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HETEROGENEITY OF ESTROGEN RECEPTORS IN THE CYTOSOL AND NUCLEAR FRACTIONS OF THE RAT UTERUS

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Summary: Multiple species of estrogen binding sites have been demonstrated in cytosol and nuclear fractions of uteri from immature and adult ovariectomized female rats. Equilibrium binding analyses of uterine cytosol yielded two binding sites, I and II, with dissociation constants of 0.8 and 33 nM respectively. The high affinity cytosol site (0.8 nM - Type I) translocated to the nuclear compartment following estrogen treatment in vivo and appears to represent the classical estrogen receptor which can be measured by ^{3}H-estradiol exchange. Type II sites remain in the cytosol after estrogen injection. A third binding component was observed in the nuclear compartment (nuclear Type II) which binds ^{3}H-estradiol at 4° and displayed cooperative binding characteristics. The presence of these sites in both cytosol and nuclear compartments complicates the accurate measurement and differentiation of these sites. Valid estimates of binding parameters for cytosol Type I and II sites may be obtained by saturation analyses over a wide range of ^{3}H-estradiol concentrations (0.05-40 nM). Nuclear Type I can be differentiated from nuclear Type II by performing saturation analysis under exchange conditions which measure both Type I and II sites and comparing the values obtained when the assay is performed at 4°C which measures only Type II sites.

INTRODUCTION

Estrogen receptors are cytoplasmic macromolecules that bind estrogen and form receptor estrogen complexes which accumulate in the nuclear compartment of estrogen sensitive cells (1-3). The nuclear accumulation and subsequent

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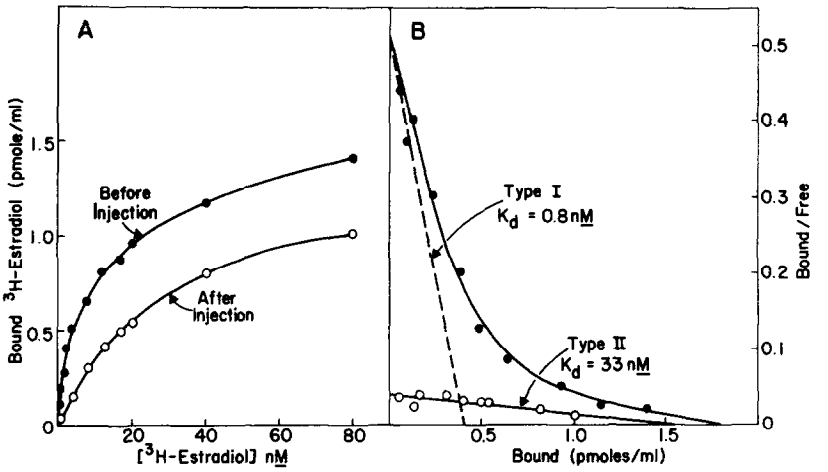

Fig. 1. Estrogen Binding in Rat Uterine Cytosol

A. Saturation Analysis. The quantity of ^{3}H-estradiol bound in a manner displaceable by excess DES ("specific binding") was determined in uterine cytosols from non-injected rats (●) and rats injected with 2.5 μg of estradiol 60 min. prior to sacrifice (○).

B. Scatchard Analysis of the Data in A. The amount of ^{3}H-estradiol bound to uterine cytosols from estrogen treated animals (○-Type II) was subtracted from the total binding in the system (●) to yield the dashed line labeled Type I.

retention of these receptor estrogen complexes at specific acceptor sites on chromatin appear to be involved in increasing genomic activity which ultimately results in uterine growth (4).

Cytoplasmic and nuclear estrogen receptors are generally considered to have one binding site for estrogen (5); however, sufficient variability and disparity between the reported dissociation constants, K_d, have caused us to reinvestigate this question (6-8).

MATERIALS AND METHODS

Mature ovariectomized (60-80 days old) and immature rats (21-23 days old) were used in these experiments. Cytosol and nuclear fractions were prepared as previously described (4). Estrogen binding in the cytosol was measured by the hydroxylapatite method following incubation of cytosol plus tritium-labeled hormone for 20 hr at 4°C or under exchange conditions (4). Nuclear binding sites were measured by the ^{3}H-estradiol exchange assay (4,9) or by incubating nuclear fractions at 4°C for 60 minutes. The tissue to volume ratio for all assays was maintained at 25 mg/ml of buffer or 180 μg DNA/ml for nuclear studies.

RESULTS AND DISCUSSION

Most investigators have measured the binding properties of the estrogen receptor via saturation analyses from 0.1-20 nM which constitutes an adequate range for the measurement of a binding site with a K_d of $\sim$ 1 nM. We have extended the saturation analysis of the cytoplasmic receptor by using concentrations of ^{3}H-estradiol ranging from 0.05 nM to 80 nM (Fig. 1). In non-injected animals the cytosol receptor displays two binding sites when analyzed by the method of Scatchard (10). For simplicity we refer to them as Type I and II sites which have K_d's of 0.8 nM and 33 nM respectively. When rats are injected with 2.5 µg of estradiol, the Type I sites are depleted from the cytosol while the Type II sites remain. Thus, two types of estrogen binding sites exist in the cytosol: Type I, which corresponds to the estrogen receptor as previously defined, and a second site which has a lower affinity for estrogen.

It should be noted that the data shown in Fig. 1 were obtained under equilibrium conditions, i.e., incubation of hormone with cytosol for 20 hr. at 4°C. When incubations of shorter periods are performed, the Type II site does not reach equilibrium and the saturation curve will appear to have a biphasic shape which consists of two components: a hyperbolic function due to Type I sites and a sigmodial function due to Type II sites. Since receptor assays are often performed under non-equilibrium conditions with respect to Type II sites, the error introduced by the presence of Type II will depend upon the incubation period.

The binding of ^{3}H-estradiol to the Type II site is stereospecific for estrogens and this site is not found in non-target tissues (to be published elsewhere). The Type II site does not appear to be α-feto protein since it is found in the adult rat uterus and is competitively inhibited by diethylstilbestrol. Thus this Type II site manifests some of the properties of a hormone receptor.

After an injection of estradiol, the estrogen receptor accumulates in the nucleus. This accumulation is assumed to result from depletion of the cytoplasmic

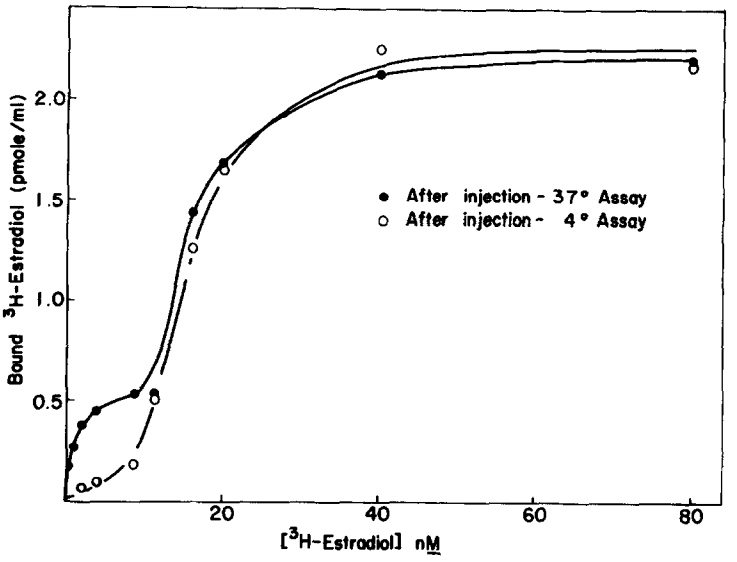

Fig. 2. Saturation Analysis of Estrogen Binding in the Nuclear Fraction of the Rat Uterus.

Estrogen receptive sites in the nuclear fraction of the rat uterus were determined by the ^{3}H-estradiol exchange assay to 37°C after injection of 2.5 µg of estradiol (●). Specific ^{3}H-estradiol binding was also determined by exchange at 4°C in nuclei from estrogen treated rats (○).

receptor. As shown in Fig. 2, analysis of nuclear fractions for estrogen binding sites by the ^{3}H-estradiol exchange assay reveals a complex picture which involves at least two sites. One site conforms to the Type I site which was depleted from the cytosol and is undoubtedly identical to the classically described estrogen receptor. The other site displays a sigmoidal saturation curve. When the quantity of this second site, which we will call nuclear Type II, is subtracted from the total quantity of nuclear bound hormone as measured by exchange, one obtains only the amount bound to Type I. Scatchard analysis of nuclear Type I reveals a K_d of 0.60 nM and a maximal number of sites of 0.36 pmoles/ml. These values do not differ significantly from those of the cytosol receptor, Type I, which was depleted by estrogen treatment and conform to the usually accepted properties of the estrogen receptor.

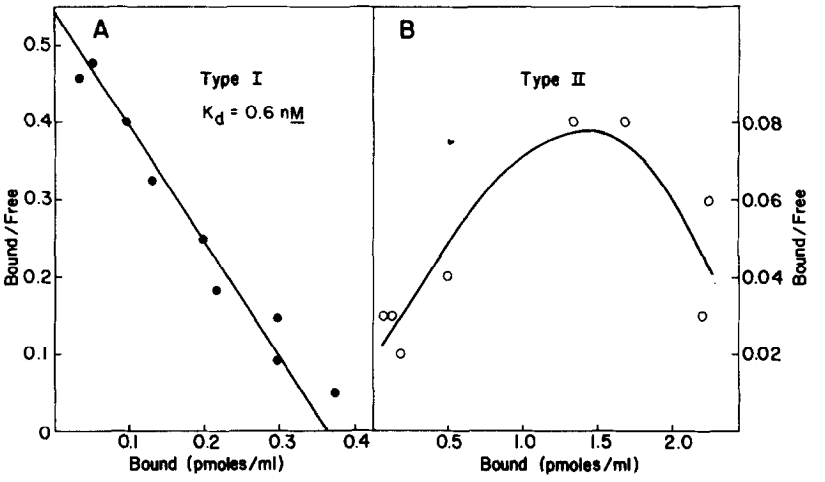

Fig. 3. Scatchard Analysis of Type I and II Estrogen Receptive Sites in the Rat Uterine Nuclear Fraction.

A. Scatchard Analysis for Type I Nuclear Sites. The amount of ^{3}H-estradiol bound to Type I sites was obtained by subtracting the amount of ^{3}H-estradiol bound at 4°C from that observed at 37°C in Fig. 2 (●-● minus O-O).

B. Scatchard Analysis of Type II Nuclear Sites. The quantity of bound ^{3}H-estradiol that was observed by exchange at 4°C in Fig. 2 was used in this Figure.

The nuclear Type II sites do not appear to be identical to the cytosol Type II sites and display cooperative binding behavior with a Hill coefficient of approximately two (Fig. 3,B). Nuclear Type II sites are stereospecific for estrogens, are absent or in very low quantities in non-target tissues and, as with the cytosol Type II, are found in the adult rat uterus (to be published elsewhere). Therefore, this second estrogen binding site, present in the nuclear fraction, possesses some of the properties of a receptor-like molecule. The cooperative behavior of these nuclear Type II sites appears real and not due to an artifact resulting from non-equilibrium conditions during the assay. Uterine nuclei have also been purified by the hexylene glycol procedure as previously described (11). These nuclei were analyzed for estrogen binding sites by the ^{3}H-estradiol exchange assay as described above. Patterns of estradiol binding were observed in these highly purified nuclei which were

identical to those seen in crude nuclear preparations (results to be published elsewhere). These data support the finding that following an injection of estradiol, uterine nuclei contain two distinct binding sites for the hormone.

The complexities of estrogen binding introduced in this report have important implications with respect to the validity of receptor measurement. Valid estimates of Type I and II binding sites in cytosol fractions can be obtained *via* complete saturation analyses with the appropriate corrections as shown in Fig. 1B (also see reference 2). Assays for nuclear Type I and II are readily accomplished by saturation analyses of nuclear fractions *via* exchange at both 37°C and 4°C. At 37°C ^{3}H-estradiol exchanges with the Type I sites and binds to Type II sites; hence at 37°C both sites are measured. Since exchange of occupied Type I sites occurs very slowly at 4°C, only Type II sites are measured at this temperature. Subtraction of Type II sites as measured at 4°C from those sites measured *via* exchange at elevated temperature (Types I plus II) yields the contribution made by Type I alone. This evaluation is a necessity if accurate assessments of individual receptive sites are required. Previous work from our laboratory and others have undoubtedly over estimated the quantities of Type I sites in nuclear fractions. This over-estimate is also true for assays of cytosol receptor employing hydroxylapatite or other protein adsorbant procedures. The charcoal adsorption assay is less likely to be in error since long exposure to charcoal strips estradiol from Type II sites (to be published elsewhere). We have also observed variable quantities of nuclear Type II sites in non-treated uterine nuclear fractions. These could be mistakenly identified as Type I sites unless saturation analyses are performed at both 4°C and 37°C.

We do not understand the functional significance of these various forms of estrogen binding sites; however, it is possible to make some suggestions concerning their function. The cytosol Type II site may be involved in the retention of estrogens within the uterus, thus creating an estrogen rich

environment for the Type I site which translocates estrogen to the nucleus. The nuclear Type II receptive site may be a component of the machinery for "processing" the Type I nuclear complex. Previously we suggested that receptor estrogen complexes bind to a small number of nuclear sites and undergo "nuclear processing" (4). This processing may be a part of the mechanism by which RNA synthesis is stimulated and the receptor estrogen complex is eventually detached from nuclear sites. Studies are currently in progress to examine these possibilities.

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