BINDING PROPERTIES OF AN ANTI-ESTROGEN TO THE ESTRADIOL RECEPTOR OF UTERINE CYTOSOL

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1. Introduction

The drug nafoxidine (U.11,100) behaves *in vivo* as an anti-estrogen as well as a slight estrogenic compound on the rat uterus [1–3]. Its mechanism of action is unknown although it was suggested that it could act by competing with estradiol for binding to its receptor(s) in the target organ [4]. This assumption was based on the fact that nafoxidine inhibits ^{3}H-estradiol uptake by uterus both *in vivo* [2] and *in vitro* [4]. The complexity of the system makes the results difficult to interpret in molecular terms, since this drug could act on the cytosol as well as on the nuclear estradiol receptor [5] and/or modify estradiol transport through cellular or nuclear membranes.

The purpose of this work is to define more precisely the binding characteristics of nafoxidine to the estradiol receptor of uterine cytosol. Our results show that nafoxidine is a competitive, reversible and eventually complete inhibitor of estradiol binding to the cytosol receptor in its dissociated and non-dissociated form. The affinity for the cytosol receptor is about 30 times less than that of estradiol ($K_i \simeq 7$ nM).

2. Methods

(6-7)^{3}H-estradiol was obtained from CEA (specific activity = 48 Ci/nM). Nafoxidine (fig. 1) (U. 11,100:1-(2-(P-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl) phenoxy)-ethyl) pyrrolidine hydrochloride) was a gift of Dr. O. Kadruka, research laboratories of the Upjohn Company. Non-radioactive estradiol was from Roussel. Calf uteri were collected on dry ice and homogenized in 10 mM Tris HCl pH 7.4–1.5 mM EDTA buffer and the 105,000 *g* supernatant (cytosol) prepared as described previously [6].

Similar results were obtained using prepubertal rat or calf uteri. The cytosol containing 2 mg protein/ml was incubated with ligand at 0–2° for 90 min, a time long enough to reach binding equilibrium. The specific binding of ^{3}H-estradiol to the receptor was assayed by adsorption of the unbound ligand on dextran-coated charcoal [7]. The cytosol was shaken overnight at 0–2° with a suspension of Dextran 0.05% and charcoal 0.5% in Tris-EDTA buffer. At the end of this incubation, the loosely bound ^{3}H-estradiol, dissociated from non-specific protein(s) and adsorbed onto the charcoal, was spun down by a 10 min centrifugation at 700 *g*. Aliquots of the supernatant were then assayed for specifically bound estradiol (Bs) by counting in a toluene scintillation mixture in the presence of ethanol with

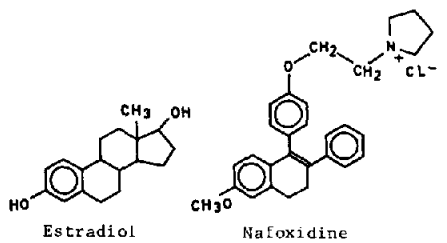

Fig. 1.

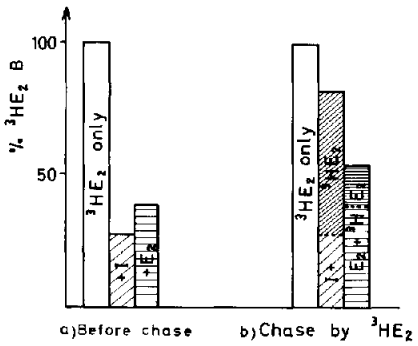

Fig. 2. Reversibility: hot chase experiments. Percentage of estradiol binding sites (% $^3HE_2B$) are shown before or after chase experiments. In both cases, 3 lots of uterine cytosol were incubated at 0° for 60 min with the inhibitor (I = 100 nM), non-radioactive estradiol (E_2 = 10 nM) or without ligand. a) Before chase: Half of each lot was subsequently incubated with 5 nM ^{3}H-estradiol for 60 min at 0°, and the specific estradiol binding sites were measured by charcoal adsorption (see Methods). □: Control bound ^{3}H-estradiol in the absence of inhibitor taken as 100% (= 0.2 pmoles/mg protein). ▨: % ^{3}H-estradiol bound in the presence of inhibitor. ▤: % ^{3}H-estradiol bound in the presence of non-radioactive estradiol. b) Chase by ^{3}H-estradiol: The other half were treated with a dextran-coated charcoal suspension at 0° for 20 min in order to adsorb unbound ligand. The mixtures were then centrifuged and the supernatants incubated with 5 nM ^{3}H-estradiol for 60 min at 0° and then treated successively at 25° for 25 min and at 0° for 60 min. The estradiol binding sites were tested by charcoal adsorption (see Methods). □: Control without inhibitor taken as 100% (= 0.1 pmoles/mg protein). The 50% inactivation was due to the 25° treatment. ▨: % of ^{3}H-estradiol binding sites freed from inhibitor (I). ▤: % of ^{3}H-estradiol binding sites freed from non-radioactive estradiol (E_2).

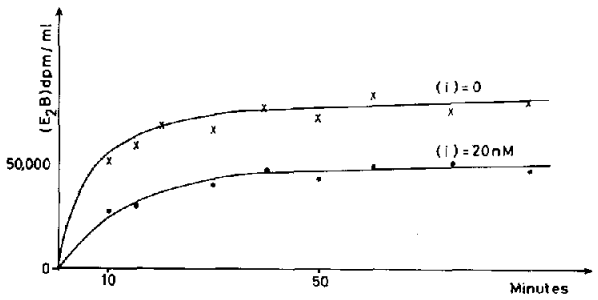

Fig. 3. Reversibility: time course experiments. Uterine cytosol was incubated at 0–2°, with 2 nM ^{3}H-estradiol in the presence (•) or absence (×) of 20 nM nafoxidine. Aliquots were withdrawn at different times and assayed for specifically bound ^{3}H-estradiol as described under Methods.

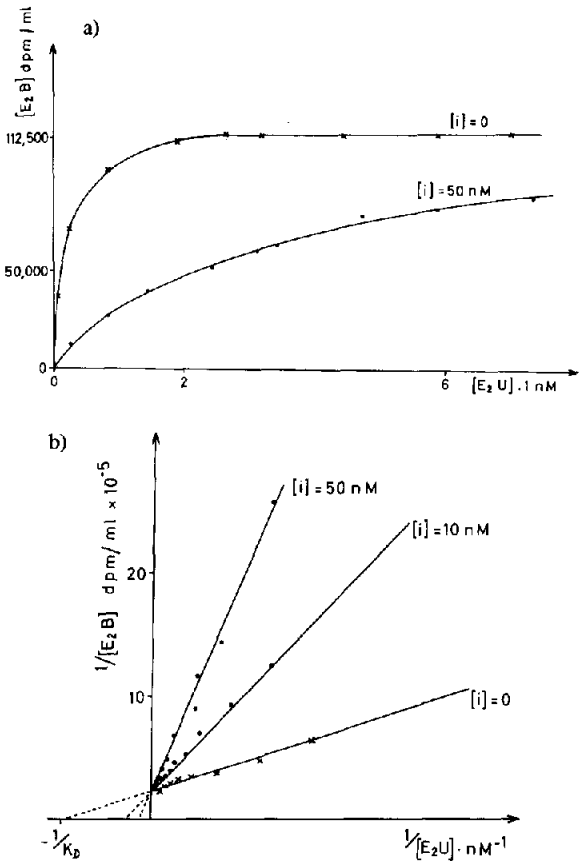

Fig. 4. Competitive inhibition. Three lots of uterine cytosol were incubated for 90 min at 0° with increasing concentrations of ^{3}H-estradiol (×). In 2 of these lots the inhibitor (I = 10 nM (○) and I = 50 nM (•)) had been simultaneously added with the radioactive ligand. At the end of incubation, specifically bound ^{3}H-estradiol was measured as described under Methods. a) Direct plot of bound (E_2B) versus unbound (E_2U) ^{3}H-estradiol, calculated as indicated under Methods. b) Double reciprocal plot according to Lineweaver-Burk [9]. The 2 plots are from 2 different cytosols.

30% efficiency. In order to determine the concentration of unbound estradiol at equilibrium (U), the non-specific binding of estradiol (Bns) was measured by equilibrium dialysis at 0–2° with ^{3}H-estradiol diluted with high concentration of non-radioactive estradiol (1–10 μM). The concentration of unbound estradiol was calculated according to $U = (T - Bs)/(1 + P)$ where P is a partition coefficient ($P = Bns/(U)$) [8] determined for each experiment. P was found to range between 0.5–2 with different

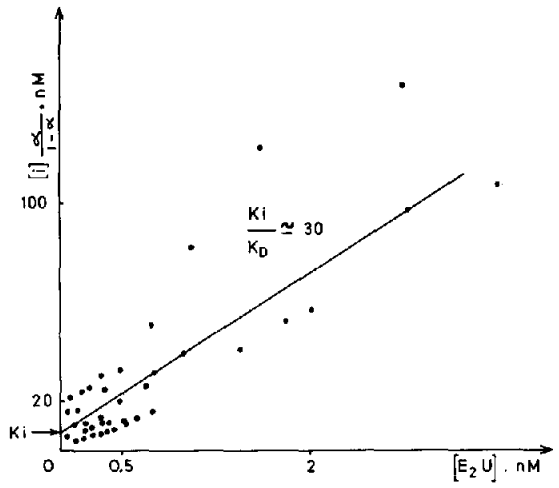

Fig. 5. Measurement of K_i: Hunter and Downs plot [10]. Five different competition experiments were carried out as described under fig. 4. Results were expressed according to the equation of Hunter and Downs:

$$(I) \times \frac{\alpha}{1-\alpha} = K_i + \frac{K_i}{K_d} \times (U) \text{ where } \alpha = \frac{\text{inhibited binding of estradiol}}{\text{uninhibited binding of estradiol}}.$$

Intersection of the linear regression with the vertical axis gives the apparent dissociation constant of nafoxidine ($K_i \simeq 7$ nM). The slope of the linear regression (K_i/K_d = 30 ± 10) indicates the relative affinity of the inhibitor as compared to that of estradiol.

cytosol and was not modified by the presence of nafoxidine.

The purity of estradiol and nafoxidine was checked by thin layer chromatography on Silica gel F 254 Merck using, respectively, benzene–ethyl acetate (3:2, v/v) and butanol–acetic acid–water (60:20:20, v/v/v). No metabolism or alteration of these compounds was observed after incubation with cytosol.

3. Results

3.1. *Reversible inhibition*

In order to discriminate between reversible and irreversible inhibition, 2 series of experiments were carried out: in the first series of experiments (hot chase) we determined the amount of estradiol binding sites initially occupied by the inhibitor and the fraction of those which were liberated through dissociation of the complex. The detailed experimental set up is given under fig. 2a. Under these conditions, 75% of the sites were occupied by inhibitor before dissociation (fig. 2a). During the thermal dissociation step about half of binding sites became available for ^{3}H-estradiol (fig. 2b). This indicates that inhibitor binding to receptor is reversible. A complete chase could be obtained using longer incubation time or higher temperature (45°), but under these conditions, thermo inactivation of receptor was very important. When non-radioactive estradiol was used instead of inhibitor, only 15% of the binding sites occupied by estradiol were liberated, suggesting that estradiol dissociated more slowly than the inhibitor.

The second type of experiments (time course), performed in the presence of both inhibitor and ^{3}H-estradiol, confirmed that nafoxidine was reversibly bound since inhibition did not increase with time (fig. 3).

3.2. *Competitive inhibition*

Saturation curves were made at different inhibitor concentrations and plotted on direct and double reciprocal coordinates, in order to decide whether this inhibitory effect reflects a change in the affinity, or in the number of estradiol binding sites. In the absence of inhibitor a saturation plateau was reached at 2 nM ^{3}H-estradiol, and no non-specific binding was detected up to 8 nM (fig. 4a). The number of specific binding sites* varied from 0.1–0.5 pmoles/mg protein according to preparation. Fig. 4 shows a typical pattern of competitive inhibition on direct and double reciprocal plots [9]. The apparent dissociation constant of the inhibitor obtained according to a Hunter and Downs plot (fig. 5) [10] was K_i = 7 nM. Assuming a dissociation constant of estradiol in the absence of inhibitor of 0.2 nM, K_i was found to vary between 4–8 nM from the slope of the linear regression.

3.3. *Total inhibition*

When the cytosol was incubated with a constant

* Expressed in equivalents of bound ^{3}H-estradiol.

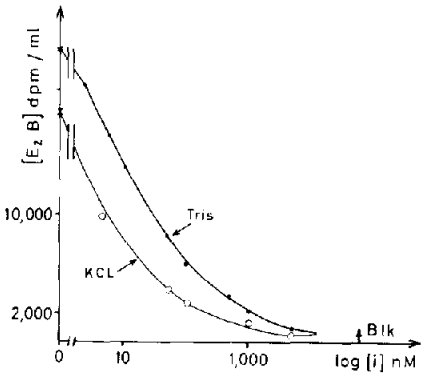

Fig. 6. Complete inhibition. Uterine cytosol made in Tris buffer (•) was incubated, for 90 min at 0°, simultaneously with ^{3}H-estradiol (2 nM) and increasing concentrations of inhibitor (I). The specifically bound estradiol (E_2B) was then tested as described under Methods. Similar blank values (Blk) were obtained using non-radioactive estradiol (10 μM) as an ideal competitor or Tris buffer instead of cytosol. Similar experiments were carried out in the presence of 0.5 M KCl (○).

amount of ^{3}H-estradiol and increasing concentrations of inhibitor, complete inhibition of estradiol binding was obtained with 5 μM of inhibitor (fig. 6) indicating that the cytosol receptor could not simultaneously bind estradiol and nafoxidine. In the presence of 0.5 M KCl, which dissociates the cytosol receptor into subunits [6], similar inhibition was observed. These findings support the existence of a common binding site for oestradiol and nafoxidine.

4. Discussion

The presented results show that nafoxidine inhibits estradiol binding to its receptor decreasing the affinity without changing the number of sites. In addition, the inhibition is fully competitive suggesting that nafoxidine binds to the same site as estradiol. However, the existence of 2 different sites for estradiol and the inhibitor cannot be ruled out. In this case, the complete inhibition observed would imply that binding of the inhibitor to its site drastically decreases the affinity of estradiol for its own. On the grounds that the inhibitory effect of nafoxidine survives the dissociation of the binding protein by KCl, we conclude that inhibitor and estradiol bind to same protein subunit.

At high concentration, nafoxidine has an anti-estrogenic activity since it is both a partial agonist *in vivo* on uterine wet weight [11] and a complete inhibitor of estradiol binding to its receptor *in vitro*, as shown in this paper. Several hypotheses can account for the difference in estrogenic action *in vivo* between estradiol and nafoxidine: they could be carried differently in blood since estradiol, but not nafoxidine, binds to specific plasma protein [12]. Another possibility is that the U.11,100 compound would inhibit estrogen action in target cells at other site(s) than estradiol receptor(s). However, the simplest hypothesis is that the interaction of the inhibitor with the cytosol receptor is less efficient than estradiol in inducing the first molecular events leading to uterus hypertrophy. This inefficiency can be due to the low affinity for the receptor (about 1/30 that of estradiol) and particularly to its rapid dissociation rate and/or to a deficient transfer of the cytosol receptor to the nucleus. This last possibility is under study.

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