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Hepatic Estrogen Responsiveness

Possible Mechanisms for Sexual Dimorphism

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SUMMARY

In addition to estrogen receptors, liver contains a second class of estrogen-binding proteins referred to as higher-capacity, lower-affinity (HCLA) binding sites which are distinct from estrogen receptors. HCLA sites comprise two classes of proteins: moderate-affinity (K_D = 0.45 μM and 0.24 μM) estrogen-binding sites unique to male cytosol and a low-affinity, nonsaturable estrogen-binding site present in both sexes. The sex differences observed in HCLA sites are apparently a consequence of imprinting by testicular androgen during a critical neonatal period. Neonatal castration causes a reduction in the concentration of HCLA sites in the subsequent adult male. Furthermore, the moderate-affinity sites detected by Scatchard analysis in adult male liver are not observed in neonatal castrates. Cell-free nuclear translocation assays demonstrate that nuclear uptake of cytosolic receptor-ligand complexes is more efficient in females than in males. This sex difference in nuclear uptake can be minimized when the concentration of the ligand is increased to a level necessary to saturate the estrogen receptor in the presence of HCLA sites. Nuclear uptake of receptor-ligand complexes in neonatally castrated males (deficient in HCLA sites) is similar to that seen in adult females. Elevations of serum triglyceride following estradiol exposure have been monitored as an indicator of hepatic responses to estrogen. Our studies have shown that female liver appears more responsive to estrogen exposure than does male liver. While a dose of 20–30 μg of estradiol per kilogram of body weight per day was sufficient to produce a 3- to 4-fold increase in the concentration of triglyceride associated with the very low-density lipoprotein fraction in females, a dose of 100 μg of estradiol per kilogram of body weight per day was needed to obtain a similar response in males. However, following neonatal castration, estrogen responsiveness in the subsequent adult male rat was similar to that in females, suggesting a role for neonatal androgens in regulating sex differences in the action of hepatic estrogen.

INTRODUCTION

High-affinity binding sites in rat liver have been characterized which, on the basis of accepted criteria, have been termed estrogen receptors (1–4). The ability of this protein to translocate to the nucleus (5, 6) suggests a functional role for the liver estrogen receptor in promoting direct estrogenic effects. Studies have demonstrated that increases in hepatic synthesis of renin substrate correlate well with nuclear uptake of receptor-hormone complexes (7). Furthermore, in avian species, increases in specific mRNA sequences for a major lipoprotein, apo VLDL[2]-II, were preceded by elevated nuclear concentrations of estrogen receptors following estrogen treatment (8). Reports in the literature (9–11) have demonstrated that estrogen influences hepatic secretion of VLDL in rats. Studies in our laboratory have attempted to correlate the increases in serum triglyceride associated with VLDL with the presence of hepatic estrogen receptors (12).

In addition to estrogen receptors, rat liver cytosol contains a second class of estrogen binding proteins which sediments in the 4 S region of sucrose gradients and binds androgens as well as estrogens (13–16). Upon further examination, it has been shown that multiple forms of estrogen-binding proteins appear to comprise the 4 S region of sucrose gradients (16). These sites have been termed HCLA binding sites. Unlike estrogen receptors, which are present in equal amounts in males and females, HCLA binding proteins exist in much greater quantities in male cytosol than in female cytosol. The sex differentiation of HCLA binding sites occurs postpubertally (17), and maintenance of sex differences is depend-

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[2] The abbreviations used are: VLDL, very low-density lipoprotein; HCLA, higher capacity, lower affinity; E_2, 17β-estradiol; DCC, dextran-coated charcoal; DES, diethylstilbestrol; HDL, high-density lipoproteins; LDL, low-density lipoproteins.

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ent upon an intact pituitary (14, 16). Several hepatic enzyme systems also exhibit large postpubertal sex differences, and this sex differentiation is apparently imprinted by neonatal androgens. The imprinting phenomenon is a result of neonatal exposure to testicular androgens during a critical period of development which irreversibly programs (imprints) the hypothalamic-pituitary axis to be responsive to postpubertal hormone changes, resulting in sex differentiation of various aspects of hepatic protein synthesis (18).

The presence of HCLA binding sites may affect nuclear translocation of hormone-receptor complexes (14), suggesting the possibility that these sites may influence hepatic estrogen action. The present studies were undertaken (a) to evaluate sex differences in the response of rat liver to E_2 using triglycerides associated with VLDL as a marker, (b) to characterize the effects of neonatal castration on cytosolic and nuclear estrogen binding proteins, and (c) to evaluate the role of separate classes of estrogen-binding proteins in nuclear uptake of ligand-receptor complexes.

MATERIALS AND METHODS

Animals. The source of Sprague-Dawley rats (CD strain) was Charles River Breeding Laboratories (Wilmington, Mass.). Rats used in the studies were in-house bred and maintained in a controlled environment (21°, 12-hr light/12-hr dark cycle) with food and water available ad libitum. Animals were weaned at 21–23 days of age and housed five animals per cage. For estrogen responsiveness studies, female animals were ovariectomized at 63 days of age. Seven days later, E_2 was administered by s.c. implantation of 8-mm sections of Silastic tubing containing E_2/cholesterol combinations. Measurements of uterine wet weight 7 or 14 days after ovariectomy were similar, indicating that a 7-day period after ovariectomy is sufficient to lower endogenous levels of estradiol before initiating hormone treatment. An 8-mm section of tubing holds approximately 3 mg of material. Assuming a constant and complete release of hormone over a 6-week period, approximately 20–30 μg of E_2 per kilogram of body weight per day (200–250 g rat) will be released when the capsules contain 10% E_2 and 90% cholesterol (used as carrier). The possibility exists that release of E_2 would not be complete within 6 weeks, which means that the estimated daily dose would be lower than indicated. Following 2 weeks of E_2 exposure, animals were killed by decapitation, and livers were quickly excised and chilled on ice. Blood was collected and serum prepared.

Chemicals and buffers. The following buffers were used: 10 mM Tris (Ultrapure, Bethesda Research Laboratories, Rockville, Md.)/1 mM EDTA/1 mM dithiothreitol (Buffer A); 0.5 M sucrose/5 mM $MgCl_2$/10 mM Tris (Buffer B); 0.25 M sucrose/5 mM $MgCl_2$/25 mM KCl/1 mM dithiothreitol/50 mM Tris adjusted to pH 7.55 at 4° (Buffer C); 1 mM dithiothreitol/0.4 M KCl/10 mM Tris (Buffer D); 0.5% (w/v) activated, untreated charcoal powder and 0.05% (w/v) dextran (clinical grade) in Buffer A (DCC solution). Buffers were adjusted to pH 7.4 at 4° unless otherwise stated.

[2,4,6,7-^{3}H]Estradiol-17β (94–113 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Unlabeled steroids were supplied by Steraloids (Wilton, N. H.). Purity (>98%) was checked periodically by thin-layer chromatography using silica-coated plates and benzene/ether (1:1, v/v) as solvent. Biofluor was purchased from New England Nuclear Corporation. Cholesterol (enzymatic) bulk set and triglyceride reagents for automation were purchased from Dow Chemical Company (Indianapolis, Ind.). Other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

Preparation of cytosol. Samples of liver were minced and homogenized in the appropriate buffer system by five passes using a Potter-Elvehjem homogenizer. The liver preparation was centrifuged at 10,000 × *g* for 60 min. Following centrifugation, the supernatant cytosolic fraction was removed, avoiding the fat layer. All procedures were performed at 4°. Protein concentrations were assayed by the method of Lowry *et al.* (19).

Quantitation of cytosolic estrogen binding sites. Cytosol (500 μl of 10–12 mg of protein per milliliter of cytosol) was incubated with an equal volume of 60 nM [^{3}H]E_2 for 2 hr at 4°. Following the incubation, unbound ligand was separated from bound by DCC treatment as previously described (16). Aliquots (100 μl) of supernatant fluid containing bound [^{3}H]E_2 were added to Biofluor liquid scintillation fluid (10.0 ml) and assessed for amount of radioactivity in a Beckman LS 9000 liquid scintillation counter.

In some cases, aliquots (200 μl) of cytosol were incubated (2 hr at 4°) with increasing concentrations of [^{3}H]E_2 (0.1 nM–5 μM) to assess the cytosolic content of both receptor and nonreceptor (nonspecific) estrogen-binding sites. Following the incubation, aliquots (400 μl) of DCC suspension were added to the [^{3}H]E_2 cytosol mixtures (30 min at 4°), and bound ligand was quantified as described above. We had previously determined that the amount of detectable [^{3}H]E_2 binding remained constant when charcoal adsorption times of 10–30 min were used. The binding data were analyzed by the method of Scatchard (20) using weighted nonlinear squares to determine the presence of multiple sites and to derive the best fit of the observed values. This method subtracts the contributions of all other sites when deriving the K_D value or when quantifying each detected binding site.

Preparation of purified nuclei. Liver minces (4-g samples) were homogenized in 10 volumes of Buffer B. The homogenates were filtered through Miracloth (Chicopee Mills Inc., New York, N. Y.) and centrifuged at 1,500 × *g* for 15 min. The crude nuclear pellet was washed once in Buffer B; the pellet was then resuspended in 10 ml of 2.2 M sucrose containing 1 mM $MgCl_2$ and purified by centrifugation (27,000 × *g* for 1 hr) through 25 ml of 2.2 M sucrose in an SW 27 swinging bucket rotor. The nuclear preparation was intact and essentially free of cytoplasmic or microsomal contamination as determined by transmission electron microscopy. The DNA content of nuclear suspensions was quantitated by the method of Burton (21), using calf thymus DNA as a standard. The nuclear suspensions contained 700–800 μg of DNA per gram of liver.

Quantitation of nuclear estrogen receptors. Nuclear receptor concentrations in estrogen-treated animals were measured by an exchange assay (5). Purified nuclei were resuspended (1–1.5 ml/g equivalent of liver, 0.9–1.1 mg of DNA per milliliter) in Buffer C. Parallel sets of polypropylene tubes containing equal volumes (200 μl) of resuspended nuclei and 60 nM [^{3}H]E_2 ± 100-fold excess of DES were incubated for 30 min at 4° to saturate unoccupied receptor sites. One set of tubes was then incubated for an additional 30 min at 29° (exchange assay) while the second set remained at 4°. Following incubations, nuclei were removed by centrifugation (800 × *g* for 5 min). The washed nuclear pellets (12) were drained until dried, and the tips of the polypropylene tubes were cut with a hot wire. The tip containing nuclei was dissolved in a scintillation cocktail {4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (Spectrofluor PPO-POPOP; AmershamCorporation, Arlington Heights, Ill.) per liter of toluene} overnight and then assessed for levels of radioactivity. Total receptor binding sites were determined by the 29° exchange assay whereas amounts of unoccupied receptor sites were measured by the 4° assay. The difference between total available and unoccupied sites is equal to the number of occupied sites. The nuclear content of specific estrogen rceptor binding was determined as the difference between radioactivity in samples incubated in the absence (total binding) or presence (nonspecific binding) of DES. Approximately 5%–10% of total nuclear binding sites represented nonspecific binding.

In some instances the binding affinity of nuclear receptor sites for E_2 was determined. Purified nuclei (200 μl) were incubated with increasing concentrations of [^{3}H]E_2 (0.1–10 nM) ± 100-fold excess of DES, under conditions identical with those described for the nuclear exchange assay (29°). Following incubations, nuclei were processed as described above and the binding data obtained for both total and occupied nuclear receptor sites were analyzed by the method of Scatchard (20).

Sedimentation analysis of nuclear estrogen receptors following cell-free nuclear translocation. Cytosol prepared in 2.5 volumes of Buffer C (25–30 mg of protein per milliliter) was incubated with an equal volume of 8 nM [^{3}H]E_2 for 2 hr at 4°. The prelabeled cytosol (5.0 ml) was added to purified nuclei (prepared from 4 g of liver), resuspended in 2.0 ml of Buffer C (1.3 mg of DNA per milliliter), and incubated at 29° for 20 min with continuous shaking. Following the incubation, nuclei were removed by centrifugation (800 × *g* for 5 min) and then washed once in Buffer C containing 0.2% Triton X-100 and twice with Buffer C. The nuclear pellet was resuspended in 600 μl of Buffer D (0.4 M KCl) and incubated on ice for 30 min. The resulting viscous salt extract was centrifuged (1,500 × *g* for 20 min), and a sample (400 μl) of the supernatant fluid was layered on a 5%–10% linear sucrose gradient (4.0 ml) prepared in Buffer D. Gradients were centrifuged in an SW 60 Ti swinging bucket rotor at 100,000 × *g* for 18 hr. Bovine serum albumin (4.6 S) was used as a sedimentation marker. After centrifugation, gradients were fractionated and each fraction (100 μl) was assessed for radioactivity. Metabolism studies in our laboratory had demonstrated that 90% of nuclear bound radioactivity was [^{3}H]E_2 and 10% [^{3}H]estrone (14).

Separation and analysis of lipoprotein classes. The separation of lipoprotein classes for cholesterol and triglyceride analyses was performed according to the micromethod described by Bronzert and Brewer (22). Blood was obtained from animals which had previously been fasted for 14–18 hr, and serum was prepared by centrifugation at 1,500 × *g* for 30 min at 4°. Aliquots (175 μl) of serum were added to duplicate sets of tubes, one set of which contained 13.8 mg of solid KBr. KBr was dissolved by gentle shaking of serum samples. Both sets of tubes were then centrifuged in a Beckman Type 42.2 Ti rotor at 200,000 × *g* for 2.5 hr at 4°. Following centrifugation, 26% of the volume was removed by aspiration from samples which were at serum density, leaving HDL and LDL in the infranatant. The top 46% of the volume was removed by aspiration from samples which had the density adjusted to 1.06 by the addition of solid KBr, leaving only HDL in the infranatant. These samples, in addition to unfractionated serum, were analyzed for cholesterol and triglyceride content. Analysis of cholesterol and triglyceride content was performed "in tandem" on a Gilford 3500 computer-directed analyzer as described by Smith *et al.* (23).

RESULTS

Saturation analysis of whole cytosol prepared from male and female rat livers was used to differentiate cytosolic proteins which might vary in their affinities toward E_2 (Fig. 1). A high-affinity (0.31 nM), low-capacity binding protein (specific estrogen receptor) was demonstrated in cytosol prepared from female livers (Fig. 1A). In addition, a nonsaturable, low-affinity binding site (nonspecific binding) was present. Scatchard analysis of cytosol prepared from male liver revealed at least two saturable, moderate-affinity binding sites (Fig. 1B) for E_2 with equilibrium dissociation constants of 0.44 μM and 0.24 μM, respectively. Similar to the results obtained for female liver, nonsaturable, low-affinity binding sites were present in male liver. The moderate- and low-affinity sites are referred to as HCLA binding sites. The apparent absence of high-affinity receptor sites in male cytosol was due to the masking of receptor by high concentrations of HCLA (approximately 5 pmoles/mg of cytosolic protein) binding sites. However, if these estrogen-binding components were removed by ammonium sulfate precipitation, the resulting partially purified preparation contained estrogen receptor proteins having properties identical with estrogen receptors in female liver cytosol (17).

Since it has been shown that sex-specific HCLA sites are present in male liver cytosol, we were interested in determining whether sex-related differences in other aspects of hepatic estrogen action could be distinguished. An *in vitro* assay system was used to study the translocation of receptor [^{3}H]E_2 complexes from the cytosol into

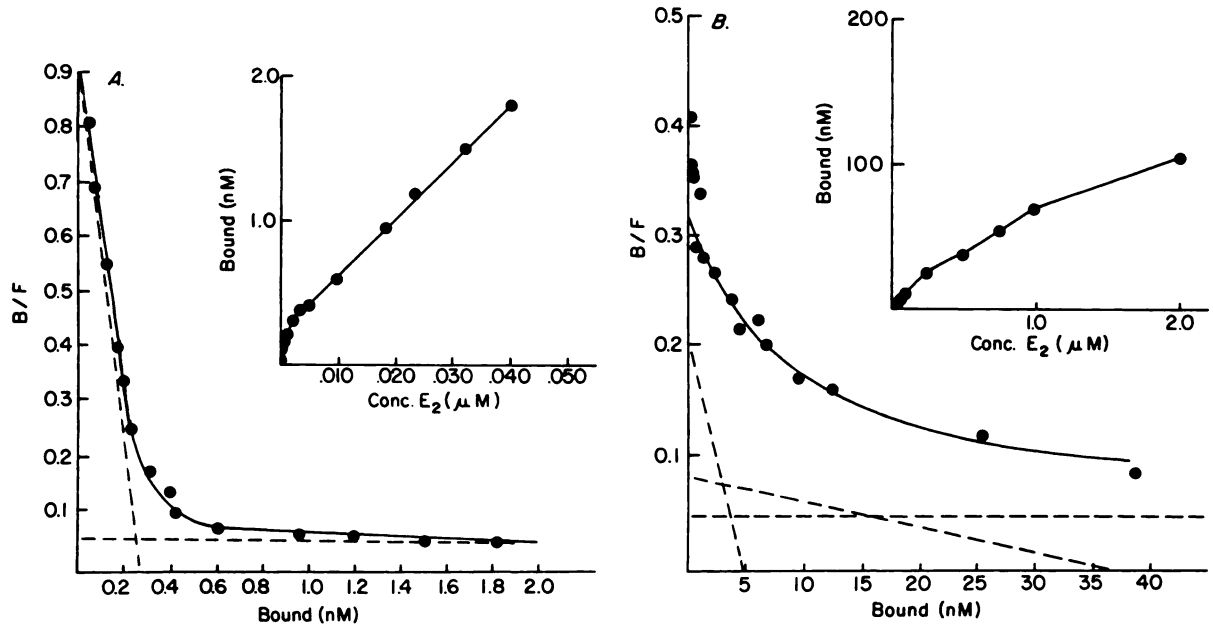

FIG. 1. *Scatchard analysis of hepatic estrogen-binding proteins prepared from female or male cytosol*

Cytosol (200 μl) prepared from female liver (A) was incubated (2 hr at 4°) with an equal volume of [^{3}H]E_2 (0.1–30 nM). Cytosol (200 μl) prepared from male liver (B) was incubated (2 hr at 4°) with an equal volume of [^{3}H]E_2 (0.1–6000 nM). Following the incubation, unbound ligand was removed by DCC treatment. Aliquots of the supernatant were assessed for bound radioactivity, and values obtained were plotted according to the method of Scatchard. The *dashed lines* were derived from a weighted nonlinear least-square analysis. (Analysis was performed on cytosol that was pooled from livers of at least three animals.)

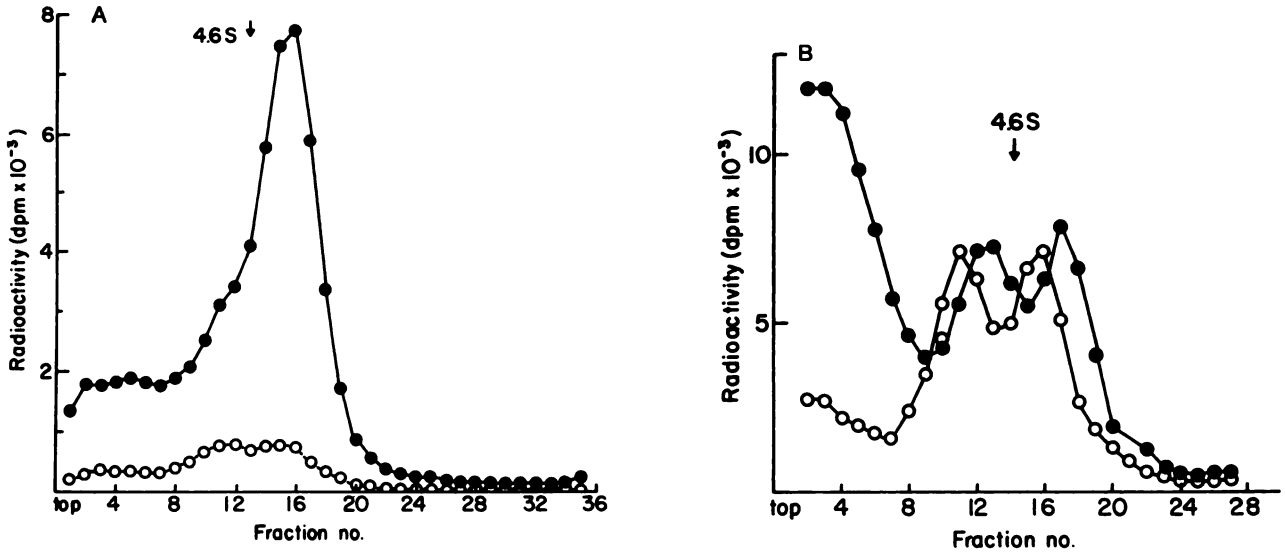

FIG. 2. *Translocation of [^{3}H]E_2-receptor complexes into rat liver nuclei*

Nuclei purified from female or male liver were incubated (20 min at 29°) with cytosol prepared from the same sex which had been preincubated (2 hr at 4°) with either 4 nM (A) or 30 nM (B) [^{3}H]E_2. The salt extracts of female (●) or male (○) nuclei were analyzed on 5%–20% sucrose gradients containing 0.4 M KCl. Cytosol protein concentrations were 20 mg/ml for A and 10 mg/ml for B. Nuclear concentrations of DNA were 1.0–1.3 mg/ml.

the nucleus. Representative sedimentation profiles of salt-extractable (0.4 M KCl) nuclear receptor sites from male or female liver preparations are illustrated in Fig. 2A. It was evident from this study that a marked sex difference exists in the ability of cytosolic receptors to translocate to the nucleus under the defined experimental conditions (4 nM [^{3}H]E_2). The levels of [^{3}H]E_2 retained in the 4 S and 5 S regions of the gradients were approximately 8 times greater in female nuclei than in male nuclei. In contrast, when cytosol was prelabeled with [^{3}H]E_2 at a final concentration of 30 nM, nuclear retention of cytosolic receptors was equivalent in both sexes (Fig. 2B) and also equivalent to female levels when 4 nM E_2 was used in the *in vitro* translocation assay.

In addition to monitoring sex differences in the nuclear uptake of receptor-[^{3}H]E_2 complexes, the comparative responsiveness of the liver to estrogen stimulation was explored. Male and ovariectomized female animals were administered E_2 continuously for 2 weeks by s.c. implantation of Silastic capsules containing specified quantities of E_2 (Table 1), and the amount of total circulating triglyceride in serum and triglycerides associated with individual lipoprotein classes was quantitated. We have previously established (12) that this treatment protocol was effective in releasing E_2 into the circulation sufficient to increase uterine wet weight in ovariectomized rats. To ensure that the triglycerides measured were of hepatic origin, animals were fasted for 14–16 hr prior to use.

TABLE 1

Effect of estradiol on serum triglycerides in male and female rats

Adult females were ovariectomized at 63 days of age. At age 70 days, both male and female rats were administered E_2 by s.c. implantation of Silastic tubing containing E_2/cholesterol mixtures (0, 10%, 25%, 50%, and 100%, respectively) that would release the appropriate amount of hormone into the circulation. Following 2 weeks of E_2 exposure, animals were fasted 14–16 hr prior to sacrifice. Serum was prepared and fractionated into different lipoprotein classes as described under Materials and Methods, and the concentration of triglyceride was measured. The values represent the mean ± standard deviation (n = number of animals per group).

Dose of E_2 administered	Triglycerides			
	Males		Females	
	Serum	VLDL	Serum	VLDL
μg/kg body wt/day	*mg/dl serum*			
0	56.3 ± 19.3 (n = 15)	29.4 ± 11.7 (n = 15)	52.3 ± 11 (n = 12)	16.5 ± 4.9 (n = 12)
20–30	70.4 ± 22.7 (n = 15)	35.8 ± 18 (n = 15)	125 ± 30.1[a] (n = 15)	70.8 ± 15.3[a] (n = 15)
50	72.5 ± 32.8 (n = 15)	37.3 ± 15 (n = 15)	—	—
100	163 ± 66.7[a] (n = 6)	145 ± 40[a] (n = 6)	—	—
200	212 ± 44.5[a] (n = 6)	140 ± 41.8[a] (n = 6)	—	—

[a] Significantly different from controls ($p < 0.05$ as determined by Student's t-test).

Concentrations of triglyceride in serum and VLDL fractions were similar in control males and ovariectomized females (Table 1). Furthermore, no observable sex difference in the concentrations of triglycerides associated with HDL and LDL was noted (data not shown). Dose-response experiments (Table 1) suggested that female liver was more responsive to estrogen than was male liver. A 3- to 4-fold increase in the concentration of circulating triglycerides associated with the VLDL fraction (16.5 ± 4.9 to 70.8 ± 5.3) was observed in female animals following E_2 administration (20–30 μg of E_2 per kilogram of body weight per day) for 2 weeks. Male liver was unresponsive at the same dose of estrogen. When the administered dose of E_2 was increased to 100 μg of E_2 per kilogram of body weight per day in males, significant increases in triglyceride levels associated with the VLDL fraction were observed. The dose of estrogen required to elicit a hepatic response in males was approximately 4-fold greater than the dose required in females.

Effect of gonadectomy on HCLA binding sites. It has been shown that sex-dependent differences exist in the composition of estrogen-binding proteins present in rat liver (Fig. 1). Preliminary investigations (14, 16) had suggested that the development of this sex difference might involve a mechanism which requires exposure to androgen during a critical neonatal period. The effect of neonatal castration of male rats on the sex differentiation of HCLA sites in the subsequent adult was analyzed by Scatchard analysis. Neonatal castration prevented the postpubertal development of HCLA sites (Fig. 3). The composition of hepatic HCLA sites in neonatal castrates was similar to that observed in adult females. Moderate-affinity sites, characteristic of cytosol prepared from adult male liver (Fig. 1B), were not detected in adult males castrated at 1 day of age. Moreover, high-affinity receptor sites (K_D = 0.49 nM) were detected in neonatal castrates.

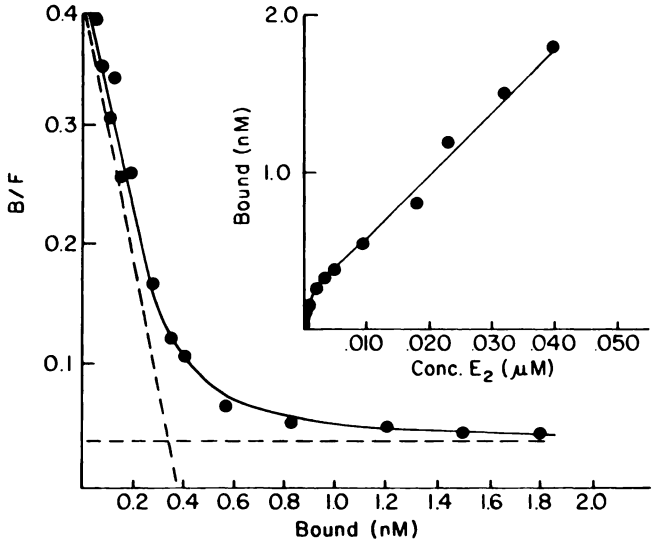

FIG. 3. *Scatchard analysis of hepatic estrogen-binding proteins of adult males castrated neonatally*

Cytosol prepared from liver of adult males castrated neonatally were incubated (2 hr at 4°) with an equal volume (200 μl) of [^{3}H]E_2 (0.1–6000 nM). Following DCC treatment, aliquots were assessed for bound radioactivity and the values obtained were plotted according to the method of Scatchard. The *dashed lines* were derived from a weighted nonlinear least-squares analysis.

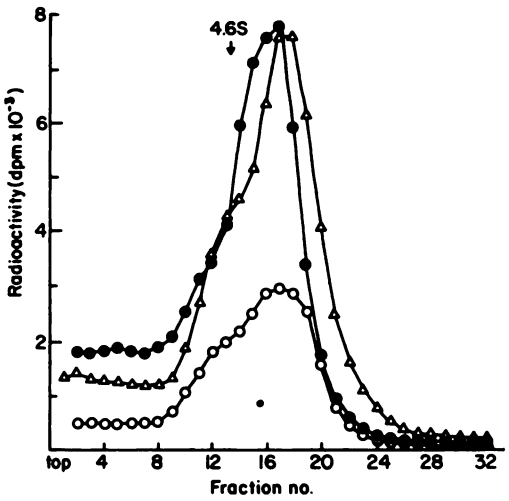

FIG. 4. *Effect of neonatal castration on the translocation of [^{3}H]E_2-receptor complexes into rat liver nuclei*

Nuclei purified from liver were incubated (20 min at 29°) with cytosol which had been preincubated (2 hr at 4°) with [^{3}H]E_2 (4 nM). The salt extracts of nuclei from females (●), males (○), or neonatal castrate males (△) were analyzed on 5%–20% sucrose gradients containing 0.4 M KCl. The cytosolic protein concentration was 20 mg/ml. Nuclear concentrations of DNA were 0.9–1.1 mg/ml.

Effect of gonadectomy on the translocation of [^{3}H]E_2-receptor complexes from cytosol to nucleus. The sedimentation profiles of salt-extractable nuclear receptor sites in various groups of animals (Fig. 4) were investigated by employing the *in vitro* assay system described in Fig. 2. Concomitant with lower concentrations of HCLA sites in adult males which had been castrated neonatally was the enhanced ability of cytosolic receptors to translocate to the nucleus under the described experimental conditions. In this group the quantity of [^{3}H]E_2 retained in the 4 S and 5 S regions of the gradient was similar to that found in intact females.

Effect of gonadectomy on hepatic estrogen responsiveness. Studies were undertaken to ascertain possible age-dependent effects of androgens on hepatic estrogen responsiveness. Female animals were ovariectomized at 42 days of age. Administration of E_2 (20–30 μg of E_2 per kilogram of body weight per day for all experimental groups) or vehicle (cholesterol) was begun at 49 days of age. Following 2 weeks of estrogen treatment, concentrations of triglyceride in serum and the VLDL fractions were determined; the results are presented in Fig. 5. The responsiveness of liver to estrogen was enhanced in adult males that had been castrated on day 1. Neonatal castration had no effect on the concentration of triglycerides associated with the VLDL fraction. However, estrogen administration to an adult male, castrated at 1 day of age, produced 3-fold increases in the concentration of circulating triglycerides. Changes observed in the concentrations of circulating triglycerides following estrogen treatment reflected increases in triglycerides associated with the VLDL fraction. Triglycerides associated with HDL and LDL fractions were not affected by estrogen treatment in any experimental group (data not shown).

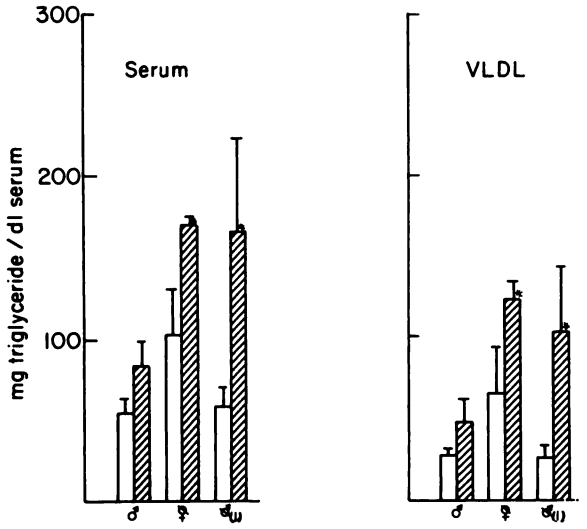

FIG. 5. *Effect of gonadectomy on hepatic estrogen responsiveness*
Adult females were ovariectomized at 42 days of age. At age 49 days, adult males (♂), ovariectomized females (♀) and neonatal castrate males (♂(1)) were administered estradiol (20–30 μg/kg body wt/day) by s.c. implantation of Silastic tubing containing 10% E_2 and 90% cholesterol as the vehicle, or vehicle (100% cholesterol) alone. Following 2 weeks of E_2 exposure, animals were fasted 14–16 hr before sacrifice. Serum was prepared and fractionated as described under Materials and Methods, and the concentration of triglyceride was measured in vehicle-treated (*open bars*) and E_2-treated (*hatched bars*) animals. The bar graphs represent the mean ± standard deviation ($n = 5$ animals per group). An *asterisk* indicates significant values as compared with controls ($p < 0.05$ as determined by Student's *t*-test).

Scatchard analyses of total nuclear receptor sites in control and estrogen-treated intact males, intact females, and adult males castrated neonatally were compared (Fig. 6). An approximate 2-fold increase in nuclear receptor sites (Fig. 6A) was observed following E_2 treatment of males, whereas in females (Fig. 6B) and neonatal castrates (Fig. 6C) there was either no change or a slight decrease in receptor sites following estrogen treatment. The binding affinities for E_2 by specific receptor sites present in nuclei prepared from male, female, or neonatal castrate male livers are summarized in Table 2. A single class of receptor sites was detected having a K_D of approximately 0.5 nM, and no observable sex differences were noted in the binding affinities of the nuclear sites for E_2.

DISCUSSION

Results from our study suggest that neonatal androgen could play an important role in initiating biochemical processes which in the postpubertal animal culminate in hepatic estrogen action typical for males. Age-dependent modulation of HCLA sites, hepatic steroid metabolism, or the type and location of nuclear estrogen receptors could result in the modification of hepatic estrogen responsiveness in subsequent adults.

The presence of a second class of hepatic estrogen-binding proteins (HCLA sites) which is higher in male liver than in female liver has been reported (13, 14). The properties which have distinguished these binding proteins from estrogen receptors include (a) a sedimentation coefficient of 4 S on sucrose gradients prepared in low salt as compared with the 8 S form characteristic of estrogen receptors, (b) lack of binding to nonsteroidal estrogens, and (c) their capacity to bind androgens as well as estrogens. Although the functional significance of the HCLA sites has yet to be clearly defined, the results of the present study indicate a correlation between the presence of cytosolic HCLA sites and sex differences in the action of hepatic estrogen.

Our findings demonstrate that female liver is more responsive to estrogen than is male liver (Table 1) when using the concentration of serum triglyceride associated with VLDL as a biological end-point. These findings confirm earlier reports which have demonstrated that the increase in triglyceride associated with VLDL following estrogen treatment is greater in plasma prepared from females than in plasma prepared from males (9). The sex difference was due, in part, to a rate of VLDL secretion in females exceeding that seen in males (9, 10). Although there is no compelling evidence that estrogens induce changes in transcriptional processes in mammalian liver, it is thought that estrogen-mediated alterations in liver function, including VLDL synthesis, might be mediated through a receptor mechanism (12, 24). Since the cytosolic concentrations and K_D values of hepatic estrogen receptors are equivalent in males and females (17), other factors must be involved in the observed sex differences in hepatic estrogen responsiveness. Following neonatal castration, the responsiveness of liver to estrogens in subsequent adult males was similar to that observed for adult females (Fig. 4). Moreover, the composition of HCLA sites in these animals was similar to that in adult females (Figs. 1 and 3); high-affinity and low-affinity sites were detected by Scatchard analysis, whereas the male-specific moderate-affinity sites (K_D = 0.44 μM and 24.3 nM) clearly were not detected.

The theory that increases in VLDL production result from receptor-genomic interactions suggests that factors which alter nuclear uptake of cytosolic receptors ultimately could render the liver more or less susceptible to biological and/or toxic effects of endogenous estrogens or estrogenically active xenobiotics. To assess whether the presence of HCLA sites could affect nuclear translocation, a cell-free nuclear translocation assay system was used. This experimental design essentially eliminates influences of microsomal steroid-metabolizing enzymes on nuclear uptake, since only cytosolic and purified nuclear preparations were used.

Previous *in vitro* studies (14) had demonstrated sex differences in the amount of ligand necessary to saturate hepatic estrogen receptor. Whereas 4 nM [^{3}H]E_2 saturated receptors in female liver cytosol, 30 nM [^{3}H]E_2 was required to saturate the receptor in males. Moreover, nuclear uptake of cytosolic receptor was more efficient in females than in males when 4 nM [^{3}H]E_2 was used. Several lines of evidence have suggested that the presence of HCLA sites may affect the translocation process by altering the availability of ligand for the receptor. First, the use of partially purified receptors (absence of HCLA sites) prepared from male or female liver eliminates observable sex differences in the extent of nuclear uptake (14). Second, when hormone concentrations are

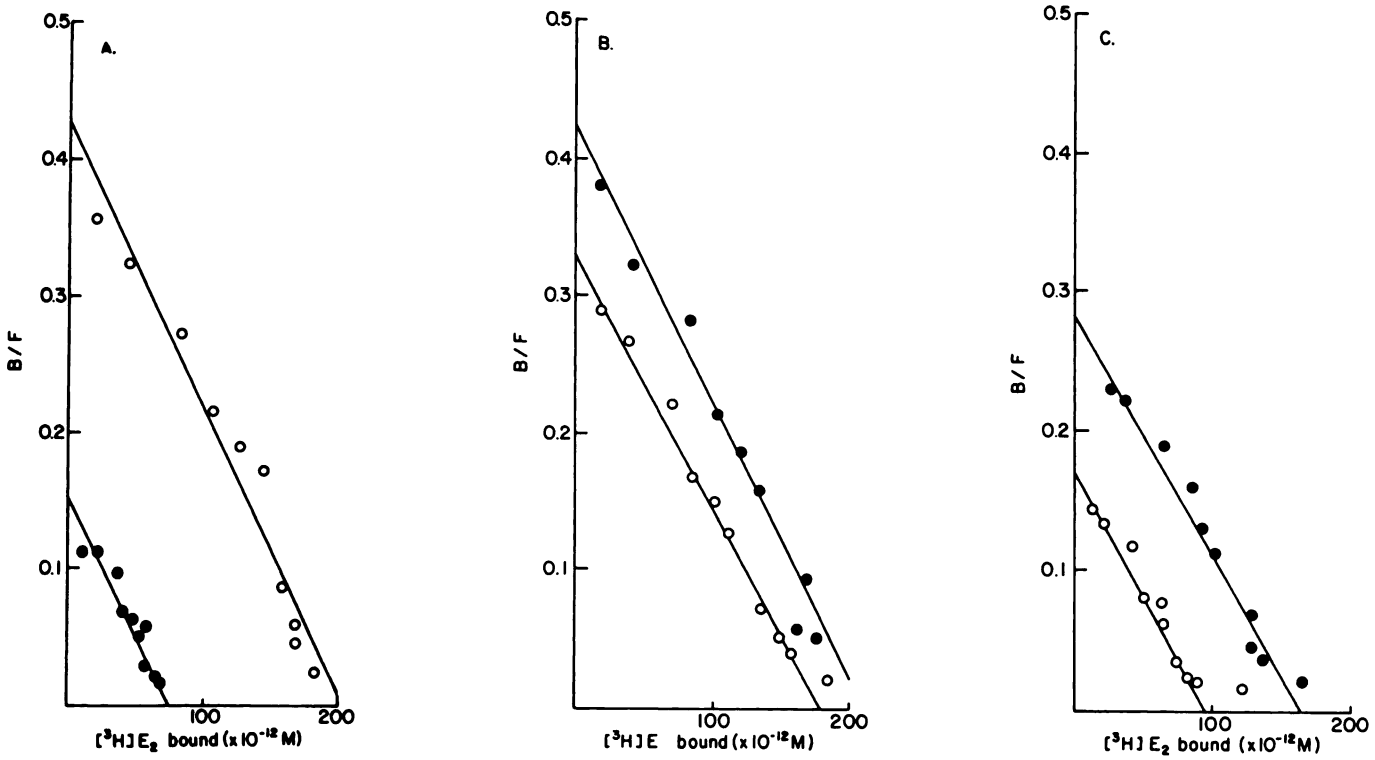

FIG. 6. *Scatchard analysis of hepatic estrogen receptors prepared from males, females, and neonatal castrate males*

Livers from at least five animals per group were pooled. Nuclei were prepared from males (A), females (B), or neonatal castrate males (C) as described under Materials and Methods. Animals were treated with vehicle (●) or 10% E_2 (○) for 2 weeks. The dose of E_2 was 20–30 μg/kg of body weight per day. The nuclei were incubated (30 min at 4°, then 30 min at 29°) with an equal volume of increasing concentrations (0.1–10 nM) of [^{3}H]E_2 ± 100-fold excess of DES. The nuclei were then assessed for radioactivity (see Materials and Methods) and the values obtained were plotted according to the method of Scatchard. The lines were derived from least-squares regression analysis.

increased sufficiently (30 nM) to saturate cytosolic receptors (14) in males, sex differences in nuclear retention in the 4 S and 5 S regions are virtually abolished (Fig. 2B). Third, cell-free nuclear translocation studies using liver cytosol prepared from male rats which been castrated at 1 day of age (deficient in HCLA sites) show a marked enhancement of nuclear uptake of cytosolic receptors, and nuclear translocation rates in this group were indistinguishable from nuclear translocation rates measured in adult females (Fig. 4).

TABLE 2

Effect of neonatal castration on the binding affinities of nuclear receptor for estradiol

Livers from at least five animals per group were pooled; nuclei were prepared as described under Materials and Methods and incubated (30 min at 4° and 30 min at 29°) with an equal volume of [^{3}H]E_2 (0.1–10 nM) ± 100-fold excess of DES. The nuclei were then assessed for radioactivity and the K_D values obtained by Scatchard analysis.

	Dissociation constant
	nM
Males	
Control	0.49
+10% E_2	0.48
Females	
Control	0.50
+10% E_2	0.55
Neonatal castrates	
Control	0.57
+10% E_2	0.55

Our *in vitro* data demonstrate the importance of HCLA sites in modulating amounts of ligand bound to cytosolic receptors and subsequent translocation of ligand-receptor complex into the nucleus. However, *in vivo* hepatic steroid metabolism, which influences the concentration of parent E_2 and its metabolites, would also be expected to play a role in the observed sex differences in hepatic responsiveness to estrogens. Certain of the steroid-metabolizing enzyme systems in rat liver demonstrate large postpubertal sex differences (25, 26); adult male liver generally has higher metabolic activities for estrogens than does female liver (27). It has been well documented that sex differentiation of hepatic enzymes (18) is a consequence of imprinting by neonatal hormones. Androgen exposure, during a critical neonatal period, imprints the hypothalamic-pituitary-hepatic axis to undergo postpubertal sexual differentiation. An intact pituitary is required for the initiation and maintenance of sex-related differences. In addition, sex differentiation of HCLA sites closely parallels the situation observed for steroid-metabolizing enzymes. Recent reports from our laboratory (14, 16, 17) have shown that (a) sex differentiation of HCLA sites occurs postpubertally; (b) neonatal castration prevents sexual development of HCLA sites in adult males; (c) once full expression of HCLA sites has been obtained, circulating androgens are not required for their maintenance; and (d) the pituitary plays a dominant role in maintenance of sex differences in HCLA sites. Changes in concentrations of HCLA sites or steroid-metabolizing enzymes as a consequence of altered pitui-

tary-hepatic interactions could modulate the potency of the E_2 administered. Characterization of nuclear-bound metabolites in isolated rat liver parenchymal cells exposed to [^{3}H]E_2 have shown that the principal nuclear-bound material in females is parent E_2. In contrast, the principal material in males is an unidentified metabolite which co-chromatographs with 2-hydroxy-E_2 (28, 29). The biological potency of this metabolite has yet to be determined.

Our results from the *in vitro* studies demonstrated a correlation between the relative efficiency of nuclear uptake of cytosolic receptors and estrogen responsiveness in the liver. In contrast, an apparent lack of correlation between the concentration of nuclear receptors and liver estrogen responsiveness was observed following *in vivo* estrogen administration. A 2.5-fold increase in total nuclear sites was observed in intact males (Fig. 6) following estrogen treatment. In contrast, this increase was not observed in neonatal castrates even though the responsiveness of the liver to estrogens was greater in neonatal castrates than in intact males (Fig. 6). Furthermore, it was apparent from Scatchard analysis (Table 2) that the nuclear receptor protein(s) in males, females, and neonatally castrated males exhibits the same affinity for E_2, suggesting that differences in hepatic estrogen responsiveness were not due to differences in the binding properties of receptor. However, heterogeneity of nuclear sites has been described in uterine nuclei (30) but the functional significance of different receptor forms is still unclear. Likewise, multiple forms of nuclear binding sites are present in rat hepatic nuclei (12). The possibility exists that female liver contains a form of receptor or nuclear binding site, not present in males, that is especially effective in stimulating gene expression.

In summary, our studies have demonstrated sex differences in hepatic estrogen responsiveness. A good correlation between the presence of HCLA sites, *in vitro* nuclear uptake of cytosolic receptors, and responsiveness is evident. However, a correlation does not exist between responsiveness of the liver to estrogens and nuclear occupancy of receptor following chronic *in vivo* estrogen treatment.

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