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FEMALE AND MALE GREEN MONKEY LIVER ESTROGEN RECEPTOR *

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Abstract—Adult female and male green monkey liver cytosol contains tritiated 17β-estradiol ([^{3}H]E_2) binding sites. The binding of [^{3}H]E_2 to these sites is reduced substantially by diethylstilbestrol. Unlike adult male rate liver cytosol, neither male nor female green monkey liver cytosol contains moderate affinity high capacity [^{3}H]E_2 binding sites. The green monkey liver cytosol [^{3}H]E_2 sites are precipitated by ammonium sulfate at 30% of saturation. The properties of the redissolved ammonium sulfate precipitated female and male [^{3}H]E_2 binding sites are similar: they have a high [^{3}H]E_2 affinity, a low [^{3}H]E_2 capacity, are estrogen specific, and are sensitive to sulfhydryl inactivation and protease digestion. Although the female and male sites are similar, there appears to be a 2- to 3-fold sex difference in the affinity (female K_d, 0.8 to 1.3 × 10^{-10} M; male K_d, 0.4 × 10^{-10} M) and capacity (female, 2.3 to 3.7 fmoles/mg of liver; male, 1.2 fmoles/mg of liver) of the [^{3}H]E_2 binding sites. Accordingly, adult female and male green monkey liver cytosol contains putative estradiol receptors.

The liver cytosol of adult female mammals contains estradiol binding sites which have been shown to be estrogen specific and of high affinity [1–6]. In the adult female rat, evidence has also been presented that the liver cytosolic estradiol binding sites can be translocated to the nucleus by estrogen administration *in vivo* [7]. The translocation was shown to be dose dependent [7]. The above properties are similar to those of the presumed uterine estrogen receptor and suggest that the estradiol binding sites in liver are estrogen receptors [1, 2].

The cytosol of adult male rat liver also contains estradiol binding sites. Unlike that of the female estradiol binding sites, the male sites have a moderate affinity, high capacity, and a different steroid specificity [8]. Male rat liver cytosol has been fractionated by 30% ammonium sulfate precipitation [9]. Ammonium sulfate precipitation at 30% of saturation has been used to purify partially the presumed estrogen receptor of adult female rat liver [7, 10]. The amount and properties of the redissolved 30% ammonium sulfate precipitated male rat liver cytosol estradiol binding sites are the same as those of the partially purified female rat liver cytosol estradiol binding sites [9]. Accordingly, it appears that adult male rat liver as well as adult female rat liver contains estrogen receptors and that the male liver receptor is demonstrable only after separation from an unusual estradiol binding protein.

When estrogens are administered to female rats, changes in the levels of plasma renin substrate are observed. This estrogen-induced increase in the levels of plasma renin substrate has been developmentally correlated with the concentration of liver estradiol binding sites [10]. When estrogens are administered to green monkeys, the levels of the plasma protein cortisol-binding globulin and thyroxine-binding globulin increase and the level of haptoglobin decreases [11, 12]. These same changes are observed in humans after estrogen administration [11].

The presence of estradiol binding sites in adult female green monkey liver cytosol has been indicated previously [2]. This paper describes the estradiol binding sites in female and male green monkey liver cytosol before and after partial purification by ammonium sulfate precipitation.

MATERIALS AND METHODS

Animals and materials. The green monkeys (*Cercopithecus aethiops*) were obtained from Primate Imports (Port Washington, NY). They were housed and cared for by the Animal Science Division. The monkeys were young adults weighing approximately 3 kg when killed. The stage of the menstrual cycle of the females was unknown. Two females and two males were anesthetized with sernylan and sodium phenobarbital. The animals were perfused with normal saline through the left ventricle, after incising the right atrium, until the livers were cleared of blood. The livers were then excised and retained in ice.

[2,4,6,7-^{3}H]Estradiol (102 Ci/m-mole) was obtained from New England Nuclear (Boston, MA). Before each experiment a portion of the tritiated estradiol ([^{3}H]E_2) stock was evaporated to dryness under nitrogen and redissolved in distilled H_2O to give the required concentration. Radioactivity was determined in a Packard 3380 liquid scintillation spectrometer at 50 per cent efficiency.

All nonradioactive steroids were of the highest purity available from commercial sources. Bovine pancreatic protease (containing tryptic and chymotryptic activity) was obtained from Worthington Biochemical Corp. (Freehold, NJ). (Code no. ATT). Parachloromercuriphenyl sulfonic acid was obtained from Sigma (St. Louis, MO).

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Cytosol preparation. All subsequent steps were performed at 0–4° unless otherwise stated. The livers were weighed, minced and homogenized in 6 vol. (w /v) of TEN buffer (0.01 M Tris–HCl, pH 7.4, 0.0015 M disodium EDTA, and 0.001 M NaN_3) using conical glass homogenizers. The liver homogenates were centrifuged at 25,000 *g* for 10 min. The homogenate supernatant fractions were then centrifuged at 105,000 *g* for 60 min and the clear supernatant fractions (cytosol) retained.

Ammonium sulfate precipitation. A neutralized saturated ammonium sulfate solution was added to the liver cytosol to a final concentration of 30% (v/v); the suspensions were stirred for 45 min, and then centrifuged at 25,000 *g* for 10 min. The supernatant fractions were discarded and the precipitates were redissolved in TEN buffer to a final volume equal to that of the original cytosol.

Binding assays and thin-layer chromatography. Unless otherwise stated, 25 μl portions of the aqueous [^{3}H]E_2 solutions were transferred to assay tubes. When competition for [^{3}H]E_2 binding with nonradioactive steroids was examined, the competitors (dissolved in 5 μl ethanol) were added before the [^{3}H]E_2. The binding assays, at least in triplicate, were commenced by the addition of 200 μl of the fractions to the assay tubes. The samples were incubated in ice for 1 hr (2 nM [^{3}H]E_2 binding incubations) or 22 hr (equilibrium [3H]E_2 binding incubations) and then the macromolecular bound radioactivity was determined with small polyacrylamide gel filtration columns (Biogel P10, exclusion mol. wt 20,000, 1.1 × 10 cm) maintained in a 4° cold room. Tris–HCl (0.01 M, pH 7.4) was used for column equilibration and sample elution [1].

When green monkey liver cytosol was assayed for the unusual estradiol binding sites detected in adult male rat liver cytosol [8], the binding assay was modified as follows: the [^{3}H]E_2 solution which was added to the assay tubes contained [^{3}H]E_2 with a specific activity of 0.4 Ci/m-mole, the final concentration of [^{3}H]E_2 was 1 μM, and the Biogel P10 column buffer contained 1.5 mM EDTA as well as 10 mM Tris–HCl pH 7.4 [8]. The samples were incubated for 1 hr in ice and then were gel filtered.

Upon completion of the assay incubations, the identity of the radioactivity in the incubation mixtures and in the macromolecular bound fractions was determined by thin-layer chromatography using silica gel. The elution solvent was 80% chloroform—20% ethylacetate. When the cytosols and redissolved ammonium sulfate fractions were incubated with 2 nM [^{3}H]E_2 for 60 min, most of the radioactivity present in the incubation mixtures was toluene extractable, and greater than 90 per cent of the toluene extractable radioactivity remained unmetabolized [^{3}H]E_2. The same was true of the binding at equilibrium incubations. In addition, 70 per cent of the toluene soluble macromolecular bound radioactivity remained unmetabolized [^{3}H]E_2 when cytosols were incubated with 1 μM [^{3}H]E_2.

Protein determination and data analysis. The protein concentrations of the fractions were determined by the method of Lowry *et al.* [13]. The liver cytosols contained 10–15 mg/ml of protein. Five to 7 per cent of the cytosol protein was recovered in the redissolved 30% ammonium sulfate precipitated fractions.

The standard error of the mean (S.E.M.) (triplicate

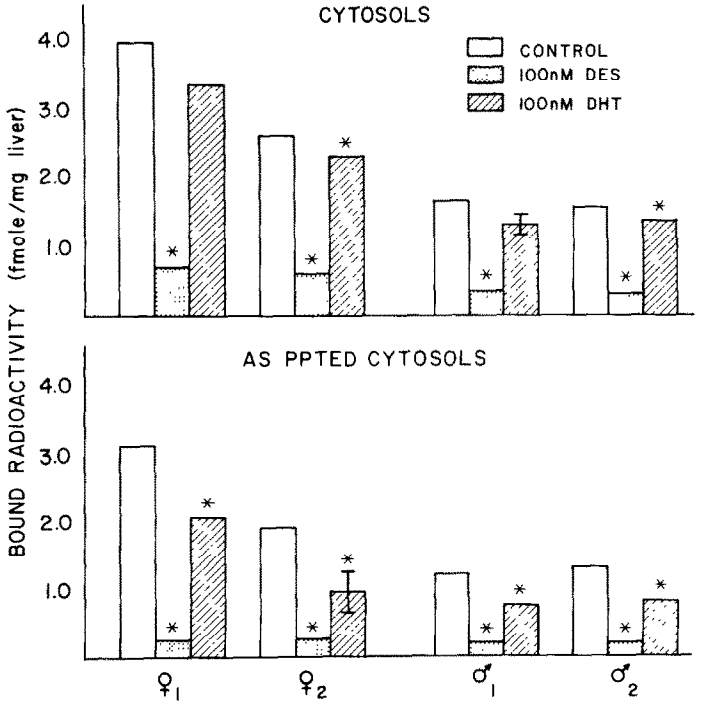

Fig. 1. [^{3}H]E_2 binding per mg of liver of cytosol and redissolved 30% ammonium sulfate precipitated cytosol of adult female and male green monkeys. The fractions were incubated with 2 nM [^{3}H]E_2 in the absence or presence of a 50-fold excess of DES or DHT for 1 hr at 0° and then the macromolecular bound radioactivity was determined by gel filtration. The asterisk (*) indicates $P < 0.05$ less than control.

Table 1. Macromolecular binding of [^{3}H]E_2 by green monkey cytosol after incubations with 1 μM [^{3}H]E_2*

Addition	Bound radioactivity (fmoles/mg liver)			
	Female 1	Female 2	Male 1	Male 2
None	57 ± 4	37 ± 2	40 ± 2	37 ± 3
Estradiol (10 μM)	46 ± 3	30 ± 1	33 ± 2	30 ± 4

* Cytosols were incubated with 1 μM [^{3}H]E_2 in the absence or presence of 10 μM nonradioactive estradiol for 1 hr at 0°; then the macromolecular bound radioactivity was determined by gel filtration. The gel filtration columns were equilibrated and eluted with 0.010 M Tris–HCl, pH 7.4, and 0.0015 M disodium EDTA. Values are means ±S.E.M.

values) is indicated in the figures by brackets. If a bracket is not depicted, the S.E.M. was smaller than could be clearly shown in the figures.

RESULTS

Estradiol binding of female and male liver cytosol. The macromolecular binding of tritiated 17β-estradiol ([^{3}H]E_2) by the cytosols of adult female and male green monkeys after incubations with 2 nM [^{3}H]E_2 is presented in Fig. 1. Both female green monkey cytosols bound more [^{3}H]E_2 than the male cytosols. The [^{3}H]E_2 binding of each cytosol was reduced, by a 50-fold excess of nonradioactive diethylstilbestrol (DES), to approximately 20 per cent of the control binding. A 50-fold excess of 5α-dihydrotestosterone (DHT) reduced the binding to approximately 85 per cent of control.

The macromolecular binding, after incubations with 1 μM [^{3}H]E_2 in the absence or presence of a 10-fold excess of nonradioactive estradiol, was also determined. A concentration of 10 μM estradiol is sufficient to saturate the moderate affinity estradiol binding sites found in adult male rat liver cytosol [8]. The results for the green monkey liver cytosols are presented in Table 1. Male rat liver cytosol contains 1540 ± 75 fmoles/mg of liver of [^{3}H]E_2 binding sites which are reduced to 180 ± 11 fmoles/mg by 10 μM estradiol [8]. Unlike male rat liver cytosol, none of the green monkey cytosols contained high levels of [^{3}H]E_2 binding sites which were reduced substantially by 10 μM estradiol. The levels of sites observed in the green monkey liver cytosols were the same as those observed in female rat liver cytosol (33 ± 1 reduced to 26 ± 2 fmoles/mg of liver by 10 μM estradiol) [8].

Table 2. Purification of green monkey cytosol [^{3}H]E_2 binding by ammonium sulfate precipitation*

Sample	[^{3}H]E_2 binding (fmoles/mg protein)	
	Cytosol	Partially purified cytosol
Female 1	32 ± 0.3	530 ± 10
Female 2	25 ± 0.3	260 ± 3
Male 1	16 ± 0.3	200 ± 4
Male 2	11 ± 0.2	290 ± 4

* Fractions were incubated with 2 nM [^{3}H]E_2 for 1 hr at 0°, and the macromolecular bound radioactivity was determined by gel filtration. Values are means ±S.E.M.

Comparison of the estradiol binding properties of the partially purified cytosols. Each cytosol was fractionated by 30% ammonium sulfate precipitation and the [^{3}H]E_2 binding of the redissolved precipitates determined. The results are presented in the lower portion of Fig. 1. Eighty to 90 per cent of the binding (which is reduced by 100 μM DES) was recovered in the redissolved precipitates and the binding per mg of protein was increased 10- to 26-fold by the fractionation (Table 2). Five to 7 per cent of the cytosol protein was recovered in the redissolved ammonium sulfate precipitates. The [^{3}H]E_2 binding of the partially purified cytosols was reduced by DES to approximately 12 per cent of control. DHT reduced the binding to approximately 60 per cent of control.

In addition to the nonradioactive competitors presented in Fig. 1, the [^{3}H]E_2 binding of the redissolved precipitates was examined in the presence of other competitors. The results are presented in Table 3. The binding was reduced substantially by estradiol, estrone and estriol but not by testosterone, cortisol or progesterone. In addition, the binding was reduced by sulfhydryl inactivation and proteolytic enzyme digestion (Table 3).

The [^{3}H]E_2 binding at equilibrium, of the partially purified female and male cytosols, was also determined. The fractions were incubated with various [^{3}H]E_2 concentrations ranging from 0.1 to 5 nM for 22 hr. The equilibrium binding results are presented as Scatchard plots (Fig. 2). The data for one of the females and one of the males are presented. Least squares fits of the data were employed to calculate the equilibrium dissociation constants and binding capacities of the fractions. Since the lowest binding values significantly deviated from linearity in each fraction, they were excluded. The basis for these deviations is unknown (see Discussion). The highest binding value of both males also significantly deviated and was excluded (see Discussion). The calculated dissociation constants, the binding capacities, the number of data points used, and the r values of the fits were as follows: Female 2 (data shown—K_d 0.8 × 10^{-10} M, capacity 22 fmoles/mg of cytosol protein, 6, 0.97; Male 1 (data shown)—K_d 0.3 × 10^{-10} M, capacity 12 fmoles/mg of cytosol protein, 5, 0.99; Female 1—K_d 1.3 × 10^{-10} M, capacity 29 fmoles/mg of cytosol protein, 5, 0.94; Male 2—K_d 0.4 × 10^{-10} M, capacity 9 fmoles/mg of cytosol pro-

Table 3. Specificity and sensitivity of partially purified female and male green monkey liver cytosol [^{3}H]E_2 binding*

Treatment	Percent of control [^{3}H]E_2 binding			
	Female 1	Female 2	Male 1	Male 2
None	100 ± 3	100 ± 1	100 ± 2	100 ± 1
Steroid competition (10^{-7} M)				
Estradiol	7 ± 1†	12 ± 1†	20 ± 3†	21 ± 1†
Estrone	11 ± 1†	19 ± 4†	20 ± 1†	24 ± 1†
Estriol	25 ± 1†	26 ± 1†	37 ± 3†	32 ± 1†
Testosterone	93 ± 7	89 ± 7	119 ± 3	99 ± 6
Cortisol	100 ± 3	94 ± 5	103 ± 6	90 ± 3
Progesterone	96 ± 6	92 ± 5	108 ± 6	119 ± 5
PCMS (5×10^{-3} M)	7 ± 2†	8 ± 4†	14 ± 4†	20 ± 2†
Protease (0.25 mg/ml)	5 ± 1†	6 ± 1†	25 ± 5†	17 ± 1†

* Partially purified cytosols were incubated with 2 nM [^{3}H]E_2 in the absence or presence of the nonradioactive steroids for 1 hr at 0°, with parachloromercuriphenyl sulfonic acid (PCMS) for 30 min at 0° and then 2 nM [^{3}H]E_2 for 1 hr at 0°, or with bovine pancreatic protease and 2 nM [^{3}H]E_2 for 1 hr at 25°; the macromolecular bound radioactivity was determined by gel filtration. Values are means ±S.E.M.

† $P < 0.05$ less than control.

tein, 5, 0.95. It appears, using the reduced number of data points, that the equilibrium dissociation constants and binding capacities of the females are 2- to 3-fold those of the males. This relative difference is also observed if all the data points are included in calculating the dissociation constants and binding capacities.

DISCUSSION

Both female and male green monkey liver cytosols contain estradiol binding sites which are partially purified by ammonium sulfate precipitation. Neither male nor female green monkey liver cytosol contains the

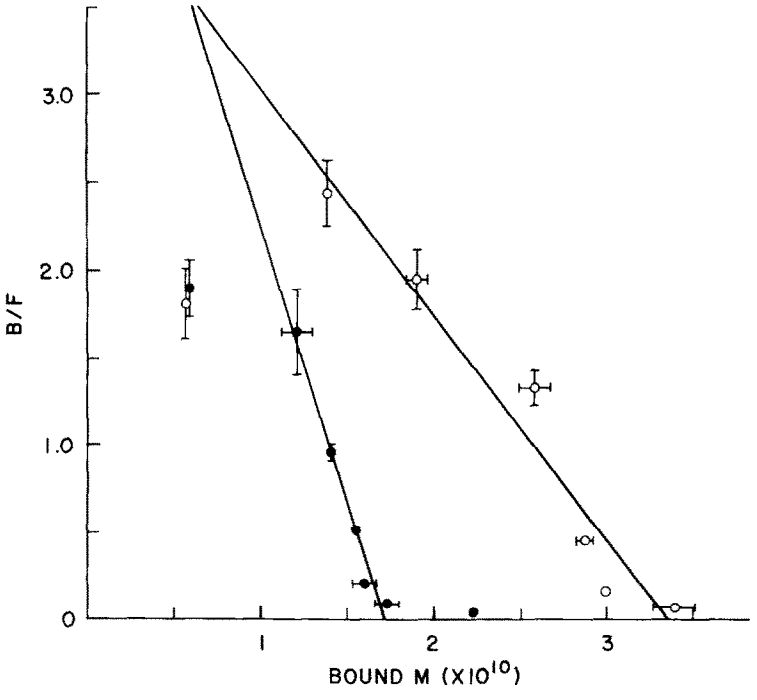

Fig. 2. Scatchard plots of the [^{3}H]E_2 binding of partially purified female and male green monkey liver cytosol. Redissolved 30% ammonium sulfate precipitated cytosols were incubated with seven concentrations of [^{3}H]E_2 ranging from 0.1 to 5 nM for 22 hr at 4° and then macromolecular bound radioactivity was determined by gel filtration. The data for a female (○) and a male (●) are presented. The lines are least squares fits of the data after points which significantly deviated from linearity were excluded (the lowest binding value for female and male and the highest binding value for male).

unusual sex steriod binder present in adult male rat liver cytosol [8]. The partially purified female and male liver cytosol estradiol binding sites appear to be protein(s) which contains sulfhydryl groups and appears to be estrogen specific, although 1×10^{-7} M DHT partially reduces the binding in these fractions as well as in the cytosols. DHT at 10^{-7} M does not reduce the estradiol binding by adult female rat liver cytosol [1] or partially purified female or male rat liver cytosol [9]. In addition, these sites have an apparent high affinity and low capacity for estradiol. The values for the affinity and capacity of the female sites appear to be 2- to 3-fold larger than the values for the male sites. Such a sex difference was not observed in partially purified female and male rat liver estradiol binding sites [9]. At present this difference can only be considered apparent. The affinities and capacities were determined from Scatchard plots which contained data points that deviated from linearity at low (0.1 nM) and high (5 nM) estradiol concentrations. Deviations from linearity at low estrogen concentrations have also been observed in Scatchard plots of rat uterine cytosol estradiol binding sites [14, 15]. For the uterine estrogen receptor the deviations were suggested to be an indication of cooperativity [15]. It is possible that the monkey liver binding sites may also be unstable at low ligand concentrations. Deviations from linearity at high ligand concentrations have also been observed for rat uterine cytosol estradiol binding sites [14, 15]. These deviations have been considered to be non-specific and of weak affinity. The basis for the deviations by the green monkey liver estradiol binding sites is unknown. Such deviations have also been observed for the partially purified female and male rat liver estradiol binding sites [9].

The presence of estradiol binding sites in female green monkey liver cytosol has been reported previously [2]. In that study fewer competitors were examined and the equilibrium dissociation constant and binding capacity were not determined. The properties of the partially purified female and male green monkey liver estradiol binding sites examined in the present study are the same as those that were reported for adult female green monkey liver cytosol [2]. In addition, the properties of the partially purified green monkey liver estradiol binding sites are similar to those of the partially purified female and male rat liver cytosol sites [9, 10]. It has been proposed that the rat liver cytosol sites are estrogen receptors [7, 9, 10]. It would appear that green monkey liver contains estrogen receptors.

Estrogen receptors have also been reported in human liver cytosol [16]. The cytosol was prepared from livers obtained post-mortem from postmenopausal women. Estrogen binding sites which appeared to be estrogen specific protein(s) and had a K_d of 2.4×10^{-9} M were observed.

A major reason for examining mammalian liver estrogen receptor(s) has been their potential role in modulating plasma protein levels and other aspects of liver function [17–20]. This modulation may contribute to the side effects which have been reported for women taking estrogens alone or in oral contraceptives [21–27]. In the rat, evidence has been presented which suggests that the liver estrogen receptor modulates at least one liver function, the synthesis of renin substrate [10]. The increased levels of plasma renin substrate appear to be a direct effect of estrogens on the rat liver [28]. It has been demonstrated that estrogen administration to green monkeys results in changes in the levels of several plasma proteins: thyroxine-binding globulin (TBG) and cortisol-binding globulin (CBG) levels were increased and haptoglobin levels were decreased [11, 12]. In humans, the same changes in levels of these plasma proteins (TBG, CBG and haptoglobin) were also observed after estrogen administration [11]. It is possible that these plasma protein changes in green monkey and human are a direct estrogen effect (via the estrogen receptor) on the liver.

Continued study of the possible modulatory role of mammalian liver estrogen receptors on liver functions (including changes in the synthesis of plasma proteins) may provide principles useful for improving the safety of estrogens taken alone or in oral contraceptives.

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