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The Formation at 37 C of a Nondissociable Receptor-Estradiol Complex

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SUMMARY: The receptor-estradiol complex formed in rat uterine cytosol when heated at 37 C converts from a dissociable to a nondissociable form. The conversion is best observed in cytosols pretreated with charcoal at 0 C which renders the subsequently formed receptor-estradiol complexes thermostable at 37 C. In the presence of dithiothreitol the heated complex remains dissociable. Tamoxifen does not form nondissociable complexes with the estradiol receptor. It is proposed that the nondissociable form of the receptor complex is a required phase in the mechanism of estradiol action.

The in vitro study of hormone binding by the estradiol receptor has been so far restricted to temperatures below the physiological temperature, most generally 2-4 C, because of the thermal instability of the receptor and its hormone complex (1, 2). Recent work in this laboratory has uncovered that treating the target tissue cytosol with charcoal prior to incubation with the hormone imparts thermal stability to the receptor-estradiol complex (ER) (3, 4). Using such charcoal pretreated cytosol, it has now been established by several experimental criteria that the ER complex which is readily dissociable at lower temperatures undergoes a transformation to a nondissociable form (NER) at 37 C. By contrast, the complex formed with the antiestrogen Tamoxifen (Tam) is not transformed to a nondissociable form.

This communication deals with the experimental evidence for the transformation phenomenon and its possible role in the mechanism of hormone action.

MATERIALS AND METHODS

Phosphate buffer (PBS), pH 7.3, was composed of 0.01 M sodium phosphate, 0.15 M NaCl, and 0.01% sodium azide. Uteri were excised from mature Sprague-Dawley rats at random stage of the estrous cycle. They were slit along the horns, rinsed in 0 C saline and shattered to fine powder in a steel mortar cooled with liquid nitrogen. The powder was homogenized at 0-2 C in PBS, 2 ml buffer/uterus, and the homogenate centrifuged at 105,000 x g to obtain the cytosol. A minimum of 6 uteri were pooled in each cytosol preparation.

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Dextran-coated charcoal (DCC) was prepared by dispersing 5 g of Norit A (Fisher) charcoal in 100 ml PBS containing 0.5 g of dissolved Dextran T70 (Pharmacia).

Cytosol was pretreated with charcoal, 15 mg/ml, by gently dispersing in it a DCC pellet with a spatula, and then incubating the quiescent system on ice for 90 min with occasional redispersion of the sedimented charcoal. Vigorous agitation of the mixture causes losses in receptor binding and should be avoided. The DCC was removed by two successive centrifugations at 2000 x g.

Aliquots of cytosol, 0.2 ml, were incubated with 5 nM [^{3}H]estradiol (New England Nuclear) in the presence and absence of a 200-fold molar excess of diethylstilbestrol (DES) (Sigma) or of a 1000-fold excess of Tam citrate (ICI America) in a total incubation volume of 1.0 ml. Incubations at 37 C were preceded by a 16 hr incubation at 4 C and were followed by a 2 hr incubation also at 4 C.

In hormone binding assays, to remove the free ligand in the incubated solution, 0.1 ml of the dispersed DCC was added to each solution at 0 C. After 15 min the mixture was centrifuged and the supernatant decanted directly into a scintillation vial. All tests were carried out in triplicate.

The integrity of the [^{3}H]estradiol following 37 C incubations with the cytosol was verified by TLC and reverse isotope dilution tests. Similarly, the preservation of Tam properties at 37 C was verified by heating Tam with cytosol for 5 hr at 37 C and then showing that aliquots of this solution inhibited effectively estradiol binding to the receptor in 4 C incubations.

RESULTS

The results in Fig. 1 provide evidence that the receptor-estradiol complex, ER, is transformed to a nondissociable form (NER) at 37 C. When excess DES is added at 4 C to DCC pretreated cytosol which has been preincubated with [^{3}H]estradiol at 4 C and the new mixture is incubated at 37 C, the DES, as expected, substitutes for the labeled hormone in the receptor complex. When however the DES is added to a solution which has been preincubated at 37 C with [^{3}H]estradiol and the new mixture is further incubated at 37 C, the DES now fails to displace the bound ligand. Similar results were obtained with unlabeled estradiol as the competing ligand. This transformation at 37 C of ER to NER is completed within 30 min. In sucrose gradient centrifugations (results not shown) the NER was found to sediment at 4S.

Excess Tam just like excess DES displaces the labeled hormone from the ER complex at 37 C when it is added to the solution prior to incubation at 37 C. However, in contrast to DES, with continuing 37 C incubation the Tam in the receptor complex is gradually displaced back by the [^{3}H]estradiol (Fig. 1).

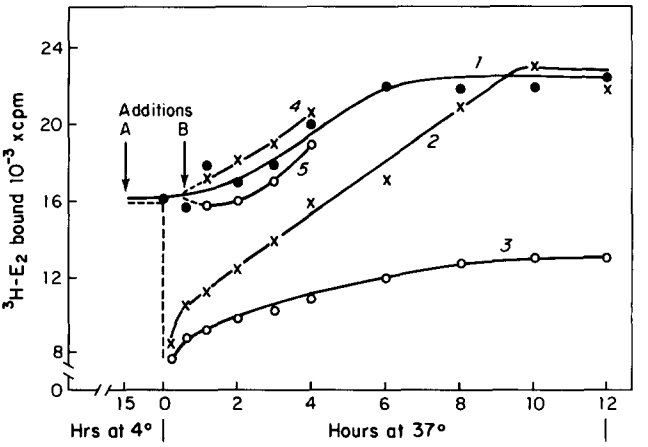

Fig. 1. Excess DES or Tam added to cytosol preincubated at 37 C with [^{3}H]estradiol fails to displace the labeled ligand from the receptor complex. DCC pretreated cytosol was incubated with 5 nM [^{3}H]estradiol for 16 hr at 4 C, then at 37 C, and hormone binding was assayed after various periods at that temperature. The control set of data so obtained is shown in Curve 1. In a parallel set of incubations 1 μM DES or 5 μM Tam, at final concentration, was added at 4 C to cytosol plus [^{3}H]estradiol mixtures which had been preincubated at 4 C for 15 hr. The point of addition is shown by the arrow marked A. The new mixtures were incubated at 4 C for 1 hr then at 37 C for the same periods as the solutions in the control set. Binding of [^{3}H]estradiol in the presence of Tam is shown in Curve 2 and in the presence of DES in Curve 3. In another set of parallel incubations the Tam and DES additions were made to cytosol plus [^{3}H]estradiol mixtures preincubated 16 h at 4 C and then 30 min at 37 C. The point of addition is shown by the arrow marked B. The new mixtures were incubated at 37 C and bound [^{3}H]estradiol was assayed at the same time intervals as in the other sets. The Tam results are shown in Curve 4 and the DES results in Curve 5. The dashed lines connect, for orientation, [^{3}H]estradiol binding at the point of competitor addition with the first measured binding after this addition.

Since as mentioned earlier the properties of Tam are preserved in the cytosol at 37 C, the observed changes cannot be attributed to Tam metabolism. This suggest that unlike the estrogenic ligands, estradiol and DES, the antiestrogen Tam does not form nondissociable complexes with the receptor and consequently despite its excess quantity in the incubaton mixture, it is eliminated from receptor binding by the progressive formation of NER at 37 C.

The results in Fig. 2 provide further evidence for the formation of NER at 37 C. Rat uterine cytosol, DCC pretreated, was incubated with [^{3}H]estradiol ± DES at 4 C. When the test solutions were then further incubated but now in the presence of newly added DCC (5 mg/ml) the [^{3}H]estradiol was substantially removed from the solution within 10 min when the temperature was raised to 37 C (left panel). This is because at 37 C the ER complex dissociates, not

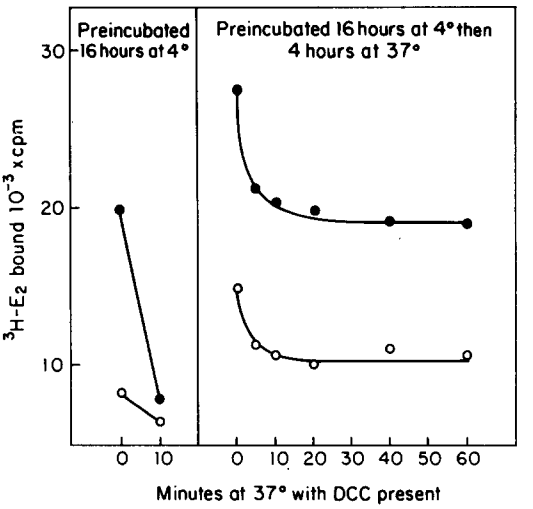

Fig. 2. Charcoal added to cytosol preincubated with [^{3}H]estradiol at 37 C fails to strip the ligand from the receptor complex. DCC pretreated cytosol was preincubated with 5 nM [^{3}H]estradiol ± 1 μM DES for 16 hr at 4 C only or additionally for 4 hr at 37 C. To each test solution (1.0 ml) was then added 0.1 ml of DCC dispersion (5 mg charcoal). The systems were incubated for various periods at 37 C and finally at 0 C for 15 min. Charcoal was removed by centrifugation and the solution counted. The closed circles show [^{3}H]estradiol binding in the absence of DES, the open circles in the presence of excess DES. The left panel shows results with solutions which were preincubated at 4 C only, the right panel with solutions which were preincubated additionally at 37 C.

having had time to be converted to the nondissociable form, and the free hormone is sequestered by the DCC. If, however, the DCC is added to solutions which were preincubated at 37 C and the incubation then continued at 37 C the DCC now removes only some non-specifically bound hormone but specific binding, as reflected by the difference in binding in the presence and absence of DES, is preserved (Fig. 2).

In a separate set of experiments the DCC pretreated cytosols included 10 mM dithiothreitol (Calbiochem). Such cytosols also provide thermostable ER complexes but with the distinction that these complexes appear to remain dissociable following 37 C incubation. Excess DES or Tam when added to such cytosols after these were preincubated with [^{3}H]estradiol at 37 C, routinely displaced the estradiol from the ER complex. Also, in these cytosols excess Tam appears to inhibit specific estradiol binding in extended incubations at 37 C without decrease in inhibiting capacity.

DISCUSSION

It is a reasonable hypothesis that the mechanism of steroid hormone action should include a stage where at 37 C the receptor-hormone complex assumes a less dissociable form when it is residing at or activating the genomic site.

The results offered here reveal the existence of such a state of the ER complex. The physiological significance of this state is supported by the distinction that the receptor complex with the synthetic antiestrogen Tam fails to convert to a nondissociable state.

The effect of DTT on the system points to the probable involvement of sulfhydryl groups in obtaining the tightly bound ER configuration. Since DCC pretreatment in the presence or in the absence of DTT leads equally to a thermostable ER, the achievement of thermostability and the transformation to NER must be regarded as two separate processes where the former represents an *in vitro* process permitting the observation of the latter which is physiological in nature.

It has been noted in this laboratory and by others (1) that 20-30% of the ER produced in incubations with untreated cytosol appears to be thermostable at 37 C for at least 24 hr. We have now confirmed that this thermostable fraction is also of the NER form. This is evidence that charcoal pretreatment of the cytosol is not directly related to the ER transformation process.

It should be pointed out that DCC pretreatment of the cytosol imparts thermostability only to the ER complex. In the absence of hormone the receptor binding capacity is destroyed at 37 C. This distinction confirms that the binding observed at 37 C is not artifactual but is specific for the estrogen receptor.

Our idea that nondissociable complexes are the functioning entities at the nuclear site finds support in experimental results from other laboratories. To start with, the ability of tightly bound hormones to function in the nucleus is illustrated by 11β-chloromethyl estradiol which binds covalently to the estradiol receptor and is found to be fully estrogenic in rat uteri and in MCF-7 cell cultures (5, 6). Rossini and Liao (7) show that steroids are not

exchanged when the receptors are bound to chromatin. Jakesz et al (8) report that following continuous *in vivo* exposure of the rat to estradiol the receptor-estradiol complex found in the uterine cytoplasm is nondissociable and so is the KCl nonextractable complex in nucleus.

Our results bring new considerations to the mechanism of hormone action and underscore the point that receptor studies conducted at 0-4 C may have limited relevance to processes occurring at physiological temperature.

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