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ANTIESTROGEN SPECIFIC, HIGH AFFINITY SATURABLE BINDING SITES IN RAT UTERINE CYTOSOL

Jean-Charles FAYE, Bernard LASSERRE and Francis BAYARD

INSERM U 168, Department of Endocrinology, C.H.U. Rangueil
Université Paul Sabatier, Toulouse 31054 F R A N C E

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SUMMARY. Analysis of rat uterine cytosol for Tamoxifen binding reveals that the saturable binding sites are only partially inhibited by estradiol-17β. Partial thermal denaturation of the cytosol at 30° C for 2 h 30 allows the characterization of a high affinity (Kd = 3.3 x 10^{-9}M) saturable Tamoxifen class of binding sites insensitive to estradiol-17β while remaining sensitive to the antiestrogens CI628 and Nafoxidine. The uterine concentration of these binding sites is lower in the uterus of immature or castrated animals, increases from metestrus to proestrus and reaches a peak on the day of estrus.

INTRODUCTION

Sutherland and Foo have recently reported (1) that, in chick oviduct cytosol, the antiestrogens Tamoxifen (2) and CI628 (3, 4) are bound to three components i.e. a non-specific binding, the estrogen receptor and a specific antiestrogen binding site. In the uterine cytosol of the immature rat and calf (1, 5, 6), in contrast, these antiestrogens are apparently bound to only two components i.e. the non-specific binding and the estrogen receptor site. These binding characteristics might eventually explain the different effect of Tamoxifen in the chick oviduct-where it behaves as a pure antagonist of estradiol-17β (7) - and in rat uterus - where it acts as a partial agonist of estradiol-17β (8). However, in the present report , we show that a specific antiestrogen binding site is also present in rat uterine cytosol.

MATERIALS AND METHODS

Chemicals : Tritiated Tamoxifen (^{3}H-Tx ; 19.52 Ci/mmole) and unlabeled Tamoxifen (Tx) base were gifts of ICI Pharmaceuticals. Unlabeled estradiol-17β (E_2) was from Sigma. Unlabeled CI628 and Nafoxidine (4) were gifts from Parke-Davis Co. and from Upjohn Co. respectively.
Cytosols. Uteri were from immature (21-23 day old) and mature (3 month old) Sprague-Dawley rats housed under controlled photoperiods (14 hr light cycle) and temperature (21-22° C). Adult animals were used after at least two successive 4 day cycles. Some of them were ovariectomized through the dor-

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sal route at least 15 days before the study. The uteri were homogenized in 1/16 (w/v) 20 mM Tris HCl, 1.5 mM EDTA buffer, pH 7.4, containing 1 mM dithiothreitol. Cytosols were prepared as previously described (9). Prior to use, each cytosol was treated with dextran-coated charcoal (10) to remove any uncomplexed or weakly bound endogenous hormone. The cytosols were then used immediately (fresh) or after thermal denaturation at 30° C for variable length of time.

Methods. One hundred µl aliquots of cytosol were incubated in triplicate with 10 µl tritiated Tamoxifen in presence or absence of a 100 fold excess or unlabeled ligand for 18-20 h at 4°C. ^{3}H-Tx concentrations ranged from 0.1 to 10 x 10^{-9}M. Following incubation, the protein bound and unbound ligand were separated using dextran-coated charcoal. An aliquot of supernatant was removed and counted in a liquid scintillation spectrometer using external standardization. The binding parameters, dissociation constant (Kd) and binding capacity of the saturable binding component, were estimated by Scatchard plot (11) using the correction of Rosenthal (12) and least-squares regression analysis of the data. Only data where the least-squares derived correlation coefficient, r, was $\geq$ 0.96 with a minimum of 9 experimental points were taken into consideration. To determine ability of E_2, CI628 and Nafoxidine to compete for saturable Tx binding sites, ^{3}H-Tx (2 x 10^{-9}M) was incubated with cytosol and increasing concentrations of the different competitors for 18-20 h at 4° C. The protein bound and unbound ^{3}H-Tx was separated by charcoal absorption and the data plotted as percent tracer bound versus log of the competitor concentration.

RESULTS

Binding of Tx to fresh uterine cytosol. Competitive studies done on fresh uterine cytosols from mature rats during the different phases of the estrous cycle show that E_2 can only partially inhibit ^{3}H-Tx binding (fig. 1). The

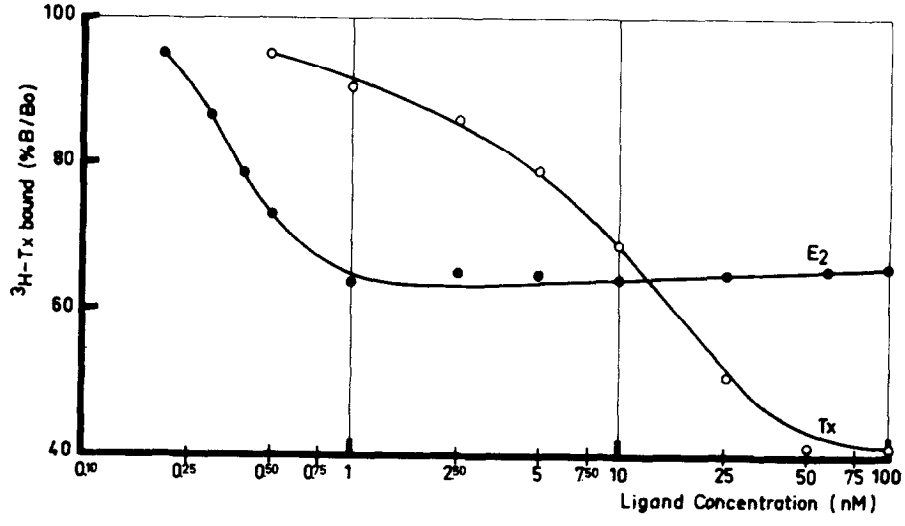

Fig. 1 : COMPETITION OF ESTRADIOL-17β FOR TAMOXIFEN BINDING SITES IN FRESH UTERINE CYTOSOL OF MATURE RATS. ^{3}H-Tx (2 x 10^{-9}M) was incubated with cytosol and increasing concentrations of Tx or E_2 for 18-20 h at 4°C. Protein bound and unbound ^{3}H-Tx was separated by charcoal absorption. Data are plotted as percent tracer bound versus log of the competitor concentration. The cytosol was from a rat in estrus in the present experiment, similar data were obtained in the other phases of the estrous cycle and in castrated animals.

ability of E_2 to compete with ^{3}H-Tx binding varies as a fonction of maturity, phase of estrous cycle and castration, being greatest in immature animals and lowest during proestrus and estrus (data not shown). In these conditions, the estimate of antiestrogen binding parameters derived from saturation analysis include antiestrogen binding to estrogen receptor sites and also to probable specific antiestrogen binding sites. Conditions were then investigated for the specific measurement of this second class of sites.

Binding of Tx to uterine cytosol after thermal denaturation. Fresh uterine cytosols from mature animals were incubated in absence of ligand at 30° C. A various times, aliquots were taken and incubated with ^{3}H-Tx in presence

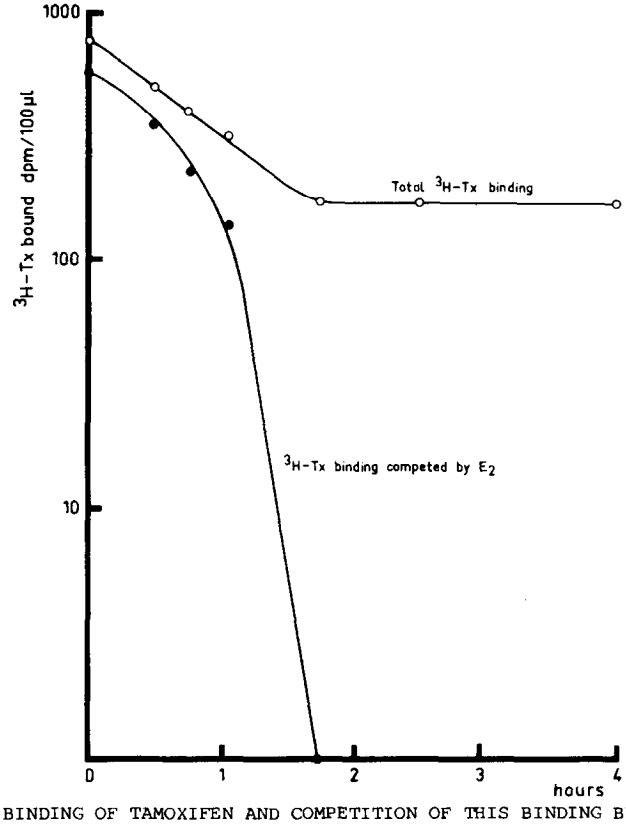

Fig. 2 : BINDING OF TAMOXIFEN AND COMPETITION OF THIS BINDING BY ESTRADIOL-17β IN UTERINE CYTOSOL PREINCUBATED AT 30°C FOR VARYING PERIODS OF TIME. The binding was measured in presence of 2 x 10^{-9}M tritiated Tamoxifen for 18-20 h at 4°C. Total ^{3}H-Tx binding ○ represents the difference between the binding in absence and the binding in presence of 200 x 10^{-9}M unlabeled Tx. ^{3}H-Tx binding competed by E_2 ● represents the difference between the binding in absence and the binding in presence of 200 x 10^{-9}M unlabeled E_2.

or absence of a hundred fold molar excess of unlabeled Tx for 18-20 h at 4° C to measure the total ^{3}H-Tx binding. Similar aliquots were incubated with ^{3}H-Tx in presence of a hundred fold molar excess of unlabeled E_2 to measure the concentration of the total ^{3}H-Tx which could be competed by E_2. As shown in fig. 2, after an initial decrease of the concentration of Tx binding sites during the first 2 h of incubation, a plateau is reached and the remaining ^{3}H-Tx binding sites appear to be stable for at least 4 h. The competitive ability of E_2 decreases progressively from a maximum in fresh cytosol to become undetectable after 2 h of incubation. Data derived from saturation analysis using uterine cytosol incubated at 30° C for 2 h 30 show only one class of binding sites for Tx with a Kd of 3.3 ± 0.9 x 10^{-9} M (mean ± SD) which does not change during the estrous cycle or in castrated animals. The low concentration of these sites and a high non-specific binding did not allow an accurate determination of the Kd in immature animals ; the binding capacity was then evaluated at the concentration of 5 x 10^{-9}M. More detailed competitive studies reveal that E_2 cannot inhibit the binding of ^{3}H-Tx to this category of high affinity and satura-

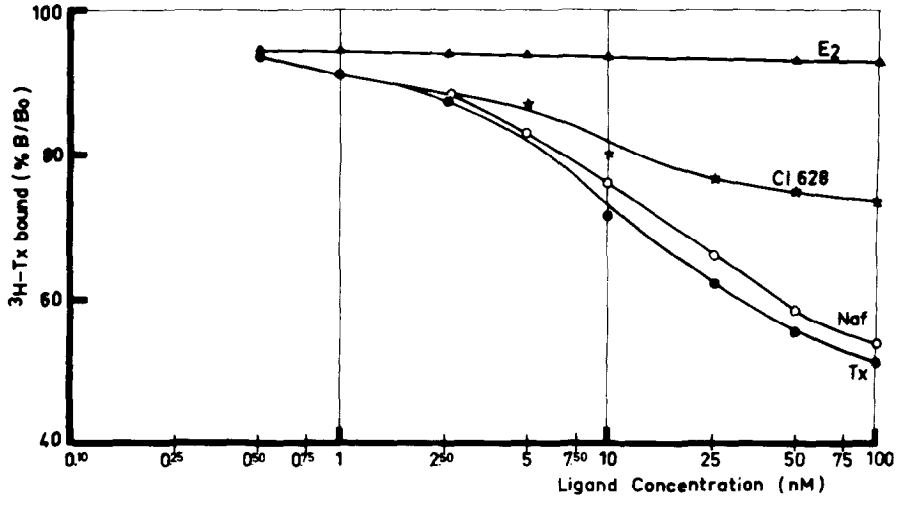

Fig. 3 : COMPETITION OF ESTRADIOL-17β ▲ CI628 ★ AND NAFOXIDINE ○ FOR TAMOXIFEN BINDING SITES IN UTERINE CYTOSOL PREINCUBATED AT 30°C FOR 2 H 30. Data were obtained and plotted as described in Fig. 1.

ble binding site, but Nafoxidine inhibits completely and CI628 50 % of this binding (fig. 3). As shown in fig. 4, the specific activities (fmol/mg cytosol protein) of these binding sites in mature castrated animals and in animals during estrus are similar and significantly higher then those recorded during other phases of the estrus cycle and in immature animals ($p < 0.025$) The concentration of binding sites per uterus is low in immature and castrated animals and increases significantly in animals during metestrus and diestrus ($p < 0.005$). This concentration increases further in proestrus ($p < 0.005$) and reaches a peak during estrus ($p < 0.05$).

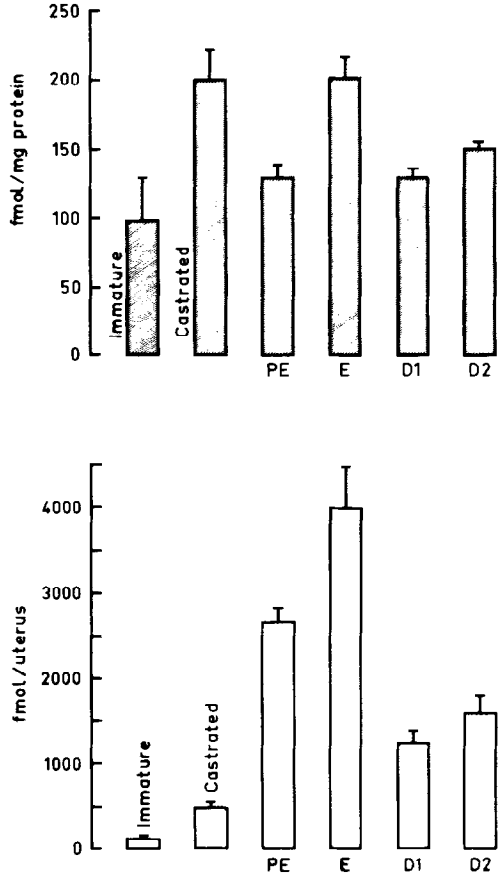

Fig. 4 : CONCENTRATIONS OF TAMOXIFEN BINDING SITES IN UTERINE CYTOSOL OF IMMATURE OR MATURE RATS AT DIFFERENT PHASES OF THE ESTROUS CYCLE OR AFTER CASTRATION (mean ± SEM of at least 5 experimental points) AFTER PREINCUBATION OF THE CYTOSOL AT 30°C FOR 2 H 30.
A - Concentration expressed per mg of cytosol proteins.
B - Concentration expressed per uterus.

DISCUSSION

The results presented here confirm earlier observations that Tx binds to cytoplasmic estrogen receptor binding sites in the rat uterus (1, 4, 5, 6). In addition our data demonstrate the coexistence of high affinity, saturable binding sites specific for antiestrogens of the triphenylethylene class, which do not bind E_2. From the data presented in fig. 2, it appears that the Tx specific binding sites, which exist in fresh cytosol, are recovered after thermal denaturation of this cytosol at 30° C for 2 h 30. However their physicochemical characteristics may have been influenced by this treatment.

Under our conditions of incubation, the uterine concentration of accessible Tx binding sites at 4° C (fig. 4) varies greatly for different animals as a function of maturity, phase of estrous cycle or castration. Such variations should be taken into consideration when interpreting data suggesting the presence of two distinct high affinity, low capacity E_2 binding components with different sensitivity to Tx (13). Such variations would also suggest that these binding sites have some physiological function, however their role in the mechanism of antiestrogenic action and the existence of a natural antiestrogen remain the object of inquiry.

ACKNOWLEDGEMENTS

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