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ESTROGEN RECEPTOR IN PURIFIED NUCLEI FROM DECIDUOMATA OF THE PSEUDOPREGNANT RAT

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SUMMARY

A specific estrogen receptor was identified in purified nuclei from deciduomata of pseudopregnant rats. Sucrose density gradient centrifugation of 0.4 M KCl extracts of deciduomal nuclei from rats injected with [^{3}H]-17β-estradiol ([^{3}H]-17β-E_2) revealed the presence of saturable estrogen–receptor complexes sedimenting at 4.6 *S*. A nuclear exchange assay was used to determine the association constant (K_a) for the binding of 17β-E_2 by purified deciduomal nuclei and the concentrations of specific nuclear estrogen receptor sites on different days of decidualization. The mean K_a of nine determinations was $4.0 \times 10^9\ M^{-1}$ indicating that deciduomal nuclei bind 17β-E_2 with high affinity. The concentration of specific estrogen receptor sites in deciduomal nuclei on day 3 of decidualization was determined to be 0.21 pmol/mg DNA or approximately 1300 sites/nucleus. Between days 3 and 7 of decidualization there is a significant linear decrease ($P < 0.01$) in the number of estrogen receptor sites in deciduomal nuclei; on day 7 there are approximately 60% as many sites as on day 3. The significance of the nuclear estrogen receptor and its decrease as a function of decidualization time are discussed with regard to the growth and regression of deciduomata.

INTRODUCTION

The development of deciduomata is dependent upon the concerted action of estrogen and progestin. A priming dose of estrogen, or estrogen secreted at proestrus, followed by an increase in the concentration of circulating progesterone is required for decidualization to occur [1, 2]. Progesterone plays a major role in regulating the sensitivity of the uterus to decidualization but estrogen modulates the changes that occur [3]. Maximal growth of the deciduomata in the rat requires the presence of a continued low level of estrogen in addition to an elevated level of progesterone [2]. Even when high levels of progesterone are maintained for a prolonged period the deciduomata degenerate near the end of their normal life span [4]. These findings suggest that factors other than the levels of circulating steroid hormones have a regulatory role in decidual regression.

Recent reports from this laboratory have described the presence of cytoplasmic estrogen [5] and progestin [6] receptors within the deciduoma of the pseudopregnant rat and decreases with time in the concentrations of both types of receptor sites. These may be important in the control of the growth and development of deciduomal tissue by these steroid hormones. These decreases in receptor concentrations may explain why the administration of exogenous progesterone does not prevent or delay deciduomal regression in the pseudopregnant rat [4].

The interaction between estrogen and progestin in controlling the concentrations of their specific uterine receptors has been well established [7, 8]. The cytoplasmic estrogen receptor decreases before the progesterone receptor in rat deciduomata [5, 6]. The temporal relationship of these changes suggests the possibility that the decreased progesterone receptor concentration may be a result of the decreased number of estrogen receptors. The regression of the deciduomata might then be a consequence of the depressed number of progestin receptors, an amount insufficient for maintenance of the tissues.

The currently accepted theories of steroid hormone action indicate that, following the binding of steroid hormones to their specific cytoplasmic receptors, the complexes undergo translocation to nuclear sites where biological actions of the hormone are elicited [9]. The concentration of nuclear estrogen–receptor complexes has been demonstrated to change in parallel with estrogen induced changes in uterine metabolism and growth [10, 11]. In the present studies we have identified estrogen–receptor complexes within purified deciduomal nuclei and determined their concentration as a function of the day of decidualization. A decrease in the concentration of nuclear estrogen receptors with time was detected which might be related to decreases in cytoplasmic estrogen and progestin receptors and subsequent tissue regression.

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EXPERIMENTAL

Animals. Virgin Sprague–Dawley rats (Charles River, Wilmington, MA), weighing 180–300 g were maintained under a program of 14 h light and 10 h darkness. Pseudopregnancy was induced on the morning of estrus by vagino-cervical vibration for 1 min [12, 13]. The day of estrus was designated as day 0 of pseudopregnancy. Uterine decidualization was induced unilaterally by transcervical intraluminal instillation of 50 μl olive oil on the afternoon of day 4 of pseudopregnancy [13, 14]. Therefore, day 4 of pseudopregnancy was designated as day 0 of decidualization. On days 3–7 after the instillation of oil the decidualized horns were slit open and the deciduomata were removed from the myometrium by gentle scraping with a glass slide. All tissue samples were placed immediately on ice and further treatments performed at 0–4°C except where otherwise noted.

Nuclear purification. Deciduomal nuclei were purified according to the method of McCormack and Glasser[15] with some modification. The tissue was homogenized in 10 vol of 50 mM Tris–HCl, 0.34 M sucrose, 0.002 M $MgCl_2$, 0.025 M KCl, 0.001 M dithiothreitol, pH 7.5 buffer (0.34 M sucrose–TKD) using 10 strokes of a Teflon-on-glass homogenizer followed by 10 strokes with a Dounce homogenizer. The homogenate was centrifuged at 2500 *g* for 10 min and the supernatant discarded. The nuclear pellet was resuspended in 10 vol of 0.34 M sucrose–TKD and homogenized again with 10 strokes of the Dounce. The nuclear suspension was centrifuged as described above and the supernatant discarded. The nuclear pellet was resuspended in approximately 10 vol the original tissue weight of 1.9 M sucrose–TKD. The nuclear suspension was placed on top of 1.9 M sucrose–TKD and centrifuged at 20,000 *g* for 45 min in SW 25.1 rotor. The purified nuclear pellet was washed two times with 10 vol of 0.34 M sucrose–TKD followed by centrifugation at 2500 *g* for 5 min. The nuclei were then resuspended in the final volume of 0.34 M sucrose–TKD by gentle homogenization with a Teflon-on-glass homogenizer. Nuclei prepared in this manner were examined by phase contrast microscopy and found to be intact and free of contamination by cytoplasmic organelles.

Scatchard analysis. 1 ml aliquots of the nuclear suspension were incubated in triplicate in polystyrene culture tubes (Falcon No. 2025) in the presence of $1–10 \times 10^{-10}$ M [2,4,6,7-^{3}H] 17β-estradiol [^{3}H]-17β-E_2, New England Nuclear, 102 Ci/mmol with or without a 100-fold molar excess of diethylstilbestrol (DES) at 20°C for 2 h. After incubation the nuclei were washed three times with 1 ml of 0.34 M sucrose–TKD followed by centrifugation at 1000 *g* for 10 min. The bottoms of the tubes containing the washed nuclear pellets were cut off with a hot blade and dissolved in 4 ml of a toluene scintillation fluid containing 0.4% Omnifluor (New England Nuclear). The radioactivity of samples was determined with a 42% efficiency for tritium. Specific binding was taken as the difference in bound radioactivity in the presence and absence of a 100-fold molar excess of DES. The data were analyzed according to the method of Scatchard[16] to determine the equilibrium association constant for the binding reaction and the concentration of nuclear 17β-E_2 receptor sites.

The amounts of specific binding of 1×10^{-10} M [^{3}H]-17β-E_2 were determined as described above at various dilutions of nuclear suspension in order to insure that the assays were performed within a range where specific binding is linear with respect to DNA concentration. It was found that specific binding of 17β-E_2 was linear up to a concentration of at least 0.17 mg DNA/ml in nuclei isolated from 3 day deciduomata. Therefore, all assays were performed at DNA concentrations within this range.

Identification of nuclear estrogen receptor in deciduomata in vivo. Pseudopregnant rats which were given a decidualization stimulus 3 days earlier were castrated and injected subcutaneously with 20 mg Depo-provera (The Upjohn Co.). 24 h later the animals were injected intraperitoneally with 0.2 μg [^{3}H]-17β-E_2 with or without 20 μg unlabelled 17β-E_2 in 1 ml of 0.15 M NaCl containing 4% ethanol. After 1 h the animals were sacrificed, deciduomata removed and nuclei isolated as described above.

The nuclei were extracted with 0.4 M KCl in 10 mM Tris–HCl, 1.5 mM EDTA pH 7.4 buffer (TE) at 0°C for 2 h. The suspensions were centrifuged at 2500 *g* for 10 min and the supernatants removed. 0.4 ml of the extracts were layered on to 5–20% sucrose density gradients prepared in TE buffer containing 0.4 M KCl. The gradients were centrifuged at 190,000 *g* for 16 h in a SW 50.1 swinging bucket rotor. [^{14}C]-bovine serum albumin (~3000 DPM) prepared according to the method of Siiteri *et al.*[17] was centrifuged on a separate gradient for determination of sedimentation coefficient.

Gradients were fractionated by collecting three drop aliquots (~0.2 ml) directly into liquid scintillation vials. 2 ml of scintillation fluid containing toluene–Triton X-100 (2:1, v/v) and 0.6% Omnifluor were added to gradient fractions and radioactivity measured at 34% efficiency for tritium.

Statistical analysis. The association constant (K_a) and the number of binding sites (n) were estimated from Scatchard plots using a linear regression process. The quantity n was obtained as the ratio of the ordinate intercept and the slope ($-K_a$); therefore the standard error is approximate. The mean values for n and K_a are weighted means and their standard errors are pooled estimations. The significance of the rate of change in deciduomal receptor concentration as a function of decidualization time was tested with the t-test procedure.

Miscellaneous. The protein content of nuclear extracts was measured according to the method of Lowry *et al.*[18] using bovine serum albumin as a standard. The DNA concentration of purified nuclear

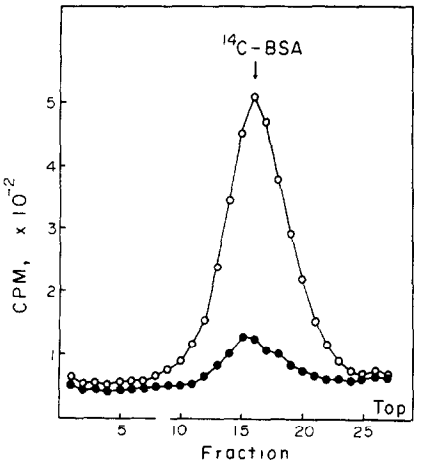

Fig. 1. Sucrose gradient profiles of 0.4 M KCl extracts of deciduomal nuclei from 4 day decidualized rats injected with 0.2 μg [^{3}H]-17β-E_2 (O——O) or 0.2 μg [^{3}H]-17β-E_2 ± 20.0 μg 17β-E_2 (●——●). The nuclear extracts contained 0.8 (O——O) or 1.2 (●——●) mg protein/ml.

suspensions was determined by the method of Burton[19] using calf thymus DNA as a standard.

RESULTS

The presence of estrogen–receptor complexes in the nuclei of deciduomata of pseudopregnant rats was demonstrated *in vivo*. Sucrose density gradient centrifugation of the 0.4 M KCl extract of nuclei isolated from deciduoma of 4-day pseudopregnant rats injected with [^{3}H]-17β-E_2 showed that the estrogen–receptor complexes sediment at 4.6 *S* (Fig. 1). The labelled hormone is apparently complexed to a macromolecule of low capacity since the simultaneous injection of a 100-fold excess of unlabelled 17β-E_2 resulted in a marked reduction in the amount of bound radioactivity. Extraction of the purified nuclei

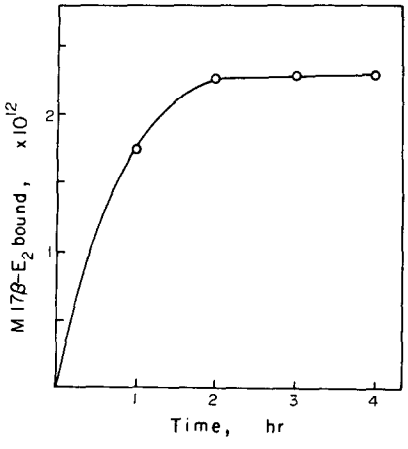

Fig. 2. Specific binding of 1 × 10^{-10} M [^{3}H]-17β-E_2 with time by purified nuclei of 4 day deciduomata. Assays were performed as described in materials and methods for Scatchard analysis. The DNA concentration of nuclear suspensions was 44 μg/ml.

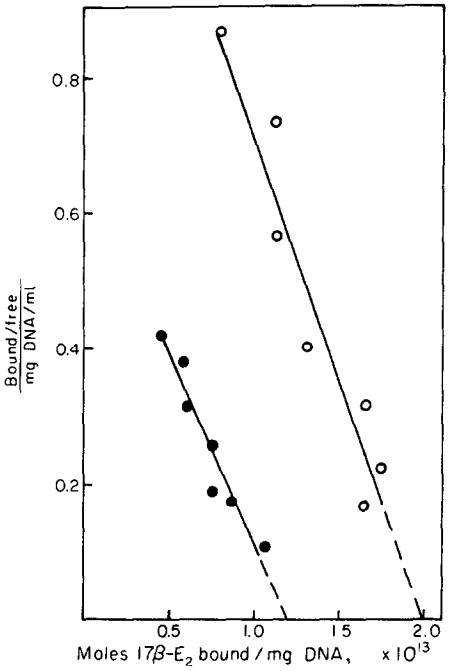

Fig. 3. Scatchard plots of specific 17β-E_2 binding by purified nuclei from day 3 (O——O) and day 7 deciduomata (●——●). The DNA concentrations of the nuclear suspension were 84 μg/ml (O——O) and 77 μg/ml (●——●). The K_a values and concentrations of binding sites (*n*) were: (O——O) K_a = (7.1 ± 0.3) × 10^9 M^{-1}, *n* = (2.0 ± 0.3) × 10^{-13} mol/mg DNA; (●——●) K_a = (5.6 ± 0.6) × 10^9 M^{-1}, *n* = (1.2 ± 0.2) × 10^{-13} mol/mg DNA.

with 0.4 M KCl solubilized 85% of the specifically bound radioactive estrogen.

The nuclear exchange assay described by McCormack and Glasser[15] was used in order to quantitate the number of 17β-E_2 receptor sites in purified deciduomal nuclei. Under the assay conditions employed the binding of 17β-E_2 to nuclear receptor sites was maximal after 2 h at 20°C (Fig. 2). This binding remained stable up to at least 4 h of incubation. Therefore, nuclear exchange assays were performed at 20°C for 2 h.

The data obtained from saturation analysis of 17β-E_2 binding to purified nuclei were expressed according to Scatchard[16]. Representative Scatchard plots for the specific binding of 17β-E_2 to receptor sites of nuclei isolated from deciduomata of 3 and 7 day decidualized animals are presented in Fig. 3. The plots indicate that a single class of high affinity sites with a limited capacity for 17β-E_2 is present in purified deciduomal nuclei during the period of 3–7 days of decidualization.

The mean values for the concentrations of nuclear estrogen receptor sites in deciduomata decreased from day 3 to day 7 of decidualization (Fig. 4). On day 3, the concentration of specific estrogen receptor sites in deciduomal nuclei was 0.21 ± 0.02 pmol per mg DNA. This concentration represents approximately 1300 sites per deciduomal cell based on a DNA content of 10.3 pg/nucleus [15]. The concentration of

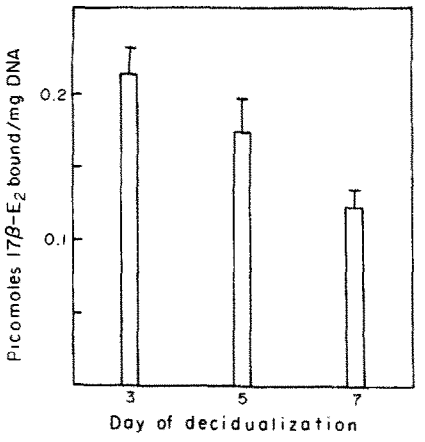

Fig. 4. The concentration of specific 17β-E_2 receptor sites in nuclei from day 3, 5 and 7 deciduomata. The mean values ± standard error of three determinations for each day were as follows: Day 3, $n = (2.1 \pm 0.2) \times 10^{-13}$ mol/mg DNA. Day 5, $n = (1.7 \pm 0.2) \times 10^{-13}$ mol/mg DNA. Day 7, $n = (1.2 \pm 0.1) \times 10^{-13}$ mol/mg DNA.

nuclear estrogen receptor sites underwent a significant linear decrease ($P < 0.01$) between days 3 and 7 of decidualization; the concentration of high affinity estrogen receptor sites on day 7 was about 60% of that on day 3. The mean values for the association constants for the binding of 17β-E_2 by nuclei from day 3, 5 and 7 deciduomata did not differ significantly ($P > 0.05$).

DISCUSSION

These studies have identified a high affinity, low capacity estrogen receptor within purified deciduomal nuclei of the pseudopregnant rat. The characteristics of this receptor are quite similar to those reported for the nuclear estrogen receptor of other estrogen target tissues. The presence of saturable estrogen receptors in deciduomal nuclei was demonstrated *in vivo* by the injection of pseudopregnant rats with [^{3}H]-17β-E_2 with or without a 100-fold molar excess of unlabelled 17β-E_2. Extraction of the purified nuclei with 0.4 M KCl solubilized 85% of the specifically bound hormone. Other authors [20, 21] have reported similar recoveries of estrogen–receptor complexes by 0.4 M KCl extraction of uterine nuclei from immature rats which were injected with [^{3}H]-17β-E_2 1 h prior to sacrifice. The estrogen–receptor complex of deciduomal nuclei sediments at 4.6 *S* on high salt sucrose density gradients which is in reasonable agreement with the 5 *S* value obtained by other workers for the nuclear estrogen receptor of rat uterus [9, 21].

The apparent association constant K_a for the binding of 17β-E_2 by purified deciduomal nuclei was $(4.0 \pm 0.2) \times 10^9\ M^{-1}$ (9 determinations). In comparison, McCormack and Glasser[15] have reported a K_a of $5.4 \times 10^9\ M^{-1}$ for the binding of 17β-E_2 by nuclei isolated from the basal zone trophoblast of pregnant rats. These K_a values are approximately an order of magnitude higher than those reported for the binding of 17β-E_2 by the rat uterine nuclear receptor [20, 21]. Whether this dissimilarity is due to differences in the actual affinities of the receptors for 17β-E_2 or in the techniques employed is not known.

The estrogen receptor site concentration in deciduomal nuclei from 3 day decidualized rats (day 7 of pseudopregnancy) was 0.21 ± 0.02 pmol/mg DNA or approximately 1300 sites/cell. This level of nuclear estrogen receptor is quite similar to that measured by Martel and Psychoyos[22] in the endometrium of rats pregnant 7 days (~1400 sites/cell). In their studies nidation was prevented by sectioning of the oviducts on day 2 of pregnancy. The close agreement in the number of nuclear estrogen receptor sites in endometrial cells and deciduomal cells on days 7 of pregnancy and pseudopregnancy, respectively, seems particularly relevant since decidual cells are derived from the stromal fibroblasts of the endometrium.

The concentration of nuclear estrogen receptor in deciduomata on day 3 is also comparable to that in uteri at metestrus or estrus but considerably less than that at diestrus or proestrus [10, 11]. It has been demonstrated that the fluctuation in nuclear estrogen receptor concentration in rat uteri during the estrous cycle [10, 11] closely parallels the rate of ovarian estrogen secretion [23]. Welschen *et al.*[24] have determined that the concentration of plasma 17β-E_2 in the pseudopregnant rat is within the range of those at metestrus until day 10. On day 12 of pseudopregnancy the concentration of plasma 17β-E_2 is about twice that on day 10. Therefore, the low levels of nuclear estrogen receptor present during deciduomal development are associated with low concentration of circulating estrogen, as is also the case in the uterus at metestrus or estrus in the cycling rat.

The number of nuclear estrogen receptor sites per deciduomal cell decreases from approximately 1300 at day 3 to 800 on day 7 of decidualization. This decrease occurs during the period of rapid formation of deciduomal cells by proliferation and transformation of stromal fibroblasts of the endometrium. The deciduoma of the pseudopregnant rat achieves its maximum growth potential on day 7 of decidualization after which the tissue regresses [5]. Clark and Peck[20] have conducted studies which indicate that the induction of uterotrophic responses requires the long term retention of a low number (~1400/nucleus) of estrogen receptor sites in rat uterine nuclei. Such a mechanism may be operative in deciduomata so that the decreased levels of nuclear estrogen receptor at day 7 of decidualization are unable to maintain deciduomal development and tissue regression occurs.

We have previously detected decreases in the concentrations of cytoplasmic estrogen [5] and progesterone [6] receptor sites in deciduoma following days 3 and 5 of decidualization, respectively. No significant changes were observed in the concentrations of estrogen and progesterone receptors in contralateral untreated uterine horns. Thus, the decreases in

receptor levels in the deciduoma were not the result of a general alteration in the endocrine status of the animal [5, 6]. The decreases in cytoplasmic and nuclear estrogen receptors of the deciduomata occur during the same period of decidualization, but the cytoplasmic sites decrease at a faster rate than those in the nucleus. The number of cytoplasmic estrogen receptor sites per deciduomal cell is approximately 30,000 on day 3 of decidualization and decreases to about 6000 by day 7 [5]. One might speculate that the decrease in nuclear sites is due to a decreased availability of cytoplasmic receptors for binding of estrogen and subsequent translocation of the complexes to the nucleus. However, a decreased level of nuclear receptor sites might also lead to a fall in the concentration of cytoplasmic receptor since estrogen induces the synthesis of its own receptors [25]. The reason why estrogen receptor concentrations decrease during deciduomal development is not known but is apparently not due to changes in the concentration of circulating 17β-E_2 or progesterone which are maintained at relatively constant levels during this period [24, 26, 27].

The decrease in estrogen receptor levels in the deciduomata precedes that of the cytoplasmic progesterone receptor which does not change until after day 5 of decidualization [6]. The concentration of cytoplasmic progesterone receptor has been shown to be under estrogenic control in both birds [28] and mammals [7, 29]. The mechanism by which estrogen controls the synthesis of specific proteins appears to involve the regulation of nuclear RNA synthesis by estrogen–receptor complexes [30]. The decreased concentration of estrogen receptor complexes within deciduomal nuclei after day 3 of decidualization may be insufficient to maintain the synthesis of progesterone receptor. The depressed level of progesterone receptor might then lead to regression of the deciduoma.

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