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BINDING OF ESTRADIOL-PEROXIDASE CONJUGATE TO ESTROGEN RECEPTOR

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Summary—The binding of estradiol-horseradish peroxidase conjugate to rat uterine cytosolic estrogen receptor was studied. The conjugate having a steroid to enzyme ratio of 2.8:1 was allowed to bind to protamine precipitated receptor in presence or absence of 100-fold excess of free estradiol. The bound enzyme activity was measured and the data subjected to Scatchard analysis to obtain the dissociation constant and the number of binding sites. Although the binding parameter so obtained differed from values obtained using radiolabelled estradiol, the method may be used for comparative studies.

INTRODUCTION

The level of estrogen receptor (ER) in human breast cancer is a useful marker for predicting response to endocrine therapy [1, 2]. Various assay systems have therefore been developed for detecting and quantitating ER in breast tumour or other target tissues. Radiometric methods, using radioligand with high specific activity have been most widely used. Alternative assay systems using fluorescent ligand have also been developed for ER measurement either in intact cells [3] or in solution [4–8]. The monoclonal antibody against purified ER have also been used for ER assay by conventional enzyme immunoassay (EIA) technique [9–11]. However, these methods have some limitations. The fluorescent steroid conjugates, in general, have low affinity for the receptor and are quite lipophilic. In case of EIA, highly purified ER preparations are necessary for antibody production and for various standards. Since receptors are very labile proteins, their preservation for an extended time period is problematic.

The growing interest in replacement of radioactive isotopes with non-isotopic labels prompted us to investigate the feasibility of receptor assay using enzyme labelled estradiol (E_2). Estradiol-BSA-peroxidase conjugate has been earlier used for histochemical detection of ER in tissue sections [12], but there are no reports so far of receptor assay in solution using enzyme labelled ligand. Use of enzyme labelled E_2 for ligand binding assay has the problem that, conventional charcoal or hydroxylapatite adsorption methods are not applicable for bound-free separation. However, we have found that protamine sulphate precipitated ER, as used by Chamness *et al.* [13], is capable of binding E_2-HRP conjugate, and that it is possible by Scatchard analysis [14] of the data to determine the receptor site concentration and the dissociation constant (K_d).

EXPERIMENTAL

Materials

[1,2,7-^{3}H]Estradiol-17β (sp. act. 101 Ci/mmol) was obtained from Radiochemical Centre (Amersham). All other reagents were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Preparation of uterine cytosol

Normal female Sprague–Dawley rats weighing 120–150 g were used. Cytosol was prepared in TED buffer, (0.01 M Tris–HCl, pH 7.4, 0.0015 M Na-EDTA, 0.5 mM DTT and 0.001 M Na-azide) according to the procedure of Chamness *et al.* [13]. The protein concentration of cytosol was assayed by the method of Lowry [15] using BSA as standard and adjusted to 1–3mg/ml with the homogenization buffer.

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Abbreviations: ER: estrogen receptor; 17β-E_2: 17β-estradiol; Tris: tris (hydroxymethyl) aminomethane; EDTA: ethylenediamine tetraacetic acid; DTT: dithiothreitol; HRP: horseradish peroxidase (EC 1.11.1.7); BSA: bovine serum albumin; E_2-3CME: 17β-estradiol-3-*O*-carboxymethyl ether; [^{3}H]E_2: [^{3}H]17β-estradiol.

Preparation of E_2-HRP conjugate

Estradiol-3-*O*-carboxymethyl ether [16] was conjugated to horseradish peroxidase (HRP) by the method of Mattox *et al.* [17] using 0.25 mg of steroid for 2 mg of HRP. The E_2-HRP conjugate was purified by overnight dialysis followed by Sephadex G-50 gel filtration using 0.1 M Na-phosphate buffer, pH 7.0. The active fractions were pooled, concentrated and enzyme activity was tested before its storage in small aliquots at −20°C. The degree of conjugation was determined by the ratio of absorbance at 280 and 403 nm. The conc. C of E_2 in E_2-HRP conjugate was determined by the following equation,

$$C = \left[A_{280} - \left(\frac{A_{403}}{RZ}\right)\right] \Big/ \epsilon$$

where A_{280} and A_{403} are the absorbances of the conjugate at 280 and 403 nm respectively, RZ is the experimentally determined ratio $A_{403}:A_{280}$ of the unmodified HRP and ϵ is the molar extinction co-efficient of estradiol-3-*O*-carboxymethyl ether.

The only assumption made here was that there is no haeme loss during the conjugation. The working solution of E_2-HRP conjugate, was made in TE buffer (0.01 M Tris–EDTA, pH 7.4) to give a final E_2 concentration of 14.4×10^{-9} M which was suitable for measurement of peroxidase activity.

Protamine sulphate precipitation and estrogen receptor assay

Cytosol (0.2 ml) was precipitated with 0.25 ml of protamine sulphate (1 mg/ml) according to the method of Chamness *et al.* [13]. The protamine sulphate precipitated pellets were washed twice with 1 ml of ice-cold TE buffer followed by thorough suction. The pellets were then incubated in TE-buffer containing various concentration of either E_2-HRP or radiolabelled E_2.

The radiolabelled assay, was done by the procedure described in the literature [13] using $[^3H]E_2$ concentrations varying from 0.1 to 2.0 nM. For assay with E_2-HRP the pellets were incubated with 500 μl of buffer containing various concentrations of E_2-HRP (0.072–1.44 nM) with or without 100-fold excess of unlabelled E_2. After overnight incubation at 4°C, the incubation fluid was removed, pellets were washed with ice-cold TE buffer (4 × 1 ml) with careful draining. Then 2 ml of *O*-phenylenediamine hydrochloride solution (0.5 mg/ml) in 0.1 M Na-citrate buffer, pH 5.1 containing 0.02% H_2O_2 was added, and incubated for 20 min in the dark at room temperature. The reaction was stopped with 0.6 ml $4(N)H_2SO_4$ and the colour intensity was recorded with a Beckman spectrophotometer at 492 nm. Total added enzyme activity was also determined in each case simultaneously. To determine the endogenous peroxidase activity, we always did a control enzyme activity test in protamine sulphate precipitated cytosol with buffer only, without addition of enzyme conjugate. The value obtained was then subtracted from both the total and non-specific binding. The amount of E_2 bound was obtained from a standard curve prepared by plotting the enzyme activity of known amounts of enzyme conjugate under identical condition. The observed optical density (O.D.) was found to be linear in the concentration range used.

RESULTS

The E_2-3CME was conjugated to horseradish peroxidase (HRP) by activated ester method and the steroid: protein ratio of the conjugate as determined by u.v. spectral analysis was found to be 2.8:1. The concentration of E_2 in the stock solution of E_2-HRP conjugate was found to be 21.6×10^{-6} M.

Incubation of increasing concentration of E_2-HRP with protamine precipitated cytosol showed the binding of the enzyme conjugated E_2 to the precipitate as evidenced by the increase in bound enzyme activity with the increase in concentration of E_2-HRP used.

Figure 1 shows a typical saturation curve obtained in presence and absence of 100-fold

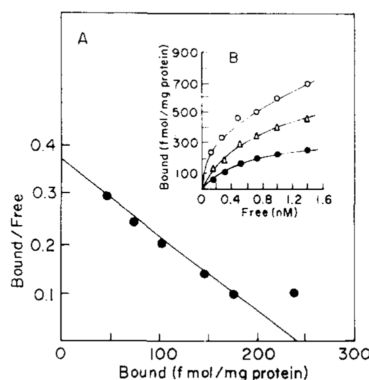

Fig. 1. Binding of E_2-HRP to rat uterine estrogen receptor. Cytosol (200 μl) was precipitated with protamine sulphate and the precipitate incubated at 0° for 20 h with 0.072–1.44 nM of E_2-HRP with or without 100-fold excess of cold E_2. After removal of supernatant and washing, the bound enzyme activity was measured. (A) Scatchard plot (B), saturation curve. (—○—) total binding; (—△—) non specific binding and (—●—) specific binding.

excess of free estradiol. The non-specific (control) activity due to endogenous peroxidase in the protamine sulphate precipitate varied from 0.04 to 0.138 O.D. in different lots of cytosol preparations and was subtracted from both total binding and the binding with 100-fold molar excess of unconjugated E_2. In the concentration range used, the bound activity after subtraction of non-specific activity was found to be in the range of approx. 0.1–1.0. Scatchard analysis of the data produced a fairly linear plot indicating a single class of binding sites. Analysis of the binding data from nine separate samples of cytosols from mature virgin rats revealed the dissociation constant (K_d) in the range $4.60–8.32 \times 10^{-10}$ M, the binding site concentration (n) was in the range of 185–305 fmol/mg cytosol protein. Cytosols from fertility proven rats (ten samples) were found to have K_d values in the range of $2.74–4.52 \times 10^{-10}$ M while n varied from 230 to 695 fmol/mg cytosol protein.

For comparison, six samples of protamine precipitated cytosol of mature virgin rats were incubated parallelly with [^{3}H]estradiol and the binding parameters were obtained as described by Chamness *et al.* [13]. Typical saturation and Scatchard curves are shown in Fig. 2. As can be seen from Table 1, with [^{3}H]E_2, the dissociation constant (K_d) was found to be in the range of $2.72–3.89 \times 10^{-10}$ M, while binding site concentration varied from 228–370 fmol/mg cytosol protein. The enzyme label gave K_d values 1.6–2.5 times higher than the radiolabel while the number of binding sites estimated were

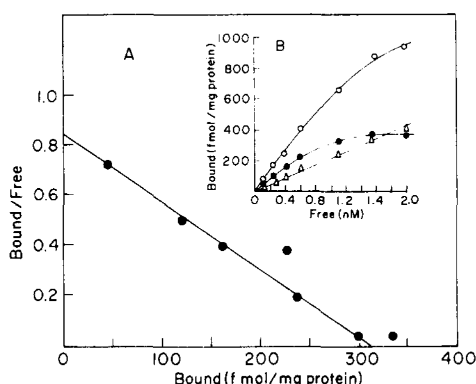

Fig. 2. Binding of [^{3}H]E_2 to rat uterine estrogen receptor. Cytosol (200 μl) was precipitated with protamine sulphate and the precipitate incubated at 0° for 20 h with 0.1–2.0 nM of [^{3}H]E_2 with or without 100-fold excess of cold E_2. After removal of supernatant and washing, the bound radioactivity was measured after ethanol extraction. (A) Scatchard plot. (B) Saturation curve. (—○—) Total binding; (—△—) non specific binding and (—●—) specific binding.

Table 1. Comparison of binding parameters of virgin rat uterine cytosolic ER obtained using E_2-HRP and [^{3}H]E_2

	K_d (10^{-10} M)			Binding site (fmol/mg protein)		
Experiment	E_2HRP	[^{3}H]E_2	Ratio*	E_2HRP	[^{3}H]E_2	Ratio*
(1)	6.80	2.72	2.50	238	310	0.77
(2)	6.16	2.84	2.17	185	235	0.78
(3)	8.13	3.89	2.09	305	370	0.82
(4)	8.32	3.72	2.24	208	295	0.70
(5)	6.52	3.02	2.16	201	228	0.88
(6)†	4.60	2.90	1.59	236	308	0.76

*Ratio of values obtained using enzyme and radioactive label.
†Scatchard and saturation curves of this experiment are shown in Figs 1 and 2.

12–24% lower than the values obtained from radiolabelled experiments.

The time dependence of E_2-HRP uptake by protamine precipitated uterine cytosol at 0°C was analysed. It was found that saturation of specific binding sites in protamine precipitated cytosol was achieved in 4–5 h at 0°C and that binding remained stable for at least 20–22 h.

To find out the effect of freezing tissue supernatant on the binding of E_2-HRP, supernatant from virgin rat uteri were stored at −20°C for 24 and 48 h and then analysed at 4°C. The results showed no significant difference in values for K_d and receptor concentration when analysed immediately or after storage for 24 h. Further keeping, however, resulted in significant loss of receptor binding property. It has been observed that storage of cytosol as such or in protamine precipitated form led to increase in endogenous peroxidase activity and it was impossible to measure true specific binding of E_2-HRP to cytosolic ER after 48 h of storage.

DISCUSSION

The paper presents evidence that estradiol-peroxidase conjugate can compete with free estradiol for binding to estrogen receptor and analysis of the binding data permits the simultaneous assessment of ER concentration and dissociation constant (K_d) in cytosols.

The method is simple and unlike the other non-isotopic method, the fluorescence assay needs no extraction of bound ligand. Furthermore as the method is based on direct measurement of the bound enzyme ligand conjugate to its receptor, antibody against the receptor is not necessary. The stability of the enzyme conjugate permits its use even after 4–5 months storage.

Although there are some discrepancies between the values of binding parameter (n, K_d) obtained by the present method with the values obtained by radiolabelled receptor analysis, the

values still lie in the normal biological range. This discrepancy may therefore be acceptable for comparative study of receptor concentration in estrogen target tissues. Similar discrepancies between results obtained from radio-ligand and alternative methods have been reported by other workers [18]. Another difficulty encountered was the variable and occasionally high background colour obtained due to endogenous peroxidase present in cytosols. Change of the enzyme label may obviate this difficulty.

We have observed that storage of tissue supernatant either as such or in protamine precipitated form at −20°C for more than 24 h gave rise to anamolous results. Similar anamolous results for receptor assay in frozen cytosols has been reported by other methods also [19].

In conclusion, the present study indicates that enzyme-labelled assay of estrogen and other receptors may be a viable alternative to isotope labelled and fluorescent labelled assay or enzyme immunoassay.

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