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DIETHYLSTILBOESTROL AND THE BINDING OF TRITIATED OESTRADIOL IN PLASMA AND UTERINE CYTOSOLS*

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SUMMARY

In pregnant rat plasma tritiated oestradiol ([^{3}H]-E_2) binds to a single class of sites with relatively high affinity (K_D 4°C 3×10^{-9} M). By multipoint competition studies, diethylstilboestrol (DES) is shown to have a much lower affinity for plasma [^{3}H]-E_2 binding sites (K_D 4°C 3×10^{-7} M). In contrast, oestrogen receptors in uterine cytosols from immature rats show a higher affinity for DES (K_D 4°C 10^{-9} M), demonstrated by multipoint competition studies for [^{3}H]-E_2 binding in both low and high salt conditions. It has been previously suggested that the high salt uterine oestrogen receptor is identical with the plasma globulin alphafoetoprotein (AFP). The finding of a difference in affinity for DES, of at least two orders of magnitude, makes it unlikely that the high salt uterine oestrogen receptor is unmodified AFP or any other pregnancy associated plasma protein.

INTRODUCTION

Rat uterine cytosols, incubated with tritiated oestrogens and subjected to density gradient centrifugation, have commonly revealed two major oestrogen-binding macromolecular complexes, with sedimentation coefficients of ~4*S* and 8*S*; these are currently termed the 4*S* and 8*S* receptors. In hypotonic solutions the 8*S* form predominates; in hypertonic media the 4*S* form constitutes the major binding species.

In a recent publication[1], Uriel and co-workers claimed that "alphafoetoprotein (AFP), a serum globulin, accounts mainly, if not entirely, for the high oestrogen-binding properties of uterine cytosols from immature rats". Given the potential importance of these authors' conclusion—that the classical uterine oestrogen receptor consists of AFP linked with some other intracellular constituent(s)—their hypothesis appeared worthy of further examination.

One such re-examination—using density gradient centrifugation techniques, purified AFP and anti-AFP antibodies—has been recently reported in abstract form[2], and failed to reproduce the findings of Uriel *et al.* The second line of evidence adduced by these latter authors for the identity of AFP and the 4*S* uterine receptor was on the basis of the affinity of the two binding species for various ligands. Their studies on relative affinities were made by comparing the areas under the peaks of density gradient curves, at a single (twelve-fold) concentration of each unlabelled, competing steroid.

It therefore seemed appropriate to conduct a more stringent examination of the most striking of the reported changes in affinity, that of diethylstilboestrol (DES) for the various binding species (AFP, 4*S*, 8*S* uterine binders). In AFP-rich plasma from pregnant rats we find DES to bind with low affinity to oestrogen binding sites ($\leqslant 1\%$ that of E_2), a finding in agreement with the low affinity of DES for purified AFP shown by Uriel *et al.* Unlike these authors, however, we find that both high and low salt uterine cytosols from immature rats have a high affinity for DES, judged by its ability to compete with [^{3}H]-E_2 for oestrogen binding sites.

MATERIALS AND METHODS

In the series of experiments on plasma binding of [^{3}H]-E_2, four female Wistar rats (17–19 days pregnant) were stunned and exsanguinated. Heparinized plasmas were diluted with TEK (Tris 10 mM, EDTA 2 mM, mercaptoethanol 2 mM, KCl 0.4 M, pH 7.4) to a final protein concentration of mg/ml. One ml aliquots of this diluted plasma were incubated with [^{3}H]-E_2 (Amersham Searle, 51 Ci/mmol) at a concentration of 10^{-9} M, either alone, or in the presence of a range of concentrations of radioinert E_2 or DES. Samples were incubated at 4°C for 16 h, at the end of which period protein-bound [^{3}H]-E_2 was separated from residual free steroid by gel exclusion chromatography on Sephadex G50 (fine) mini-columns, bed vol. 3.6 mls.

In the studies on the cytosol binding of [^{3}H]-E_2, forty female Wistar rats were killed in rapid sequence by decapitation, and their uteri removed and placed in 0.9% NaCl on ice. Four pools of ten uteri were homogenized in 2 ml of TE buffer (Tris 10 mM, EDTA 2 mM, mercaptoethanol 2 mM, pH 7.4) with

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an Ultra-Turrax (3 × 5 s bursts at 20 s intervals, in ice). Homogenates were centrifuged at 4°C and 150,000 *g* for 30 min to yield TE buffered, low salt cytosols.

To study [^{3}H]-E_2 binding under low salt conditions, 200 μl aliquots of these TE cytosols were added to 50 μl aliquots of TE buffer containing [^{3}H]-E_2 either alone or with radioinert DES. For high salt cytosols, the vehicle used was TE 5K buffer (Tris 10 mM, EDTA 2 mM, mercaptoethanol 2 mM, KCl 2 M, pH 7.4) to give a final KCl concentration in the 250 μl incubate of 0.4 M. The use of TE 5K buffer, and the final vol. of 250 μl, were the only differences between our tissue extract preparation procedures and those of Uriel *et al.*, who used solid KCl and a final incubation vol. of 200 μl.

In all cytosols, the final [^{3}H]-E_2 concentration was 10^{-9} M, and the final concentration of ethanol from the stock solutions of steroids $\leq 0.1\%$. Cytosols were incubated at 4°C for 16 h, at the end of which period bound [^{3}H]-E_2 was separated from residual free steroid by gel chromatography. In both sets of studies—on plasma and cytosol—the Sephadex columns were equilibrated with, and samples eluted with, the appropriate (TE or TEK) buffer. In both studies aliquots of the excluded vol. containing protein bound steroid, vol. 1 ml, were added to 10 mls of Unisolve and counted to 10^4 counts in a Packard 3320 liquid scintillation counter.

RESULTS

Figure 1 shows the results of a series of experiments in which pregnancy plasma, diluted 1:100 with TEK

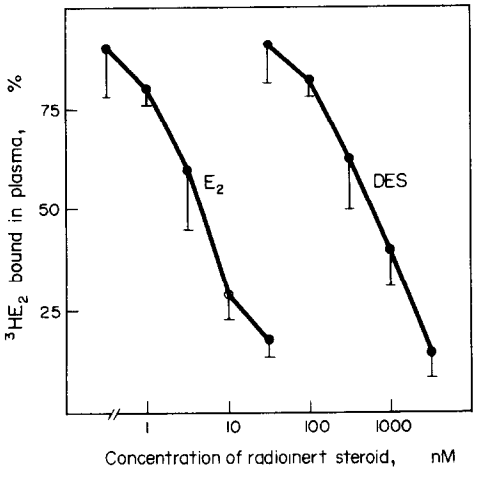

Fig. 1. Relative affinity of oestradiol (E_2) and diethylstilboestrol (DES) for [^{3}H]-E_2 binding in plasma from pregnant rats. In four plasma samples, binding of [^{3}H]-E_2 in the absence of radioinert steroid was 8, 18, 29 and 32% of the total [^{3}H]-E_2 (10^{-9} M). For each plasma, the binding in the absence of radioinert steroid has been taken as 100%, and the binding in the presence of E_2 or DES expressed as a percentage thereof. Each point represents the mean ± S.D. of determinations made in four different plasmas.

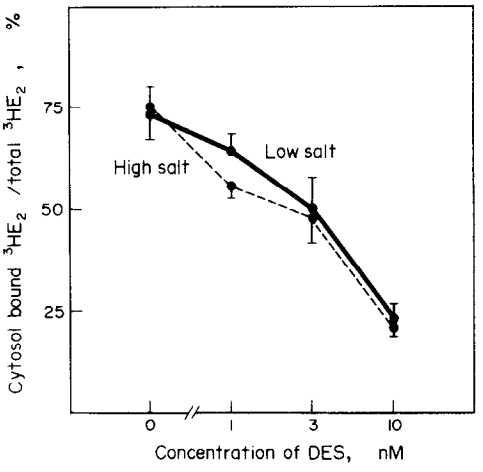

Fig. 2. Effect of increasing concentrations of diethylstilboestrol (DES) on the binding of [^{3}H]-E_2 in uterine cytosols under low salt (●——●) and high salt (●- -●) conditions. Binding is expressed as a percentage of the total added [^{3}H]-E_2 (10^{-9} M); each point represents the mean ± S.D. of determinations made in four different cytosol preparations.

buffer, was incubated with [^{3}H]-E_2 alone, with [^{3}H]-E_2 plus increasing concentrations of radioinert E_2, and with [^{3}H]-E_2 plus increasing concentrations of DES. There are a number of indirect indices supporting the assumption that the observed plasma binding of [^{3}H]-E_2 is to AFP. First, such binding is necessarily to sites with relatively high affinity ($K_D < 10^{-6}$ M) in that it survives Sephadex chromatography. When the data for radioinert E_2 competition shown in Fig. 1 are represented as a Scatchard plot, a single rectilinear plot is obtained, with an apparent dissociation constant K_D 4°C $\approx 3 \times 10^{-9}$ M. That such a single class of binding sites are a pregnancy associated macromolecule is suggested by parallel experiments on similarly diluted male and non-pregnant mature female rat plasma, where minimal, non-displaceable [^{3}H]-E_2 binding was found. Finally, the variations in the total amount of tracer bound in the four different plasmas, in the absence of radioinert steroid (8, 18, 29, 32%) corresponds with the number of foetuses found at sacrifice (2, 5, 9, 10).

As was not unexpected, DES—using a five point competition assay, spanning a hundred-fold range of concentrations—was found to have ~1% the affinity of radioinert E_2 itself for [^{3}H]-E_2 binding sites in plasma. Such a finding is in accord with the results obtained by Uriel *et al.* using purified AFP and a single concentration of competing DES, and with the literature sources cited by these authors for the low affinity of AFP for DES.

In contrast with the findings on [^{3}H]-E_2 binding in pregnant rat plasma, low concentrations of DES compete strongly for [^{3}H]-E_2 binding sites in uterine cytosol preparations, as can be seen in Fig. 2. More importantly, there is no marked difference to be seen between low salt cytosols (●——●) and high salt

cytosols (●—·—●). In other studies (not shown) DES appeared of similar potency as radioinert E_2 itself in competing for [^{3}H]-E_2 binding sites, under both sets of ionic conditions. In the series of studies shown in Fig. 2, where nanomolar concentrations of [^{3}H]-E_2 were used, ~70% of the added tracer was bound in the absence of competing radioinert steroid. When the data are represented by a Scatchard plot, a single predominant class of [^{3}H]-E_2 binding sites are found. Based on the ability to compete for these [^{3}H]-E_2 binding sites, the affinity of DES for the oestrogen receptor, in both high and low salt cytosols, is of the order K_D 4°C 10^{-9} M. The calculated dissociation constant, for DES binding to plasma $^3HE_2$ binding sites, is 3×10^{-7} M. In contrast, then, to Uriel *et al.* who found DES to have a similar, low affinity for both AFP and the high salt oestrogen receptor, we find a clear difference in affinity of at least two orders of magnitude.

DISCUSSION

Uriel *et al.*[1] suggested that AFP accounted mainly, if not entirely, for the high oestrogen-binding seen in immature rat uterine cytosols on the basis of two lines of evidence. First, using specific immunoabsorption of AFP, these workers had previously reported that at low salt concentrations AFP accounted for most of the oestrogen-binding capacity of the 4*S* macromolecular complex[3, 4]. In their most recent study, they presented data in support of the attractive hypothesis that in hypotonic cytosols AFP is present partly as a free protein with a sedimentation coefficient of 4*S*, and partly in association with some intracellular constituent(s) to form the 8*S* binding entity.

As reported, the consequences of this association to form the 8*S* receptor were, at least, two-fold; a loss in antigenic reactivity to anti-AFP antibodies, and a significant change in binding specificity. This change in specificity was shown for oestradiol, oestriol, diethylstilboestrol, nafoxidine and oestrone; the most marked alteration in affinity was that for diethylstilboestrol (DES) vis-à-vis oestrogen (E_2). For the 8*S* receptor, the two molecules appeared equipotent as ligands; in high salt cytosols, DES appeared much less potent than E_2.

The interpretation of the data reported in the present paper would appear relatively simple. The studies on [^{3}H]-E_2 binding on pregnant rat plasma are in a sense preliminary, in that they serve to establish the validity of the methods of studying high affinity [^{3}H]-E_2 binding, and their appropriateness for competition experiments using an acceptable range of concentrations of competing steroids. Using such techniques, we find DES to have $\leq 1\%$ the affinity of E_2 for the single class of high affinity [^{3}H]-E_2 binding sites in pregnant rat plasma, which sites are presumably AFP. Using identical techniques of separation of bound and free steroid, binding of [^{3}H]-E_2 in both high and low salt uterine cytosols from immature rats is found to be predominantly to a single class of sites, with an affinity for E_2 characteristic of oestrogen receptor binding. Using a range of concentrations of DES, in both low and high salt cytosols, we find that DES has a high affinity for oestrogen receptors, an affinity at least two orders of magnitude higher than its affinity for AFP. By the criterion of steroid spacificity, therefore, it appears unlikely that the high salt uterine cytosol receptor is identical to unmodified AFP. Similarly, the claim that the 8*S* receptor is simply AFP in association with some as yet unidentified intracellular component, would appear without foundation in terms of steroid specificity. The possibility that both the 4*S* and the 8*S* receptors represent AFP modified in some way remains an intriguing though unproven hypothesis.

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