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DIFFERENTIATION OF ESTRADIOL RECEPTORS IN RAT UTERINE CYTOSOL BY SENSITIVITY TO TAMOXIFEN

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Summary

Analysis of rat uterine cytosol by means of immobilized antibody discloses the presence of two distinct high affinity, low capacity estradiol binding components. One of these is readily saturable by the antiestrogen tamoxifen (TS = tamoxifen sensitive), the other is tamoxifen insensitive (TI). Only TS-estradiol binding shows positive cooperativity at low ligand concentration. TS but not TI is lost when frozen tissue is thawed at 4°C then refrozen and stored for an additional 12 hours. Experiments with ovariectomized rats show that TI is formed only in the presence of estradiol. In the estrus cycle TS increases in the order: metestrus, diestrus, proestrus and estrus. The quantity of TI is the same in metestrus and diestrus and also the same but fractionally higher in proestrus and estrus.

Estradiol antagonists of the triphenylethylene class induce estrogenic changes in the rat uterus but in distinction to estrogen agonists this activity is not sustained by additional administration of the antagonist and it does not extend to the full complement of estrogen sensitive cells in the uterine tissue. Some of the explanations for this behavior hinge on the long residence time of the antagonist-receptor complex in the nucleus which either prevents replenishment of the cytosolic receptor (1) or blocks further entry of receptor complexes into the nucleus (2). We considered the possibility that the antiestrogens bind to only some of the existing receptors in the cytoplasm and that they fail to exhibit normal estrogenic action because this requires the translocation to the nucleus of all types of cytosolic estrogen receptors. We now report evidence that tamoxifen, the antiestrogen studied by us, does indeed bind to only some of the cytosolic estradiol receptors in the rat

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uterus. The receptors which do not bind tamoxifen still bind estradiol with high affinity and the presence of the female hormone is required for their formation.

Materials and Methods

The method used in this work is a modification (3) of the one we reported on earlier (4, 5). Tritium labelled estradiol is complexed in a prior incubation with estradiol antibody immobilized on poly(vinylidene flouride) film. Upon incubation in a second solution comparisons are then made of the degree of dissociation of this complex in the presence and absence of vacant estradiol binders.

Cytosols. Uteri were removed from mature Sprague-Dawley rats. Cytosols were prepared as described by McGuire (6) and by McGuire and DeLaGarza (7). Six uteri were pooled for each cytosol preparation. Volume was adjusted to 4 ml per gram wet tissue. Prior to use each cytosol was treated with dextran coated charcoal (8) to remove any uncomplexed or weakly bound endogenous estradiol.

Tamoxifen Solution. Tamoxifen citrate (courtesy L. French, ICI America) was dissolved in ethanol, 25 mg/ml, and this alcoholic solution used to prepare a 2×10^{7}M solution in 0.01M phosphate buffer, pH 7.4, containing 9.0 g NaCl per liter (PBS).

Test Procedure. Each strip of polymer containing the same amount of immobilized antibody, IA, is incubated at 4°C for a minimum of 12 hours in 1.0 ml of ^{3}H-estradiol solution at one of several concentrations in the range from 10 to 200×10^{-12}M. The difference in the radioactivity of aliquots of the estradiol solution before and after incubation with IA is a measure of the amount of hormone complexed with the immobilized antibody. In the second stage, the strips with the immobilized complex, IAE, are removed from the tubes and each strip inserted in 0.8 ml of ice cold PBS (0.1% gelatin). One set of tubes receives an additional 0.2 ml of PBS per tube, in a second parallel set each tube receives 0.1 ml cytosol plus 0.1 ml PBS and in a third parallel set each tube receives 0.1 ml cytosol plus o.1 ml tamoxifen solution.

All systems are briefly agitated, incubated at 4°C for 12 hours or longer, and solution radioactivity determined. From the results as explained later, the equilibrium quantity of free hormone and of hormone complexed to the dissolved binder is evaluated. All experiments were in triplicate.

Results and Discussion

The immobilized antibody method differs importantly from other methods used for the study of estradiol receptors in that the procedure does not require a mechanical separation of receptor bound hormone from unbound hormone and the measurements are therefore made at unperturbed equilibrium and they are free of any possible artifactual distortions introduced by the separation step. Also in these tests the uncomplexed hormone is restricted to low concen-

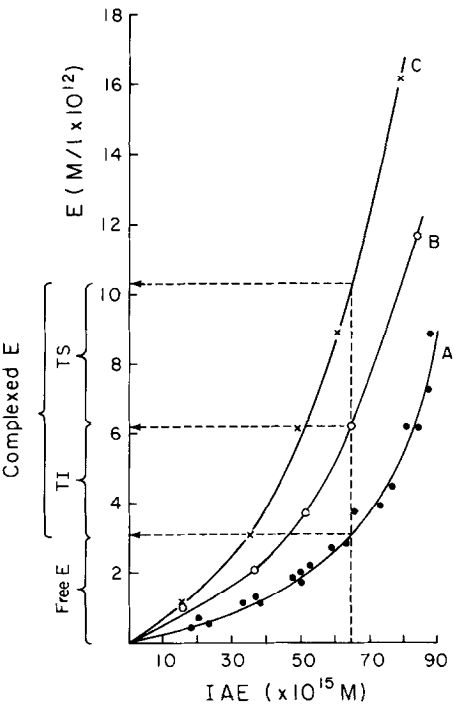


Figure 1. Estradiol complexed with immobilized antibody (IAE) in equilibrium with estradiol in solution. Each immobilized antibody strip carrying a known quantity of complexed tritium labeled estradiol was incubated at 4°C with one ml of physiological saline buffered at pH 7.4 and containing either (A) no additives, or (C) 0.1 ml rat uterine cytosol, or (B) 0.1 ml cytosol plus 20 picomoles of tamoxifen. The equilibrium quantity of estradiol released from IAE into the incubating solution is plotted here versus IAE corrected for the released estradiol. The manner of deriving information from these curves is discussed in the text. The results shown here are for uteri excised at the estrus stage. Similarly shaped curves were obtained with uteri at the other stages of the estrus cycle.

trations in the test solution with the desirable result that low affinity binders contribute negligibly to the overall hormone binding in the test solution (3).

Fig. 1 shows ^{3}H-estradiol dissociation equilibria at different levels of ^{3}H-estradiol complexed to the antibody. The tamoxifen present at concentration 2×10^{-8}M in one set of test solutions exceeds the concentration of free estradiol by a factor of at least 1000. If the tamoxifen had successfully inhibited the estradiol from binding to all available receptor sites, curve B would have been coincident with curve A. Since that is not the case we conclude that the cytosol contains both tamoxifen sensitive (TS) and tamoxifen insensitive (TI)

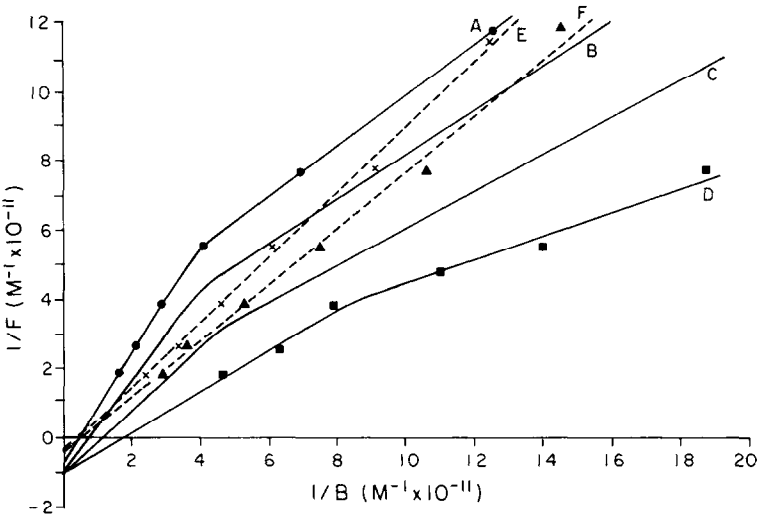


Figure 2. Double reciprocal plots for the tamoxifen sensitive (TS) and tamoxifen insensitive (TI) fractions in rat uterine cytosol at various stages of the estrus cycle. The TI curve for estrus was coincident with the TI curve for proestrus (curve E, dashed). Similarly, the TI curve for metestrus was coincident with the TI curve for diestrus (curve F, dashed). The designations of the TS curves are (A) estrus, (B) proestrus, (C) diestrus, and (D) metestrus. For clarity the points on curves B and C are omitted.

estradiol binders. In curve A the hormone released represents only free estradiol in solution, while in curves B and C it comprises free estradiol plus estradiol complexed to receptors and/or other high affinity binders in solution. At equilibrium the quantity of free estradiol in solution is uniquely related to the amount of estradiol remaining bound to the immobilized antibody (IAE). As a consequence, at the same value of IAE on curves A, B, and C, the quantity of free hormone in solution is the same and is specifically given by the curve A values. The quantity of hormone complexed in solution in equilibrium with a particular quantity of uncomplexed hormone is then simply, as illustrated in Fig. 1, the difference in the values at the ordinate axis between curve A and curves B and C at a particular IAE value. The values for complexed and for free estradiol in solution obtained from graphs such as those on Fig. 1 are plotted as reciprocals (Fig. 2) to obtain the total quantity and the association constant of estradiol binders in solution. The change of slope in the curves for TS in Fig. 2 is attributed to positive cooperativity, a phenomenon fre-

quently observed in the study of estradiol receptors at low concentrations of free estradiol (9,10). In contrast, the curves for TI do not exhibit such cooperativity.

The association constant found for TS is 1.0 x $10^{11}M^{-1}$ and that for TI is 0.5 x $10^{11}M^{-1}$. The values reported in the literature for the estradiol receptor range from 10^{9} to $10^{12}M^{-1}$ (9, 11-13). Because of the low concentration of free estradiol in the test solution the amount of complexed material did not exceed 30% saturation which limits the accuracy of the determinations (14). To check the reliability of the method at these conditions, sets of parallel tests were run with single and double volumes of antiestradiol antiserum and of pooled rat uterine cytosol. The results reflected well the doubling of the quantity of high affinity binding in the test solution. With cytosol the binding ratio found was 2.3 and with the antiserum it was 2.2.

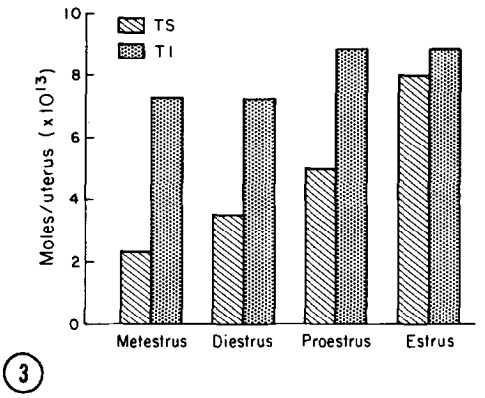
The possibility that the TI fraction represents non-specific low-affinity binding is discounted on three grounds. Firstly, as reported above, the experimental data extrapolates to a high value for the association constant for the TI-estradiol (TI-E) complex. Secondly, test solutions containing ^{3}H-estradiol, cytosol and tamoxifen when preincubated for 4 hours at 4°C before contact with IA show a much higher estradiol binding by solution components than do solutions incubated from the start with the IA present. This is evidence that the TI-E complex has a low rate of dissociation. Thirdly, at the low concentration of free estradiol (2 x 10^{-11}M) maintained in the present experiments, low affinity hormone binders such as albumin, even at several thousand fold molar excess over receptor or immobilized antibody should bind only negligible quantities of the hormone. Even for estradiol binders with affinity constant $10^{8}M^{-1}$ the share of hormone bound by them should not exceed 10% of the total bound if the molar ratio of these binders to receptors does not exceed 10.

These latter statements, based on hypothetical conclusions discussed in another report (3), have been verified experimentally. A portion of a rat uterine cytosol was mixed with a solution of albumin to provide a mixture containing 50 x 10^{-15}M of TS + TI binding sites and about 10^{-10}M of albumin per ml. This represents a molar ratio of albumin to TS + TI of at least 2 x 10^{3}. Tests conducted on this cytosol-albumin mixture yielded curves of the type shown in Fig. 1, which did not differ significantly from those obtained with albumin free cytosol.

Uteri frozen at -20°C for 24 hours show little change in TS and TI content compared to immediately tested unfrozen uteri. However, if the frozen uteri are thawed at 4°C and then refrozen and kept at -20°C for 12 hours or longer all TS binding is lost while TI content remains unaffected. We think that due to cell lysis and the loosening of cytoplasm structure caused by freezing and thawing, in the subsequent period of storage some TS is lost by proteolysis and the remainder is excluded from the cytosol by tight complexing with various cell components including sites at the cell wall.

The quantities of TS and TI in rat uterine cytosol at various stages of the rat estrus cycle as determined by the IA procedures are compared in Fig. 3. The pattern of changes in the TS quantity is similar to the one found by others for cytoplasmic estradiol binders (15, 16). The quantity of TI, however, appears to be the same in proestrus and estrus and lower but again about equal in metestrus and diestrus.

To explore the formation of the uterine TS and TI receptors we examined their relative content in ovariectomized rats at various times following estradiol and tamoxifen subcutaneous injection. In the control group (Fig. 4) TS is virtually the only estrogen binder present. In the estradiol treated animals 15 min after the injection there is significant reduction in the quantity of TS compared to the controls. This reflects rapid translocation of this receptor to the nucleus induced by the influx of estradiol (2, 17). At the same time, however, TI makes a strong appearance, surging to



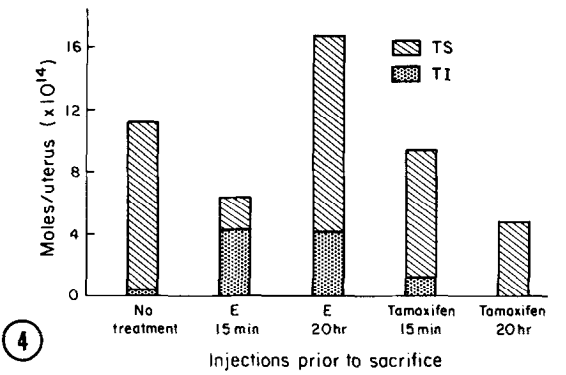


Figure 3. Comparison of the quantity of TS and TI fractions in rat uterine cytosol at various stages of the estrus cycle. The values were derived from the double reciprocal plots in Fig. 2.

Figure 4. Comparison of the quantity of TS and TI fractions in uterine cytosol of ovariectomized rats following estradiol and tamoxifen administration. All rats were primed with 1 µg estradiol injection at 92 and at 72 hr prior to sacrifice. The control group (6 rats) was left without additional treatment. Rats in the second group were injected with 5 µg of estradiol. Rats in the third group were injected with 50 µg of tamoxifen. In both groups half of the rats (6 rats) were sacrificed 15 min after the injection and the remainder 20 hours later.

over 60% of the unoccupied binding sites in the cytosol. The replenishment of cytosolic receptors with time (2) is clearly manifested by the sharp increase in binding capacity in rats sacrificed 20 hours after estradiol treatment. TI remains constant and the increase is restricted to TS.

In the tamoxifen treated animals, 15 min after injection, TI is a minor fraction of the overall binding while 20 hr later it is completely absent. The small reduction in binding 15 min after tamoxifen compared to the same time after estradiol injection reflects the slower translocation of the receptor to the nucleus under the influence of tamoxifen (2,17). The further reduction of binding at 20 hr may mean that the receptor under the particular experimental condition is not replenished (1) or that the rate of replenishment is slower than the rate of receptor translocation to the nucleus by residual tamoxifen in the organism (18). Another possibility is that TI is necessary for the replenishment process to be effective.

The presence of substantial quantities of TI only in the estradiol treated animals suggest that TI is induced by estradiol, possibly by the transformation of TS to TI, and that its translocation to the nucleus may be a slower process than that of TS.

Several investigators have already suggested that there may be more than one type of estradiol receptor in the uterine cytoplasm (9, 10, 19-21). Observations on the thermal stability of the receptor-estradiol complex (22,33) also hint at this possibility.

We now think that both TS and TI are necessary for sustained estrogenic activity in the uterus and that the antiestrogenic property of tamoxifen (and perhaps of other antiestrogens also) is insured by its twin disability: (a) to produce TI, and (b) to transport TI to the nucleus. Thus, even in the presence of estradiol, tamoxifen will insure that the TI and TS receptors will fail to translocate to the nucleus in their regular physiological concert.

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