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Cytoplasmic and Nuclear Estradiol Receptor in the Corpus Luteum of the Pseudopregnant Rabbit (40672)

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Several laboratories have demonstrated that estradiol is the principal luteotropin in the corpus luteum of the rabbit (1–3). As expected, specific estradiol receptors are present in such an estrogen-responsive tissue (4). The pattern of luteal cytosol receptor activity through early and late pseudopregnancy has been reported by several investigators. Estradiol receptor activity was reported to rise from Day 4 to a maximum on Day 12 of pseudopregnancy, declining toward Day 16 and then falling abruptly as pseudopregnancy ends at Days 16–18 (5). The initial appearance of estrogen receptor in the rabbit corpus luteum was the subject of two recently published studies. Mills and Osteen (6) reported elevated levels of receptors on Day 4 of pseudopregnancy indicating that receptor levels are elevated at least 24 hr before the tissue becomes estrogen dependent on Days 5–6 (7). On the other hand, Miller and Keyes (8) concluded that significant levels of estrogen receptor appear only coincidently with the onset of estrogen dependence on Days 5–6.

The present study was performed to examine both cytoplasmic and nuclear estrogen receptor activity in the rabbit corpus luteum over the early stages of pseudopregnancy. Specifically, the experiments sought to confirm the previous findings of a significant rise in cytosol binding on Day 4 of pseudopregnancy. Furthermore, the levels of nuclear receptor over the first 6 days of pseudopregnancy were examined. The results of these experiments show that the receptor is present in the nucleus after estrogen administration due to translocation from the cytoplasm.

Materials and Methods. Animals and treatment. Sexually mature, white female rabbits of the New Zealand strain weighing 4–5 kg were used. Animals were housed in individual cages with compressed food and water available *ad libitum*. Pseudopregnancy was induced in these animals by a single i.v. injection of 100 IU human chorionic gonadotropin, hCG (APL-Ayerst). In some of the studies, rabbits on Days 2, 4, or 6 of pseudopregnancy were selected in pairs with one receiving a single i.v. injection of 100 μg estradiol suspended in 1 ml of physiological saline. The animal receiving the estradiol was sacrificed 30 min after injection. The remaining rabbit in the pair received no injection. Rabbits were sacrificed by cervical dislocation, and the ovaries were quickly removed and placed in ice-cold saline for transportation to the laboratory.

Steroids. [2,4,6,7-^{3}H]Estradiol (sp. act. 91.3 Ci/mmole) was purchased from New England Nuclear Corporation. The purity of this steroid was checked prior to use using either paper or column chromatography. Purification was accomplished where indicated. Nonradioactive steroid was obtained from Schwarz/Mann.

Tissue preparation. The following procedures were completed simultaneously for the corpora lutea from all rabbits whether estrogen treated or untreated. In the laboratory under ×7 magnification, clearly discernible corpora lutea were dissected out and cleaned of adhering interstitial tissue. All procedures from this stage to completion of the binding assay were performed at 0–4°. Corpora lutea were counted, weighed, and combined with sufficient Tris–EDTA buffer (0.0015 *M* disodium EDTA and 0.01 *M* Tris, pH 7.4) to give a final concentration of 20 mg corpus luteum/ml. The tissues were homogenized in a sintered glass homogenizer and then centrifuged at 800*g* for 10 min. The supernatant from this low speed spin was then centrifuged at 150,000*g* for 40 min in a Beckman L2-65B centrifuge. The supernatant fraction (cytosol) was decanted and used to assess cytoplasmic estradiol binding activity as described below. The low-speed pellet from the 800*g* centrifugation was resuspended in Tris–EDTA buffer and utilized in the determination of nuclear estrogen receptor as described below.

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Determination of estradiol binding in the cytosol. Estrogen binding in the cytosol was measured by the method of Cidlowski and Muldoon (9) and Abney (10) and as previously described (6). Briefly, the technique was as follows: 200-μl aliquots of cytosol were added to prepared glass tubes containing five concentrations of [^{3}H]estradiol ranging from 170,000 to 10,000 dpm/tube (758 to 45 fmole/tube) in a final volume of 1 ml of Tris–EDTA buffer. A second series of tubes contained, in addition, a 100-fold *M* excess of nonradioactive estradiol which gave an estimate of nonspecific steroid binding. The nonspecific binding values were eventually subtracted from total binding to yield specific estradiol receptor activity. After an 18-hr incubation at 4°, the estrogen which was not bound to the cytoplasmic receptor was removed by the addition of 1 ml of a suspension which contained 0.3% activated charcoal (Norite A previously washed to remove fines) and 0.03% dextran (Nutritional Biochemical Company) in Tris–EDTA buffer. Immediately after addition of the charcoal, the contents of the tubes were mixed and then incubated for 15 min in an ice bath. The charcoal was then separated centrifugally and the supernatant fractions transferred to counting vials for determination of radioactivity by liquid scintillation spectrometry. The results of these counts were used to determine the association constant (K_a) and the number of estradiol binding sites/mg corpus luteum according to the method of Woosley and Muldoon (11). In some studies, there was insufficient tissue available from the rabbit ovaries to complete analysis at all five steroid concentrations. In these cases, the binding results are based on fewer points.

Determination of binding of estradiol in the nuclear fraction. Nuclear binding sites were quantified by the exchange method described by Clark *et al.* (12) with some modification based on preliminary studies. The pellet resulting from the low-speed (800*g*) centrifugation of the homogenate was washed three times with 3 ml Tris–EDTA buffer followed each time by centrifugation at 800*g* for 10 min. The resulting washed, crude, nuclear preparation was combined with sufficient buffer to yield a concentration equivalent to 40 mg luteal wet wt/ml buffer.

In order to determine the optimal conditions under which to perform the exchange assay, corpora lutea were collected 6 days after hCG injection and 30 min following estradiol injection. Four-hundred-microliter aliquots of the resulting nuclear preparation were incubated at 37° for 15, 30, 60, and 90 min with [^{3}H]estradiol (486 fmoles/tube) with and without a 100-fold *M* excess of nonradioactive estradiol. Following incubation, the nuclear pellets were washed three times with 3 ml buffer followed by centrifugation at 800*g* to remove unbound [^{3}H]estradiol. Two milliliters of ethanol was then added to each sample in order to extract the bound [^{3}H]-estradiol and the ethanolic extract transferred to counting vials for determination of ^{3}H content once the solvent had been evaporated. Tritium was quantified in a Beckman LS-250 liquid scintillation spectrometer with external quench correction.

A second preliminary study was designed to show that with increasing amounts of the nuclear preparation, a proportional increase in the binding of [^{3}H]estradiol could be demonstrated. The 200-, 400-, and 800-μl aliquots of crude nuclear preparation were incubated for 60 min at 37° in the presence of [^{3}H]-estradiol (486 fmoles/ml) with and without a 100-fold *M* excess of nonradioactive estradiol. At the end of the incubation, the nuclear preparations were washed and [^{3}H]estradiol binding was determined as described above.

In a third preliminary study, 400-μl aliquots of the nuclear preparation were incubated with [^{3}H]estradiol in the presence of a 100-fold *M* excess of estradiol, progesterone, 20α dihydroprogesterone (20α-hydroxy-4-pregnen-3-one), or testosterone for 60 min at 37°. Following incubation, the ^{3}H binding was determined as described above.

In a final initial experimental series, the association constant (K_a) for the nuclear binding was calculated in the following manner: 400-μl aliquots of the nuclear suspension were transferred to prepared glass tubes containing four concentrations of [^{3}H]estradiol ranging from 775 to 84 fmoles/tube. Another set of tubes contained a 100-fold *M* excess of nonradioactive estradiol to give an estimate of nonspecific steroid binding. The tubes were incubated at 37° for 60 min and binding was analyzed as previously described. The kinetic parameters were calculated according to the direct linear plot method of Woosley

and Muldoon (11).

Expression of results. Estradiol binding in the cytosol and nuclear fractions is expressed as fmole bound/mg corpus luteum. Some results are presented as mean ± SE of all determinations on corpora lutea of the same group from the same day after hCG injection. The results have been analyzed statistically using linear regression analysis and Students' *t* test.

Results. The first 6 days of pseudopregnancy are marked by rapid growth of the corpus luteum in the rabbit. As demonstrated in Fig. 1, luteal weights virtually triple over this interval with 4.0 ± 0.2 mg/CL on Day 2, 8.0 ± 0.4 mg/CL on Day 4, and 11.6 ± 0.4 mg/CL on the 6th day after hCG injection. Because of this marked change in luteal weight, it was necessary to correct all receptor measurements for this increase in weight of the corpora lutea. This has been accomplished by expressing the results as fmole [^{3}H]estradiol bound/mg corpus luteum. However, this method may slightly underestimate the nuclear receptor concentration since a small increase in the receptor might be obscured by the accompanying increase in luteal weight.

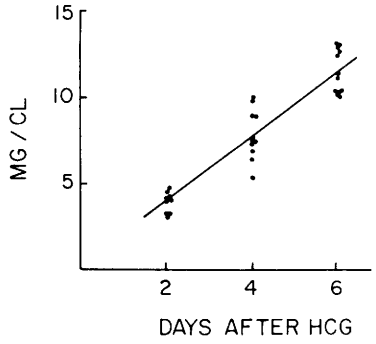

FIG. 1. The weights of corpora lutea collected 2, 4, and 6 days after a single injection of hCG. Each point is the mean weight of at least eight corpora lutea. The weight increase is linear over the 6 days ($r = 0.94$, $P < 0.05$). 100 I U hCG caused an average of 10.7 ± 0.43 ovulations/rabbit.

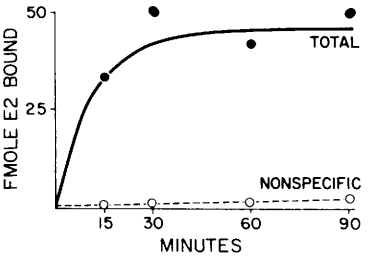

FIG. 2. The effect of duration of incubation at 37° on the binding of [^{3}H]estradiol by receptors in a nuclear fraction prepared from rabbit corpora lutea 6 days after hCG injection and 30 min after injection of estradiol. Nonspecific binding is the [^{3}H]estradiol bound in the presence of a 100-fold *M* excess of nonradioactive estradiol. Each point represents an individual determination of binding activity.

The results in Fig. 2 demonstrate that incubation of luteal nuclei for periods of 15 to 90 min at 37° does not result in any decline in estrogen binding activity. Accordingly, an incubation period of 60 min was selected for all subsequent exchange assays.

The binding activity in the nuclei of corpora lutea proves to be specific to estradiol (Fig. 3). No significant inhibition of [^{3}H]estradiol binding occurred in the presence of a 100-fold *M* excess of progesterone, 20α-dihydroprogesterone, or testosterone; these hormones were selected as representative steroids which are present in elevated concentration in rabbit corpora lutea (13) and blood (14).

Using an incubation period of 60 min, a linear relationship between the amount of luteal nuclear preparation and exchangeable estradiol binding activity can be demonstrated (Fig. 4). The data in this figure also establish that nonspecific binding activity remains low (<4% of the total activity) during the 60-min incubation.

Having established the appropriate conditions for the measurement of nuclear receptor activity in the rabbit corpus luteum a study was performed to determine how quickly after estradiol injection the cytoplasmic receptor was depleted. The results, depicted in Fig. 5, show that within 30 min after injection, cytosol receptor levels are nearly 0 and little recovery is noted by 60 min after injection. Nuclear estrogen receptors have the opposite pattern with maximal levels present at 30 min postinjection and little decline from this level by 60 min. Since depletion appears to be total by 30 min, this interval between estrogen injection and sacrifice of the rabbits was selected for all subsequent studies.

Kinetic analysis proves that the nuclear estrogen receptor in the rabbit corpus luteum is of high affinity and low capacity. The association constant (K_a) was found to be 3×10^9 *M* before estradiol injection and 3.3 ×

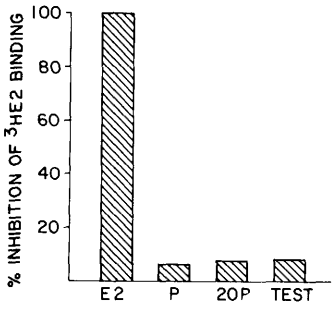

FIG. 3. The inhibition of [^{3}H]estradiol binding during the incubation of a nuclei preparation in the presence of a 100-fold *M* excess of estradiol (E2), progesterone (P), 20αdihydroprogesterone (20P), or testosterone (TEST). The inhibition of [^{3}H]estradiol binding by nonradioactive estradiol was arbitrarily set at 100% and the inhibition caused by the other steroids compared to it. The nuclei were prepared from corpora lutea 6 days after hCG injection and 30 min after estradiol administration. Each bar represents the average of two separate determinations.

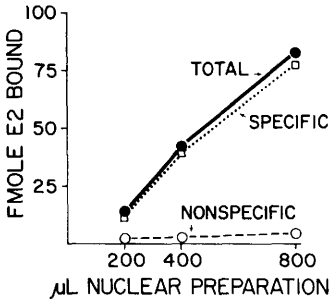

FIG. 4. The relationship between the quantity of luteal nuclear preparation and the amount of [^{3}H]estradiol bound. The 200-, 400-, and 800-μl aliquots nuclear preparation from corpora lutea collected 6 days after hCG injection and 30 min after estradiol injection were incubated alone or in the presence of a 100-fold *M* excess of nonradioactive estradiol. Specific binding is the difference between total binding and nonspecific binding. Each point represents an individual determination of binding activity.

10^9 *M* at 30 min following injection of 100 μg estradiol. In a recent preliminary communication, Yuh and Keyes (15) reported the existence of two classes of nuclear binding sites: A high-affinity site with a K_a of 7×10^9 *M* with a lower-affinity binding component of $K_a = 2 \times 10^7$ *M*. The value of 3×10^9 *M* for the association constant in the present study compares very favorably with the K_a for the high-affinity site as reported by Yuh and Keyes (15).

A portion of the results in Fig. 6 confirm findings previously published from this laboratory (6). As was reported, by Day 4 after hCG, levels of cytoplasmic estrogen receptor in animals not receiving estradiol injection are significantly increased over Day 2 levels (Day 2: 0.40 ± 0.09 fmole estradiol bound/mg CL; Day 4: 2.88 ± 0.79 fmole/mg CL, $P < 0.05$). The increase continues with even greater receptor at Day 6 (8.99 ± 2.83 fmole/mg CL). Despite this dramatic rise in cytosol receptor activity, nuclear receptor activity in the corpora lutea of rabbits not injected with

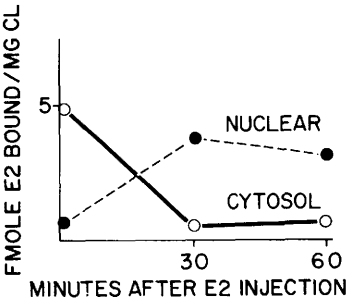

FIG. 5. The effect of time after estradiol injection on the levels of estradiol receptor in the cytosol and nuclear fractions prepared from corpora lutea 6 days after hCG injection. Each point represents an individual determination of receptor activity.

FIG. 6. Estradiol receptor activity in the cytosol and nuclear fractions of corpora lutea collected 2, 4, and 6 days after hCG injection and before (0 min) and 30 min after estradiol injection. Each bar represents the mean of three to five determinations with brackets equaling 1 SE. Solid bars—cytosol receptors, hatched bars—nuclear receptors.

estradiol changed little over the period of Days 2, 4, and 6 of pseudopregnancy. Figure 6 also demonstrates that cytoplasmic receptor disappears by 30 min after the intravenous injection of 100 μg of estradiol; on Days 2, 4, and 6 of pseudopregnancy, the estrogen injection leads to a sharp decline in levels of cytosol receptor and a concomitant increase in nuclear binding activity.

Discussion. The results of these experiments are in full agreement with the previously published finding that luteal cytoplasmic estradiol receptor increases by the 4th day of pseudopregnancy (6). The data presented in Fig. 6 show significant estrogen binding on Day 4 as compared to Day 2 after hCG ($P < 0.05$). The increase continues with elevated cytoplasmic receptor on Day 6 as well (Fig. 6). The finding of elevated cytosol receptor on Day 4 supports our previous conclusion that the receptor is increased at least 24 hr before the onset of luteal estrogen dependence reported to occur between Days 5 and 6 (7, 8). However, Miller and Keyes (8) arrive at a different conclusion in their studies. These authors feel that the onset of estrogen dependence is coincident with (and possibly due to) the appearance of estradiol receptor on the 5th day of pseudopregnancy. However, this conclusion is not fully supported by their published results; examination of Fig. 4 in their study (8) reveals a discernible increase in cytoplasmic receptor well before the onset of estrogen dependence.

Miller and Keyes (8) measured nuclear receptor activity on Days 3 through 6 of pseudopregnancy but found little change in activity except for a transient rise on the 5th day. Since the estrogen receptor is present in the nucleus only by virtue of being translocated from the cytoplasm, the elevation of nuclear receptor on Day 5 can only follow increased secretion of estradiol on the 5th day. Hilliard and Eaton (16) reported an elevation in estrogen secretion on Day 4 of pregnancy, while Challis *et al.* (17) found an increase on Day 6 of pregnancy although levels were not measured on Days 4 or 5. However, despite this apparently estrogen-mediated increase in nuclear receptor, Miller and Keyes (8) did not report the expected concomitant decline in cytosol receptor in early pseudopregnancy. In the present study, there was no measurable change through Day 6 in nuclear receptor in the corpora lutea of rabbits which did not receive exogenous estradiol although marked increases were observed in cytosol receptor over this period (Fig. 6). Possibly the estrogen secreted on the 4th and 6th day of pseudopregnancy was insufficient to cause the extensive translocation of receptor into the nucleus which follows injection of 100 μg estradiol (Figs. 5 and 6).

The studies reported here demonstrate the presence of a luteal estradiol receptor which can be translocated from the cytoplasm into the nucleus. The levels of both the nuclear and cytoplasmic receptor have been followed as the corpus luteum develops through the first 6 days after injection of an ovulatory dose of hCG. The results support the contention that the appearance of an authentic, translocatable estrogen receptor is a normal, programmed event in the sequence of luteal development and occurs prior to the onset of estrogen dependence in the rabbit corpus luteum.

Summary. Estradiol binding activity has been measured in the rabbit corpus luteum on Days 2, 4, and 6 of pseudopregnancy. The results show that receptor activity is significantly greater on Day 4 than on Day 2 with continued increases on Day 6. In addition, the estradiol receptor activity is translocated into the nucleus within 30 min of injection of 100 μg of estradiol. Appreciable receptor translocation is observed following estradiol injection on Days 2, 4, and 6 of pseudopregnancy. It is concluded that estradiol binding activity in the rabbit corpus luteum is due to an authentic estradiol receptor and that a marked increase in this activity occurs by the 4th day of pseudopregnancy.

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