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DEVELOPMENTAL CORRELATION OF HIGHER LEVELS OF ESTROGEN BINDING BY MACROMOLECULES IN RAT LIVER SUPERNATANT AND OF INCREASES IN PLASMA RENIN SUBSTRATE LEVELS AFTER ESTROGEN ADMINISTRATION

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Abstract—The adult female liver supernatant contains a high affinity estrogen specific binding protein. The liver of the prepubescent female rat has less estrogen specific binding macromolecules than the adult. This difference in quantity is maintained when binding activities are partially purified by precipitation with ammonium sulfate at 30% saturation. After administration *in vivo* of ethinyl estradiol, plasma renin substrate (PRS) levels increase 167 per cent above control in the adult female rat. The corresponding increase is only 15 per cent in the prepubescent rat. In contrast, renin substrate levels are markedly increased in both the prepubescent and adult by administration of the glucocorticoid, dexamethasone. The substantial increase in the amount of estrogen binding and PRS responsiveness to estrogen administration with sexual maturation suggests that the estrogen binding protein may be an estrogen receptor involved in modulating synthesis of at least one plasma protein.

The preceding companion paper [1] indicates that the liver supernatant of the adult female rat contains estrogen specific binding proteins. The present study shows that this binding is relatively low in the prepubescent rat. In earlier studies from other laboratories, prepubescent animals were used and substantial estrogen binding in liver supernatant could not be demonstrated [2–6].

Estrogen may act in the mammalian liver to change its biochemical composition and function [7]. It has been known that estrogen administration to mammals can change the level of selective plasma proteins, presumably by modulating their hepatic synthesis. In women, estrogen administration increases several plasma proteins and decreases others [8]. Fewer plasma protein levels are changed in some lower mammals [8]. In adult rat, the plasma protein that undergoes the largest change is renin substrate. Renin substrate increases several-fold after administration of high doses of estrogens [9, 10]. The present study shows that, unlike the adult, the prepubescent female rat has little, if any, increase in plasma renin substrate (PRS) after estrogen administration.

This report extends the data we have previously presented [11]. Estrogen binding in the prepubescent and adult rat liver supernatant is further studied. Plasma renin substrate responses after administration of an estrogen or of a glucocorticoid are compared.

MATERIALS AND METHODS

Macromolecular binding of radioactive estradiol to tissue supernatants was studied using gel filtration columns to separate bound from free radioactivity. The procedures followed are described in the preceding companion paper [1].

Ammonium sulfate fractionation of liver supernatant and plasma was accomplished by the slow addition of a neutralized saturated ammonium sulfate solution. The mixture was maintained on ice and constantly stirred during and for 45 min after the ammonium sulfate addition to 30% saturation. The precipitate was separated by centrifugation at 23,000 *g* for 10 min and then redissolved in the Tris–EDTA buffer in a volume corresponding to the initial volume of the supernatant. Radioactive estradiol was then incubated with the partially purified preparations and assayed for radioactive estradiol binding in a fashion similar to supernatant preparations.

The studies of estradiol binding and of administration with ethinyl estradiol used 200 g adult or 25 to 29-day-old female (60 g) Sprague–Dawley rats. They were obtained from Charles River and were of known birthdate. Ethinyl estradiol (Mann Research) was dissolved in propylene glycol at a concentration of 1 mg/cm^3; 100 μg (0.1 cm^3) was injected in the subcutaneous space between the scapulae at 0 and 24 hr. The controls received the vehicle alone. Blood samples were obtained at 48 hr for renin substrate determination.

For the studies with dexamethasone treatment, the Sprague–Dawley rats were obtained from Zivic Miller. Blood samples were obtained from the jugular vein under light ether anesthesia. (0.5 ml/Blood 100 g of body weight) was aspirated into a syringe containing 0.05 ml of 2% disodium EDTA. These blood samples were obtained just prior to steroid administration so that each animal served as its own control. The rats then received a single subcutaneous injection

Table 1. [^{3}H]E_2 binding in tissue supernatants of the prepubescent and adult female rat *

	Bound radioactivity in supernatants (dis./min)			Bound radioactivity in supernatants (dis./min/mg cytosol protein)		
	Liver	Uterus	Plasma	Liver	Uterus	Plasma
Prepubescent	1,900 ± 600	65,000 ± 400	4,200 ± 390	930 ± 120	99,000 ± 600	3,600 ± 300
Adult	9,600 ± 1,800†	42,000 ± 1,300‡	230 ± 20‡	4,800 ± 900†	59,000 ± 1,800‡	130 ± 10‡

* [^{3}H]estradiol (2×10^{-9} M) was incubated with 0.2 ml of tissue supernatant from 28-day-old or mature female rats for 1 hr at 0° before gel filtration. In this experiment, the uteri were homogenized in 12 vol. buffer. Results are expressed as dis./min bound/0.2 ml of supernatant and/mg of cytosol protein ± S.E.M.

† $P < 0.01$, significantly higher in adult.

‡ $P < 0.001$, significantly lower in adult.

of 4 mg/kg of dexamethasone phosphate dissolved in isotonic saline. A second blood sample was obtained 24 hr later. The blood samples were collected into chilled tubes containing EDTA. The plasma was immediately separated in a refrigerated centrifuge and frozen until analyzed. Plasma renin substrate was determined by radioimmunoassay of angiotensin I. Angiotensin I was generated by incubating the plasma with an excess of purified rat kidney renin in the presence of inhibitors of converting enzyme and angiotensinases as previously described [12].

RESULTS

The prepubescent rat liver supernatant has less macromolecular-bound estradiol than the adult expressed as either per mg of tissue or per mg of supernatant protein (Table 1). This contrasts with higher levels bound in the uterine supernatant or diluted plasma from the 28-day-old rat. The lower binding in the prepubescent liver supernatant is not due to more rapid metabolism of the [^{3}H]estradiol. As shown by thin-layer chromatography, approximately 75 per cent of the radioactivity in the supernatant remains unchanged estradiol after adult or prepubescent liver supernatant was incubated 1 hr at 0°. Mixing of prepubescent and adult liver supernatants before incubation with the estradiol leads to approximately the binding expected from the separate incubations (Table 2). Accordingly, the prepubescent liver does not seem to contain an inhibitor of the binding process.

The top panel of Fig. 1 illustrates that the low level of [^{3}H]E_2 binding in prepubescent liver supernatant is partially diminished by 10^{-7} M non-radioactive E_2 or diethylstilbestrol (DES). The macromolecular binding of [^{3}H]E_2 in prepubescent plasma differs from that of the liver in that estradiol but not DES competes. The estrogen binding was further characterized by ammonium precipitation at 30% saturation. The adult estrogen binding macromolecules are partially purified without loss of binding activity. As illustrated in the bottom panel of Fig. 1, the adult has an 8-fold higher level of binding (subject to estradiol or DES competition) than the prepubescent liver. The binding in prepubescent liver remains partially inhibited by addition of either E_2 or DES, while that of plasma is inhibited only by E_2. Figure 2 illustrates the same

Table 2. Effect of mixing prepubescent and adult female liver supernatants*

Prepubescent (cm^3)	Adult (cm^3)	Binding (dis./min)
0.2	0	1050 ± 10
0	0.2	6170 ± 100
0.1	0.1	3150 ± 130

* Prepubescent and adult female liver supernatants were incubated separately or mixed before adding 2×10^{-9} M [^{3}H]E_2 for 1 hr at 0° followed by gel filtration. The expected binding in the mixture based upon the separated incubations is 3600 dis./min.

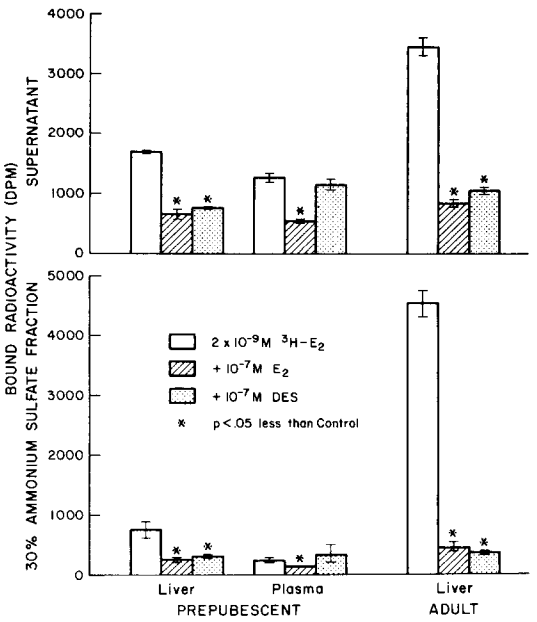

Fig. 1. Lower levels of [^{3}H]estradiol binding in the prepubescent female liver supernatant before and after $(NH_4)_2SO_4$ fractionation. The top panel illustrates the macromolecular binding of 2×10^{-9} M [^{3}H]estradiol to 0.2 ml of liver supernatant or plasma after 1 hr at 0°. The prepubescent females were 28 days old and the adults (200 g) 60 days old. The columns in each group represent the control binding (open) and in the presence of 10^{-7} M E_2 (hatched) or 10^{-7} M DES (dotted). The results in the bottom panel were obtained by precipitation of a fraction of supernatant with $(NH_4)_2SO_4$ at 30% saturation, redissolving the precipitate in the same volume as the original supernatant and then incubating with [^{3}H]estradiol as described above. An asterisk indicates $P < 0.05$, lower than corresponding control.

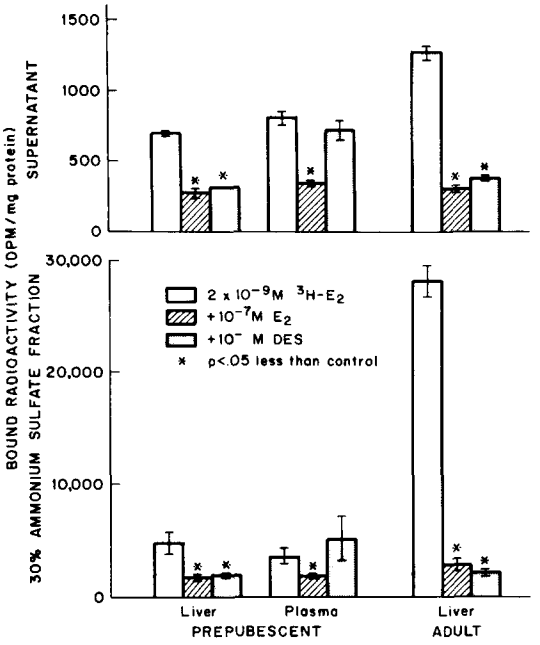

Fig. 2. Binding of [^{3}H]estradiol in liver supernatant and the 30% ammonium sulfate fraction/mg of protein. The conditions of the experiment are described in the legend to Fig. 1.

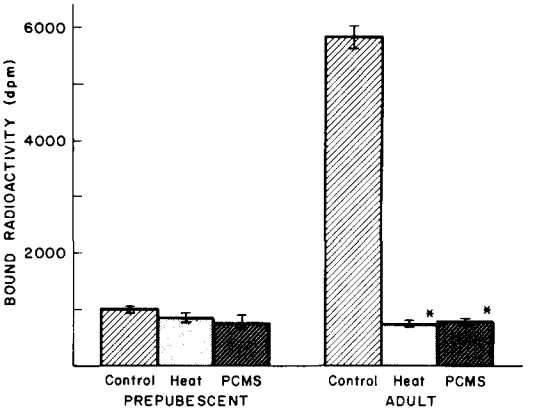

Fig. 3. Properties of supernatant liver binding in the prepubescent and adult female. Temperature stability was determined by reacting 2×10^{-9} M [^{3}H]E_2 with the supernatant for 1 hr at 0° followed by heating to 50° for 10 min (heat). Sensitivity to a sulfhydryl reacting reagent was determined by adding 5×10^{-3} M *p*-chloromercuriphenylsulfonate (PCMS) to the supernatants for 30 min before adding the radioactive estradiol. The values are expressed as dis./min bound in 0.2 ml supernatant ± S.E.M. An asterisk indicates $P < 0.05$, less than control.

pattern of results when expressed as binding per mg of protein. The radioactive estradiol binding of adult liver subject to competition with DES is concentrated more than 25-fold/mg of protein by this ammonium sulfate fractionation of cytosol.

The estradiol binding in the adult liver supernatant is sensitive to heat and the sulfhydryl reagent, *p*-chloromercuriphenylsulfonate (PCMS) (Fig. 3). The low level of binding in the prepubescent rat liver supernatant does not exhibit this sensitivity. The binding studies shown in Fig. 3 used livers from the control groups of prepubescent and adult rats of the following experiment. Figure 4 compares the effect of estrogen or glucocorticoid administration *in vivo* upon PRS levels. Groups of prepubescent and adult rats received two daily injections of 100 μg 17-alpha ethinyl estradiol or the vehicle alone as control. For the prepubescent rats, this represents a four times higher dose/g of body weight. Plasma renin substrate levels were

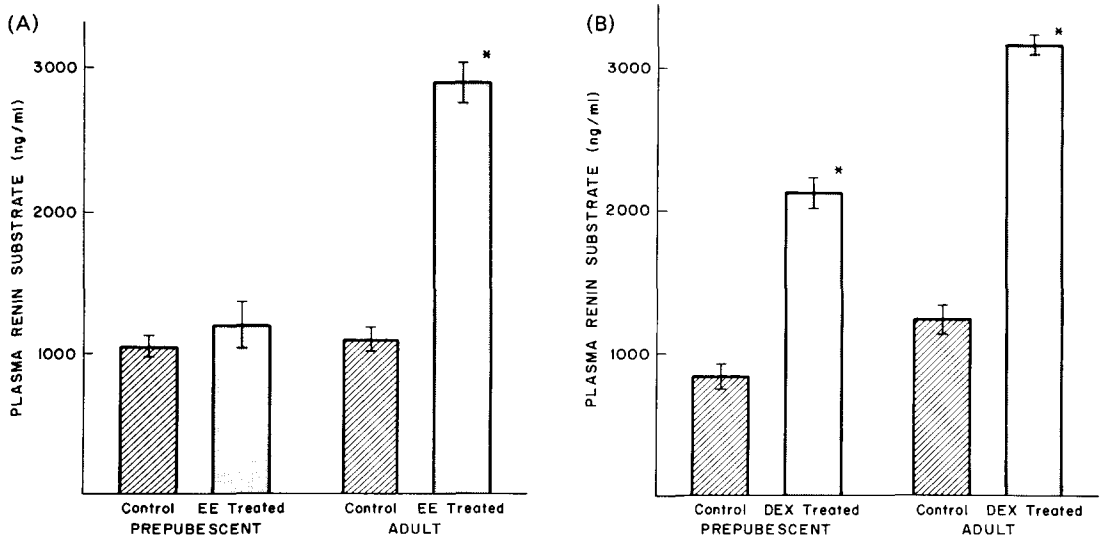

Fig. 4. Effect of administration of ethinyl estradiol or dexamethasone on plasma renin substrate levels in prepubescent and adult female rats. (A) Ethinyl estradiol (100 μg) was given s.c. in 0.1 ml propylene glycol to 25-day-old and to adult (200 g) female rats. The controls received the vehicle alone. The estrogen was injected at 0 and 24 hr with plasma renin substrate determined at 48 hr. (B) Prepubescent and adult female rats were treated with 4 mg/kg of dexamethasone at 0 hr. Plasma renin substrate was measured at 0 and 24 hr. An asterisk indicates $P < 0.05$, compared to control.

determined by radio-immunoassay 48 hr after the first ethinyl estradiol injection. As anticipated from previous reports, administration of high doses of ethinyl estradiol increases plasma renin substrate in the adult. In this experiment, the increase in PRS in the adult was 169 per cent above control. As previously described [13], estrogen administration had little, if any, effect on the plasma renin substrate level in the prepubescent rat. In addition, little plasma renin substrate response was observed when prepubescent rats were given four daily injections of ethinyl estradiol and the PRS level was measured at 96 hr.

Plasma renin substrate is also increased by glucocorticoid administration to adult rats. Figure 4B indicates that dexamethasone administration increased plasma renin substrate levels of both prepubescent and adult rats. The increase in PRS was 156 per cent in the prepubescent and 156 per cent in the adult.

DISCUSSION

Early studies did not demonstrate substantial levels of estrogen specific binding in mammalian liver. These studies examined prepubescent, not adult, mammalian liver supernatants [2–6]. Our results indicate that lower concentrations of a [^{3}H]estradiol-binding macromolecule are found in the prepubescent rat liver supernatant and 30% ammonium sulfate fraction than in the adult. By DES competition, the low levels in the prepubescent liver can be distinguished from a plasma binder which is known to have a poor affinity for DES [14]. At the moment it is unclear if the low levels of binding in the prepubescent liver represent the same binding protein as in the adult liver, since there is an apparent difference in heat and sulfhydryl reagent sensitivity.

In addition to examining the levels of estrogen specific binding, we have examined the levels of plasma renin substrate after administration of ethinyl estradiol or glucocorticoid to adult and prepubescent rats. Our evidence, as well as that of others, indicates that plasma renin substrate levels in the adult female rat are substantially increased by either estrogen or glucocorticoid administration. We have also found that the prepubescent rat has a substantial response to glucocorticoid administration; however, a poor response was observed after ethinyl estradiol administration.

There is evidence which suggests that the estrogen induction of plasma renin substrate levels is most likely a direct effect on the liver, resulting in increased hepatic synthesis of the plasma protein. Administration of an estrogen *in vivo* or directly in the perfusate increases the synthesis of renin substrate in isolated perfused rat liver [15].

Based on the above evidence, we propose that the number of occupied estrogen or glucocorticoid binding sites present per liver cell determines the level of plasma renin substrate response. Although the estrogen-binding sites appear to be deficient in the prepubescent liver, the presumed glucocorticoid receptor is known to be present in high concentrations in prepubescent rat liver [16]. However, this model is not the only possibility. It may be that the rate of metabolism of ethinyl estradiol to less active derivatives is more rapid in the hepatocyte of the prepubescent than in the adult. The following evidence suggests that this possibility is unlikely. Ethinyl estradiol is predominantly metabolized to hydroxylated derivatives by the liver microsomal mixed-function oxidase system [17]. When estradiol was used as the substrate for mixed-function oxidase activity, little difference was observed between prepubescent and adult female rats [13].

We propose that the estrogen binding protein in liver supernatant may be the estrogen receptor. Its effects may include modulating hepatic synthesis of selective plasma proteins. Consistent with current concepts of steroid hormone receptor action, we have preliminary evidence that the liver estrogen binding protein can translocate to the nucleus and can attach to chromatin [11].

A major reason for our interest in a possible estrogen receptor in the mammalian liver is its potential role in modulating plasma protein levels and other aspects of liver function of women taking estrogens alone or oral contraceptives containing estrogens. Estrogen administration to women is known to increase the plasma concentration of several plasma proteins and decrease that of others [8]. Women on the estrogen-containing birth control pills are known to have increased clotting factors [18, 19], decreased clotting inhibitor anti-thrombin III [18–20], increased renin substrate [21, 22], and increased triglycerides and prebetalipoproteins [21, 22]. These proteins are synthesized in, and secreted from, the liver. Although their relative importance as initiating factors remains unresolved, increased clotting factors and decreased anti-thrombin III may contribute to thromboembolism, increased renin substrate may contribute to hypertension, and increased triglycerides and lipoproteins might accelerate atherosclerosis [19, 21–24]. It has recently been reported that oral contraceptives increase the incidence of hepatomas [25]. Estrogens might also act in the liver to change the composition of bile secreted and thereby contribute to the observed increased incidence of gall stones and inflammation of the gall bladder [26, 27]. Possibly an estrogen interaction with its receptor in the liver might contribute to these side effects.

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