0–16 h after estrogen treatment [2, 10] while the term "modulation" will be used to describe the changes in estradiol-17β cytosolic receptor (E_2–R_c) concentration determined *via* Scatchard analysis. This report considers the changes in receptor concentration which occur after restoration of E_2–R_c to pretreatment levels. Factors involving synthesis, degradation, or sequestrational changes in E_2–R_c cannot be separately distinguished by current methodology.

To date little or no information is available to correlate the known effects of progesterone on the rat estrous cycle with the potential effect of progesterone on E_2–R_c concentrations in the four primary target tissues. In this preliminary report, we have examined the "modulation" of estrogen receptor concentration in uterine, vaginal, pituitary, and hypothalamic tissue following stimulation by estradiol-17β (E_2) and progesterone (P_4) for 24–48 h. The results are consistent with a differential effect of these two sex steroids on estrogen receptor concentration in the uterus and a differential response between the 4 target tissues.

EXPERIMENTAL

Animals. Female Sprague–Dawley rats (190–250 g) were bilaterally ovariectomized and used four weeks post-operatively. The atrophied uterus of the four week castrate is referred to as the "long term castrate" uterus. Animals were sacrificed under light etherization and cervical dislocation. The four target tissues were removed (uterus, vagina, pituitary and hypothalamus) and placed in Hank's balanced salt solution at 0–2°C. The "hypothalamus" was defined to extend

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posterior from the mammillary bodies to the preoptic region, approximately 3 mm anterior of the optic chiasma, bounded laterally by its obvious morphological termination. This tissue was excised to a depth of approximately 2 mm. The pituitary was teased from the sella turcica and used intact.

Cytosol preparation. Tissue homogenates were prepared as previously described [8–12, 15]. Tissues were debrided of connective tissue and fat. Small pieces of minced tissue were carefully homogenized at 0–2 C in $TEMK_{50}$ buffer (0.04 M Tris, pH 7.2, 0.0015 M EDTA; 0.014 M mercaptoethanol; 0.05 M KCl) with a tissue to buffer ratio of ($N = 4$)/ml for uterine and vaginal tissue, and ($N = 8$)/ml for pituitary and hypothalamic tissue (N = number of animals used per determination). All procedures were performed at 0–2 C except where indicated. The homogenate was centrifuged at 800 *g* for 15 min. The pellet was saved for DNA determinations performed according to Burton[13]. The low speed supernatant was then centrifuged at 106,000 *g* for 90 min in a 50 Ti fixed angle rotor. (Beckman Indust.) yielding the high speed supernatant or "cytosol" fraction. Protein determinations were performed according to Lowry *et al.*[14] on this cytosol and samples diluted to approximately the same protein concentration prior to receptor assay. (Tables 2 and 3).

HTP assay. Cytosols were assayed for E_2-R *via* the "hydroxylapatite batch assay" [15] employing modifications of the column techniques of Erdos *et al.*[16] and Williams and Gorski[2]. Cytosolic aliquots (225 μl) were incubated with various concentrations of [^{3}H]-estradiol-17β ([^{3}H]-E_2), S.A. = 80–100 Ci/mM (Amersham-Searle), for 18 h at 0–2°C. Hydroxylapatite (DNA grade Biogel HTP, BIORAD Inc.) was prepared in $TEMK_{50}$ buffer (10 g/100 ml) and mixed overnight. Cytosolic receptors were then adsorbed to the hydroxylapatite (HTP) by adding 1.2 ml of HTP to each aliquot of cytosol and shaking at 0–2 C for 30 min. The HTP was then centrifuged (800 *g*, 4 min.) and washed six times with $TEMK_{50}$ buffer at 0–2 C. Bound estradiol was removed from the HTP with two 4 ml ethanol extractions. The ethanol extract was air dried in scintillation vials and liquid scintillation counting was performed in a Beckman LS-230 liquid scintillation counter utilizing a PPO-POPOP fluor[2,5 diphenyloxazole (4 mg) and 1,4 Bis (2-5 phenyloxazol) benzene (500 μg) per liter toluene]. Quench correction was determined with an external standard. Multiple samples (4 ×) were run for each steroid concentration with seven concentrations being generally employed for Scatchard analysis ($n = 28$). Correction was made empirically for non-specific binding by adding 100× excess non-radioactive E_2. Saturation was achieved for at least two of the concentrations employed.

In vitro *E_2 organ binding assay. In vitro* manipulations were performed in Hank's buffered MEM (GIBCO), with 2% Penn-Strep (GIBCO) at 37 C under an atmosphere of 95% O_2 and 5% CO_2. Tissues were assayed for ability to retain [^{3}H] E_2 by incubating tissue minces with varying concentrations of [^{3}H]-E_2 for 2 h in the presence and absence of a 100-fold excess non-radioactive estradiol-17β. Tissue was washed in three changes of MEM at 0–2 C and carefully homogenized. After homogenization steroidal uptake was determined by double extraction with equal volumes of ethyl acetate. Extraction efficiency was found to be at least 95%. Correction was made for nonspecific binding components in the presence of excess competitor.

Biological response. Vaginal lavage cytology was determined according to the following classification: large numbers of cornified epithelial cells = estrus; cornified epithelial cells + leucocytes = diestrus 1; large numbers of leucocytes = diestrus 2; large numbers of round epithelial cells = proestrus. Whenever cornified cells were observed their presence was recorded irrespective of the final classification of the lavage type.

In vivo experiments on long term castrate rats consisted of (a) a saline injected control group, (b) a second group receiving 25 μg or 0.1 μg E_2 intraperitoneally (I.P.) in 0.5 ml saline per rat, and (c) a third group receiving E_2 (I.P.) at 0 h and 1 mg progesterone subcutaneously (S.C.) in 0.5 ml propylene glycol [34] at 24 h. All rats were sacrificed after 48 h.

RESULTS

In vitro *E_2 organ binding assay.* Long term castrate female rats were injected with estradiol-17β (25 μg E_2 I.P.) in order to test the "priming" effect of estradiol on receptor concentration (Table 1). After 48 h, the animals were sacrificed and the tissues subjected to an "E_2 organ binding assay" *in vitro*. The results were plotted according to Scatchard[18] and subjected to linear regression analysis on an IBM 360-91 computer.

Under the conditions of the E_2 organ binding assay (Table 1), it is clear that estrogen binding per uterus is increased from 1.86 to 4.53 pmol by 48 h after prior exposure to non-radioactive estradiol *in vivo*. Studies reported by Sarff and Gorski[10] indicated non-radioactive steroid plasma levels and nuclear bound receptor have subsided to base line by 16–18 h after treatment. A decrease in association constant (K_a) was seen perhaps due to some competition from non-radioactive estradiol. However, the presence of non-radioactive competitive hormone (E_2) as a consequence of the E_2 pretreatment would not affect the numbers of binding sites when determined by Scatchard analysis [18]. It should be noted that the events described in Table 1 are tissue phenomena and may (or may not) reflect specific molecular or synthetic events. These tissue phenomena or binding events [19] could include changes in cytoplasmic receptor concentration, in nuclear acceptor site concentration, in receptor affinity and cooperativity, or in some set of events which can alter the manner

Table 1. *In vitro* E_2-organ assay for estradiol tissue binding in uterus[a]

Tissue	In vivo Treatment	$K_a \times 10^{10}{}_M{}^{-1}$	Binding $\times 10^{-12}$ moles/uterus	r^2
Uterus	0.5 ml saline (I.P.)	0.50 ± .0112	1.86 ± 0.32	-0.937
Uterus	+25 μg estradiol -17β in 0.5 ml saline (I.P.)	0.20 ± .0050	4.53 ± 0.84	-0.927

[a]Minced uterine tissue was incubated in Hank's MEM for 2 h at 37°C with varying concentrations of ^{3}H-estradiol-17β. K_a: tissue association constant. Binding: concentration of tissue binding sites per uterus. r^2: correlation coefficient. Values are expressed ± the standard error of the regression estimate (n=28).

in which estradiol is partitioned within the uterus [20].

Biological response in vivo *to a lowered* E_2 *dose.* Since the "priming" doses (25 μg E_2, I.P.) of non-radioactive estrogen used in the previous experiment appeared to influence the K_a values, it was desirable to decrease the pretreatment steroid dosage. E_2 dosages of 0.1 μg/50 gm of rat body weight have been shown to influence E_2–R_c in the uterus [3, 22]; however, it was of special importance to be able to demonstrate a measurable biological response to these lower levels of steroid in order to justify further receptor "modulation" studies.

Vaginal lavage was monitored in two groups of long term castrate animals under low E_2 stimulation. The first group received estradiol (0.1 μg E_2/0.5 ml saline, I.P.) at $t = 0$. The effect of E_2 treatment alone on vaginal cell types is shown in Fig. 1a. Round epithelial cells characteristic of a proestrous lavage cell type increase maximally by 50 h after E_2 treatment with a concommitant decrease in the leucocytic (diestrus 2) lavage cell type. By 120 h after injection all lavages were again leucocytic. The second group received 0.1 μg estradiol-17β in 0.5 ml saline (I.P.) at $t = 0$ plus an injection of 1000 μg progesterone 0.5 ml propylene glycol. (s.c.) at $t = 24$ h (Fig. 1b).

Contrasting the estrogen treatment alone, cornified epithelial cells characteristic of the normal estrous lavage cell type appear in the vaginal lavage after estrogen plus progesterone treatment. While one animal showed an "estrous lavage" after E_2 treatment alone, more ($n = 9$) displayed this type of lavage only after treatment with both estrogen and progesterone. The presence of cornified cells led to a lavage classification of "estrus" and "diestrus 1" only after progesterone exposure. While not mimicking exactly the temporal sequence of vaginal cell types, these treatments did succeed in inducing the morphological cell types associated with estrogenic and progestational events in the intact rat.

Assay for cytosolic receptors. Cytosolic receptors for long term castrate uteri can be demonstrated by the conventional sucrose gradient technique *in vitro* [8]. However, sucrose gradients cannot provide the large number of data points necessary for the Scatchard analysis or the statistical significance necessary to evaluate association constants (K_a), numbers of estradiol binding sites (EBS), etc. Therefore the cytosolic receptor was assay using batch hydroxylapatite (HTP) adsorption [15].

In these experiments the dose of estradiol-17β was reduced from 25 μg/animal (Table 1) to 0.1 μg/animal in order to approximate a "physiological" dose. Progesterone (1000 μg/animal) was included in our treatment schedule in order to correlate the biological or vaginal lavage changes with the effect of this steroid on cytosolic receptor concentrations. Uterine E_2–R_c concentrations did not differ significantly in saline injected long term castrate controls, determined either by "HTP Assay" or by the *in vitro* "E_2 organ binding

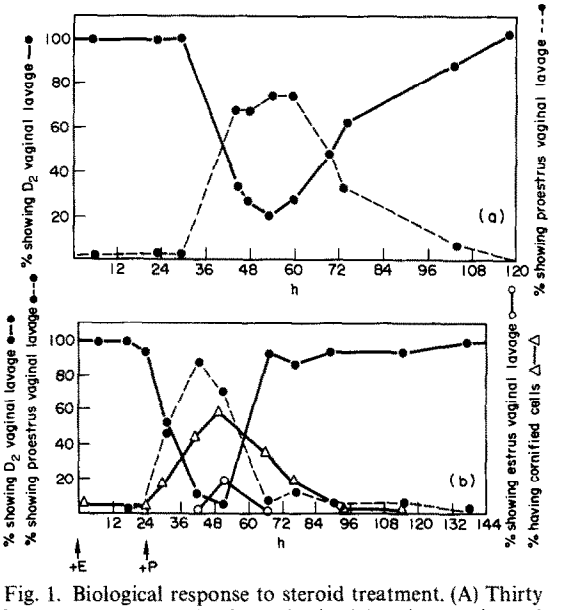

Fig. 1. Biological response to steroid treatment. (A) Thirty long term castrate animals received a 0.1 μg intraperitoneal (I.P.) injection of estradiol-17β in saline at $t = 0$. (B) Thirty long term castrate animals received a 0.1 μg (I.P.) injection of estradiol-17β in saline at $t = 0$ and a subcutaneous injection of 100 μg progesterone in propylene glycol at $t = 24$ h. Vaginas of all animals were smeared at the times indicated and lavage cell types classified with respect to cell types seen in the cycling animal. Only one animal displayed a cornified cell lavage after estrogen treatment alone. Following progesterone treatment, an increase in cornified cell types occurred in all lavage types.

assay". E_2 R concentrations in long term castrate rats were found to be $1.53 \pm 0.32^* \times 10^{-12}$ mol/uterus by HTP assay while *in vitro* E_2 organ binding assay for E_2 R indicated that the concentration was $1.86 \pm 0.32^* \times 10^{-12}$ mol/uterus [15]. *(Regression value estimate $\pm$ standard error of the regression value estimate).

Data describing changes in total protein as a response to steroid treatment are presented for cytosols assayed on HTP in Table 2. These data show the concentration of soluble protein employed in receptor assay. Little response to steroid treatment can be seen in pituitary and hypothalamic cytosols. Statistical analysis of the estimated values in Table 2 and 3 was simplified by a least squares regression analysis performed on Scatchard plots. The Scatchard determination degrees of freedom and Students t values are indicated for each of the four experimental groups. Table 2 and 3 summarize the Scatchard analysis results for E_2 R. Due to the surgery performed four weeks earlier and to the variable amounts of internal scar tissue formed around the oviducts, recovery of uterine horns of equal length tended to vary. Thus, normalization was based on protein and DNA content rather than on number of uteri.

When uterine cytosolic receptor content (Table 2a) was expressed per mg protein there was no treatment difference. However, when normalization was based on DNA content, the estradiol receptor concentration increased 2-fold in response to a 48 h pretreatment with estradiol. This suggests that the increase in estradiol receptor is not due to a simple increase in cell number but parallels an increase in cell size or protein content. When progesterone is present for the last 24 h of steroid pretreatment, the receptor concentration remained at the original untreated level (Table 2a). Similar findings have been reported for the estrogen stimulated increase in progesterone receptors in the chick oviduct [17].

Even greater changes (5–6 fold) can be seen in rat cytosolic E_2 R concentration from vaginal tissue (Table 2b). The ratio of protein/DNA in uterine and vaginal tissue parallels the cytosolic estrogen receptor response. This is in agreement with earlier work on protein/DNA changes at 24–48 h after estrogen treatment reported by Hamilton *et al.*[21]. These data suggest that vaginal tissue has a greater hypertropic response to estradiol than uterine tissue or alternatively that uterine tissue has a greater mitotic response than vaginal tissue. It is also possible that a greater percentage of the total vaginal cell population responded to the estrogen treatment. Good correlation coefficients were determined for these tissues.

Similar assays were performed on hypothalamic and pituitary cytosols. Table 3 shows that hypothalamic tissue contained 1/5 to 1/10 as many EBS/mg DNA compared to the pituitary tissue. This correlates well with recent reports in the literature [19, 24]. There were no significant differences in the brain tissue K_a or in the absolute binding site concentration of E_2 R after 24–48 h of steroid treatment normalized to either protein or to DNA (Table 3). The protein/DNA ratio remained statistically unchanged.

This evidence suggests that receptors in the hypothalamus and pituitary respond to steroid feedback modulation in a manner distinct from the system operating in the uterus or the vagina, while K_a values for E_2–R from all four tissues were indistinguishable.

Table 2. Uterine and vaginal cytosol estradiol receptor HTP assay

	Treatment I		Treatment II		Treatment III	
	Saline	"t"Test (I vs II)	0.1μg E_2, 48hrs	"t" Test (II vs III)	0.1μg E_2,48hrs 1.0mg P_4,24-48hrs	"t" Test (I vs III)
A. *Uterus:* n=35/group						
Cytosol protein, mg/ml	3.77±0.34		4.83±0.22		5.60±0.34	
$K_a \times 10^{10}{}_M{}^{-1}$	0.12±0.01[a]	(0.000)	0.12±0.02	(0.800)	0.10±0.02	(1.208
EBS$\times 10^{-12}$moles/mg protein	3.72±0.27	(0.248)	3.81±0.24	(1.345)	3.27±0.32	(1.080)
EBS$\times 10^{-12}$moles/mg DNA	47.92±3.33	(8.297)*	115.17±7.39	(6.967)*	52.57±5.11	(0.762)
Protein/DNA	12.88±1.88	(9.172)*	30.24±1.58	(8.042)*	16.01±0.97	(2.049)
Correlation coefficient=r^2	-0.993	——	-0.948	——	-0.953	——
B. *Vagina:* n=21/group						
Cytosol protein, mg/ml	3.13±0.02		4.00±0.29		4.03±0.38	
$K_a \times 10^{10}{}_M{}^{-1}$	0.07±0.01	(1.333)	0.05±0.01	(3.098)	0.27±0.07	(2.834)
EBS$\times 10^{-12}$moles/mg protein	4.94±0.39	(0.138)	4.83±0.70	(1.897)	3.46±0.19	(3.394)
EBS$\times 10^{-12}$moles/mg DNA	119.12±9.43	(0.138)*	660.40±95.23	(6.453)*	45.65±2.54	(7.523)*
Protein/DNA	24.12±9.43	(6.691)*	160.89±20.44	(7.211)*	13.20±1.28	(8.439)*
Correlation coefficient=r^2	-0.968	——	-0.877	——	-0.905	——

a. Values are expressed ± standard error of the regression value estimate.
b. Long term castrate Sprague-Dawley rats received treatments via injection in vivo. Hydroxylapatite Assay was used to retain hormone receptor complexes. Data was analyzed by linear regression analysis on Scatchard Plots.
n: Number of determinations used in Scatchard analysis.
* Students "t" test significantly different at F=0.995, p=0.0005
Degrees of freedom = m+n-2
A. uterus DF = 18
B. vagina DF = 22

Table 3. Hypothalamic and pituitary cytosol estradiol receptor HTP assay

	Treatment I		Treatment II		Treatment III	
	Saline	"t" Test (I vs II)	0.1μg E_2,	"t" Test (II vs III)	0.1μg E_2,48h 1.0mg P_4^2,24-48h	"t"Test (I vs III)
A. *Hypothalamus:* n=21/group						
Cytosol protein mg/ml	8.14±0.50		7.86±0.48		7.89±0.24	
$K_a 10^{10}{}_M{}^{-1}$	0.04±0.03[a]	(1.288)	0.15±0.08	(0.300)	0.12±0.06	(1.193)
EBSx10^{12}moles/mg protein	0.35±0.17	(0.838)	0.52±0.12	(0.536)	0.62±0.15	(1.214)
EBSx10^{12}moles/mg DNA	20.36±9.64	(0.818)	26.92±6.61	(0.971)	41.37±9.77	(1.531)
Protein/DNA	57.53±3.61	(0.032	57.57±3.53	(2.996)	70.03±2.20	(2.910)
Correlation coefficient=R	-0.748	——	-0.744	——	-0.732	——
B. *Pituitary:* n=21/group						
Cytosol protein mg/ml	3.60±0.07		3.57±0.37		3.60±0.35	
$K_a x 10^{10}{}_M{}^{-1}$	0.11±0.02	(0.282)	0.12±0.03	(1.342)	0.21±0.06	(0.063)
EBSx10^{-12}moles/mg protein	1.67±0.31	(1.871)	1.14±0.33	(0.055)	1.16±0.15	(1.470)
EBSx10^{-12}moles/mg DNA	113.0±22.40	(2.010)	57.12±16.46	(1.742)	93.01±12.39	(0.781)
Protein/DNA	67.63±2.22	(2.498)	50.12±6.15	(2.012)	80.05±13.31	(0.924)
Correlation coefficient=r^2	-0.961	——	-0.893	——	-0.859	——

a: Values are expressed ± standard error of the regression value estimate
n: Number of determinations used in Scatchard analysis.
Conditions are as described in table 2
Degrees of freedom = m+n-2
A. Hypoth. DF = 14
B. Pituitary DF =14

Another explanation may be that the techniques used in this, and other studies, were still not sensitive enough to demonstrate differences or changes which may occur. The correlation coefficients for the hypothalamus determinations were inferior to those observed for uterine and vaginal tissue and may reflect the extremely small amounts of material (E_2–R_c) which were being assayed or the possible effect of high levels of lipid interference typically derived from neural tissue.

Recent reports by several groups have shown no effect on the concentration of pituitary E_2–R_c following ovariectomy even though the fall in uterine E_2–R_c concentration was quite significant [23]. Recent reports suggest that gonadectomy and/or sex differences do not affect the K_a, the EBS concentration, or the molecular characteristics of the pituitary or hypothalamic E_2–R_c concentration [24]. This adds support to these results suggesting that the K_a for all four E_2–R_c containing tissues are almost identical before and after steroid feedback modulation.

Many factors may be responsible for the decrease in apparent uterine or vagina E_2–R_c concentration when progesterone is present. One plausible explanation may be that progesterone interacts with the estrogen receptor and thus changes the estrogen binding site on the receptor. Although progesterone does not compete directly for the estrogen binding site, it could be acting as a negative heterotropic effector and may demonstrate negative cooperativity. Since cooperativity has been reported for estrogen receptors [16, 25, 26] and deviations from linearity (reported to reflect cooperativity) can be observed in some Scatchard plots, the data has been reevaluated using the "Hill Plot" equation in order to obtain the Hill "coefficient of cooperativity" [25, 27] seen in Table 4.

Statistically neither treatment with estrogen nor progesterone significantly changed the Hill coefficient for estrogen binding. Thus, progesterone does not reduce the affinity of the estrogen receptor for estradiol by some type of heterotropic or allosteric interaction. Correlation coefficients for this data were very good.

Hill coefficients for uterine, vagina, pituitary and hypothalamus E_2–R_c measured by the HTP assay (range = 1.023 to 1.142) at 0–2°C demonstrated neither positive or negative cooperative effects. These results are in contrast to the Hill coefficients reported for uterine cytosolic receptors measured by equilibrium dialysis (range = 1.40 to 1.620) at 0–2°C [26].

DISCUSSION

Progesterone mediated decreases in E_2–R_c concentration were first suggested to us when uterine cytosols from estrogen-progesterone treated long term castrate rats demonstrated decreased binding on sucrose gradients as compared to cytosols from both untreated controls and estrogen treated long term castrates. Since these injections had 10,000 times more progesterone than estradiol (an amount 10^6 times in excess of a 3 pmol uterine receptor capacity), it was possible that the vast excess of progesterone competed successfully against [^{3}H]-estradiol for binding to the receptor.

When Scatchard analysis was employed to determine competition as well as concentration of E_2–R_c, the competitive contributions by progesterone were found to be negligible. Two separate techniques, each

Table 4. Hill coefficients for uterine and vaginal tissue[a]

Treatment	Hill coefficient	S.E.	r^2
A. UTERUS			
(1) 0.5 ml saline (I.P.)	1.069	0.028	0.998
(2) +0.1μg E_2-17β 48 hr	1.142	0.084	0.987
(3) +0.1μg E_2-17β 48 hr ; +1.0 mg progesterone hr 24 to 48	1.070	0.068	0.990
B. VAGINA			
(1) 0.5 ml saline (I.P.)	1.023	0.044	0.996
(2) +0.1μg E_2-17β 48 hr	1.079	0.101	0.979
(3) 0.1μg E_2-17β 48 hr ; +1.0 mg progesterone hr 24 to 48	1.069	0.061	0.992

[a]Conditions are as described in Table 2.

r^2=Correlation Coefficient

S.E.=Standard Error of the regression value estimate.

subject to Scatchard analysis were employed. Both the *in vitro* "E_2 organ binding assay" and the "hydroxylapatite E_2–R_c assay" yielded similar values for the receptor concentration per uterus in the saline injected controls. However, K_a values obtained *via in vitro* assay at 37°C were about 4 times greater than those determined on hydroxylapatite at 0–2°C. Contributions to the affinity constant by low affinity binding proteins and partition effects would be expected to depress the affinity constant rather than increase it. In fact, an increase in K_a after correction for non-specific binding is common to almost all assay techniques, notably charcoal adsorption [15].

The difference in K_a values between these two techniques can be interpreted as a consequence of "physiological" conditions. In the *in vitro* E_2 organ assay the binding conditions occur at physiological temperature, cellular concentration, and are not terminated by tissue disruption. This procedure obviates any concern for receptor loss during homogenization or processing. This is especially important since E_2–R_c is more labile in the absence of steroid [3]. Binding affinity may be tighter under intact conditions. Alternatively, fundamental differences might exist between techniques so that estimation of K_a values is biased. Such a bias may result when high concentrations of non-specific binding components alter the distribution of "available" steroid and can be eleviated by direct determination of unbound steroid; moreover, this bias does not seriously effect the estimation of total receptor concentration [15].

Concentrations for the nuclear estrogen receptor (E_2 R_n) have not yet been determined in this system, however, theoretically nuclear translocation has easily occurred after 48 h [10, 28]. Thus, effects due to nuclear vs cytoplasmic compartmentalization will not be sufficient to explain these E_2–R_c responses. Two recent reports support our observations on rat uterine cytosol receptors. These findings, under different dose schedules and using different assay techniques, extend progesterone mediated decreases in estrogen receptor concentration to the immature rat uterus [30] and to the monkey oviduct [31].

The present data on binding cooperativity remove the possibility of progesterone acting as a negative heterotrophic effector. It supports the emerging concept of independence between binding sites for estrogen compared to progesterone in the uterus and vagina and extends this independence to pituitary and hypothalamic estrogen receptors. Deviations from linearity were seen in Scatchard plots at the lowest concentration and can have several possible explanations: (a) interference from other cytosolic proteins is maximized at low ligand concentrations, (b) equilibrium times may be greater at low ligand concentrations, especially in heterogenous cytosolic preparations, (c) the receptor, "protected" by bound steroid, is actually labilized at low "unprotected" ligand concentrations thus altering the equilibrium parameters of the assay. The deviations from linearity in these Scatchard plots are small and do not appear to be indicators of positive cooperativity when the data are reevaluated as the Hill coefficient (Table 4).

Milgrom *et al.*[29], have detailed control mechanisms mediated by estrogen and progesterone for the uterine cytosolic progesterone receptor. They report

that progesterone limits the half life of its own progesterone receptor thus, progesterone may program uterine cell populations in some terminal fashion to block both E_2 and P_4 responsiveness.

Cidlowski and Muldoon[19], have recently reported replenishment phenomenon for pituitary and hypothalamus. It is emphasized that their results cannot be directly compared to these experiments. Their report dealt with early events (1–14 h) after steroid treatment with estradiol only and their E_2–R_c "replenishment" never exceeded control levels. Also their experimental design using a charcoal adsorption assay without Scatchard Analysis for E_2–R_c measured "relative" rather than "absolute" E_2–R_c binding site concentrations [19]. Studies are currently underway to apply the HTP E_2–R_c Assay and Scatchard plotting to the entire time period covering 0–72 h to get a better comparison of the sequence of events described here as replenishment vs modulation.

Recent data by Clark *et al.*[30, 32, 33] suggest that other known estrogen antagonists (nafoxidine, CI-628, and clomiphene) mimic the estrogen stimulated increases in polymerase I and II, as well as the increases in RNA, protein, DNA synthesis. Also they block or fail to stimulate the "replenishment" of the cytosolic estrogen receptors possibly by retaining receptor in the nucleus. Work by several groups [10, 19] using cycloheximide demonstrated an inhibition of receptor replenishment in the absence of protein synthesis. Thus, while it is tempting to speculate about receptor synthesis, transcriptional control mechanisms, and other processes such as receptor activation or decreased degradation, none can be eliminated at present. Further work is necessary to demonstrate that protein synthesis is actually involved in the replenishment processes.

The variability in the comparisons of various data on E_2–R_c currently in print [15] serve to re-emphasize the danger in trying to compare numerical values between different animals, target tissues, experimental designs, and assay systems. It is particularly significant, however, that when all four target tissues for estrogen are compared under the same experimental conditions, they do not respond to sex steroid feedback or modulation in the same fashion. Indeed, growth and cell division should not be expected to occur as a response in neural tissue. The principal response in the pituitary or hypothalamus, for instance, is probably low level synthesis and secretion, hypertrophy or even depolarization, but not mitosis. Thus, it would seem logical that estrogen receptor content in the hypothalamus and pituitary remains unchanged with respect to either protein or DNA after steroid treatment.

Vaginal tissue responds in the same relative fashion as uterine tissue but perhaps with greater hypertrophy and less cell division as shown in Table 2. These data indicate that estrogen stimulated an increase in estrogen receptor sites from 47.92 to 115.17 $\times 10^{-12}$ mol/mg DNA in the uterus; concommitantly, receptors increased from 119.12 to 660.40 $\times 10^{-12}$ mol/mg DNA in the vagina.

In summary these experiments performed on mature, long term castrate rats illustrated that the capacity of reproductive tissue to respond to low levels of sex steroids (estradiol-17β and progesterone) either by *in vivo* biological or *in vitro* E_2–R_c criteria has been retained even in highly atrophied uterine and vaginal tissue. While this response was characterized by estrogen mediated increases in cytosolic E_2–R_c concentration in uterine and vaginal tissues, the steroid treatments had no effect on long term pituitary or hypothalamic E_2–R_c concentration. Progesterone opposed the E_2 induced increases in E_2–R_c concentration. Values for K_a in all four target tissue studies were similar and were not affected by either steroid treatment.

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