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Stabilization of Estradiol-Receptor Complexes by Elimination of Cytosolic Factors

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Treating mature rat uterine cytosol with dextran coated charcoal (DCC) for 2 hrs at 0-4C in the absence of ligand causes the subsequently formed receptor-estradiol complex to be stable at 37C. Receptor binding is increased by the DCC treatment for uteri excised at metestrus or diestrus but remains nearly unchanged for uteri obtained at proestrus or estrus. Results suggest that the DCC removes or inactivates factor(s) present in the cytosol which render the receptor complex thermolabile.

The hormone binding capacity of the estradiol receptor in in vitro conditions suffers loss on exposure to 37C and so most experimental data are generated at 0-4C. The belief is that the binding loss is caused by accelerated enzymatic degradation of the receptor at the physiological temperature (1-7). This report deals with newly uncovered features of receptor-hormone binding which suggest the involvement of other factors besides enzymes in the binding loss.

Although in the reported experiments the mature rat uterus was used as the model estradiol target tissue, quite similar results were obtained recently with receptor positive human breast cancer and ovarian cancer tissues.

The simple experimental procedure which yielded the new results should enlarge significantly the scope of investigations of estrogen receptors.

Materials and Methods

Uteri were excised from mature Sprague-Dawley rats (150-200 g) on the morning of a particular stage of the estrus cycle. Experiments were also performed on pooled uteri excised at no particular stage of the estrus cycle. A minimum of four uteri were pooled in each cytosol preparation. To obtain the cytosol, the uteri were shattered in liquid nitrogen, the powder homogenized with 2 ml buffer per uterus and the homogenate centrifuged at 105,000 x g for 60 min (2). The buffer, pH 7.3, was composed of 0.01M sodium phosphate, 0.15M NaCl, 0.1% gelatin and 0.01% sodium azide.

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

Cytosol was used intact or pretreated with DCC. In pretreatment, the DCC dispersion was centrifuged at 2000 x g for 10 min, the supernatant discarded, cytosol added, the DCC pellet redispersed and the system incubated on ice for 2 hrs, which was enough for maximum effect. The system was then centrifuged at 2000 x g in the cold, the supernatant cytosol decanted and centrifuged again to assure removal of any residual charcoal particles. One ml of the original DCC dispersion was used in preparing a pellet for treating 2 ml of cytosol.

Incubation solutions contained 0.2 ml of cytosol and 5 nM tritiated estradiol (6,7 ^{3}H-E_2, New England Nuclear) with or without 0.8 uM diethylstilbestrol (DES, Sigma Chemical) in a final volume adjusted to 1.0 ml with buffer. At the above ^{3}H-E_2 concentration receptor sites are saturated and the DES excess, when present, is sufficient to displace all the specifically bound hormone without materially affecting nonspecific binding (8).

In binding assays, 0.1 ml of the DCC dispersion was added to each test solution, the mixture incubated on ice for 15 min, centrifuged at 2000 x g for 10 min and the supernatant decanted directly into a scintillation vial. All determinations were performed in triplicate.

Nonspecific hormone binding is defined as that measured in the presence of excess DES, and specific binding as the difference in binding in the presence and absence of excess DES.

Results and Discussion

The experimental results provide evidence for two hypotheses:

1) At low temperature (4C) a fraction of the proteins which can bind estradiol specifically and nonspecifically is present in the cytosol in the form of inert agglomorates which disperse at higher temperatures with exposure of active hormone binding sites.

2) The rat uterine cytosol contains one or more factors which render the receptor-estradiol complex (RE) thermolabile at physiological temperature and may also inhibit the dispersion of binding protein agglomorates at higher temperature. These factors can be inactivated or removed by charcoal (DCC).

Fig. 1 shows an example of a time course curve for the thermal degradation of RE at 37C. As already noted by others (1, 9, 10) there is always a residue of specific binding which is temperature stable and this suggests the presence of at least two species of RE in the cytosol. Also shown in Fig. 1 is the time course for nonspecific binding which in contrast to specific binding increases at 37C. This increase is thought to reflect the gradual dispersion of binding protein agglomorates.

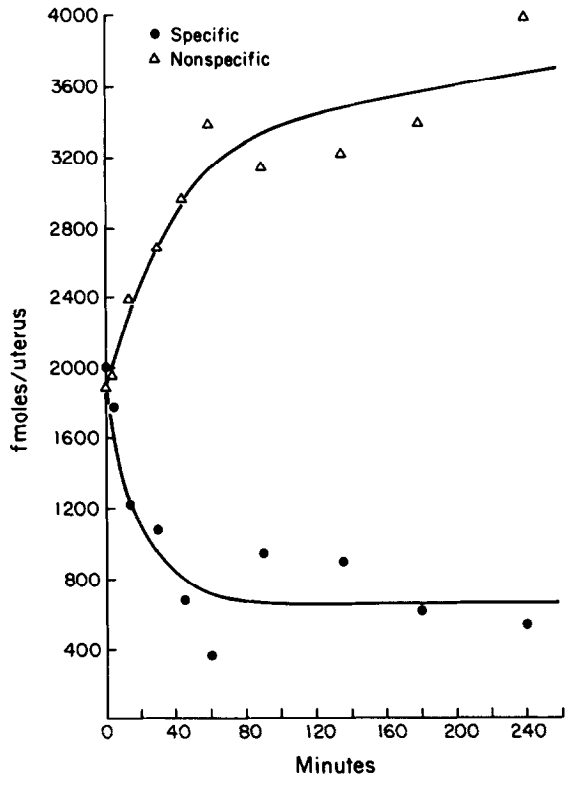

Fig. 1. Time course for the change in specific and nonspecific $^{3}H\text{-}E_2$ binding in untreated cytosol solutions on heating at 37C. Test solutions were preincubated at 4C for 16 hrs then heated for various periods at 37C and finally incubated for another 16 hrs at 4C.

Estradiol binding in untreated and DCC pretreated cytosol is compared in Fig. 2 for incubations at 4C and 37C. On incubation at 4C only (A), DCC pretreated cytosol shows often, but not always, a higher binding capacity which may be due to the removal of some endogenous estradiol.

Incubation at 37C (B, Fig. 2) causes a drastic drop in RE in untreated cytosol (as already illustrated in Fig. 1) while surprisingly with DCC pretreated cytosol it causes an increase in RE. In terms of the hypotheses stated above, the DCC pretreatment imparts thermostability to the RE and in so doing permits also the emergence of additional binding sites with the dispersal of receptor agglomorates.

Heating the cytosol at 37C in the absence of ligand destroys specific binding capacity (C, D, Fig. 2). Adding such a heated cytosol to untreated or DCC pretreated cytosol does not change specific binding at 4C (compare A and

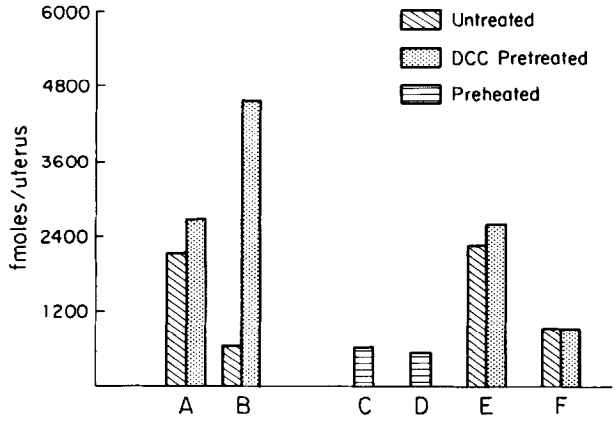

Fig.2. Specific ^{3}H-E_2 binding in cytosol test solutions following incubation at 4C and at 37C. Cytosols were: untreated, DCC pretreated, preheated (C, D), preheated mixed with untreated (E, F), preheated mixed with DCC pretreated (E, F). The total volume of each test solution was 1.0 ml and it contained 0.2 ml of cytosol except in the mixed systems (E, F) where it contained 0.2 ml of preheated cytosol in addition to 0.2 ml of either untreated or DCC pretreated cytosol. In calculating hormone binding in solutions with mixed cytosols, the presence of the preheated component was ignored. Preheated cytosol was prepared by heating untreated cytosol at 37C for 3 hrs in the absence of ligand. The results A, C, E are for incubations at 4C for 32 hrs. The results B, D, F are for 16 hr incubations at 4C followed by 7 hrs at 37C and a final 16 hrs at 4C.

E, Fig.2). However, when these mixtures are incubated at 37C the protective effect of DCC pretreatment on RE is completely eliminated (compare B with F, Fig. 2). The heated cytosol, therefore, retains in active form the factor or factors which impair RE thermostability.

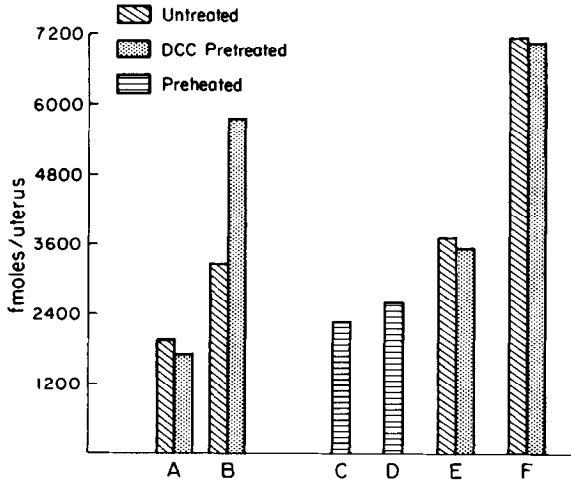

Fig. 3. Nonspecific ^{3}H-E_2 binding in cytosol solutions following incubation at 4C and at 37C. The test solution systems and incubations are described in legend to Fig. 2.

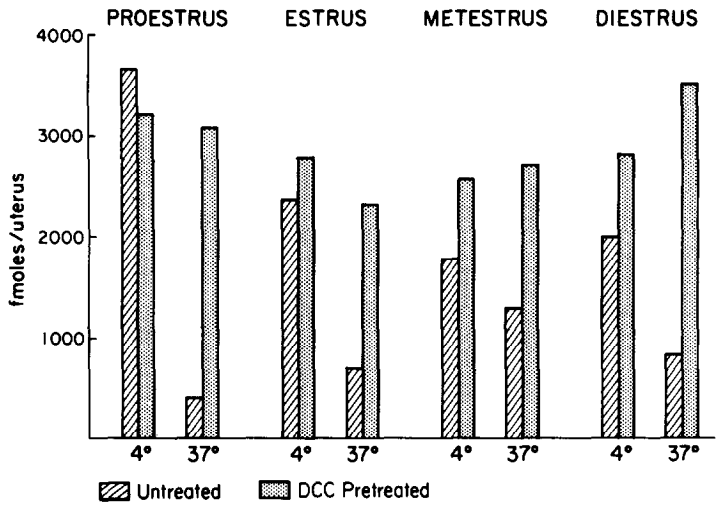

Fig. 4. Specific binding of ^{3}H-E_2 in cytosols from uteri excised at various stages of the estrus cycle. Comparison is made between untreated cytosol and DCC pretreated cytosol One set of test solutions was incubated for 32 hrs at 4C only. A second set was incubated 16 hrs at 4C followed by 7 hrs at 37C and a final 16 hrs at 4C.

In contrast to specific binding, nonspecific binding in untreated cytosol is increased by incubation at higher temperature (Figs. 1, 3) and DCC pretreatment of the cytosol enhances markedly this increase (A, B, Fig. 3). The assumption is that one of the factors removed or inactivated by DCC promotes the agglomoration of hormone binding proteins.

Heating the cytosol in the absence of ligand does not destroy nonspecific binding capacity (A, C, Fig. 3) and additions of heated cytosol to untreated cytosol test solutions increase nonspecific binding (A, E and B, F, Fig. 3).

Thermal stability of the cytosolic RE appears to be related to the estrus cycle stage at which the uterus was excised (Fig. 4). On incubating at 37C for 7 hrs, specific binding loss in untreated cytosol is 89%, 71%, 25%, and 51% for uteri at proestrus, estrus, metestrus, and diestrus respectively and the amount of RE stable at 37C seems to be inversely related to the amount of RE found in 4C incubation. As mentioned in the discussion of Fig. 1, the uterine cytosols may contain one variety of receptors which forms thermostable and another which forms thermolabile RE complexes. Fig. 4 results would indicate that the thermolabile variety comes to a maximum at proestrus and the thermostable at metestrus.

Pretreatment with DCC produces the most significant increase in specific hormone binding at 4C in metestrus and diestrus cytosols. This increase may reflect the removal of endogenous estradiol from the cytosol, although this view is difficult to reconcile with the mean plasma concentrations of estradiol in the rat which are reported to reach maximum in proestrus (11). In incubations at 37C the largest effect of DCC pretreatment on RE is seen with diestrus and proestrus cytosols. The reason for this may be that the diestrus and proestrus stages are in the preovulatory period of the cycle where in addition to the rise in receptor content in the uterus there is also possibly a rise in the content of factors which control receptor activity at 37C and which the DCC succeeds in removing from the cytosol.

No conclusions can be drawn from the present data whether the thermostable RE generated by DCC treatment is the same as the thermostable RE fraction in untreated cytosol. Another open question is whether the DCC treatment changes thermolabile RE to thermostable RE or whether it activates hormone binding by a species of receptor capable of forming thermostable RE.

Acknowledgment

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