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OESTROGEN RECEPTORS IN THE RAT ADRENAL GLAND

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SUMMARY

Cytosol from the adrenal gland of male and female rats contains a specific binding protein for oestradiol-17β. This protein has all the characteristics of a cytoplasmic oestrogen receptor. It is excluded by Sephadex G-200 gel filtration, has a sedimentation coefficient of 8–9 *S* by sucrose density gradient centrifugation in low salt and dissociates into a 4 *S* form by centrifugation in high salt (0.5 M KCl). The binding protein is heat sensitive and oestradiol-17β binding is eliminated by protease and by sulphydryl blocking reagents (2 mM *p*-chloromercuriphenylsulphonate). The bound oestradiol dissociates very slowly at 0°C. The adrenal oestrogen receptors have a very high affinity for oestradiol-17β, but lower affinity for oestradiol-17α and do not bind testosterone, androstene-3,17-dione or corticosterone. Scatchard analysis of the saturation data for oestradiol revealed one class of high affinity binding sites with an apparent equilibrium constant of dissociation (K_D) at 0°C of 5.8×10^{-10} M. The number of binding sites was calculated to be 70 fmol/mg cytosol protein. Cytosol fractions from androgen insensitive (*tfm*) male rats contain oestrogen receptors in amounts very similar to that of the normal littermates.

INTRODUCTION

Numerous studies have established that gonadal hormones play a major role in the regulation of adrenal cortical secretion in rats [1, 2], and increasing evidence indicates that sex hormones and pituitary peptide hormones interact in producing their effects on adrenal function [3, 4]. Thus, increased adrenal production of corticosterone has been demonstrated in response to oestradiol in ovariectomized, hypophysectomized rats maintained with ACTH. This effect, which is observed only in the presence of ACTH, indicates that oestradiol also acts directly on the adrenal to enhance corticosterone secretion [4]. This concept is in agreement with reports of selective accumulation of [^{3}H]-oestradiol in adrenal cortex of both mice [5], and rats [6]. Recently van Beurden-Lamers *et al.* [7] have reported on the presence of a 8 *S* oestradiol–protein complex in the adrenal of male rats. However, these receptors have not been extensively characterised.

The purpose of the present studies was to confirm the presence of oestrogen receptors in the adrenal gland of rats, and to further characterize these receptors. In addition we wanted to examine if there was a major difference in the adrenal oestrogen receptor concentration between the sexes and between normal rats and rats with the testicular feminization syndrome (*tfm*).

MATERIALS AND METHODS

Chemicals

[2,4,6,7-^{3}H]-Oestradiol 17β (S.A. 90 Ci/mmol) was obtained from New England Nuclear Corporation (U.S.A.). The solvents (benzene and ethanol) were evaporated under a nitrogen atmosphere at 50°C and the hormones dissolved in ethanol. The purity of the hormones, tested by thin-layer chromatography, was greater than 98%. Unlabelled steroids, Tris-(hydroxymethyl)-aminomethane (Tris), *p*-chloromercuriphenyl sulphonate (PCMPS), bovine serum albumin (BSA) and thioglycerol were obtained from Sigma Chemical Company (U.S.A.). Human immunoglobulin G (IgG) was purchased from A.B. Kabi (Sweden). The tetrasodium salt of ethylenediamine tetracetic acid (EDTA) was obtained from Fluka AG (Buchs SG, Switzerland). Silica gel plates (F 1500 LS 254) were purchased from Schleicher & Schüll (Dassell, Germany).

Animals

Male and female Sprague–Dawley rats 3 months of age were used. Rats with testicular feminization syndrom (*tfm*) and their normal littermate male (NL) rats were obtained from the colony established by Stanley and Gumbreck, University of Oklahoma. At the time of the experiments the animals were 12 months old. The experimental animals were kept under standardized conditions of temperature (23°C) and light (12 h light and 12 h darkness) on a diet consisting of commercial pellets and water *ad libitum*. Certain rats of both sexes were castrated one day before the experiments.

Preparation of cytosol and incubation procedures

The rats were killed by decapitation. The adrenals and in some cases the spleen and pieces of abdominal muscle were immediately removed, cooled on ice and

weighed after being carefully freed of adherent fat and connective tissue. Following mincing, the pooled tissues were transferred to 3 vol. of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 0.001 M EDTA. The adrenals were homogenized in an ice bath using round-bottomed glass homogenizers with Teflon pestles. The spleen and muscle tissues were homogenized with a X-1020 homogenizer (Intern. Laborat. App. GmbH, 7801 Dottingen, Germany) using 3 pulses of 10 s and at least one min between each of the bursts. The cytosol fractions were prepared by centrifugation of the homogenates at 105,000 *g* for 60 min at 0°C.

Incubation procedures

Aliquots of the cytosol fractions were incubated at 0°C for 16 h with 2 nM of [2,4,6,7-^{3}H]-oestradiol-17β with or without unlabelled steroids at varying concentrations above that of the radioligand. In addition, samples of the cytosol fractions were subjected to (1) heating for 60 min at 30° or 45°C after the incubation, or (2) treatment with protease (1 mg/ml) or *p*-chloromercuriphenyl sulphonate (PCMPS) (2 mM) for 3 h at 4°C.

The samples were finally analysed by sucrose gradient centrifugation.

Characterization of radioactive steroids

Steroids in the cytosol. Aliquots of the cytosol fractions were taken when the incubations were terminated for determination of possible metabolites of the radioligands. The aliquots were extracted 3 times with 2 vol. of diethyl-ether. The ether phase was evaporated and the residue subjected to thin layer chromatography (t.l.c.) on silica gel plates which were developed in benzene–ethylacetate (3:1, v/v). Oestradiol-17β ($R_F \sim 0.36$) and oestrone ($R_F \sim 0.60$) were completely separated in this system.

Protein bound steroids. The steroids bound by 8–9 *S* components from 5 gradients were extracted with ether. After evaporation the residues were examined by t.l.c.

Sucrose density gradient centrifugation

Linear 5–20% (w/v) sucrose gradients were prepared in 0.01 M Tris–HCl buffer at pH 7.4, containing 0.001 M EDTA, 0.012 M thioglycerol and 10% glycerol. To some gradients 0.5 M KCl was added. Following incubation, the cytosol fractions (250 μl) were layered on the top of the gradients and centrifuged at 265,000 *g* (av) for 20 h at 0°C in a Beckman L2-65B ultracentrifuge using a SW-56 rotor. After centrifugation the gradients were fractionated (10 drops per fraction) by perforating the bottom of the tubes.

The sedimentation coefficients were determined by the method of Martin and Ames [8] with Bovine serum albumin (BSA) (4.6 *S*) and human IgG (6.9 *S*) as reference standards. Radioactivity was measured after adding 5 ml of Bray's scintillation solution [9].

Other analytical methods

Protein was determined by the method of Lowry *et al.* [10], using BSA as reference standard. Radioactivity was measured in a Nuclear Chicago, Mark II liquid scintillation spectrometer with a ^{3}H-counting efficiency of 44%.

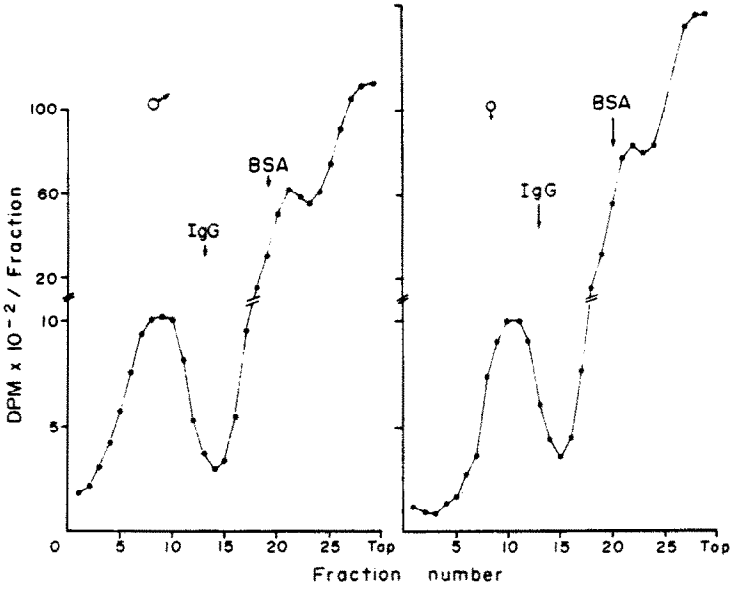

Fig. 1. Sucrose gradient centrifugation of male and female adrenal cytosol fractions incubated with 2 nM of [2,4,6,7-^{3}H]-oestradiol for 16 h at 0°C. Aliquots of 250 μl at equal protein concentration (12–14 mg/ml) were layered on the top of the gradients and centrifuged at 265,000 *g* (av) for 20 h at 0°C. Human gamma globulin (IgG) (6.9 *S*) and bovine serum albumin (BSA) (4.6 *S*) were used as markers. Data from the male is shown on the left, that from the female is shown on the right.

Table 1. Competition for [^{3}H]-oestradiol binding by unlabelled steroids

[^{3}H]-Steroid	Unlabelled steroid	% Binding
[^{3}H]-Oestradiol +	none	100
[^{3}H]-Oestradiol +	17β-oestradiol × 10	3
[^{3}H]-Oestradiol +	17β-oestradiol × 100	0
[^{3}H]-Oestradiol +	17α-oestradiol × 100	15
[^{3}H]-Oestradiol +	Testosterone × 20	95
[^{3}H]-Oestradiol +	Testosterone × 100	88
[^{3}H]-Oestradiol +	Androstenedione × 100	95
[^{3}H]-Oestradiol +	Corticosterone × 100	103

Aliquots of adrenal cytosol were incubated with 2 nM of [^{3}H]-oestradiol with and without unlabelled steroids at varying excess concentrations above that of the radioligand and analysed by sucrose gradient centrifugation. Bound radioactivity in the 8–9 *S* area of the gradients is expressed as per cent of the control.

RESULTS

Sedimentation pattern of bound radioactivity by sucrose density gradient centrifugation

The sedimentation profile of the bound radioactivity in adrenal cytosol fractions subjected to sucrose density gradient centrifugation after incubation with [^{3}H]-oestradiol was the same in male and female rats as shown in Fig. 1. The major part of the bound radioactivity sedimented in the 8–9 *S* area of the gradients while only a minor part migrated as a 4 *S* component. The amounts of radioactivity in the 8–9 *S* region of the gradients were very similar for both sexes. Very similar to what has been shown for oestradiol–receptor complexes in other target tissues, the 8–9 *S* binding component was dissociated into a 4 *S* form when the cytosol and sucrose gradients were made 0.5 M in KCl. Furthermore, when cytosol fractions labelled with [^{3}H]-oestradiol were subjected to Sephadex G-200 gel filtration at low ionic strength, the bound radioactivity was eluted in the void volume of the column (not shown).

No 8–9 *S* oestrogen binding component could be demonstrated in the cytosol of spleen or muscle tissue (not shown).

Specificity of the binding

The specificity of the 8–9 *S* oestrogen binding component of the adrenal cytosol was determined by measuring the degree of competition of [^{3}H]-oestradio binding by various unlabelled steroids. Samples of cytosol were incubated at 0°C for 16 h with [^{3}H]-oestradiol in the absence or presence of 10, 20 or 100-fold excess of unlabelled steroid. Oestradiol had a much higher affinity for the 8–9 *S* binding component than the other steroids tested. A 10 and 100 fold excess of unlabelled oestradiol-17β caused a complete inhibition of [^{3}H]-oestradiol binding (Table 1). Oestradiol-17α had lower affinity for the 8–9 *S* binding component and a 100-fold excess reduced binding by only approximately 85%. The binding affinity of testosterone, was very low and a 100-fold excess reduced [^{3}H]-oestradiol binding by less than 20%. Androstenedione and corticosterone did not affect the binding at any of the doses tested (Table 1).

Binding affinity and capacity

The binding affinity and capacity of the 8–9 *S* oestrogen binding component of the adrenal cytosol were determined by incubating aliquots of adrenal cytosol fraction from male rats with varying concentrations of [^{3}H]-oestradiol for 16 h at 0°C.

The binding to the 8–9 *S* component was almost saturated at 2 nM of [^{3}H]-oestradiol as shown in Fig. 2. The increase in binding obtained by higher concentration of the radioligand was very small. Scatchard analysis [11] of the saturation data indicate an apparent equilibrium constant of dissociation (K_D) of 5.8×10^{-10} M. The number of binding sites calculated from the intercept with the abscissa was 70 fmol per mg cytosol protein.

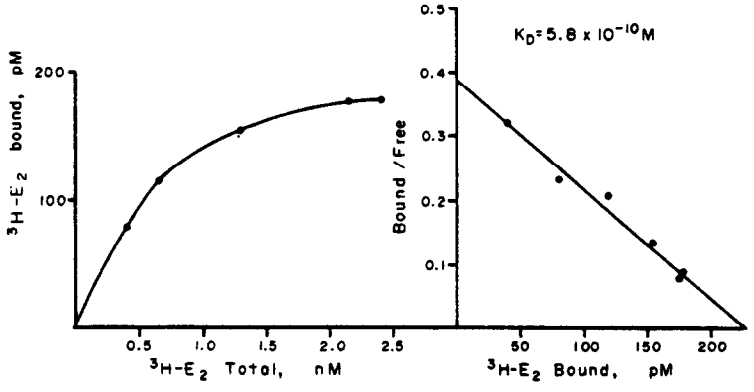

Fig. 2. Binding affinity and capacity of the 8–9 *S* adrenal oestrogen binding protein. Aliquots of the cytosol were incubated with increasing concentration of [^{3}H]-oestradiol-17β for 16 h at 0°C. Bound radioactivity was subsequently determined after sucrose gradient centrifugation performed as described in Fig. 1. Left: Bound [^{3}H]-oestradiol versus the concentration of [^{3}H]-oestradiol. Right: Scatchard plot of the data. Equilibrium constant of dissociation (K_D): 5.8×10^{-10} M. Number of binding sites: 70 fmol/mg cytosol protein.

Other characteristics of the oestrogen binding component

The 8–9 *S* oestradiol–receptor complex was destroyed when the adrenal cytosol was treated with protease or PCMPS as shown in Fig. 3. After heating of the cytosol for 60 min at 30°C a substantial decrease in steroid binding by the 8–9 *S* component was observed, and the binding was completely eliminated by heating to 45°C for 60 min.

The slow rate of dissociation of the oestradiol–receptor complex was demonstrated by adding a 1000-fold excess of unlabelled oestradiol-17β to samples of adrenal cytosol which had been equilibrated overnight with 2 nM [^{3}H]-oestradiol at 0°C. The amount of radioactivity bound by the 8–9 *S* component was determined by sucrose density gradient centrifugation, started three h after the addition of the unlabelled hormone. Only a marginal reduction in the 8–9 *S* complex was noticed after this treatment (Fig. 3).

Metabolism of [^{3}H]-oestradiol during incubation

Table 2 shows the metabolism of [^{3}H]-oestradiol taking place during incubation of adrenal cytosol at 0°C for 16 h. Of the added radioligand, 49% remained unchanged, whereas 44% was converted to oestrone and 3% behaved as polar compounds. In contrast, 95% of the radioactive material bound by the 8–9 *S* component was still oestradiol-17β.

Table 2. Metabolism of [^{3}H]-oestradiol during incubation for 16 h at 0°C and identification of bound oestrogens

[^{3}H]-Metabolites (%)	Cytosol metabolism	Bound
Polar compounds	3.0	5.3
Oestrone	44.3	0
Oestradiol-17β	49.0	94.7
Unidentified	3.7	0

Adrenal cytosol from intact female rats was incubated with 2 nM of [^{3}H]-oestradiol for 16 h at 0°C. Metabolism was examined in the cytosol and the pooled fractions from 5 gradients corresponding to the 8–9 *S* area. The radioactive steroids were extracted with 10 vol. of diethyl ether which was then evaporated. The residues were subjected to thin layer chromatography in duplicate. All values are corrected for blanks and presented as per cent of total radioactivity counted.

Binding of [^{3}H]-oestradiol in the adrenal cytosol of tfm *rats*

When samples of adrenal cytosol from *tfm* rats and their normal littermate (NL) male rats were incubated with [^{3}H]-oestradiol and subjected to sucrose density gradient centrifugation, the sedimentation pattern of the bound radioactivity was the same as that found in the Sprague–Dawley rats (Fig. 4). The amount of radioactivity bound by the 8–9 *S* component in the *tfm* and NL rats was very similar.

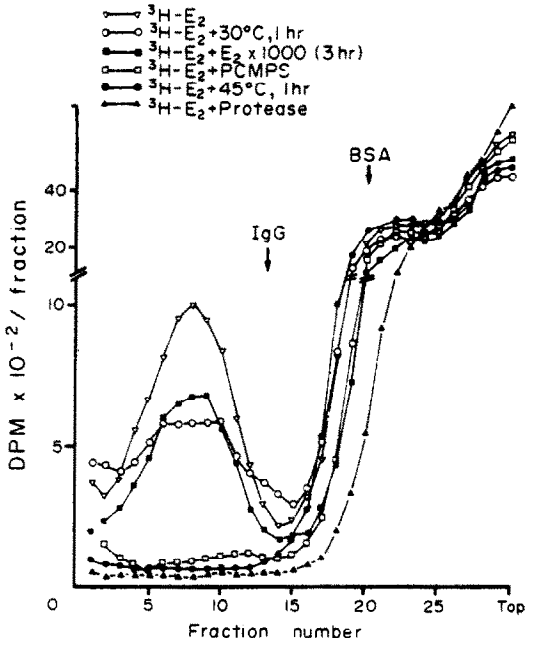

Fig. 3. Aliquots of adrenal cytosol equilibrated with 2 nM of [2.4.6.7-^{3}H]-oestradiol-17β for 16 h at 0°C were subjected to: (1) heating for 60 min at 30° and 45°C, (2) treatment with protease (1 mg/ml) or *p*-chloromercuriphenyl sulphonate (2 mM), (3) incubation for additional 3 h after adding a 1000-fold excess of unlabelled oestradiol-17β. Thereafter, aliquots of 250 μl were examined by sucrose gradient centrifugation as described for Fig. 1.

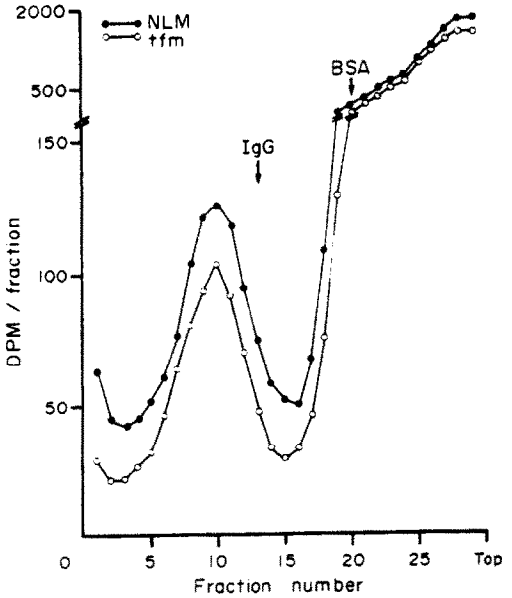

Fig. 4. Sucrose gradient centrifugation of adrenal cytosol fractions from androgen insensitive rats (*tfm*) and their normal littermates (NLM). The samples were incubated with 2 nM of [2.4.6.7-^{3}H]-oestradiol for 16 h at 0°C and aliquots (250 μl) analyzed by sucrose gradient centrifugation.

DISCUSSION

The present study demonstrates that the adrenal cytosol of both male and female rats contain a specific protein that binds oestradiol-17β with high affinity and low capacity and which has the characteristics of an oestrogen receptor. The presence of such a receptor implies that oestrogens may have a direct effect on the rat adrenal. By autoradiography Ullberg *et al.* [5] were able to demonstrate a selective concentration of radioactivity in the adrenal cortex of mice after administration of tritium labelled oestradiol-17β *in vivo*. Furthermore, our studies confirm and extend the results of van Beurden-Lamers *et al.* [7] demonstrating an 8 *S* oestradiol–protein complex in the adrenal gland of male rats.

The present results, demonstrate some additional characteristics of the oestrogen receptor of the rat adrenals, and establish the protein nature, the temperature instability, the slow dissociation and the inhibitory effect of alkylating reagents like PCMPS. The sedimentation coefficient of 8–9 *S* and the fact that the receptor proteins were excluded by Sephadex G-200 gels indicate a molecular weight of more than 200,000. The physicochemical properties, and steroid specificity are very similar to what has earlier been demonstrated for oestrogen receptors in the uterus [12] and anterior pituitary and hypothalamus of male and female rats [13, 14]. Furthermore, the binding affinity (K_D 5.8 $\times$ 10^{-10} M) and the number of cytoplasmic binding sites (70 fmol/mg cytosol protein) are of the same order of magnitude as has been reported for the oestrogen receptors in other male and female oestrogen target tissues [12, 13].

Almost 50% of the added [^{3}H]-oestradiol was metabolized into oestrone during the 16 h of incubation at 0°C. This finding probably means that the K_D of 5.8 $\times$ 10^{-10} M is an underestimation of the affinity of the receptor for oestradiol. However, in spite of the high degree of metabolism during incubation, nearly all of the radioactivity bound by the receptor was unchanged oestradiol. These results, which are in agreement with earlier observations [12], indicate that receptor bound oestradiol-17β is protected against metabolic conversion and in addition that the receptor has much lower affinity for oestrone than for oestradiol.

The affinity of the adrenal oestrogen receptor for testosterone was very low and only negligible displacement of the radioligand was observed by a 100-fold excess of unlabelled testosterone. Recently the rat adrenal has also been shown to possess specific androgen receptors [16]. However, the different binding specificities, and the present finding of a normal oestrogen receptor concentration in the adrenal cytosol of the androgen insensitive (*tfm*) rat (androgen receptors are absent in the *tfm* adrenals [16]), clearly show that the adrenal oestrogen receptors are entirely different from the androgen receptors.

The physiological significance of the adrenal oestrogen receptors is not yet clear. Ovariectomy in rats results in a significant reduction in adrenal corticosterone secretion, independent of the changes in ACTH secretion. These effects are reversed by oestradiol-17β administration [15]. Furthermore, adrenal corticosterone production is increased by oestradiol both in the presence and absence of the pituitary gland. This effect, observed only in the presence of ACTH, indicates that oestradiol also acts directly in enhancing adrenal corticosterone secretion [4]. The finding of specific oestrogen receptors in the adrenal gland of the rat adds further support to the concept that oestradiol can affect adrenal steroid secretion by direct action.

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