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SPECIFIC MACROMOLECULAR BINDING OF ESTRADIOL IN THE MAMMALIAN LIVER SUPERNATANT

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Abstract—The liver supernatant of adult female rats contains proteins with a high specificity for binding radioactive estradiol and other potent estrogens. Several properties of the liver macromolecules are similar to those of the presumed estrogen receptor found in the uterus. Specific estrogen binding is also present in the liver supernatant of the rabbit and green monkey. Estrogens are known to influence liver composition and function in mammals. The estrogen binding protein observed in the mammalian liver may be an estrogen receptor.

High affinity binding of estradiol has been demonstrated in the supernatant fraction of target organs such as the uterus, hypothalamus and pituitary. This is thought to represent the initial interaction of the steroid with its receptor. The complex may then cross the nuclear membrane, attach to chromatin and modulate RNA synthesis [1–3].

In non-mammalian vertebrates, estrogens act in the liver to increase the synthesis of egg yolk proteins which are secreted into the plasma and incorporated into the egg by the ovarian follicles [4–7]. It is also known that estrogen administration modulates the composition and function of the mammalian liver [8]. It is not established if this is a direct effect of estrogen on the liver. The effect may be indirect, for example, via the hypothalamic-pituitary axis.

Early studies did not show substantial levels of high affinity estrogen specific binding in a mammalian liver supernatant after the neonatal period [9–13]. We have described in abstracts [14–16] and in a summary report [17] that the adult mammalian liver does contain binding proteins with a high affinity for estrogens. The present report extends the data in particular comparing the estrogen binding properties found with liver supernatant to those of the presumed estrogen receptor from the uterus.

MATERIALS AND METHODS

The animals were intact adult females. The rats were obtained from Charles River (CD strain) and weighed 200–300 g. The rabbits were New Zealand Whites. The green monkeys (*Cercopithecus aethiops*) were obtained from Primate Imports. The animals were housed and cared for in the Animal Science Division.

To diminish the blood contained in the tissue samples, the anesthetized rats and rabbits were perfused with normal saline through the left ventricle after incising the right atrium. The monkeys were anesthetized with fluothane and nitrous oxide and liver portions obtained by open wedge resection at laparotomy. The monkey liver samples were sliced and rinsed briefly in the cold saline.

The tissues were homogenized in 6 vol. of 0.01 M Tris–HCl, pH 7.4, with 0.0015 M EDTA in a conical all-glass homogenizer. After centrifugation in a Sorvall RC2-B at 5000 *g*, the supernatant below the floating lipid phase was transferred to a clean tube and recentrifuged at 100,000 *g* for 1 hr in a Beckman L5-65 ultracentrifuge. The clear supernatant was obtained with a Pasteur pipette, once again excluding the lipid layer.

Radioactive estradiol-2,4,6,7-[^{3}H] (100 Ci/m-mole) was obtained from New England Nuclear. Aqueous solutions from the benzene methanol stocks were prepared by addition of water and flash evaporation at 40°. All non-radioactive steroids and enzymes were of the highest purity available from commerical sources.

Unless otherwise specified, 25 μl of radioactive estradiol in water was added to 200 μl of the supernatant to a final concentration of estradiol of 2×10^{-9} M. Each experimental group contained at least triplicate samples. The tubes were briefly shaken and then placed in ice for 1 hr. Macromolecular-bound radioactivity was separated from free with small polyacrylamide gel filtration columns (Biogel P10, exclusion mol. wt. 20,000, 1.15 cm in diameter and 10 cm in height) maintained at 4° in a cold room. One hundred μl of 1% dextran blue was added to the incubated supernatants immediately before application to the column. The column eluate (3 cm^3) was collected starting with the appearance of the dye marker. Organic solvent soluble radioactivity in this macromolecular-bound fraction was extracted into 10 ml of 95% toluene–5% isoamyl alcohol. Five ml of the organic phase was added to 10 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole and 50 mg *p*-bis[2-(5-phenyloxazolyl)]benzene/liter of toluene. The samples were counted in a Packard 3380 at 50 per cent efficiency. This experimental procedure has been previously described [18]. The protein content of the supernatants was determined by the assay

of Lowry *et al.* [19] using bovine serum albumin as the standard.

The identity of radioactivity in the toluene extract of the supernatant after incubation and also after gel filtration was determined by thin-layer chromatography using silica gel with 80% chloroform–20% ethyl acetate as solvent. The radioactivity in the toluene extract after gel filtration was further characterized by derivative formation using conditions for methylation described by Brown [20]. The methylated products were identified with the 80% chloroform–20% ethyl acetate thin-layer chromatographic system.

The sedimentation coefficient of the macromolecular-bound radioactivity was determined after gel filtration. In some experiments, a sedimentation coefficient marker, 1 mg of albumin, was added to the gel-filtered supernatant immediately before layering the sample onto the sucrose gradients. The 5–20% gradients contained 0.01 M Tris–HCl, pH 7.4; 0.01 M KCl; 0.001 M NaN_3; and 0.0015 M EDTA. The samples were centrifuged in a Spinco L5-65 in the SW56Ti rotor at 36,000 rev./min (126,000 g R_{ave}) for 16 hr. Eight-drop fractions were collected after puncturing the bottom of the tubes. The albumin peak was localized by measuring the 280 nm optical density of the fractions; then radioactivity was measured after the addition of 10 cm^3 Aquasol.

To determine the equilibrium dissociation constant, a dialysis system was devised to minimize the effect of metabolism on the [^{3}H]estradiol concentrations. In preliminary experiments, it was found that the radioactive estradiol was extensively metabolized to estrone in prolonged incubations even at 4°. Liver was homogenized in 24 vol. of Tris–EDTA buffer and the cytosol was obtained by ultracentrifugation. A portion of the supernatant (1 ml) was mixed with eight concentrations of [^{3}H]E_2 with and without 10^{-6} M diethylstilbestrol (DES) in dialysis bags. Each of the sixteen dialysis bags was suspended in 50 ml of the buffer containing the corresponding concentrations of radioactive estradiol with and without DES. After magnetic stirring for 24 hr in the cold room (4°), binding (in quadruplicate 0.2-ml samples of the dialyzed cytosol) was measured by gel filtration. Another portion of the supernatant and a portion of the dialysis buffer were extracted with toluene, and the extract was counted and the radioactivity identified by thin-layer chromatography. Only slight metabolism was found; the free radioactive estradiol levels were near the initial concentrations.

RESULTS

The macromolecular binding of radioactive estradiol by rat liver supernatant is compared to other tissue supernatants in Table 1. The supernatants of the tissues were incubated with 2×10^{-9} M [^{3}H]E_2 for 1 hr in ice before gel filtration. The liver binding is lower than that found with uterine supernatants but higher than plasma, spleen or heart. Upon thin-layer chromatography, the bound radioactivity in the liver is 90 per cent estradiol and 10 per cent estrone. After methylation with dimethyl sulfate, which converts estradiol to 3-methoxyestradiol, 90 per cent of the radioactivity has a similar chromatographic mobility to authentic 3-methoxyestradiol.

Table 1. [^{3}H]E_2 macromolecular binding in liver and other organs*

Supernatant	Bound radioactivity (dis./min)	Bound radioactivity (dis./min/mg cytosol protein)
Liver	6200 ± 350†	1900 ± 110†
Spleen	1500 ± 50	720 ± 20
Heart	740 ± 20	560 ± 20
Plasma	300 ± 20	110 ± 10
Uterus	30,800 ± 470†	23,000 ± 350†

* [^{3}H]E_2 (2×10^{-9} M) was incubated with 0.2 ml supernatant for 1 hr at 0° followed by gel filtration.
† $P < 0.01$, significantly higher than other organs.

The specificity of the binding of 2×10^{-9} M radioactive estradiol was studied by adding 2×10^{-7} M non-radioactive compounds to the incubation mixture. The potent estrogens–estradiol, ethinyl estradiol or DES—decrease liver [17] and uterine radioactive estradiol binding to 10 per cent of control or less. There is no significant decrease with testosterone, dihydrotestosterone (DHT), progesterone, corticosterone or dexamethasone with either liver [17] or uterus. As shown in Table 2, estrone, estriol or mestranol partially decreases both liver and uterine estradiol binding. Using higher concentrations (10^{-5} M), only dihydrotestosterone of the steroids studied diminishes liver estradiol binding. Dihydrotestosterone (10^{-5} M) diminishes liver and uterine binding to the same extent. Thus, the steroid specificity for estradiol binding in liver and uterine supernatant is similar. The binding of estradiol to liver or uterine supernatant macromolecules is also equally sensitive to the proteolytic enzymes papain, trypsin or chymotrypsin.

The binding of estradiol in liver is inhibited by the addition of a sulfhydryl reacting reagent [17]. The concentration of *p*-chloromercuriphenylsulfonate (PCMS) required for similar levels of inhibition is higher for the liver than the uterus (Table 3).

Table 2. Steroid specificity of radioactive estradiol macromolecular binding*

Steroid	Conc (M)	Per cent of control Liver	Uterus
Estrone	10^{-7}	17 ± 4†	8.5 ± 0.7†
Estriol	10^{-7}	17 ± 3†	14.0 ± 0.6†
Mestranol	10^{-7}	27 ± 0.5†	39.0 ± 1.0†
Dihydrotestosterone	10^{-5}	54 ± 3†	53.0 ± 1†
Testosterone	10^{-5}	102 ± 2	
Progesterone	10^{-5}	120 ± 3	
Corticosterone	10^{-5}	123 ± 2	
Dexamethasone	10^{-5}	110 ± 2	
Cortisol	10^{-5}	112 ± 5	

* [^{3}H]E_2 (2×10^{-9} M) was mixed with 1×10^{-7} or 1×10^{-5} M non-radioactive steroids. After adding the tissue supernatants and incubating for 1 hr at 0°, the samples were gel filtered. Values are the per cent of control binding ± S.E.M. Control binding has been set at 100 per cent.
† $P < 0.05$, lower than controls.

Table 3. Sensitivity of [^{3}H]E_2 liver and uterine binding to a sulfhydryl reacting reagent*

PCMS concn (M)	Per cent of control binding	
	Liver	Uterus
10^{-3}	67 ± 4†	28 ± 1
5×10^{-3}	16 ± 1†	2 ± 0.2
10^{-2}	9 ± 1†	1 ± 0.1

* Liver or uterine supernatants were incubated at 0° with *p*-chloromercuriphenyl-sulfonate (PCMS) for 30 min followed by the addition of [^{3}H]E_2 for 1 more hr before gel filtration.

† $P < 0.05$, less inhibited than uterus.

The liver is the major site of metabolism of steroids including estrogens. Upon incubation of the supernatant at higher temperatures, most of the estradiol is converted to estrone. Conceivably, the enzyme responsible for this conversion, 17-hydroxysteroid oxidoreductase (EC 1.1.1.-) could be the binding protein. This possibility is ruled out by the data shown in Table 4. Fifty per cent of the radioactive estradiol is metabolized to estrone in 1 hr at 22°. Addition of non-radioactive DES almost completely abolishes the macromolecular binding but does not diminish the oxidation of estradiol to estrone.

The sedimentation coefficients for the estradiol binding macromolecules from liver and uterine supernatants were determined by ultracentrifugation in sucrose gradients. The supernatants were first incubated with 5×10^{-9} M radioactive estradiol in the presence and absence of 1×10^{-7} M DES for 1 hr in ice and then gel filtered. For liver supernatant, the specific binding (the difference between the binding in the presence and absence of DES) was distributed as 38 per cent in the 8S region and 62 per cent in the 4S region [17]. For uterus, the specific binding was distributed as 55 per cent in the 8S region and 45 per cent in the 4S region. The ratio of 8S to 4S specific binding is 0.6 for the liver supernatant and 1.2 for the uterine supernatant.

As previously described [17], the dissociation constant and binding capacity for the liver were determined using dialysis conditions which maintain the radioactive estradiol at its initial concentrations. The dissociation equilibrium constant is approximately 1×10^{-10} M and the binding capacity for estradiol is 4.7 fmoles/mg of liver (58 fmoles/mg of cytosol protein). The corresponding values found with the uterine supernatant using this dialysis system were a K_D of 1.4×10^{-10} M and a binding capacity of 15 fmoles/mg of uterus.

Table 4. Dissociation of [^{3}H]estradiol macromolecular binding from 17-oxidoreductase activity in liver supernatant*

	Macromolecular binding (dis./min)	Conversion of supernatant radioactivity to estrone (%)
Control	13,760 ± 1500	51 ± 2
DES	586 ± 40	63 ± 2

* Rat liver supernatant was incubated at 22° for 30 min with 2×10^{-9} M [^{3}H]estradiol with or without 10^{-7} M DES; 0.5 ml of the supernatant was gel filtered to measure macromolecular binding. Another portion of the supernatant was extracted with toluene and the radioactivity identified by thin-layer chromatography.

Table 5. [^{3}H]E_2 binding in rabbit liver supernatant*

	Liver	Plasma
Control	11,400 ± 760	380 ± 90
5×10^{-3} M PCMS	12 ± 1†	101 ± 13
Heat (50°)	12 ± 2†	134 ± 4
Estradiol	18 ± 1†	
DES	21 ± 2†	
Cortisol	96 ± 4	

* Studies of adult female rabbit liver supernatant were conducted as described for the rat. The control represents binding of 2×10^{-9} M [^{3}H]E_2 in 0.2 ml supernatant; values are expressed as dis./min. The other results are expressed as per cent of control binding. Temperature stability of the preformed complex was determined by a 10-min incubation at 50°. For competition, the concentration of estradiol was 2×10^{-7} and 10^{-7} M for DES or cortisol.

† $P < 0.05$, lower than control.

The liver supernatant of other mammals also binds estradiol. The adult female rabbit liver supernatant has high levels of binding which are diminished in the presence of non-radioactive E_2 or DES (Table 5). The adult female green monkey liver supernatant binds high levels of [^{3}H]estradiol (Table 6), and 70 per cent of the bound radioactivity is estradiol. When the radioactivity from the toluene extract of the macromolecular-bound fraction reacts with dimethyl sulfate, the estradiol peak is converted to a derivative with the mobility of authentic 3-methoxyestradiol. The specificity of the green monkey liver binding clearly distinguishes it from the sex hormone-binding globulin found in green monkey blood. Serum sex hormone-binding globulin is known to bind estrogens and androgens, but not DES. In liver, DES [17] is an effective binding competitor; testosterone and DHT are relatively ineffective. The estradiol binding to macromolecules in the supernatant from rabbit or

Table 6. [^{3}H]E_2 binding in monkey liver*

	Bound radioactivity
Control	13,500 ± 3400
Estradiol (10^{-7} M)	14 ± 1†
Estrone	18 ± 1†
Testosterone	101 ± 3
Dihydrotestosterone	87 ± 2†
Cortisol	103 ± 4
PCMS (10^{-3} M)	63 ± 2†
(5×10^{-3} M)	11 ± 1†
Heat 50°, 10 min	11 ± 1†

* Adult female green monkey liver supernatant (0.2 ml) was incubated with 2×10^{-9} M [^{3}H]estradiol for 1 hr followed by gel filtration. The concentration of the non-radioactive hormones was 10^{-7} M. Values for the control are expressed in dis./min. The other results are expressed as the per cent of control binding.

† $P < 0.05$, less than control.

monkey liver is diminished by heating to 50° or by addition of a sulfhydryl reacting reagent.

DISCUSSION

This paper demonstrates a high affinity estrogen specific binding protein in the adult female rat liver with several similar properties to the presumed estrogen receptor in the uterus. The only apparent differences between the liver and uterine binding are that the liver macromolecule is predominately 4S in sucrose gradients using low salt conditions and that higher concentrations of PCMS are required for comparable inhibition. The adult female rabbit and green monkey liver supernatants also contain estrogen specific binding macromolecules.

The characteristics of this rat liver estrogen binder do not correspond to some systems with a poorer affinity which have been described previously [21–23]. Although earlier reports could not demonstrate the present system, two recent reports confirm its presence in the adult female rat liver supernatant [24, 25].

There are several factors which contribute to the previous experimental difficulties in demonstrating estrogen receptor-like interaction in the mammalian liver. The concentration of the binding protein in the adult liver is lower than the uterus. The liver contains metabolizing enzymes that will metabolize the [^{3}H]estradiol especially at elevated temperatures. A major factor in previous studies using rat supernatant *in vitro* is that the liver supernatant was obtained from prepubescent rats. The accompanying paper [26] indicates that the concentration is low in the liver before puberty and correlates this with a liver-mediated response to estrogen administration.

Work in progress in this laboratory further supports the concept that the present system may be the estrogen receptor in the rat liver. There is temperature-dependent transfer of the binding protein from the cytosol to nucleus in liver slices. In a cell-free system, the cytoplasmic-binding protein–estradiol complex can attach to the liver chromatin [17]. Furthermore, we have preliminary evidence that the binding macromolecules can be translocated to the liver nucleus by estrogen administration *in vivo*.

The biologic and pharmacologic significance of this potential estrogen receptor in the mammalian liver is further discussed in the accompanying paper [26].

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