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ESTROGEN RECEPTOR IN MAMMARY GLANDS AND UTERUS OF RATS DURING PREGNANCY, LACTATION AND INVOLUTION*

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SUMMARY

Specific estrogen receptor (ER) in both mammary gland and uterus of Sprague–Dawley rats were measured during the course of pregnancy, lactation and involution by sucrose gradient sedimentation, and Dextran-charcoal adsorption methods. During these physiological changes, uterus and mammary gland have ER molecules similar in nature to that reported by other investigators: cytoplasmic ER has a high affinity to estradiol-17β, sediments at the 8–9 S region of a low salt sucrose gradient and is heat denatured. Its bound estradiol is competitively displaced by estrogenic and anti-estrogenic compounds. Significant variation of dissociation constant derived from Scatchard analysis was noted from early to late lactation in the uterus; and, in the case of the mammary gland, changes were also observed during involution. Cytoplasmic ER levels of the uterus or mammary gland from different animals vary considerably during the latter part of pregnancy. Both tissues exhibit a continual increase of ER binding sites during the first half of lactation. Rapid decline of ER binding sites in the mammary gland was not accompanied by a similar decrease in the uterus during post lactation. The increase of ER binding sites was not due to an impairment of the translocation pathway for the steroid–receptor complex, since most of the radioactivity was found in the nucleus of explants incubated with [^{3}H]-estradiol. Among other possibilities discussed, it is suggested that changes in hormonal milieu during pregnancy and lactation may be a major contributing factor for the differences in ER binding capacities observed. A positive or negative regulatory mechanism for ER replenishment or its eventual fate may be at work at different times during these major physiological changes.

INTRODUCTION

In recent years, much evidence has accumulated to indicate the importance of estrogen receptor (ER) as a molecule in mediating estrogen action [1–2]. The mechanism of estrogen action consists of a series of events initiated by the formation of receptor–steroid complex in the cytoplasm. Molecular transformation of cytoplasmic ER then occurs followed by the translocation of modified ER molecule to the nucleus. Interaction of this steroid–receptor complex at the gene site is believed to trigger transcriptional and translational processes analogous to but much more complicated than that occurring in bacterial cells.

Although much is known about the mechanism of action, the regulation of the synthesis of the ER molecule and its eventual fate after entering the nucleus are poorly understood. It is intended in this investigation to elucidate the changes of ER binding capacity in the uterus and mammary gland while animals are undergoing major hormonal changes during time of pregnancy, lactation and involution. By correlating the hormonal states to the ER binding capacity, we hope to be able to determine if there exists a positive and/or negative regulatory mechanism governing the synthesis or the eventual fate of the ER molecule.

MATERIALS AND METHODS

Tissue preparation. Uteri and abdominal mammary glands were excised from pregnant, lactating or post-lactating Sprague–Dawley rats. The tissue was weighed, finely minced, and homogenized in varying amounts of buffer containing 0·01 M Tris and 0·0015 M EDTA, pH 7·5. A four-volume homogenate (w/v) was prepared for Dextran-charcoal and sucrose density gradient centrifugation; a 15-vol. homogenate of mammary tissue and a 30-vol. of uterus was used for Scatchard plot analysis. Tissues were homogenized with Polytron Pt 10 tissue disintegrator (Brinkman Instruments, Inc.), for two 10-s periods at setting 3·5 with a 30-s cooling interval. Post-mitochondria supernate (PMS) was obtained after centrifuging the homogenate at 40,000 ***g*** for 10 min. The cytosol fraction for sucrose gradient analysis was prepared by centrifugation of the PMS at 105,000 ***g*** for 1 h with an SW 50·1 rotor in the ultracentrifuge (Beckman L2-65).

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ER Assay by Dextran-charcoal (DC). A 0·2 ml aliquot of the PMS solution was used as the source of ER. Assay was carried out simultaneously in 2 sets of test tubes, one containing 0·2 nM estradiol [2,3,6,7-^{3}H]-Estradiol, 105 Ci/mmol, New England Nuclear) and the other 0·2 nM ^{3}H-estradiol with a 5000-fold excess of nafoxidine hydrochloride* in a final incubation vol. of 0·5 ml. Immediately after addition of [^{3}H]-estradiol, the mixture was vortexed and incubated at 30°C for 30 min. To the total solution was then added 0·5 ml of a buffer suspension containing 0·5% charcoal (Norit A) and 0·05% Dextran 70 (Pharmacia). The final mixture was vortexed and incubated for 10 min at 30°C. The unbound and loosely bound [^{3}H]-estradiol which adsorbed to the DC surface was removed by centrifugation at 1000 *g* for 10 min. A 500 μl aliquot from this supernate was added to 10 ml of Liquifluor–toluene (Beckman) containing 20% of BBS-3 for estimation of bound radioactive estradiol. Counting efficiency of sample material in a Beckman LS-250 was approximately 28%. The non-specific and total bound radioactivity were derived from sets of tubes incubated with and without nafoxidine, respectively, and estrogen receptor was calculated from the differential radioactivity incorporated. The reliability and detailed description of this method was described [3]. Values obtained by this method is about 2·3 times less than the Scatchard plot technique described below [3]. When the estrogen binding capacity of samples are low, 0·2 nM estradiol provide a better sensitivity with the DC method.

Scatchard plot analysis. The number of ER binding sites and the apparent dissociation constant (K_D) of ER was estimated by Scatchard plot using conditions described by Feherty *et al.*[4] which were similar to those used for the DC method. Various amounts of ^{3}H-estradiol ranging from 0·1–1 nM were incubated with 100 μl of PMS in a final incubated volume of 200 μl at 30°C for 30 min. To these solutions 500 μl of DC was added and the resulting mixtures were incubated for an additional 10 min at 30°C. Bound estradiol was determined from 500 μl of the supernate as in the DC method. Employing the equation shown in Fig. 1, binding sites were determined from the intercept of the abscissa and the apparent K_D from the negative slope of the plot.

Sucrose density gradient. A mixture of 150 μl of cytosol and 50 μl of Tris–EDTA, pH 7·5, with or without 5000-fold nafoxidine was incubated at 0°C for 10 min with 1 nM [^{3}H]-estradiol in 50 μl buffer. Two hundred μl of this mixture was then applied to a 10–30% sucrose gradient. After centrifugation in a Spinco SW 50·1 rotor at 250,000 *g* for 14 h, 100 μl fractions were collected in a fraction collector (ISCO Company, Lincoln, Nebraska, U.S.A.) as previously described [3]. Radioactivity was determined as described for the DC method. Yeast alcohol dehydrogenase (Worthington Biochemical Corp., New Jersey) was used as marking standard. Protein in all three methods was determined by the method of Lowry[5].

* Nafoxidine hydrochloride or U-11 100A, was a gift from the Upjohn Company, Kalamazoo, Michigan, U.S.A. Nomenclature: 1-{2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-ethyl}pyrrolidine hydrochloride.

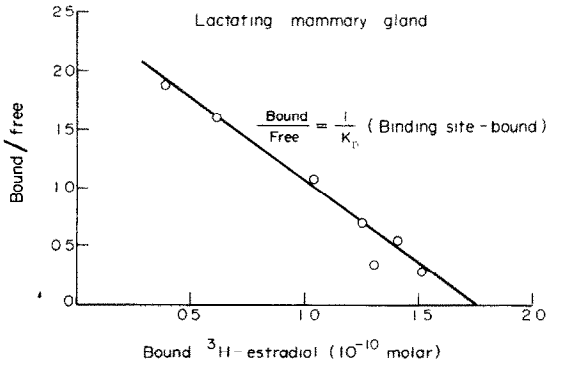

Fig. 1. Scatchard plot analysis of the titration data for ER in lactating mammary gland. Conditions and method of assay were described in the text. The dissociation constants, K_D, calculated from the negative slope, for both the mammary gland and uterus during lactation were tabulated on Table 3. Binding sites were obtained from the intercepts at the abscissa.

RESULTS

Conditions of assay for DC. In order to ascertain that the Dextran-charcoal assay and the sucrose gradient method provides a valid measurement of the

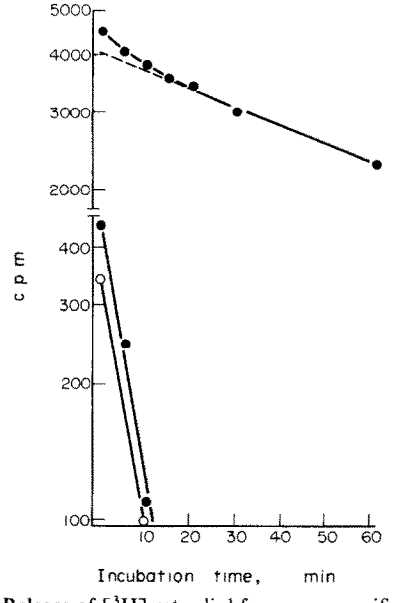

Fig. 2. Release of [^{3}H]-estradiol from non-specific binding protein by DC treatment at 30°C (upper curve) under conditions described in text. Lower line (filled circles) was derived from experimental data minus extrapolated values from the upper curve. When PMS samples were heated for 20 min at 55°C to denature ER prior to assay, a similar release of bound radioactivity (open circles) was observed. Each point represents the mean of triplicate determinations using rat uterine PMS.

Table 1. Binding sites and K_D of ER derived from Scatchard plot analysis at 0°C and 30°C of incubation with [^{3}H]-estradiol. Paired aliquots of PMS of rat uteri from young adult, estrous rats were assayed at the same time under exactly the same conditions as described in text but at different temperatures of incubation with [^{3}H]-estradiol

0°		30°	
fmoles/mg Protein	K_D (10^{-10} M)	fmoles/mg Protein	K_D (10^{-10} M)
101	0.96	104	0.16
99	1.85	95	0.61
124	2.86	132	0.75

relative ER binding capacity in mammary tissues, a series of experiments was performed.

One of the criteria for a valid assay of ER binding capacity is the resolution of specifically and non-specifically bound estradiol. In the DC assay, free estradiol adsorbed to the DC surface and was removed by centrifugal precipitation. The remaining radioactivity in the supernate represented the estradiol that was bound specifically and non-specifically to the ER molecule. After a PMS from the uterus was charged with [^{3}H]-estradiol, incubation of the resulting solution with DC at 30°C caused a rapid decrease of the bound estradiol during the first 15 min of incubation (Fig. 2). This rapid linear reduction in radioactivity (experimental minus extrapolated data) apparently represents non-specifically bound estradiol, because the non-specifically bound estradiol left in the same PMS solution after heat denaturation of ER exhibited similar phenomenon. Almost complete removal of this non-specifically bound estradiol can be accomplished by DC treatment at 30°C for 10 min (see lower curve). Furthermore, ER binding sites estimated by incubation of [^{3}H]-estradiol with PMS at 30°C were as high as those incubated at 0°C by the Scatchard analysis (Table 1).

The temperature effect on [^{3}H]-estradiol binding to ER from mammary tissue is shown in Fig. 3. During the first hour of incubation, there was no appreciable difference in [^{3}H]-estradiol binding assayed at room temperature (23°C) compared to that at 30°C. Maximal binding in either case was reached between 30 and 60 min of incubation with [^{3}H]-estradiol. Incubation at 0–4°C did not reach comparable maximum until 3 h later. In other experiments, it was observed that incubation at 0 4°C for longer periods up to 24 h did not increase bound estradiol appreciably. Incubation at 37°C is not recommended since very rapid loss of specifically bound estradiol was noted at this temperature. Temperature effect on uterine ER was similar.

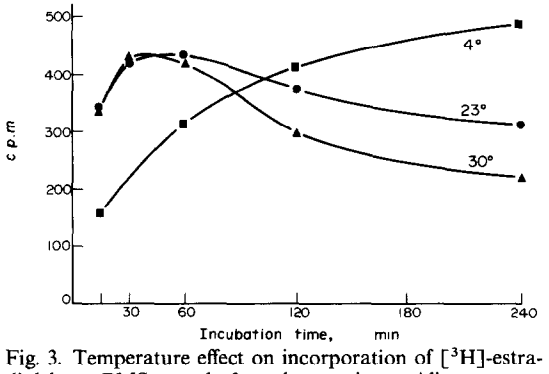

Fig. 3. Temperature effect on incorporation of [^{3}H]-estradiol by a PMS sample from breast tissue. Aliquots were processed under exact conditions described in text except for the temperature differences in incubation of [^{3}H]-estradiol with PMS. Excess free and loosely bound estradiol was removed by centrifugal precipitation with DC at 30°C for 10 min. Each point represents triplicate determinations.

Table 2. ER binding capacities of mammary and uterine tissues during pregnancy. PMS was first pretreated with DC for 10 min at 30°C to remove endogenous estradiol before ER assay was performed by the simple DC method described under method section. Each value is the mean ± SD of the number of samples in parenthesis

MAMMARY GLAND	ER (fmoles/mg protein)
11 - 12 days	4.6 ± 0.9 (6)
19 - 21 days	8.3 ± 6.7 (4)
UTERUS	
11 - 12 days	7.6 ± 0.9 (6)
15 - 16 days	14.8 ± 8.2 (6)
19 - 21 days	10.6 ± 8.9 (8)

Changes in ER levels. During pregnancy, the estrogen level is elevated. In order to remove the endogenous steroid which may compete with added [^{3}H]-estradiol, PMS was pretreated with DC for 10 min at 30°C followed by centrifugation. The resulting supernate was assayed for ER by the simple DC method. Table 2 shows the results of ER binding capacity in uterus and mammary glands during mid- and late pregnancy. The ER binding capacity in both uterus

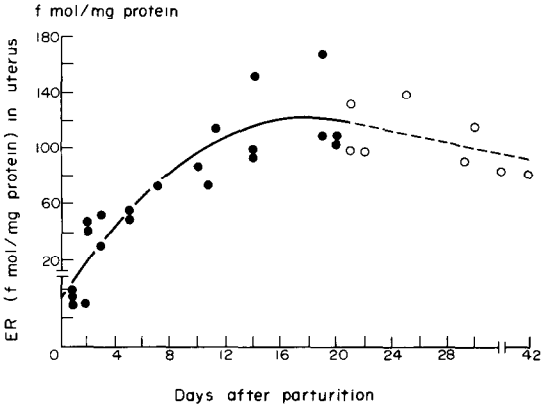

Fig. 4. ER binding sites in uterine tissue after parturition. ER was analyzed by method of Scatchard as described in text. Each value (solid circle) was from a lactating rat. Open circles represent ER values from post-lactating animals. The curve was derived from least square plot of these values.

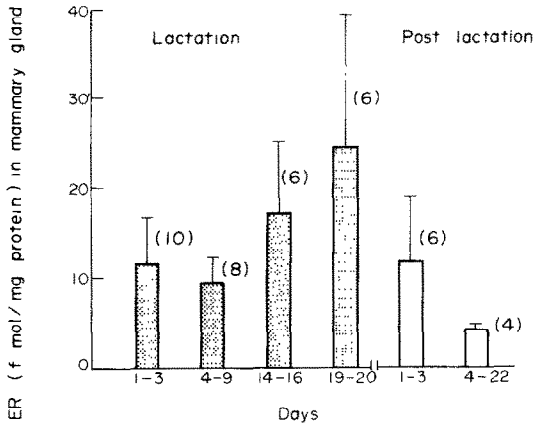

Fig. 5. ER binding sites in mammary gland tissues after parturition. Each bar shows the mean ± SD of the number of rats shown in parenthesis. Conditions of assay and analysis by Scatchard method was described in the text.

and mammary glands from different animals during late pregnancy showed appreciable degrees of variation. During this period the 8 s peak in the mammary gland or the uterus was either very small or could not be demonstrated with consistency (Fig. 6a), presumably due to endogenous estrogen or low ER binding capacity. However, the ER values for both the mammary gland and the uterus at late pregnancy were comparable to their respective values at early lactation by DC and Scatchard analysis.

Using the Scatchard technique, it was found that the binding sites increased during the course of lactation in both the uterus (Fig. 4) and the mammary gland (Fig. 5). Rapid decline of ER in the mammary gland was observed during the first three days of post-lactation and reached quiescent levels following the 4th day of weaning. This rapid degradation of ER was not observed in the case of the uterus. ER values remained high during the post-lactating period. At quiescent state, ER was only slightly lowered.

Throughout the course of lactation, ER molecules for both uterus and mammary gland sediments at about the 8 s region under the conditions of this assay (Figs. 6 and 7). However, heavy aggregates were observed occasionally during early lactation and early involution periods (Figs. 6d and 6e). The 8 s peak was abolished by the presence of estrogen antagonists such as nafoxidine and estrogenic compounds such as estriol and diethylstilbestrol or by heat denaturation (Leung, unpublished data). The estrogen bind-

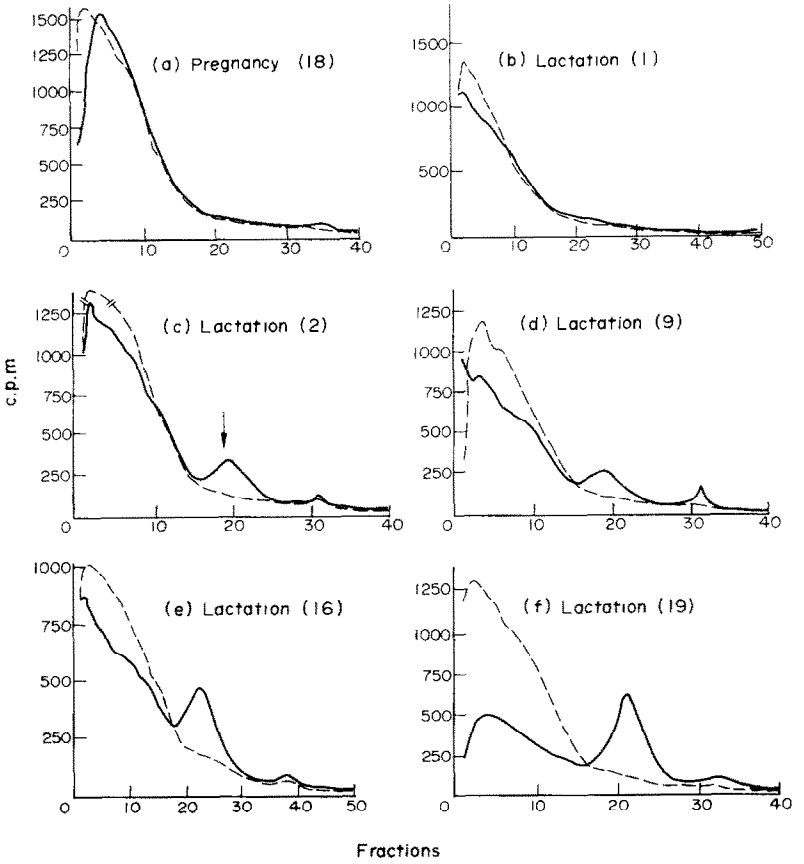

Fig. 6. Sucrose gradient profiles of ER in mammary glands at different stages of gestation and lactation. The numbers in parenthesis show the day of pregnancy or lactation. Solid lines are PMS incubated with [^{3}H]-estradiol only and dotted lines are split samples incubated with [^{3}H]-estradiol and 10,000-fold excess of nafoxidine as described in text. The ER is shown as the 8–9 S peaks (fractions 15–25) near yeast alcohol dehydrogenase (positioned by an arrow) in the sucrose gradient (10–30%).

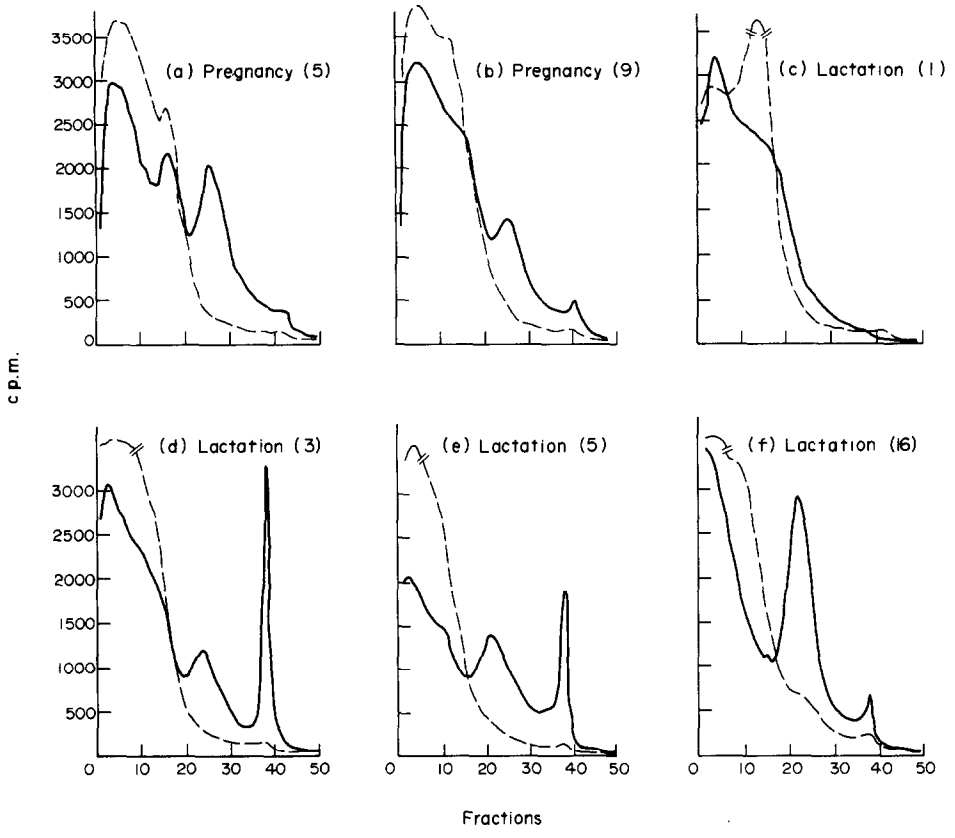

Fig. 7. Sucrose gradient profile of ER in uterus during pregnancy and lactation. See Fig. 6 for detail.

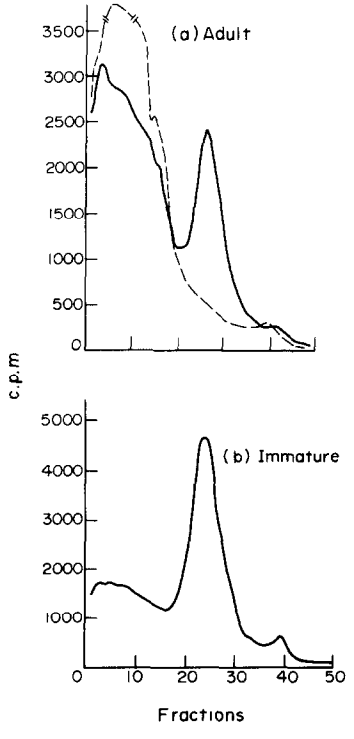

Fig. 8. Sucrose gradient profile of ER in adult and immature rat uterine tissue. See Fig. 6 for detail. A, from a young adult about 50-days-old and, B, from a 21-day-old immature rat.

ing capacity in the uterus of adult rats was similar to that during mid- or late lactation (Fig. 8a); immature uteri were very high in ER binding (Fig. 8b). It seems, therefore, during pregnancy, especially just prior to paturition, ER production might be inhibited.

Dissociation constant (K_D). The change in apparent K_D for mammary gland and uterus during the course

Table 3. Values of K_D during lactation as calculated by Scatchard plot analysis. Each value is the mean ± SD of the number of specimen shown in parentheses. L and PL designate lactation and post lactation respectively. Student t test was used in evaluating statistical significance of K_D values to that at early lactation for each tissue; ‡, not significant, *, $P < 0.01$ and **, $P \ll 0.01$. In the mammary gland, K_D during late lactation and early post lactation is highly significant $P \ll 0.01$

TISSUE	PHYSIOLOGICAL STATE	K_D (10^{-10} M)
Uterus	1 - 11 L	0.56 ± 0.14 (10)
	14 L - 21 PL	2.63 ± 0.62 (12)**
Mammary Gland	1 - 9 L	1.04 ± 0.57 (20)
	14 - 20 L	1.85 ± 1.25 (13)*
	1 - 7 PL	3.67 ± 0.85 (6)**
	9 - 22 PL	1.03 ± 0.76 (3)†

Table 4. Tissue explants were prepared and incubated in medium 199, containing [^{3}H]-estradiol (0·1 nmol) in the presence or absence of 10,000-fold excess of nafoxidine hydrochloride as described in previous publication [25]. After incubation, PMS was prepared according to method described in text. After removal of free and loosely bound estradiol from the PMS with DC, the differential radioactivity incorporated from explants incubated in solutions with or without nafoxidine was evaluated for cytoplasmic receptor values (ER_c). Nuclear receptor (ER_n) of explants was derived from the differential radioactivity incorporated into the 40,000 *g* particulates after washing them 3 times with equal vol. of Tris-EDTA buffer

TIME INCUBATION (37°C)	ER_c fmoles/mg DNA	ER_n fmoles/mg DNA
30 Minutes	< 0.1	53.3
120 Minutes	8.8	52.1

of lactation and involution is shown in Table 3. In the uterus, after day 14 of lactation, levels of ER sites remained constant at a high level while the dissociation constant increased 4-fold from that of previous days. Slight, but significant, increase of K_D was noted for the mammary gland after day 14 of lactation and was about 3-fold higher at 1 7 days post-lactation than in early lactation and K_D returned to previous early lactation levels afterwards.

Translocation process. The translocation of ER during lactation was investigated with an attempt to elucidate what brought about the increase of ER during the course of lactation. If there is an impairment in this process, ER might be expected to accumulate in the cytoplasm. This was found not to be the case from organ culture experiments (Table 4 and Table 5). Both uterine and mammary tissues (data not shown) were capable of translocating estradiol from the cytoplasm to the nucleus during pregnancy and lactation.

Table 5. Tissue explants were prepared according to previously described procedure [25]. Control tissues and explants that were incubated for 6 h in medium 199 containing no estrogen, were analysed for ER by the DC assay described in text. The 40,000 *g* particulate was used to determine nuclear receptor (ER_n) by the nuclear exchange assay described by Hsueh *et al.*[11]. Each alphabetic letter designates an individual rat

DISTRIBUTION OF ESTROGEN RECEPTOR IN UTERINE EXPLANTS DURING PREGNANCY

PREGNANCY STATE		NO INCUBATION pmoles/mg DNA		6 HR INCUBATION (37°C) pmoles/mg DNA	
		ER_c	ER_n	ER_c	ER_n
15 - 16 Days	A	3.49	0.18	0.09	0.46
	B	1.32	0.12	0.16	0.79
	C	4.05	0.96	0.22	0.63
18 Days	D	3.33	<0.01	0.43	0.87
20 Days	E	7.85	<0.04	0.56	0.58
	F	5.05	<0.01	0.70	0.40

DISCUSSION

While this study was in progress [6, 7] several investigators have reported the presence of ER in lactating mammary glands of rodents [8–11]. Our result is consistent with these reports that ER in mammary glands exhibits similar characteristics to those of other estrogen target tissues. It binds tightly and specifically to estradiol, sediments in the 8–9 s region of a low salt sucrose gradient, and is denatured by high temperature. Its bound estradiol is competitively displaced by estrogenic compounds and anti-estrogens, and the steroid–receptor complex can be translocated to the nucleus. The present study also demonstrates an interesting fact that the K_D of ER derived from the Scatchard plot analysis were different during early and late lactation. It suggests that a slight alteration in the conformation of the receptor's binding site might have occurred.

Although ER in the uterine tissue has been studied extensively in recent years, little information was provided for the changes of ER in uterus during lactation. The present result showed that ER in the uterine wall during the latter part pregnancy was low as was reported by Feherty *et al.*[12]. Also, ER binding was extremely low on the first 2 days of lactation. These results suggest that the synthesis of this molecule might have been inhibited during pregnancy. A gradual rise of ER during lactation may be the result of a recovery from the inhibitory process. ER in lactating mammary gland showed a similar pattern as to that of the uterus. The increase of ER during lactation was not attributed to an accumulation of unutilized cytoplasmic ER, since translocation to the nucleus can be well demonstrated at late lactation. Similarly, the translocation process of ER was not impaired in the mammary gland (Leung, unpublished data), as was also reported by Hsueh *et al.*[11]. It was suggested that variation of steroid–receptor binding capacities in the mammary gland might be a function of epithelial cell density [9, 13]. Although this explanation cannot be excluded, our present results indicate that another plausible explanation might be the existence of a hormonal regulatory mechanism governing the expression of estrogen in these target tissues. The abrupt changes in hormonal milieu prior to delivery and in lactation may be of paramount importance to the synthesis, the binding integrity of the ER or to its final fate after the formation of the steroid–receptor complex.

Major hormonal changes occur in animals during pregnancy and lactation. During early pregnancy, plasma prolactin level is elevated to about 3-fold that of diestrus, declines to a low level in mid-pregnancy, increases thereafter to reach a high maximum before mid- and late lactation and subsequently decreases [14–18]. Factors such as estrogen, ovariectomy, time and number of sucklings and exposure to ether,

also affect prolactin levels [14, 17–20]. In contrast, serum FSH and LH exhibit different patterns than that of prolactin [17, 21] and plasma corticosteroid remains practically constant throughout pregnancy and lactation [15, 18]. Prostaglandin release from isolated rat uterus increases during pregnancy, reaching a maximum high on the day of expected delivery, and decreased rapidly to normal levels on day 3 of lactation [22]. The period of increase of prostaglandin appears to coincide with the decline of progesterone [23] and the late "surge" of estrogen prior to the termination of pregnancy [24]. It seemed that the changes in hormonal milieu from late pregnancy to lactation might be related to the changes of ER. Recent reports from our laboratory [25, 28] and that of Vignon and Rochefort [29] have demonstrated that prolactin augments ER binding capacities. An inhibition of ER by either progesterone [25, 27] or prostaglandin [30] was also reported. These results suggest that a positive and a negative regulatory systems governing the expression of estrogen may exist in estrogen target tissues. It remains to be demonstrated, however, whether the observed changes in ER during lactation was due to: (a) a positive regulatory mechanism resulting from increase of prolactin to estrogen ratio; (b) a negative regulatory mechanism from high levels of progesterone followed by high levels of estrogen or prostaglandin just prior to delivery; and (c) the combined effects of the above. The changes in ER binding capacities during lactation may, of course, be related to a slight alteration in the conformation of the binding sites as was noted previously.

In conclusion, we have demonstrated that ER in breast and uterine tissues was very low during early lactation, increases during the course of lactation, and returns to normal levels at quiescence soon after termination of lactation. We postulate that the change in ER levels might be hormonally regulated. The exact mechanism resulting in this change and the physiological significance of the variation of ER remain to be elucidated.

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