ESTRADIOL RECEPTORS: INFLUENCE OF PLASMA PROTEINS ON DETECTION AND QUANTITATION

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SUMMARY

The plasma derived proteins albumin and sex hormone binding globulin were studied for their effects on the measurement of estradiol receptor binding parameters. The influence of sex hormone binding globulin was found to be the major factor likely to lead to erroneous values. Testosterone prevents this interference.

INTRODUCTION

The estradiol receptors present in uterus, mammary tumours and other estrogen target organs can be assayed by a host of different methods [1–6], which separate receptor-bound estradiol from free hormone. Of these, Dextran-coated charcoal adsorption, because of its reproducibility, easy operation and ability to handle a considerable number of assays is the most widely used [1, 7–10]. When estradiol binding is determined with a series of estradiol concentrations and the results are compiled in Scatchard plot form an estimate of the number of binding sites and the dissociation constant for the estradiol–receptor complex can be obtained [7].

Reports have consistently been made about the wide variability of dissociation constant values in studies with breast carcinoma [7, 10, 11] and uterine [7] extracts. These variations have been attributed to a range of factors including inaccuracy of the assay method, the influence of contaminating proteins or other substances and alteration of the receptor under pathological conditions [10, 11]. At this stage insufficient is known about receptor structure or the chemistry of its estradiol binding site to exclude this last proposal. In our hands, however, the charcoal adsorption technique has been applied with high reproducibility [12] and we decided therefore to take a closer look at the effects of plasma derived contaminants.

In this article we present data which show that plasma protein interference can lead to a false evaluation of receptor binding parameters. Our results also indicate that sex hormone binding globulin (SHBG) is the major contributor to this effect.

MATERIALS AND METHODS

Tissues. Breast tumours and myometrial tissue were homogenized immediately after collection in 0.01 M Tris–HCl, 0.001 M dithiothreitol, pH 7.4 buffer ($T_{10}D_1$, pH 7.4). The cytosols were prepared as previously described [21] and stored at −20°C until used.

Partial purification of SHBG. Third trimester pregnancy plasma (4 ml) was stripped of steroids by shaking with charcoal (Norit A, Pfanstiehl, U.S.A.; 50 mg per ml of plasma) for 1 h at room temperature. The steroid-free plasma was diluted with 6 ml of 0.01 M Tris–HCl, pH 7.4 buffer containing 0.5 M NaCl (Na) and 0.001 M $CaCl_2$ (Ca), $MgCl_2$ (Mg) and $MnCl_2$ (Mn) ($T_{10}Na_{500}Ca_1Mg_1Mn_1$, pH 7.4) and applied to a 4 ml Con-A-Sepharose (Pharmacia, Lane Cove, N.S.W.) column packed in the same buffer. Unbound proteins were removed from the adsorbent by washing with buffer (20 ml) while retained proteins, including SHBG, were recovered by elution with 4% methyl-α-D-glucopyranoside (Calbiochem, Carlingford, N.S.W.) in buffer (6 ml).

Estradiol receptor assay. The estradiol-binding properties of cytosols were determined from Scatchard plot analyses [7] as described [21]. Aliquots (1 ml) of cytosol (usually diluted 1:10 with $T_{10}D_1$, pH 7.4 buffer) were incubated with radioactive estradiol in the presence and absence of varying amounts of the unlabelled hormone. Unbound steroid was removed with Dextran-coated charcoal. The influence of plasma proteins on the binding pattern was examined in a similar way after controlled addition of non-pregnancy plasma, pure bovine albumin (Serva, Germany) and partially purified SHBG extract to cytosols containing estradiol receptors. Where indicated the assays were carried out in the presence of 1 μg of testosterone. Protein concentration was quantitated by the method of Lowry *et al.* [22].

RESULTS

We first investigated the influence of plasma addition on the shape of a Scatchard plot used to analyse the binding properties of a human myometrial cytosol. A control, with cytosol only and a test sample

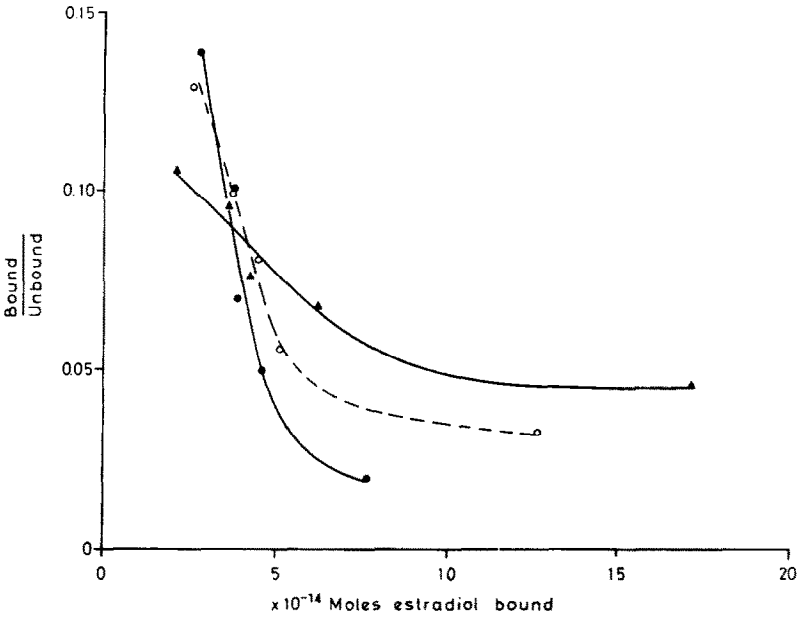

Fig. 1. Influence of plasma proteins on the Scatchard plot characteristics of a human myometrial cytosol containing estradiol receptors. ●——●, Control cytosol only; ▲——▲, test sample prepared from a 1 in 32 dilution of non-pregnancy plasma with cytosol; ○——○, test sample with the inclusion of 1 μg testosterone during incubation.

containing 15.5 ml of cytosol and 0.5 ml of human blood plasma (pooled from non-pregnant women) were prepared. Estradiol binding characteristics were determined using the charcoal technique on 1 ml aliquots at five different estradiol concentrations in triplicate-60 pg of [^{3}H]-estradiol alone and in the presence of 50, 100, 200 and 1000 pg of the non-radioactive steroid. Figure 1 shows that both control and test cytosols exhibited non-linear curves characteristic of extracts which bind estradiol specifically with high affinity. However, a difference was noted in the linear steep section of the curve where a decreased slope for the plasma contaminated cytosol was observed. This part of the curve represents high affinity saturable binding and on extrapolation can be used to determine the number of binding sites (n) and the dissociation constant (K_D) for the receptor-estradiol interaction [7, 8]. From the Scatchard plot data in Fig. 1 plasma addition caused an apparent increase in binding site concentration as well as a five fold rise in the dissociation constant. On other occasions the effect was even more pronounced. Plasma addition to cytosols containing smaller amounts of receptors produced flat curves with no indication of specific estradiol binding.

Since the presence of plasma in tissue cytosols cannot be excluded all results are the sum of tissue protein plus plasma protein influences. The plasma proteins most likely to have significant influence on the receptor-estradiol association are sex hormone binding globulin (SHBG), which has a considerable affinity for estradiol (K_D 1.7×10^{-9} M) [13] and albumin which has a large capacity though low affinity for estradiol (K_D 9.6×10^{-5} M) [14].

Purified bovine albumin was added to a breast carcinoma cytosol known to contain estradiol receptors such that albumin concentrations of 32% and 62% (amount albumin added with respect to total protein concentration) were achieved. After binding analyses were carried out in the usual way the resulting Scatchard plots (Fig. 2) revealed that increased albumin

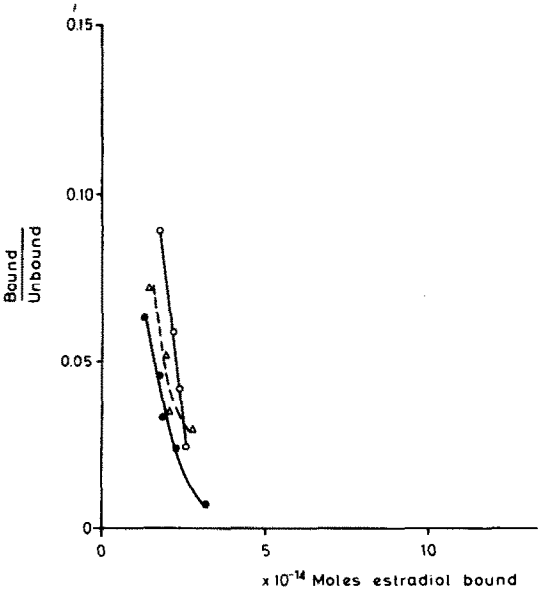

Fig. 2. Influence of albumin on the Scatchard plot characteristics of a human breast cancer cytosol containing receptors. ●——●, Control cytosol only; ○——○, Cytosol after bovine albumin addition (32% with respect to total protein concentration); △-----△, Cytosol with 62% bovine albumin.

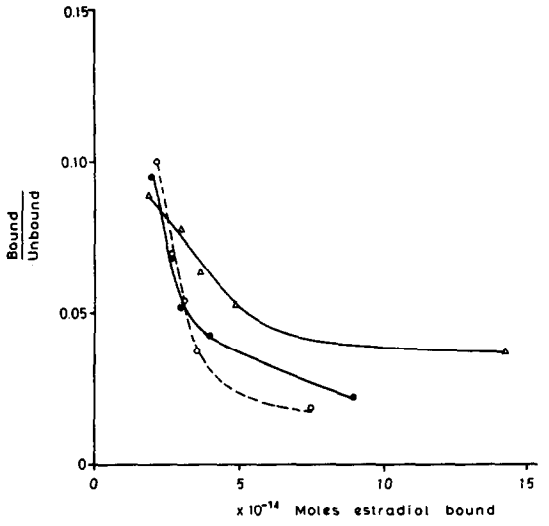

Fig. 3. Influence of SHBG partially purified by Con-A-Sepharose chromatography on the Scatchard plot characteristics of a myometrial cytosol containing receptors. ●——●, Control cytosol only; △——△, test sample, prepared by a 1 in 10 dilution of SHBG extract with cytosol; ○---○, test sample with the inclusion of 1 μg testosterone during incubation.

levels had only a marginal effect on the binding pattern. The expected upper limit of albumin contamination is around 60% in which case almost all the protein in the cytosol would be of plasma origin. This result demonstrates that the major alterations in cytosol binding characteristics depicted in Fig. 1 cannot be attributed to albumin interference.

Previously we have reported the affinity of SHBG for the glycoprotein adsorbent Con-A-Sepharose [15]. This group specific interaction can be reversed and the retained proteins recovered by washing with buffers containing 4% methyl-α-D-glucopyranoside. The adsorbent has been used to fractionate serum proteins and does not react with albumin [16, 17]. A partially purified SHBG extract, free of albumin, was obtained by Con-A-Sepharose chromatography of third trimester pregnancy plasma and the material was examined for its effects on the receptor-estradiol attachment. Myometrial cytosol was used as a control and a test sample consisting of the SHBG extract diluted 1 in 10 with cytosol was prepared. Binding studies were conducted as before except that on this occasion the test sample incubations were carried out in the presence and absence of 1 μg of testosterone. The results shown in Fig. 3 indicate a distinct change in the binding pattern on SHBG addition. Increased binding site concentration and dissociation constant were observed and the response mimicked the effect seen with plasma addition in Fig. 1. In the presence of testosterone however the influence of SHBG was completely negated. Presumably the androgen saturated the SHBG binding sites which otherwise would have been competing with the receptor for estradiol. Identical treatment of the plasma contaminated myometrial cytosol used in Fig. 1 gave parallel results and confirmed that SHBG was the major causative ingredient giving rise to variation in Scatchard plot characteristics.

DISCUSSION

The results described above illustrate that plasma proteins can interfere with the receptor–estradiol binding reaction. The interference is dependent on the degree of plasma contamination and the concentration of true receptor sites. The binding pattern observed is determined by contribution from both sources. Several workers have acknowledged the problems arising from plasma contamination of breast tumour cytosol [4, 9, 10, 18].

Our experiments have confirmed the previously reported [18] involvement of SHBG in the Dextran-coated charcoal assay for the receptors in target tissue cytosols. The inclusion of saturating amounts of testosterone during the incubation corrects this interference. Other workers have used the estrogen antagonist Upjohn 11,100 to discriminate between receptor and SHBG activity [9]. Although some interaction with SHBG has been noted the predominant effect of the antiestrogen is to suppress the binding of estradiol to receptor sites [9]. Wagner [3] with his elegant technique of argargel electrophoresis, has provided another means of differentiation between receptor- and SHBG-bound estradiol.

From our observations the presence of albumin appears to have little influence on the binding interaction at the concentrations measured (0.3 mg per ml of cytosol). This result conflicts with a report that albumin, in unspecified amounts, reduced the specific binding of estradiol [4]. Large concentrations of albumin in the assay medium (10 mg/ml) can alter the estimates from the binding parameters of high-affinity receptors [9].

The presence of high affinity estradiol-binding components in some human malignant breast tumours is related to response in endocrine therapy [12]. Studies of biochemical and clinical correlations have been based on quantitative as well as qualitative assessment of the receptors in these tissues [8, 9, 12, 20]. Excessive plasma contamination can lead to an over-estimation of tissue binding site concentration and the tumour would be wrongly classified as having a higher degree of estradiol dependence. Alternatively small amounts of receptors in a cytosol may be masked by plasma contamination and saturable binding with the associated steep slope in the Scatchard plot may not be demonstrable. A false negative estradiol dependency for the tumour could then be concluded.

The addition of testosterone provides a means of establishing accurate data concerning the receptor status of a tissue. Since 5α-dihydrotestosterone binds to SHBG with greater affinity than does testosterone [23] it may be even more efficient in eliminating interference from this protein. The results described in

this paper emphasize that the influence of plasma proteins should be given due consideration when estimations of receptor parameters are being sought.

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