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EVIDENCE THAT IN VIVO ESTRADIOL RECEPTOR TRANSLOCATED INTO NUCLEI IS DEPHOSPHORYLATED AND RELEASED INTO CYTOPLASM

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Most of the estradiol binding activity is lost after the receptor-hormone complex migrates into the nuclei. We report that at the same time an equivalent amount of receptor unable to bind hormone appears in cytosol. ATP incubated with uterine cytosol from 17β-estradiol injected mice causes an increase in hormone binding activity. This activation is catalyzed by the ATP-dependent enzyme which reactivates hormone binding of estradiol receptor previously inactivated by a nuclear phosphatase (1). The inactive receptor retains the property to be activated by the ATP-dependent enzyme after a partial purification by heparin-Sepharose. A large dose of cycloheximide does not inhibit the appearance of ATP-activated cytosol receptor. Apparently the 17β-estradiol receptor which migrates into the nucleus is inactivated by the nuclear phosphatase and then released in an inactive, dephosphorylated form into the cyoplasm.

INTRODUCTION

Estradiol binds to the receptor in the cytoplasm of target cells followed by the translocation of the hormone-receptor complex into the nuclei where it induces transcriptional changes (2). The consequent rapid nuclear loss of receptor seems to be required for various hormonal responses, e.g., replenishment of cytosol estrogen-binding activity (3,4) and induction of progesterone receptor (5). Receptor loss appears to be stimulated by progesterone (6). It has been recently observed that estradiol receptor is inactivated in vitro by a nuclear phosphatase (7,8) and reactivated by a cytosol ATP-dependent enzyme (1) Evidence for such a process of nuclear dephosphorylation and cytoplasmic phosphorylation that also regulates receptor hormone-binding activity in vivo is still lacking. However, the stability of antiestrogen-receptor complexes in nuclei of intact cells (5 and 9-13) is consistent with the stability of the same complexes in vitro in presence of the nuclear phosphatase (14) and thus supporting such a possibility.

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Here we report that in uteri of mice injected with 17β-estradiol, nuclear migration of receptor is rapidly followed by the appearance in cytosol in inactive receptor which can be reactivated in vitro by the ATP-dependent enzyme (1). These findings strongly support our hypothesis (1,15) according to which estradiol receptor, translocated into nuclei of intact cells, is inactivated by nuclear phosphatase, and then released into the cytoplasm where it could be either r cycled through phosphorylation or, as the present experiments suggest, proteolyzed.

MATERIALS AND METHODS

Materials: Reagent grade materials were used in all experiments. 17β-estradiol, activated charcoal (Norit A) and heparin, grade 2, were from Sigma Chemicals Company, St. Louis, MO, U.S.A. Dextran T70 and Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden; gelatin from Serva, Heidelberg, Germany. 2,4,6,7[^{3}H]17β-estradiol (85 Ci/mmol) was obtained from the Radiochemical Center, Amersham, Bucks, U.K. Cycloheximide was from Calbiochem, San Diego, CA.

Buffers: The following buffer solutions were used: 50 mM Tris/HCl, pH 7.4, containing 2 mM dithiothreitol, 1 mM EDTA without (TED-buffer) or with 0.25 M sucrose (TED-sucrose buffer).

Heparin-sepharose preparation: Heparin was coupled with CNBr-activated Sepharose 4B as previously described (16,17).

Tissue homogenization and fractionation: Uteri from 5 days ovariectomized mice were used immediately after sacrifice. Tissues were mixed with 10 vol of cold TED-sucrose or TED-buffer and homogenized according to a previously reported procedure (17). The 750 x g pellet (nuclei) was obtained by centrifugation of homogenate at 2°C for 10 min. Cytosol was prepared either from homogenate or from 750 x g supernatant by centrifugation at 150,000 x g for 30 min at 2°C in an L5-75 Beckman ultracentrifuge, using a Ti 50.2 or Ti 75 rotor.

Separation of receptor from ATP-dependent enzyme activating hormone binding of 17β-estradiol receptor: Cytosol (7 ml) was labelled with 12 nM [^{3}H]17β-estradiol with or without a 100-fold excess of cold hormone ([^{3}H]estradiol of high and low specific activity) overnight at 4°C, then filtered through 0.5 ml of heparin-Sepharose column. The breakthrough was used as enzyme preparation activating 17β-estradiol binding. The column was washed with 1 ml of TED buffer and the receptor eluted with 1.5 ml of 0.3 M KCl.

Specific 17β-estradiol binding activity: The specific steroid was determined as previously reported (1). Bound hormone was separated from free hormone by dextran-coated charcoal treatment.

NaSCN exchange method for the assay of cytosol and nuclear receptor: This was performed as previously reported (14 and 18).

Radioactivity assay: Samples were added to 3 ml of Instagel (Packard Instrument Co., Dowers Grave, IL., U.S.A.) in scintillation vials and the radioactivity measured in a Beckman LS-7000 counter with 45% efficiency.

RESULTS

Group of mice were killed at different times after injection of 17β-estradiol. One group was not injected. Uteri were used to prepare nuclei and cytosol.

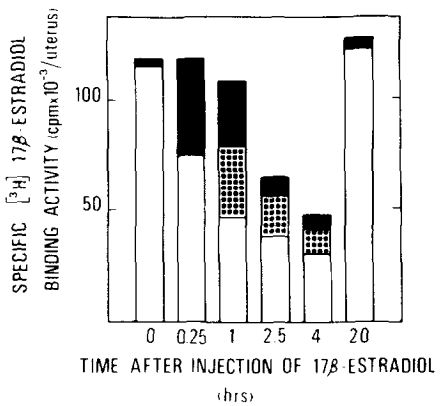

Fig. 1 Time course of subcellular distribution of 17β-estradiol receptor and of the appearance of cytosol ATP-activated receptor from mouse uterus after a single injection of 17β-estradiol. Adult 5 days ovariectomized mice were intraperitoneally injected with 2 μg of 17β-estradiol and killed in groups of 15 at the indicated times. One group of mice was not injected (0 time). Uteri were homogenized in cold TED-sucrose buffer. Nuclei were assayed for specific estradiol binding activity by the NaSCN exchange method (□). Cytosol, prepared from nuclear supernatant, was added at 0°C with 12 nM [^{3}H]17β-estradiol of high and low specific activity. After about 30 min of incubation 10 mM Na_2MoO_4 and 10 mM $MgCl_2$ were added and the cytosol was incubated at 15°C for 10 min in the absence and presence of 10 mM ATP. Samples were left overnight at 0°C, then assayed for specific estradiol-binding activity. Cytosol-binding activity in the absence of ATP (□) is reported as well as cytosol ATP-activated binding activity (□) calculated from the difference in cytosol-binding activity in the presence and absence of ATP. The values reported in the figure are averages of three experiments.

Cytosol was incubated with [^{3}H]17β-estradiol at 15°C for 10 min in the absence and presence of ATP, left overnight at 0°C and then assayed for estradiol specifi binding activity. Nuclei were also assayed for estrogen-binding activity using the NaSCN exchange methods (18). The results of these experiments are reported in Fig. 1. In untreated mice, almost all the receptor is localized in cytosol (called 0 Time in the Figure). Fifteen minutes after hormone injection, nuclear translocation of cytosol receptor causes nuclear-binding activity to drastically increase and cytosol activity to proportionally decrease. No increase in binding activity is observed after incubation of ATP with uterine cytosol from either not injected mice or from mice killed 15 min after 17β-estradiol injection. One hour after hormone injection, total receptor (cytosol plus nuclear-binding activity) appears to decrease. However, incubation of cytosol with ATP increases specific hormone binding almost restoring the total activity level present in control mice. The binding activity determined by incubation with ATP is called ATP-activated cytosol-binding activity. A decrease of nuclear and cytosol

activity, 2.5 and 4 hrs after injection including the ATP-activated cytosol activity, indicates a drastic real reduction of receptor. Twenty hours after 17β-estradiol injection almost all the receptor is localized in the cytosol as found in control mice; no increase of binding activity is observed after incubation of cytosol with ATP in mice killed 20 hrs after injection.

The effect of a large dose of cycloheximide on ATP-induced receptor activation in uterine cytosol from mice injected with 17β-estradiol was studied. Two groups of adult ovariectomized mice were injected with 17β-estradiol and killed 60 min later; one group of mice was injected with cycloheximide (1 mg per mice) 15 min before hormonal treatment. Uteri were removed and used to prepare nuclei and cytosol. As expected, incubation of cytosol from hormone-treated mice with ATP results in a significant increase of specific [^{3}H]17β-estradiol-binding activity. Cycloheximide does not reduce this increase, thereby suggesting that protein synthesis is not required for the appearance of ATP-activated receptor in cytosol of hormone-injected mice. This suggestion is supported by previous unpublished experiments from our laboratory (Rotondi, A. and Auricchio, F.) which have shown that total protein synthesis of mammary gland of adult Swiss mice is 90 and 70% inhibited 15 and 75 min respectively after intraperitoneal injection with 1 mg cycloheximide (see Table 1).

For the experiment presented in Fig. 2, uterine cytosol from untreated mice and from mice killed 60 min after an injection of 2 μg 17β-estradiol were labelled with high and low [^{3}H]17β-estradiol specific activity. Half of the cytosol from hormone-treated mice was incubated with ATP and the other without. Samples were then centrifuged through a sucrose gradient (Fig. 2). Cytosol from not injected mice shows two peaks of specific binding activity sedimenting at about 4 and 9 S. Cytosol from injected mice shows a drastic reduction of both peaks. Cytosol from injected mice incubated at ATP, shows a pattern similar to that of cytosol from untreated mice, although the heavier peak is regenerated to a major extent than the lighter peak.

Cytoplasmic estradiol receptor can be separated from the ATP-dependent enzyme, which is responsible for activating hormone binding of receptor, by heparin-Sepharose (1). Uterine cytosols from not injected mice and from mice

TABLE 1

EFFECT OF CYCLOHEXIMIDE ON THE SUBCELLULAR DISTRIBUTION OF UTERINE 17β-ESTRADIOL RECEPTOR AND ON ACTIVIATION BY ATP OF CYTOSOL BINDING ACTIVITY FROM MOUSE UTERUS AFTER 17β-ESTRADIOL INJECTION

TREATMENT	CYTOSOL BINDING ACTIVITY IN ABSENCE OF ATP	CYTOSOL ATP-ACTIVATED BINDING ACTIVITY	NUCLEAR BINDING ACTIVITY	TOTAL BINDING ACTIVITY
Mice injected with 17β-estradiol	37,014 (33)	47,134 (42)	27,966 (25)	112,114 (100)
Mice injected with cycloheximide and 17β-estradiol	41,556 (33)	49,871 (39)	35,469 (28)	126,896 (100)

A group of 15 adult ovariectomized mice was intraperitoneally injected with 2 μg of 17β-estradiol and killed 60 min later (mice injected with 17β-estradiol). A second group was treated in the same way and in addition it was intraperitoneally injected with 1 mg of cycloheximide 15 min before injection with 17β-estradiol (mice injected with cycloheximide and 17β-estradiol). Uteri were homogenized in cold TED-sucrose buffer and cytosol binding activity in absence of ATP, cytosol ATP-activated binding activity and nuclear binding activity were measured according to the procedure reported in the legend to Fig. 1 and expressed as cpm/uterus and percentage of total binding activity (in parenthesis).

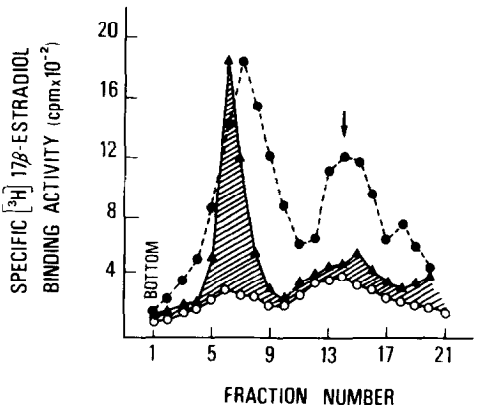

Fig. 2. Sedimentation patterns through sucrose gradient of specific [^{3}H]17β-estradiol-binding activity of uterine cytosol from mice non-injected or injected with 17β-estradiol. Two groups of 5 adult ovariectomized mice were killed without treatment, and 1 hr after injection with 2 μg of 17β-estradiol. Uteri were homogenized in TED buffer and cytosol added with 12 nM [^{3}H]17β-estradiol of high and low specific activity at 0°C. After about 30 min 10 mM Na_2MoO_4 and 10 mM $MgCl_2$ were added and the cytosol was incubated at 15°C for 10 min in the absence or presence of 10 mM ATP. Samples were left overnight at 0°C then treated with charcoal and aliquots of 0.3 ml layered on the 10-35% (w/v) sucrose gradients. Centrifugation was in a Beckman L8-55 centrifuge, rotor 65 VTi at 55,000 rpm for 2 hrs at 2°C, fraction volume was 0.25 ml. The arrow indicates the peak of the reference protein, bovine plasma albumin (4.45 S). Symbols: binding activity of uterine cytosol incubated in the absence of ATP from untreated mice (o--o) and binding activity of uterine cytosol incubated in the absence (o--o) or presence of ATP (Δ--Δ) from hormone treated mice. The shadowed area represents the ATP-activated cytosol binding activity.

killed 1 hr after 17β-estradiol injections were used as starting material. The two receptor preparations were purified by heparin-Sepharose chromatography and separately incubated with ATP in the absence and presence of each of the two enzyme preparations. The results are reported in Table 2. Addition of enzyme from non injected or injected mice has little effect on receptor from non injected mice; in contrast, both enzyme preparations have a similar and significant activating effect on receptor purified from 1 hr injected animals. The extent of this activation is similar to that observed by incubating crude cytosol from 1 hr injected mice with ATP (see Fig. 1). Therefore, hormone treatment stimulates the ATP-dependent reactivation process through a modification of receptor rather than by an increase of enzymatic activity.

DISCUSSION

Most of the estradiol receptor is rapidly inactivated after the receptor-hormone complex translocates into nuclei. The loss of estradiol-binding

TABLE 2

EFFECT OF ATP-DEPENDENT ENZYME ON ACTIVATION OF HORMONAL BINDING OF UTERINE CYTOSOL RECEPTOR

	SPECIFIC [^{3}H]17β-ESTRADIOL BINDING ACTIVITY	SPECIFIC [^{3}H]17β-ESTRADIOL BINDING ACTIVITY INCREASE DUE TO ENZYME
RECEPTOR 0 TIME + BUFFER	352,998	-
RECEPTOR 0 TIME + ENZYME 0 TIME	369,039	16,041 (5)
RECEPTOR 0 TIME + ENZYME 1 HOUR	372,519	19,521 (6)
RECEPTOR 1 HOUR + BUFFER	125,765	-
RECEPTOR 1 HOUR + ENZYME 0 TIME	222,755	97,000 (77)
RECEPTOR 1 HOUR + ENZYME 1 HOUR	230,633	104,878 (83)

Ten adult ovariectomized mice were injected with 2 μg of 17β-estradiol and killed 1 hour later. Another group of 10 adult ovariectomized served as controls and were not injected. Uterine cytosols were labelled overnight with 12 nM [^{3}H]17β-estradiol of high and low specific activity and enzymes were separated from receptors by heparin-Sepharose chromatography. Receptor preparations from non injected (receptor 0 time) and injected mice (receptor 1 hour) were added with 10 mM $NaMoO_4$, 10 mM $MgCl_2$ and 10 mM ATP and incubated at 15°C for 10 min in the absence or presence of an equivalent amount of enzyme from non injected (enzyme 0 time) or injected mice (enzyme 1 hour). Hormone-binding activity of samples was measured, referred to the entire receptor preparation and reported as cpm under specific binding activity. The difference in binding of each receptor preparation in the presence and in absence of enzyme was then calculated and reported under specific binding activity increase due to the enzyme as cpm as well as percent of binding in absence of enzyme (in parenthesis).

activity results in a decrease of total receptor ("receptor processing") (5). It has recently been reported that a nuclear phosphatase inactivates the estradiol-binding activity of receptor in vitro (7,8); this activity can be restored by a process requiring a cytosol enzyme, apparently a receptor kinase (1)

The enzymatic reactivation of receptor inactivated by nuclei has been utilized in the present paper to investigate whether in intact cells the receptor, after its translocation into nuclei, is inactivated by a dephosphorylation process and then released into the cytosplasm. Our results favor this hypothesis. The appearance of specific estrogen-binding activity, activated by incubation of cytosol with ATP, rapidly follows translocation of receptor into nuclei and parallels the initial loss of binding activity. Furthermore, 1 hr after hormonal injection, the ATP-dependent increase of uterine cytosol binding activity almost restores the total level of estrogen-binding activity present in non injected mice. Incubation of cytosol with ATP also regenerates receptor with a pattern of sedimentation through sucrose gradients similar to that of receptor from uterine cytosol of non-injected mice, whereas the pattern is drastically reduced after hormone treatment. These data suggest that the receptor activated by ATP in cytosol of treated mice is also present in cytosol of untreated mice and that it is inactivated by nuclei after nuclear translocation. The inactivation seems to be due to the dephosphorylation process previously observed in vitro (7,8) since it is reverted by ATP in the presence of the cytosol enzyme (1).

The enzyme which activates the receptor for hormonal binding in intact cells could, besides recycling the receptor "processed" by nuclei, also phosphorylates newly synthesized receptor (1). The lack of effect of cycloheximide on the amount of ATP-activated receptor seems to exclude this possiblity.

What is the in vivo fate of the receptor apparently released into the cytoplasm after nuclear dephosphorylation? The rapid disappearance of ATP-activated receptor suggests that at least after a large "non physiological" dose of hormone injected in order to obtain a massive translocation of receptor into nuclei, most of the dephosphorylated receptor is proteolyzed. It is possible that in a more physiological hormonal environment, rephosphorylation

of a small amount of receptor inactivated by nuclei occurs, which in part or completely prevents receptor proteolysis.

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