Progesterone inhibition of uterine nuclear estrogen receptor: Dependence on RNA and protein synthesis

(progesterin action/hamster/*in vitro* studies/progesterone receptor)

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ABSTRACT Progesterone treatment was found previously to reduce nuclear estrogen receptor (Re) rapidly (2-4 hr) in the hamster uterus *in vivo*. The present study was done to determine whether this inhibitory effect of progesterone on uterine nuclear Re could be demonstrated *in vitro* and whether progesterone action was dependent on RNA and protein synthesis. A uterine strip system was used in which estradiol pretreatment caused cytosol Re translocation to the nucleus and increased synthesis of cytosol progesterone receptor (Rp) during a 16-hr incubation. When progesterone was added to the medium 4 hr before the end of the incubation, cytosol Rp was depleted and nuclear Re was greatly reduced. Further experiments done with actinomycin D, puromycin, and cycloheximide indicated that the progesterone-induced loss of uterine nuclear Re was dependent on RNA and protein synthesis. These results suggest that progesterone reduced nuclear Re through a mechanism involving Rp translocation and the induction of RNA and protein synthesis, a product of which is active in degrading or otherwise inactivating nuclear Re.

Estrogen-controlled changes in uterine structure and function are altered by subsequent exposure of the uterus to progesterone (1). Recently, we discovered that progesterone action causes the rapid loss of nuclear estrogen receptor (Re) in the hamster uterus, a response that appeared to be mediated by the uterine progesterone receptor (Rp) system (2, 3). This finding suggested that the progesterone-induced decline of uterine nuclear Re may be a fundamental mechanism for progesterone regulation of estrogen action.

The purpose of this study was to further investigate the nature of progesterone's influence on uterine nuclear Re. According to the receptor hypothesis for steroid hormone action, translocation of the hormone-receptor complex from the cytoplasm to the nucleus alters gene expression, with consequent changes in RNA and protein synthesis (4). If progesterone acts through such a mechanism to cause the decline of nuclear Re, then inhibitors of RNA and protein synthesis should block the response of nuclear Re to progesterone action. A previously characterized *in vitro* uterine strip system was used to test this proposition (5, 6). In this system, estrogen action stimulates the synthesis of cytoplasmic Rp, and progesterone treatment causes Rp translocation to the nucleus (6). In the present study, the responsiveness of uterine strips to progesterone action was established by demonstrating that progesterone-induced Rp depletion was correlated with the rapid decline of nuclear Re. Then, through the use of the inhibitors actinomycin D, puromycin, and cycloheximide, it was determined that the progesterone-induced decline of nuclear Re was dependent on RNA and protein synthesis.

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MATERIALS AND METHODS

Animal Preparation. Adult female hamsters were maintained under controlled conditions with a 14-hr light:10-hr dark photoperiod, and the regularity of estrous cycles was established according to the appearance of the postestrous vaginal discharge. Animals were ovariectomized on the morning of cycle day 4 (proestrus) while they were under pentobarbital (90 mg/kg) anesthesia. Uterine tissues were collected for *in vitro* incubation after a 7-day recovery period.

Chemicals, Buffers, and Incubation Medium. Labeled steroids, [³H]estradiol (17β-[2,4,6,7-³H]estradiol, 92 Ci/mmol) and [³H]progesterone ([1,2,6,7-³H]progesterone, 97 Ci/mmol), and [³H]uridine ([5,6-³H]uridine, 37 Ci/mmol) were obtained from New England Nuclear; [³H]leucine (L-[4,5-³H]leucine, 55 Ci/mmol) was obtained from Amersham/Searle (1 Ci = 3.7 × 10¹⁰ becquerels). Radioinert steroids, actinomycin D, puromycin, and cycloheximide were obtained from Sigma. Other chemicals were obtained from commercial sources at reagent grade or better.

Incubation medium for uterine strips was medium 199 with Earle's salts (GIBCO), containing penicillin at 100 units/ml and streptomycin sulfate at 100 μg/ml and gassed with 95% O₂/5% CO₂ before use. Buffered saline was 150 mM NaCl/10 mM Tris-HCl, pH 7.5. Buffer A₃₀ contained 50 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, and 30% (vol/vol) glycerol, pH 7.5. TG buffer contained 10 mM Tris-HCl and 10% (vol/vol) glycerol, pH 7.5. Buffer B contained 10 mM Tris-HCl, 1 mM EDTA, and 12 mM monothioglycerol, pH 7.5. Dextran/charcoal suspension contained 0.5 g of Norit A (Sigma) and 50 mg of dextran-70 (Pharmacia) per 100 ml of buffer B. Scintillation counting solution was toluene/Triton X-100 (2:1, vol/vol) with 5 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter.

***In Vitro* Incubation.** Animals were killed by cervical dislocation and uteri were removed rapidly, trimmed, slit lengthwise, weighed, and placed in 30-ml beakers under a 95% O₂/5% CO₂ atmosphere at 23°C in a Dubnoff incubator. Each beaker contained 5 ml of medium 199 and 2 uterine equivalents (ca. 200-250 mg). A typical experiment involved three groups of eight animals per group, and uterine strips derived from each group were divided equally among all experimental treatments. Each experiment was replicated at least three times.

The tissues were preincubated with estradiol (30 nM) for 1 hr at 23°C, rinsed, and transferred to 8 ml of fresh medium for a 12-hr incubation at 37°C. After 12 hr, tissues were transferred to fresh medium with or without progesterone (0.1 μM), and the incubation was continued for 4 hr. In experiments done to test the dependence of progesterone action on RNA and protein synthesis, inhibitors (10 μg of actinomycin D, puromycin, or

Abbreviations: Re, estrogen receptor; Rp, progesterone receptor.

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cycloheximide per ml of medium) were added 30 min before progesterone treatment. Incubations were terminated by transferring the tissues to ice-cold buffered saline. All further procedures were carried out at 0–2°C except where specified.

Preparation of Cytosol and Nuclear KCl Extract. After incubation, the uterine strips from each incubation vessel were removed from the buffered saline, minced, and homogenized in 4 ml of buffer A₃₀, using a Polytron Pt-10 homogenizer (Brinkmann) as described (7). Cytoplasmic and nuclear fractions were prepared by centrifugation of the uterine homogenate at 800 × *g* for 15 min. The low-speed cytoplasmic fraction was centrifuged at 170,000 × *g* for 1 hr to yield the particle-free cytosol fraction. The nuclear-myofibrillar fraction was washed twice by resuspension in 4 ml of TG buffer and centrifugation at 800 × *g* for 15 min. The washed nuclear-myofibrillar pellet was resuspended in 4 ml of buffer A₃₀ plus 0.5 M KCl and incubated for 1 hr with frequent mixing. Nuclear debris was removed by centrifugation at 170,000 × *g* for 1 hr to provide the nuclear KCl extract.

Assay of Cytosol and Nuclear Receptors. Receptor concentrations were determined by Scatchard plot analysis of specific binding data (7, 8). Aliquots (300 μl) of cytosol or nuclear KCl extract were incubated in a total volume of 500 μl with increasing concentrations of [³H]estradiol (0.19–3 nM) or [³H]progesterone (0.75–12 nM) for determination of total bound ³H-labeled ligand. Identical samples were incubated with either unlabeled diethylstilbestrol (1.4 μM) or progesterone (4 μM) for determination of nonspecifically bound ³H-labeled ligand. Cytosol samples were incubated at 0°C for 18 hr to measure unoccupied Re and total Rp. Nuclear extract was incubated at 0°C for 24 hr to determine total nuclear Rp and at 30°C for 1 hr to measure total nuclear Re (3, 9). After incubation of cytosol and nuclear extract, free and loosely bound steroid were removed from each sample by treatment with 500 μl of dextran/charcoal suspension (10 min for Re; 30 sec for Rp) followed

by centrifugation at 1500 × *g* for 4 min. Radioactivity was measured in the supernatant fraction by liquid scintillation spectrophotometry using toluene/Triton counting solution at 38% counting efficiency.

Measurement of RNA and Protein Synthesis. After 14 hr of incubation, [³H]uridine or [³H]leucine (20 μCi/5 ml of medium) was added to beakers containing estradiol-stimulated uterine strips, and the incubation was terminated 2 hr later. Tissues (ca. 120 mg) were rinsed in buffered saline and homogenized in 2 ml of buffer A₃₀. Four milliliters of ice-cold 10% (vol/vol) perchloric acid was added to the homogenate; the resultant precipitate was washed with 10% perchloric acid and extracted twice with the series 90% ethanol, ethanol/diethyl ether (3:1, vol/vol), and diethyl ether. The precipitate was dried at 40°C overnight, weighed, and solubilized in 1 ml of Unisol (Isolab, Akron, OH), and radioactivity was measured using Unisol Complement scintillation counting solution at 12% counting efficiency. Incorporation of ³H-labeled precursor was calculated as dpm per mg of perchloric acid-precipitable material.

RESULTS

Estradiol and Progesterone Effects on Re and Rp. Uterine strips were preincubated for 1 hr with or without estradiol at 23°C, rinsed, and incubated for 16 hr at 37°C; one group of tissues was treated with progesterone during the last 4 hr of incubation. Estradiol pretreatment resulted in cytosol Re depletion and an increase in nuclear Re that was approximately equal to the cytosol Re decrement (Fig. 1A). Associated with the estradiol-induced increase in nuclear Re was an increase in cytosol Rp (Fig. 1B). Progesterone treatment during the last 4 hr of incubation caused cytosol Rp depletion and a marked decrease in nuclear Re (Fig. 1A). The progesterone-mediated decline of nuclear Re was not accompanied by an increase in cytosol Re, which remained at or below the sensitivity limit of the assay (Fig. 1A). Thus, Re translocation *in vitro* was corre-

FIG. 1. Estradiol and progesterone effects on Re and Rp *in vitro*. Uterine strips were preincubated for 1 hr (–1 → 0 hr) with estradiol (E₂) (30 nM) or saline/ethanol vehicle, rinsed, and then incubated for an additional 12 hr. At 12 hr, tissues were transferred to fresh medium with or without progesterone (P) (0.1 μM), and the incubation was continued for 4 hr (12–16 hr). At the end of the incubation, cytosol (open bars) and nuclear (hatched bars) Re (A) and cytosol Rp (B) concentrations were measured. Values are expressed as the mean ± SEM (*n* = 4). Cytosol Re in estradiol-treated tissues remained at or below the assay sensitivity (ca. 50 fmol/g of tissue). An analysis of variance (10) revealed that nuclear Re and cytosol Rp concentrations were enhanced (*P* < 0.01) by estradiol pretreatment and reduced (*P* < 0.01) by subsequent progesterone treatment.

Table 1. Antibiotic inhibition of incorporation of ^3H -labeled precursors into RNA and protein

Precursor	Inhibition of incorporation, %		
	Actinomycin D	Puromycin	Cycloheximide
[^3H]Uridine	90 $\pm$ 1	33 $\pm$ 3	26 $\pm$ 7
[^3H]Leucine	30 $\pm$ 5	59 $\pm$ 6	97 $\pm$ 1

Uterine strips were preincubated with estradiol (30 nM) for 1 hr, rinsed, and incubated for 12 hr, after which the strips were transferred to fresh medium containing actinomycin D, puromycin, cycloheximide (each at 10 $\mu\text{g}/\text{ml}$ of medium), or saline/ethanol vehicle (control). At 14 hr [^3H]uridine or [^3H]leucine (20 $\mu\text{Ci}/5\text{ ml}$ of medium) was added, and the incubation was terminated at 16 hr. Tissues were homogenized and radioactivity was measured in perchloric acid-precipitated material. Each value represents the mean $\pm$ SEM ($n = 3$).

lated with augmented cytosol Rp synthesis. Additionally, the cytosol Rp present in uterine strips appeared to retain biological activity *in vitro* as evidenced by the response of cytosol Rp and nuclear Re to progesterone.

Dependence of Progesterone Action on RNA and Protein Synthesis. Inhibitors of RNA and protein synthesis were used to examine the mechanism of progesterone reduction of uterine Re. The activity of the inhibitors was evaluated by measuring their influence on the incorporation of [^3H]uridine and [^3H]leucine into acid-precipitable RNA and protein. The inhibitors were present during the last 4½ hr of incubation, and the ^3H -labeled precursors were added during the final 2 hr of incubation. Actinomycin D and cycloheximide were particularly effective as inhibitors of [^3H]uridine and [^3H]leucine incorporation, respectively (Table 1). The moderate inhibition of [^3H]leucine incorporation by puromycin was consistent with its proposed mode of action; i.e., puromycin causes premature termination of polypeptide synthesis without specifically inhibiting amino acid incorporation (11). Inhibition of [^3H]uridine

FIG. 2. RNA and protein synthesis inhibitor effects on nuclear Re retention and progesterone reduction of nuclear Re. Uterine strips were preincubated with estradiol (30 nM) for 1 hr and rinsed, and the incubation was continued for 11½ hr. At 11½ hr, strips were transferred to fresh medium with or without actinomycin D (Act. D), puromycin, or cycloheximide (Cyclohex.) (each at 10 $\mu\text{g}/\text{ml}$ of medium). After 30 min, progesterone (0.1 μM) was added to some beakers (hatched bars) and saline/ethanol vehicle was added to others (open bars). The incubation was continued for 4 hr and then terminated. Nuclear Re concentration was measured and expressed as a percent of the control (estradiol pretreatment without inhibitors or progesterone). Values are means $\pm$ SEM ($n = 6$). An analysis of variance (10) revealed that nuclear Re concentrations in groups treated with the inhibitors or the inhibitors plus progesterone were significantly ($P < 0.01$) higher than in the control group treated with progesterone alone. Progesterone had no significant effect on nuclear Re in groups treated with the inhibitors.

Table 2. Effects of RNA and protein synthesis inhibitors on nuclear Re and Rp

Treatment	Nuclear receptor concentration, pmol/g tissue	
	Re	Rp
Control	1.24 $\pm$ 0.02	35.8 $\pm$ 1.3
Actinomycin D	2.43 $\pm$ 0.12*	36.7 $\pm$ 0.6
Puromycin	1.98 $\pm$ 0.24*	31.3 $\pm$ 0.4*
Cycloheximide	1.97 $\pm$ 0.14*	35.7 $\pm$ 2.5

Uterine strips were treated as described in Fig. 2. At 30 min after addition of actinomycin D, puromycin, cycloheximide or saline/ethanol vehicle (control), progesterone (0.1 μM) was added to all beakers. The incubation was continued for 4 hr and then terminated. Nuclear Re and Rp concentrations were measured; each value represents the mean $\pm$ SEM ($n = 3$). As in Fig. 2, nuclear Re concentrations were higher ($P < 0.05$) in progesterone-treated uterine strips preincubated with RNA and protein synthesis inhibitors than in uterine strips treated with progesterone alone (control). In contrast, nuclear Rp concentrations were not influenced by actinomycin D or cycloheximide and were reduced slightly by puromycin.

* $P < 0.05$, control vs. inhibitor.

incorporation by puromycin and cycloheximide, and [^3H]leucine incorporation by actinomycin D, was less marked and about 30% in all cases. These results confirm the selective inhibitory action of these compounds on RNA and protein synthesis in the uterine strip system.

Experiments were done to determine whether the inhibitors would interfere with the progesterone-induced reduction of nuclear Re. The effect of inhibitor alone was evaluated, and actinomycin D and puromycin did not significantly influence nuclear Re (Fig. 2). Cycloheximide decreased nuclear Re, but not to as great an extent as the reduction observed in response to progesterone action. However, when inhibitor and progesterone treatments were combined, the decline of nuclear Re attributable to progesterone action was blocked effectively by all three inhibitors. The inhibitors had no effect on progesterone-stimulated cytosol Rp depletion (data not shown), and they had little, if any, influence on the retention of nuclear Rp at 4 hr after progesterone treatment (Table 2). Because the inhibitors did not block progesterone action by interfering with either cytosol Rp translocation or nuclear Rp retention, these results support the conclusion that the progesterone-induced loss of nuclear Re was dependent on RNA and protein synthesis.

DISCUSSION

Previous experiments done *in vivo* with intact animals and ovariectomized, hormonally treated, animals established an early inhibitory effect of progesterone on nuclear Re in the hamster uterus (2, 3). The progesterone-induced loss of nuclear Re was not dependent on circulating estradiol levels or the availability of cytosol Re for translocation. The steroid specificity and progesterone dose dependency of the nuclear Re response indicated participation of the Rp system (3). In subsequent experiments, the effect of progesterone on uterine uptake and retention of [^3H]estradiol was studied. Progesterone had no effect on the uptake of [^3H]estradiol by the nuclear fraction, but progesterone reduced significantly the amount of [^3H]estradiol retained by the nuclear fraction at 4 hr after treatment.† Because there was no evidence for the recycling of nuclear Re to the cytoplasm, progesterone appeared to stimulate the degradation or inactivation of Re in the nucleus. The present results extend our previous observations and suggest

† Evans, R. W. & Leavitt, W. W. (1980) *Proceedings of the 62nd Annual Meeting of the Endocrine Society*, Washington, DC, June, abstr. 225.

FIG. 3. Proposed mechanism of estrogen and progesterone control of uterine Re and Rp. During estrogen (E) action, E-Re complex interacts with chromatin to stimulate estrogen-dependent RNA synthesis (RNA_E) and protein synthesis, products of which are cytoplasmic Re and Rp. After estrogen action, progesterone (P)-induced Rp translocation stimulates progesterone-dependent RNA synthesis (RNA_P) and protein synthesis, products of which are proposed to be involved in the degradation or inactivation of E-Re in the nucleus.

that the loss of nuclear Re is a result of progesterone stimulation of RNA and protein synthesis. Because RNA and protein synthesis inhibitors did not have an influence on nuclear Rp comparable to that observed on nuclear Re (Table 2), this progesterone-controlled process appears to be specific for nuclear Re. A mechanism for progesterone reduction of nuclear Re has emerged that is consistent with the generally accepted mechanism of steroid hormone action (4). Estrogen action stimulates the synthesis and accumulation of cytosol Rp, and Rp is translocated to the nucleus in response to progesterone (Fig. 3). Interaction of the progesterone-Rp complex with chromatin alters gene expression and stimulates RNA and protein synthesis. A product of this macromolecular synthesis (i.e., a regulatory factor) acts either directly on nuclear Re or indirectly through the activation of a mediator.

The nature of the proposed regulatory factor stimulated by progesterone action is not known. Calcium-activated proteases that partially degrade Re and Rp have been isolated from the calf uterus and chick oviduct (12, 13). The products of such protease action in cell-free preparations are smaller hormone-binding fragments of the receptors. However, it is possible that a calcium-activated protease or other proteolytic enzymes in intact target tissues cause more complete receptor degradation. Thus, in the hamster uterus progesterone might regulate the synthesis or activity of a proteolytic enzyme that degrades nuclear Re. Alternately, a progesterone-induced factor may bind to nuclear Re and either mask its steroid binding site or cause a conformational change in the receptor that renders it inactive.

In the rat uterus, progesterone inhibits cytosol Re replenishment measured 12-24 hr after combined estradiol and

progesterone treatment (14). Progesterone reduction of cytosol Re levels was proposed to account for diminished nuclear Re and uterine responsiveness to further estradiol stimulation (15). However, in the hamster uterus we found that nuclear Re fell within 2-4 hr in response to progesterone action without an obligatory decrease in cytosol Re (3). Cytosol Re replenishment is sensitive to inhibitors of RNA and protein synthesis (16, 17). Thus, if the loss of nuclear Re were dependent on progesterone antagonism of cytosol Re production, then RNA and protein synthesis inhibitors might be expected to mimic progesterone action on nuclear Re. Although the inhibitors used in this study were effective in blocking RNA and protein synthesis, they had little influence on nuclear Re (Table 1 and Fig. 2). This observation argues against the notion that inhibition of cytosol Re production accounts for the decline of nuclear Re. In view of the correlation between nuclear Re retention and the response of the rat uterus to estrogen action (18, 19), we suggest that progesterone inhibition of estrogen-stimulated cytosol Re replenishment proceeds through a primary inhibitory effect of progesterone on nuclear Re.

The present results support the idea that the hormonal control of receptor degradation in the nucleus is a mechanism that regulates the cell response to hormone action. However, loss of receptor from the nucleus does not necessarily indicate a reduced hormone response. Horwitz and McGuire (20) have described nuclear "processing" of Re, manifested by the decline of nuclear Re, which appears to be associated with estrogen action in cultured MCF-7 cells. Furthermore, nuclear Re processing, and hence estrogen action, was inhibited by actinomycin D (21) or anti-estrogen treatment (22). Processing of nuclear Re has also been described in suspensions of uterine cells (23). It is conceivable that uterine Re retained in the nuclear fraction for an extended period represents unprocessed receptor. Progesterone action, in causing decreased nuclear Re retention, may activate nuclear Re processing held in abeyance during estrogen stimulation. Thus, the progesterone-induced loss of nuclear Re may have either an inhibitory or a stimulatory effect on estrogen action. Clarification of this issue requires further study to determine the relationship of nuclear Re retention and processing to the expression of estrogen action.

In summary, progesterone reduction of nuclear Re appears to proceed through Rp translocation and the stimulation of RNA and protein synthesis. Nuclear Re response to progesterone action is rapid and may be an important first step in the control of estrogen action by progesterone in the uterus.

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