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REPLENISHMENT OF UTERINE ESTROGEN RECEPTOR IN CHRONICALLY ESTROGENIZED RATS

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Summary—Replenishment of uterine estrogen receptor (ER) following a single injection of estradiol-17β(E_2) was examined in chronically estrogenized rats. Subcutaneous implantation of E_2-pellet for 7 days in ovariectomized rats resulted in a significant stimulation of uteri with regard to wet tissue weight, DNA content and progesterone receptor content, with a shift of ER distribution. An intraperitoneal injection of 5 μg E_2 in the E_2-implanted rats induced a significant decrease in soluble ER (from 141.1 $\pm$ 12.6 to 69.2 $\pm$ 8.8 fmol/mg protein) with a concomitant increase in nuclear ER (from 58.2 $\pm$ 8.6 to 129.2 $\pm$ 11.6 fmol/100 μg DNA) 1 h after the injection. However, soluble ER was rapidly replenished, which was accompanied by nuclear ER reduction, and both values returned to the pre-injection levels at 4 h after the injection. An administration of 150 μg cycloheximide, that effectively blocked protein synthesis in the uterus of the E_2-implanted rats, completely inhibited the replenishment of soluble ER induced by 5 μg E_2. These findings, combined with our previous findings that replenishment of ER following a single E_2 administration in the pituitary of chronically estrogenized rats was inhibited by cycloheximide, suggest that replenishment of ER is entirely dependent on protein synthesis in chronically estrogenized rats.

INTRODUCTION

Recent studies from two laboratories have shown that cytoplasmic estrogen receptor (ER) may be artifacts of tissue homogenization and ER may reside originally in the nuclear compartment [1, 2]. However, it has been reported that studies with monoclonal antibody raised against human myometrial ER show cytoplasmic location of ER [3]. According to the current concept of steroid hormone action, estrogen binds to a cytoplasmic estrogen receptor (ER) to form an activated complex which then translocated to the nucleus and interacts with chromatin sites to modulate gene expression [4–6]. The initial translocation of receptor to the nucleus is followed by a cytoplasmic receptor replenishment which has been implicated in the ability of tissues to respond to subsequent estrogenic stimulation [7, 8]. Several studies attempting to elucidate the mechanism of replenishment of ER have been performed in immature or castrated uteri using inhibitors of protein synthesis. The findings obtained by these studies suggest that replenishment of ER depends entirely on protein synthesis [9, 10], replenishment occurs in two phases which are constituted of protein synthesis-independent and dependent process [11, 12] or replenishment does not depend on protein synthesis (may be due to receptor recycling) [13]. Their results are controversial. Since replenishment usually occurs 12 h after estradiol-17β(E_2) administration, animals should be exposed to inhibitors of protein synthesis for long periods of time in order to study the inhibition of replenishment [9–12]. The interpretation of data obtained by these studies is, therefore, complicated by the action of protein inhibitors to alter other cellular functions and the synthesis of other proteins that may be involved in degradation of ER.

On the other hand, ER in MCF-7 breast cancer cells has been reported to be synthesized within 3 h, measured by a sucrose density shift technique [14]. We recently found that ER in the pituitary was replenished rapidly following a single E_2 injection in chronically estrogenized rats [15] and that the replenishment of pituitary ER was entirely dependent on protein synthesis [16]. In this paper, therefore, we examined the effect of inhibitor of protein synthesis on replenishment of uterine ER under the same conditions.

EXPERIMENTAL

Animals and treatment

Adult Wistar female rats weighing 200 g were purchased from Shizuoka Laboratory Animal Center. Rats were ovariectomized under pentobarbital anesthesia (40 mg/kg body weight). A pellet containing 1 mg E_2 was implanted subcutaneously (s.c.) in the castrated rat. The castration was carried out 1–2 weeks in advance. The 10 mg pellet containing 10% E_2 fused with cholesterol was prepared as described by Wieder and Shimkin [17]. Seven days after the implantation, 5 μg E_2 or 5 μg E_2 combined with 150 μg cycloheximide in 0.5 ml of saline was injected intraperitoneally (i.p.) and then the rats were killed

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Abbreviations: R5020, promegestone(17,21-dimethyl-19-nor-4,9pregnadiene-3,20-dione).

by decapitation at the appropriate time. The uteri were immediately removed, weighed and stored at −80°C until use.

To evaluate the inhibition of protein synthesis by cycloheximide, [^{3}H]leucine incorporation in the uterus was determined. One or four hours after the treatment with 150 μg cycloheximide, rats were injected with 10 μCi of [^{3}H]leucine diluted with 1 ml of saline via femoral vein. One hour after the injection of [^{3}H]leucine, rats were sacrificed by decapitation. All subsequent steps were carried out at 4°C. Each uterus was homogenized in 1 ml of 0.5 M perchloric acid. Samples were washed three times with 0.2 ml of 0.3 M perchloric acid and the radioactivity incorporated in the washed pellets was counted [13].

Subcellular fractionations

All procedures were carried out at 4°C. The uteri were homogenized in 2.5 ml of TEM buffer (10 mM Tris, 1.5 mM EDTA and 2 mM mercaptoethanol, pH 7.4) with a laboratory disperser (Ystral GmbH). The homogenate was first centrifuged at 800 *g* for 10 min. The supernatant was then centrifuged at 105,000 *g* for 60 min and soluble fraction (cytosol) obtained was then incubated with 0.25 ml of 2.5% charcoal–0.025% dextran suspension for 15 min to remove unbound steroids including E_2. After centrifugation of the cytosol–charcoal mixture, the supernatant was used for the cytosol exchange assay. The pellet from the initial 800 *g* centrifugation was washed three times with 2.5 ml of TEM buffer, resuspended in 2.5 ml of TEM buffer and used without further purification for the nuclear exchange assay.

Cytosol and nuclear exchange assays of ER

Since it was found that the affinity for [^{3}H]E_2 in the uterus was not altered by chronic estrogenization, ER was quantified with 5 nM [^{3}H]E_2 in the exchange assay. For cytosol exchange assay, duplicate 0.3 ml aliquots of the cytosol were incubated with 5 nM [^{3}H]E_2 in the presence or absence of a 100-fold excess of unlabeled diethylstilbestrol (DES) in a final volume of 0.35 ml of 0°C for 1 h and then at 30°C for 1 h [18]. After incubation, 50 μl of the charcoal–dextran solution was added and the mixtures were incubated at 0°C for 15 min. The charcoal was removed by centrifugation and 0.2 ml aliquots of the supernatant were counted.

The nuclear exchange assay was performed as reported by Anderson *et al.* [19] unless specified otherwise. Duplicate 0.45 ml aliquots of the nuclear suspension were incubated with 5 nM[^{3}H]E_2 in the presence or absence of a 100-fold excess of unlabeled DES in a final volume of 0.5 ml at 0°C for 1 h and then at 37°C for 1 h. After incubation, 2 ml of TEM buffer containing 0.25% (v/v) Triton X-100 were added and the mixtures were kept in an ice bath for 10 min with intermittent mixing. The nuclear pellets were sedimented by centrifugation and washed three times with TEM buffer, and the final pellets were counted.

Assay of progesterone receptor

Duplicate 0.3 ml aliquots of the cytosol were incubated with 10 nM [^{3}H]R5020 in the presence or absence of a 100-fold excess of unlabeled R5020 in a final volume of 0.35 ml at 0°C for 4 h. After incubation, 50 μl of the charcoal–dextran solution was added and the mixtures were incubated at 0°C for 10 min. The charcoal was removed by centrifugation and 0.2 ml aliquots of the supernatant were counted.

The radioactivity for tritium was assayed in 5 ml scintillation fluid (100 mg POPOP, 3 g 2,5-diphenyloxazole in 666 ml toluene, 333 ml Triton X-100) at an efficiency of 45% in an Isocap 300 liquid scintillation counter.

Miscellaneous assays

Serum levels of E_2 were measured by radioimmunoassay as reported previously [20]. The protein concentration was measured by the method of Lowry *et al.* [21] using bovine serum albumin as standard. DNA levels were determined by the diphenylamine method [22].

Chemicals and reagents

[2,4,6,7-^{3}H]Estradiol-17β (110 Ci/mmol), [17α-methyl-^{3}H]R5020 (77 Ci/mmol), [3,4,5-^{3}H]leucine (110 Ci/mmol) and unlabeled R5020 were purchased from New England Nuclear Corp., Boston, MA. Unlabeled steroids, acid washed charcoal and bovine serum albumin were obtained from Sigma Chemical Co., St Louis, MO. Dextran T 70 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Statistics

All values were expressed as mean ± SE. To evaluate significant differences between groups, Student's unpaired *t*-test was used except for soluble ER levels after cycloheximide treatment, for which a one-way analysis of variance was applied.

RESULTS

When castrated rats were treated with E_2-pellet for 7 days, uterine wet weight as well as DNA and progesterone receptor contents per uterus increased significantly (uterine weight: 137 ± 8 to 368 ± 21 mg, $P < 0.001$, DNA content: 0.5 ± 0.1 to 1.1 ± 0.1 mg, $P < 0.001$, progesterone receptor content: 0.6 ± 0.1 to 3.5 ± 0.6 pmol, $P < 0.01$). Since these increased levels remained unchanged by an additional administration of 5 μg E_2, the uteri seemed to be fully stimulated by 7 days implantation of E_2-pellet. Serum E_2 concentration on day 7 after the implantation was found to be 152 ± 25 pg/ml, which is similar to that in normal female rats at proestrus [23].

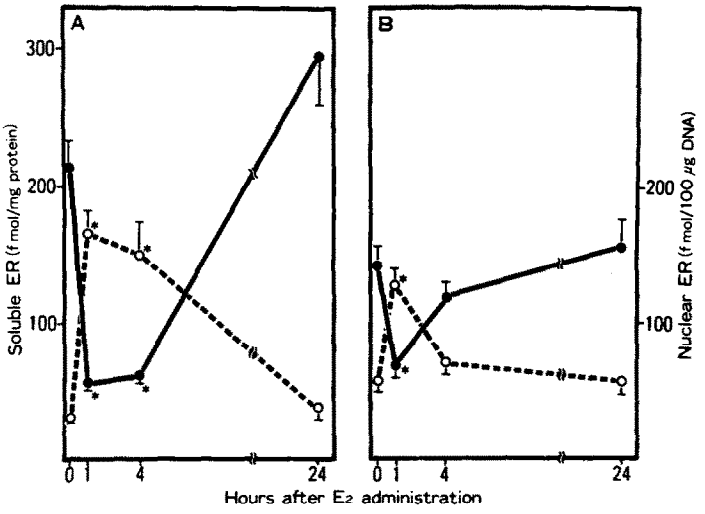

Fig. 1. Effect of a single injection of E_2 on ER distribution in rat uterus. Rats were ovariectomized (A), or ovariectomized and implanted s.c. with a single 10 mg pellet containing 10% E_2 for 7 days (B). These rats were injected i.p. with 5 μg E_2 in saline and sacrificed at indicated times. Soluble (●——●) and nuclear (○--○) ER were measured by the exchange assays. The rats for 0 h were received i.p. injection of saline. Each point represents the mean ± SE of 4 determinations. Student's t-test was used for the statistical analysis. Differences from 0 h (P): * < 0.01.

The chronic estrogenization resulted in a slight shift of ER distribution (141.4 ± 12.6 fmol/mg protein in the cytosol and 58.2 ± 8.6 fmol/100 μg DNA in the nucleus), compared with that in the uterus of castrated and non-estrogenized rats [213.8 ± 22.7 fmol/mg protein in the cytosol and 31.8 ± 4.0 fmol/100 μg DNA in the nucleus] (Fig. 1). However, the content of total ER in the uterus of E_2-implanted rats did not differ significantly from that of the castrated and non-estrogenized animals.

A single i.p. injection of 5 μg E_2 induced a rapid and significant decrease in soluble ER with a concomitant increase in nuclear ER in the estrogenized uterus. ER levels in the cytosol and nucleus were 69.2 ± 8.8 fmol/mg protein and 129.2 ± 11.6 fmol/100 μg DNA, respectively, at 1 h after the injection (Fig. 1B). However, both levels returned rapidly to the pre-injection levels at 4 h after the injection (117.6 ± 6.4 fmol/mg protein in the cytosol and 71.6 ± 8.0 fmol/100 μg DNA in the nucleus). Soluble ER increased to 156.0 ± 20.6 fmol/mg protein 24 h after the injection, but this value was not different significantly from the pre-injection value. The dynamics of ER following injection of 5 μg E_2 observed in the estrogenized rats was quite different from that in castrated and non-estrogenized rats. In the castrated and non-estrogenized rats, replenishment of ER in the uterus could not be detected at 4 h after the injection, the translocated ER in the nucleus did not diminish within 4 h, and an increase in soluble ER could be observed at 24 h after the injection (Fig. 1A).

Figure 2 shows the inhibitory effect of cycloheximide on the protein synthesis in the estrogenized uterus. A single i.p. injection of 150 μg cycloheximide resulted in a significant inhibition of protein synthesis, which was determined by [^{3}H]leucine incorporation. The inhibition rate was greater than 90% for 4 h after the administration of cycloheximide. The treatment by cycloheximide was found to have no significant effect on the amount of uterine ER in the chronically estrogenized rats (data not shown).

Since the administration of 150 μg of cycloheximide inhibited effectively the protein synthesis with no change of ER content for 4 h, replenishment of ER following a single injection of 5 μg E_2 combined with 150 μg of cycloheximide was examined in

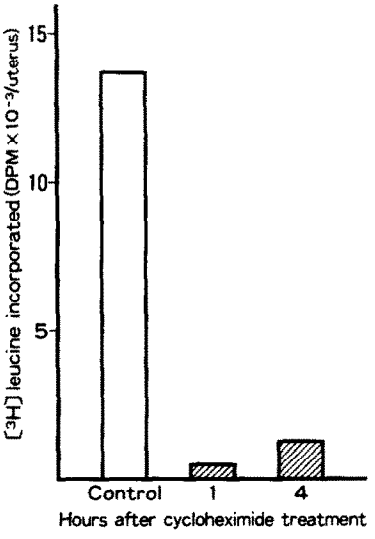

Fig. 2. Inhibition of protein synthesis in the uterus by cycloheximide in chronically estrogenized rats. Castrated and E_2-implanted rats were injected with 10 μCi of [^{3}H]leucine via femoral vein 1 or 4 h after i.p. injection of 150 μg cycloheximide or saline (Control). Values are the mean of two determinations.

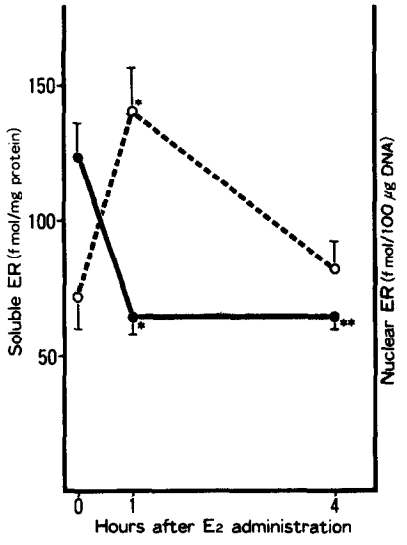

Fig. 3. Effect of cycloheximide on ER distribution in the uterus following a single injection of E_2 in chronically estrogenized rats. Castrated and E_2-implanted rats were injected i.p. with 5 μg E_2 combined with 150 μg cycloheximide. Other explanations are the same as those shown in Fig. 1. Differences from 0 h (P): * < 0.05, ** < 0.01.

the estrogenized uterus (Fig. 3). Soluble ER was translocated to the nuclear compartment at 1 h after the injection of E_2 and cycloheximide and then the increased nuclear ER level returned rapidly to the pre-injection level at 4 h. The pattern of nuclear ER shown in Fig. 3 was quite similar to that shown in Fig. 1B observed following E_2 injection without cycloheximide. However, the depleted ER level in the cytosol at 1 h after the injection of E_2 and cycloheximide (63.7 ± 5.9 fmol/mg protein) did not increase at all at 4 h (63.9 ± 4.1 fmol/mg protein) [Fig. 3].

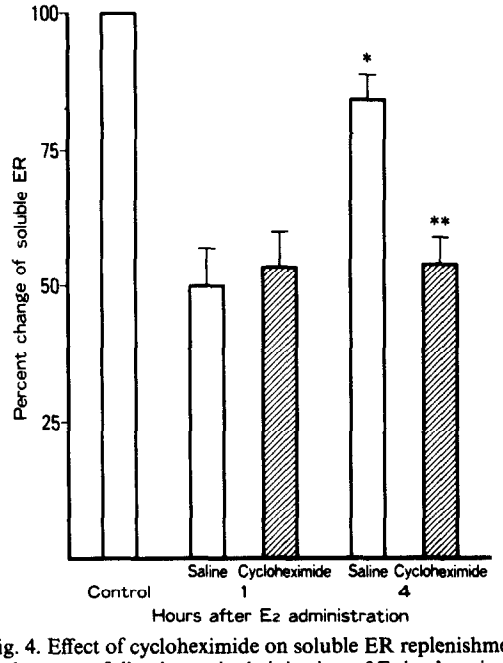

Fig. 4. Effect of cycloheximide on soluble ER replenishment in the uterus following a single injection of E_2 in chronically estrogenized rats. Castrated and E_2-implanted rats were injected i.p. with 5 μg E_2 combined with 150 μg cycloheximide or saline. The rats for control were injected i.p. with saline alone. Values are the mean ± SE of 4 determinations. A one-way analysis of variance was used for the statistical analysis. *Difference between the values at 1 and 4 h after the injection of saline, $P < 0.01$. **Difference between the values of saline and cycloheximide treated rats at 4 h after the injection, $P < 0.01$.

Changes in levels of soluble ER after the administration of 5 μg E_2 in the absence or presence of cycloheximide are summarized in Fig. 4. In the absence of cycloheximide, soluble ER decreased to 49.7 ± 6.9% of the pre-injection level 1 h after the injection of E_2, but the depeleted ER level increased significantly 4 h after the administration. In the presence of cycloheximide, on the other hand, replenishment of ER was completely inhibited at 4 h. Consequently, a great difference was noted in the levels of soluble ER at 4 h after the injection of E_2 between the two groups (84.1 ± 3.8% vs 53.9 ± 4.6% of the pre-injection level in the absence and presence of cycloheximide, respectively).

DISCUSSION

In contrast with the findings in ovariectomized and non-estrogenized rats, replenishment of soluble ER as well as reduction of accumulated nuclear ER following a single injection of E_2 were rapid in chronically estrogenized rats as shown in Fig. 1. The present results indicate that the rapid replenishment of uterine ER in the chronically estrogenized rats is entirely dependent on protein synthesis.

Sato *et al.*[24] reported similar rapid ER dynamics following an additional E_2 treatment in the uterus of DES-implanted rats. Using a density labeling technique *in vitro*, Eckert *et al.*[14] recently found that ER is synthesized rapidly in MCF-7 breast cancer cells. ER synthesis, therefore, seems to occur more rapidly than previously believed.

Earlier studies have indicated that treatment with physiological amounts of estrogen induces ER in the rat uterus [25], while high doses reduce ER [26]. Although the uteri seemed to be fully stimulated 7 days after E_2-implantation in terms of uterine weight, DNA content and progesterone receptor content, the amount of ER was not decreased by the E_2-implantation. Although serum E_2 concentration in the estrogenized rats was higher than that in normal female rats at diestrus, the concentration was similar to that at proestrus. Therefore, the uteri seem to be chronically exposed to physiological but not pharmacological doses of E_2 in the present study.

The question whether replenishment of soluble ER following nuclear translocation of the receptor pool is caused by receptor recycling or by newly synthesized receptors is a question in biochemistry of steroid receptor for a long time. Although several studies on receptor replenishment have been performed, the results obtained are inconsistent [9–13].

As previously discussed, elucidation of the mechanism of ER replenishment after E_2 injection is complicated by the following reasons. First, the replenishment is a slow process, requiring long periods. Second, E_2 causes an increase of total ER content due to newly synthesized receptor. Since a rapid replenishment of ER occurred and soluble ER was not increased by an additional administration of E_2 in the chronically estrogenized uteri, the chronically estrogenized uteri used in the present study seem to be a suitable model for studying the mechanism of ER replenishment by E_2. Cycloheximide, at a dose utilized in the present study that effectively blocked protein synthesis, was found to have no significant effect on the amount of ER content at least for 4 h after the treatment in the estrogenized rats. Furthermore, cycloheximide did not affect translocation of ER, which is consistent with the previous findings reported by others in rat uterus [10, 27].

Our findings that protein synthesis is entirely necessary for replenishment of ER are not completely agreeable with those reported previously. Previous papers [11, 12] have shown that replenishment of uterine ER following injection of E_2 is at least in part but not completely inhibited by inhibitors of protein synthesis. Kassis and Gorski[13] concluded that replenishment of uterine ER following 16α-estradiol injection does not require a new protein synthesis and the replenishment may be due to recycling. Cidlowski and Muldoon[28] reported that replenishment of pituitary ER was no more sensitive to cycloheximide treatment when ovariectomized rats were replaced with estrogen. This finding [28] is inconsistent with our present and previous findings [16]. The difference between results obtained by us and those by others may be caused by different experimental conditions as previously discussed.

Through both the present and previous findings [16] obtained in the uterus and pituitary, we have clearly demonstrated that replenishment of ER in the estrogen target tissues following E_2 injection is entirely dependent on protein synthesis in chronically estrogenized rats.

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