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Receptor-Bound Estrogens and Their Metabolites in the Nucleus of the Isolated Rat Liver Parenchymal Cell

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SUMMARY

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Recent studies have suggested that an estrogen receptor is present in rat liver and that hepatic metabolism may modulate receptor binding by estrogens. The metabolism of estrogens and the identity of receptor-bound estrogens were evaluated in the present study. Isolated hepatic parenchymal cells from rats were incubated with 10^{-7} M [^{3}H]-17,β-estradiol or [^{3}H]-17,α-ethinylestradiol for 5 min. [^{3}H]-17,β-estradiol was metabolized much more extensively than [^{3}H]-17,α-ethinylestradiol in cellular incubation mixtures. A variety of metabolites of each estrogen was detected. In incubations with [^{3}H]-17,β-estradiol, estrone was the principle nonpolar radioactivity. In incubations with [^{3}H]-17,α-ethinylestradiol, 2-hydroxy-17,α-estradiol was identified as a metabolite. The identity of the receptor-bound [^{3}H]estrogen was then studied after exposure of cells to [^{3}H]-17,α-ethinylestradiol or [^{3}H]-17,β-estradiol. After incubation of male or female liver cells with [^{3}H]-17,α-ethinylestradiol, the cells were homogenized and purified nuclei were prepared. Unchanged 17,α-ethinylestradiol and 2-hydroxy-17,α-ethinylestradiol were identified as the receptor-bound estrogens. (17,α-ethinylestradiol and 2-hydroxy-17,α-ethinylestradiol were also capable of binding to the cytosol receptor *in vitro*.) After incubation of liver cells with [^{3}H]-17,β-estradiol, the principle receptor-bound estrogen in the nucleus from female liver cells was unchanged 17,β-estradiol, in contrast to the radioactivity in the total cellular incubations, where estrone predominated. The principle nuclear-bound estrogen in male liver was an unidentified metabolite with the chromatographic mobility of 2-hydroxy-17,β-estradiol (however, it could not be methylated with catechol-*O*-methyl transferase). [^{3}H]-17,β-estradiol was apparently less effective than [^{3}H]-17,α-ethinylestradiol in binding to cellular receptors because of its more extensive metabolism, primarily to estrone. Although estrone binds cytosol receptors *in vitro*, it is much less effective than 17,β-estradiol in the promotion of receptor translocation in intact liver cells. Hepatic metabolism may modulate estrogenic potency in the liver and generate unusual estrogen metabolites which may interact with receptors. In cellular incubations, 17,α-ethinylestradiol and its metabolite 2-hydroxy-17,α-ethinylestradiol were more effective than the lower amount of unmetabolized 17,β-estradiol and the much higher levels of its metabolite estrone in the promotion of nuclear translocation of the estrogen receptor. Estrogen metabolism may contribute to the low receptor interaction and low biologic potency of 17,β-estradiol in the liver.

INTRODUCTION

When steroid hormones enter cells of a target tissue, they are exposed to receptors, other binding proteins, and enzymes of metabolism (1). In some instances, metabolites of these hormones also bind receptors and may exert a biological effect. The best-known example of this has been shown with testosterone. Testosterone is converted to dihydrotestosterone and androstanediols in some secondary sex organs (1), to androstenedione in the liver (2), and to estradiol in brain regions (3). Each of these metabolites is capable of binding to androgen (or estrogen) receptors and may exert biological activity (1–

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3). Estradiol (E_2)[1] is also metabolized in some tissues (the liver, in particular); estrone (E_1), estriol (E_3), and 2- and 4-hydroxylated forms of these estrogens have been reported (4). E_1 and E_3 possess estrogenic activity [though less than E_2 (1)], while the biological activity of the 2- and 4-hydroxylated estrogens remains controversial at present; weakly estrogenic and antiestrogenic activities have been described (5). Each of these metabolites is capable, to varying degrees, of binding to estrogen receptors of the uterus (6), pituitary, and hypothalamus (7).

The present study utilizes isolated rat liver parenchymal cells incubated with [^{3}H]-17,α-ethinylestradiol ([^{3}H]EE_2) or [^{3}H]E_2. First, the levels of receptor-bound ^{3}H are compared after treatment with either estrogen. Second, estrogen metabolism is characterized. Third, this study evaluates if either estrogen or its metabolites may bind to cellular receptors for estrogen in isolated cytosol. Fourth, the receptor-bound ^{3}H is identified after incubation of isolated liver cells with [^{3}H]E_2 or [^{3}H]EE_2.

A preliminary report has demonstrated that after incubation of [^{3}H]E_2 with female rat liver slices, E_2 and $20HE_2$ are bound to the nuclear estrogen receptors (8). Thus hepatic metabolism may regulate receptor binding. The isolated parenchymal cell preparation is longer-lived and may possess higher levels of receptors and metabolic activities for estrogens than the slice system (9). The present study uses both E_2 and EE_2 [an estrogen contained in oral contraceptives (4)] and liver of females as well as males. Male rat liver is of interest because it contains higher metabolic activities (10) and a non-receptor binder for estrogens (11).

The livers of adult male and female rats and other mammals contain probable receptors for estrogens (12, 13). In the female rat, the appearance at puberty of high levels of hepatic receptors for estrogen is correlated with the onset of inducibility of plasma renin substrate with EE_2 (14). Renin substrate is synthesized and secreted by the liver, and a high dose of estrogen increases the synthesis and release of renin substrate from the isolated, perfused rat liver *in vitro* (15).

Binding of estrogen to cellular receptors and translocation of the complexes to nuclei are thought to be essential steps in estrogen action (1). Experiments using intact male or female rats and isolated hepatic parenchymal cells *in vitro* have demonstrated that high doses of E_2 or EE_2 can effect nuclear translocation of the hepatic estrogen receptor (16–19).

The physiological role of the hepatic estrogen receptor is unknown, but it may have pharmacologic importance in humans given high doses of estrogens for contraceptive or therapeutic purposes. Estrogens increase the levels of a number of serum components of hepatic origin. Some of these changes may be related to the development of various side effects to the combined oral contraceptive (20).

[1] Abbreviations used: E_2, 17,β-estradiol; E_1, estrone; $20HE_1$, 2-hydroxy-17,β-estradiol; $20HE_1$, 2-hydroxyestrone; EE_2, 17,α-ethinylestradiol; $20HEE_2$, 2-hydroxy-17,α-ethinylestradiol; E_3, estradiol; $20HE_3$, 2-hydroxyestriol; $20MeE_2$, 2-methoxy-17,β-estradiol; $20MeE_1$, 2-methoxyestrone; $20MeEE_2$, 2-methoxy-17,α-ethinylestradiol; $20H,30MeEE_2$, 2-hydroxy-3-methoxy-17,α-ethinylestradiol; $40HE_2$, 4-hydroxy-17,β-estradiol; $40HE_1$, 4-hydroxyestrone; $17\alpha E_2$, 17α-estradiol; $20H17\alpha E_2$, 2-hydroxy-17,α-estradiol; $40H17\alpha E_2$, 4-hydroxy-17,α-estradiol; DES, diethylstilbestrol; TiA, toluene–isoamyl alcohol (19:1); 30% AS ppt, a redissolved precipitate of cytosol protein after fractionation with ammonium sulfate at 30% of saturation.

MATERIALS AND METHODS

Animals, liver cell preparation, and liver cell incubation. Mature, intact male and female rats (200–250 g; CD strain) were obtained from Charles River Laboratories. Liver cells were prepared by the collagenase perfusion technique (19). In receptor studies, 200 mg of cells was incubated in 3 ml of Waymouth MB 752/1 medium with 10^{-7} M [^{3}H]EE_2 ([6,7-^{3}H]; 42 Ci/mmol; New England Nuclear, Boston, Mass.) or 10^{-7} M [^{3}H]E_2 ([6,7-^{3}H]; 41 Ci/mmol; New England Nuclear). Replicate incubations also contained 10^{-5} M nonradioactive diethylstilbestrol (DES) to compete for binding by [^{3}H]estrogen to estrogen receptors. The female rat liver cells were incubated with estrogens for 15 min, and the male rat liver cells for 30 min [these are the times of maximal nuclear occupancy of the estrogen receptor for hepatic cells of each sex (18, 19)]. When incubations of cells were complete, cells were cooled on ice, pelleted, washed, and fractionated into partially purified cytosol receptors and purified nuclear fractions (8, 16, 19). Approximately 1 g of cells was used for each experimental group. Cells were homogenized in 0.25 M sucrose, 10 mM $MgCl_2$, 25 mM KCl, 50 mM Tris–HCl (pH 7.55) and centrifuged at 1000*g* to prepare a crude nuclear pellet. The supernatant was centrifuged at 105,000*g* for 60 min to prepare cytosol. Cytosol was partially purified by precipitation with ammonium sulfate at 30% of saturation (30% AS ppt cytosol) and resuspended in 10 mM Tris–HCl (pH 7.4), 1.5 mM EDTA, 1 mM NaN_3. Nuclei were purified by sedimentation through 2.2 M sucrose containing 0.2% Triton X-100. The nuclei appeared under light microscopy at 400× magnification to be mostly intact with no observable endoplasmic reticulum. The free and bound radioactivity in partially purified cytosol and solubilized (2 M NaCl, 5 M urea for 15 min) nuclear fractions was separated using small gel filtration columns. The bound [^{3}H]estrogen was collected in tubes containing 0.1 ml of 4% ascorbic acid with 1% glacial acetic acid in absolute methanol (standard ascorbic acid solution) to protect catechol estrogens (21).

After some incubations with [^{3}H]E_2 or [^{3}H]EE_2, cells were washed (at 0°C) with medium containing 10^{-5} M nonradioactive E_2 or EE_2. The cells were then homogenized in buffer which contained 10^{-5} mol of the same estrogen. This procedure was carried out to determine if binding of ^{3}H to receptors occurred during or subsequent to homogenization at 0°C, rather than during cellular incubations at 37°C (18, 19).

When the metabolism of estrogens in the incubation mixtures was studied, cells were prepared and incubated as stated previously, but each incubation flask contained 10^{-7} M [^{3}H]EE_2 of [^{3}H]E_2 of lower specific activity (4×10^6 dpm in each incubation flask). At the end of the incubation intervals, flasks were cooled in ice water. To each incubation mixture, 0.1 ml of standard ascorbic acid was added.

Analysis of metabolites of [^{3}H]EE_2 and [^{3}H]E_2 found in cell incubation mixtures and bound to receptors after in vitro incubations. Gel-filtered fractions from cytosol

and nuclei (which contained receptor-bound [^{3}H]estrogen) as well as [^{3}H]estrogen-containing incubation mixtures (aqueous phases) were immediately extracted 2x with 10 ml of toluene–isoamyl alcohol (19:1, v:v) (TiA), the extracts were pooled, and 0.1 ml of the standard ascorbic acid solution was added. This extract contained "nonpolar" metabolites (21) (not conjugated or protein bound). Radioactivity was determined in Liquifluor at 50% efficiency.

The aqueous phases from cellular incubation mixtures were subjected to further extraction. Ethanol (10 ml) was added to the aqueous phases. The aqueous ethanol mixture was centrifuged for 10 min at 25,000*g* to pellet precipitated macromolecules. This aqueous ethanol solution contained "highly polar" metabolites (conjugated metabolites) (21). The radioactivity in the pellets is described as "irreversibly" (presumably covalently) bound to macromolecules (21). The cell pellets were suspended in 2 ml of distilled water for sampling purposes. Five-tenths milliliter of either highly polar or irreversibly bound metabolite extracts was solubilized with 2 ml of NCS solubilizer (Amersham Searle Corp., Arlington Heights, Ill.) and counted in 10 ml of the Liquifluor at 30% efficiency.

Nonpolar metabolites in the toluene extracts were analyzed using three paper chromatography systems (21), each with a formamide–ascorbic acid stationary phase. System A (chlorobenzene–ethyl acetate, 3:1) separated the 2-hydroxylated estrogens, $20HE_2$ (R_f 0.30) and $20HE_1$ (R_f 0.55), from other components near the origin, from E_2 (R_f 0.66), and from E_1 (R_f 0.90). $20HEE_2$ (R_f 0.47) and EE_2 (R_f 0.80) were also resolved using this system. This system did not resolve E_2, E_1, and EE_2 from their *O*-methylated metabolites. System B (chlorobenzene) separated E_2 (R_f 0.4) and E_1 (R_f 0.70) from methoxylated derivatives of E_2 (R_f 0.60) and E_1 (R_f 0.87). EE_2 (R_f 0.5) and methoxylated EE_2 derivatives (R_f 0.82) were also separated with this system. (Systems A and B were run in parallel on samples for determination of metabolite levels.) System C (cyclohexane) separated methoxylated derivatives of EE_2 into 2-hydroxy,3-methoxy (20H,30MeEE$_2$; relative mobility, 0.63) and 2-methoxy (20MeEE$_2$; relative mobility, 0.82) components. Chromatographic standard E_2, E_1, EE_2, $20HE_1$, $20HE_3$, and $20MeE_1$ were purchased from a commercial source (Steraloids Inc., Wilton, N.H.). $20HEE_2$ (and [^{3}H]$20HEE_2$) were synthesized using mushroom tyrosinase (Sigma) by the method of Jellinck and Brown (22). The $20HEE_2$ was purified using ascorbic acid-impregnated silica gel (Macherneg, Naqel & Co., Düren, Germany, MN-Silica Gel G-HR) column chromatography with *n*-hexane–chloroform–acetic acid (4:1:1) as elution solvent (23). Rat liver catechol-*O*-methyl transferase (partially purified by a modification (24) of the method of Axelrod and Tomchick (25) was used to synthesize (26) 20MeEE$_2$ and 20H, 30MeEE$_2$ from $20HEE_2$. The two methylated products were chromatographed on System A and then the methylated peak was separated into its two components by rechromatography on System C (21). For detection of paper chromatographic standards, the strips containing the standards were dipped in a saturated solution of sodium periodate. This visualized the catechol estrogens. Then the noncatechol estrogens were visualized by spraying with 25% Na_2CO_3 and Phenol reagent–water (1:5). For analytical detection of cellular metabolites following chromatography, the chromatograms were cut into 1.5-cm-wide strips and then into 0.5- or 1-in. segments. These were extracted with 1 ml of methanol and the radioactivity was determined.

After preliminary identification of metabolites based on their chromatographic mobility, methylation reactions were carried out on chromatographically separated peaks (System A) for further chemical identification. E_1, E_2, and EE_2 were methylated using dimethyl sulfate (27) (Eastman Kodak Co., Rochester, N.Y.). $20HE_1$, $20HE_2$, and $20HEE_2$ were methylated using rat liver catechol-*O*-methyl transferase (26). The mobilities of methylated products from dimethyl sulfate reactions were compared with standards using chromatography (27) on silica gel G-254 plates with fluorescent indicator (Brinkman Instruments, Westburg, N.Y.) in benzene:ethanol (90:10). The mobilities of methylated products from catechol-*O*-methyl transferase reactions were compared with standards using chromatographic System A (mentioned previously).

Studies of binding by estrogen metabolites to the cytosol estrogen receptor from liver and uterus. The ability of various estrogen metabolites to bind to cytosol estrogen receptors *in vitro* was examined. $20HE_2$, $40HE_2$, $20HE_1$, $40HE_1$, $40H17\alpha E_2$, and $20H17\alpha E_2$ were synthesized using potassium dinitrosodisulfonate (ICN Pharmaceuticals, Cleveland, Ohio) (Fremy's salt) by the method of Gelbke *et al.* (28). Purification was carried out using column chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) with an elution solvent containing toluene, chloroform, and methanol (7:2:1). Separation of catechol estrogens from potentially contaminating parent estrogen was verified by adding radioactive tracer parent estrogen prior to separation on LH-20. Stock stolutions (1 mg/ml) of the catechol estrogens were maintained at −20°C in absolute methanol containing 4% ascorbic acid and 1% glacial acetic acid. When needed for experiments, more dilute solutions were made (immediately before use) in ethanol containing 0.01% ascorbic acid.

Liver cytosol (1:6, tissue wt:buffer vol) and uterine cytosol (1:15, tissue wt:buffer vol) were prepared for use in *in vitro* binding incubations (13, 16). The receptors for estrogen in liver cytosol were partially purified with ammonium sulfate fractionation (to 30% of saturation) and the protein pellets resuspended in buffer at one-half the original volume of cytosol. Two hundred microliters of uterine cytosol or partially purified liver cytosol was incubated with 2 nM [^{3}H]E_2 ([2,4,6,7^{3}H]; 90 Ci/mmol; New England Nuclear) with or without 10^{-7} M competing steroid for 1 h at 0°C (12). Bound and free steroids were separated using gel filtration. The vehicle in which catechol estrogens were dissolved did not affect binding by [^{3}H]E_2 to receptors. When [^{3}H]$20HEE_2$ (10^{-7} M) was incubated with male or female liver cytosol or uterine cytosol under the conditions used for binding, the catechol estrogen did not appear to be altered by the incubation procedure. After the binding incubation, 90% or more of the radioactivity was TiA extractable and 90%

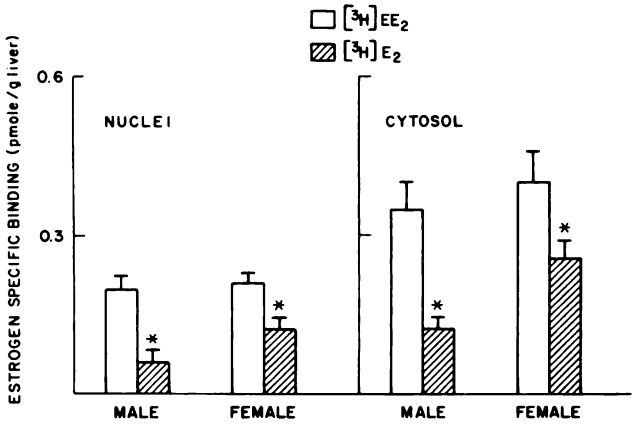

FIG. 1. *Receptor-bound ^3H after incubation of isolated rat liver cells with [^3H]E_2 or [^3H]EE_2*

Isolated liver parenchymal cells from male and female rats were incubated with 10^{-7} M [^3H]E_2 or [^3H]EE_2 (with or without 10^{-5} M DES to displace binding by receptors). Male cells were incubated for 30 min, while female cells were incubated for 15 min. The cells were then cooled on ice, pelleted, washed, homogenized, and fractionated. After gel filtration, bound radioactivity in partially purified cytosol and solubilized nuclear fractions was determined. Data shown are averages ± SE of at least two individual experiments. (*) $P<0.05$ comparing binding in each fraction after incubation in the presence of [^3H]E_2 to binding after incubation in the presence of [^3H]EE_2.

or more of the extractable material cochromatographed with authentic $20HEE_2$ on paper chromatography System A.

RESULTS

Binding of [^3H]estrogens in isolated hepatic parenchymal cells. After incubation of isolated rat liver cells with [^3H]estrogen with or without an excess of DES, the cells were fractionated into nuclear and partially purified cytosol fractions. The difference in binding without and with DES is described as "estrogen-specific" binding (binding by probable receptors for estrogen). After exposure of cells to 10^{-7} M [^3H]EE_2 or [^3H]E_2 [submaximal doses for nuclear translocation of the receptor (18, 19)], estrogen-specific nuclear and cytosol binding was measured. [^3H]EE_2 promoted higher levels of nuclear binding than E_2 in male (threefold higher) and female (twofold higher) liver cells (Fig. 1). There were also slightly higher levels of estrogen-specific binding in 30% AS ppt cytosol than in nuclear fractions of male or female rat liver cells. Levels of cytosol receptor-bound ^3H were higher with [^3H]EE_2 than with [^3H]E_2. Estrogen metabolism in the liver cell incubations was then studied to better understand these differences between estrogens.

Metabolism of [^3H]estrogens. The overall pattern of metabolites of [^3H]E_2 and [^3H]EE_2 in cells combined with their incubation medium was studied after 2, 5, and 15 min of incubation. The results after 5 min of incubation are shown in Table 1. Metabolites were first measured in three categories: nonpolar (TiA extractable), highly polar (TiA nonextractable, ethanol soluble), and irreversibly bound (nonextractable with TiA or ethanol). Conversion to highly polar and irreversibly bound metabolites was more extensive in male than female liver cells. When nonpolar metabolites were examined, E_2 was metabolized more extensively then EE_2 in male and female liver cells. For example, in female liver cells, higher levels of unchanged EE_2 (18×10^{-9} M) than E_2 (2×10^{-9} M) were observed. Levels of unchanged E_2 and EE_2 were higher in female than male liver cells. After exposure of cells to [^3H]EE_2, ^3H cochromatographing with EE_2, $20HEE_2$, and $20MeEE_2$ was observed. After [^3H]E_2 incubations, peaks of ^3H cochromatographing with E_2, E_1, $20HE_2$, $20HE_1$, $20MeE_2$, and $20MeE_1$ were observed. Levels of E_1 (30×10^{-9} M) were higher than E_2 (2×10^{-9} M).

Binding of estrogen metabolites to cytosol receptors in vitro. The ability of the partially purified liver cytosol receptors from male or female rats to bind some of these estrogen metabolites was next examined *in vitro* (Fig. 2). The results were compared with results using rat uterine cytosol. Under nonequilibrium conditions (0°C, 1-h incubations with 2 nM [^3H]E_2 ± a 50-fold excess of competitor), a variety of 2- and 4-hydroxylated estrogen derivatives was capable of competing for binding by [^3H]E_2 to cytosol receptors from rat liver and uterus.

TABLE 1

Metabolites of [^3H]EE_2 and [^3H]E_2 after incubation with isolated rat liver parenchymal cells

Cells from male or female rat livers were prepared and incubated with 10^{-7} M [^3H]EE_2 or [^3H]E_2 for 5 min. Then the cells plus media were extracted with TiA (containing ascorbic acid) and the extracts chromatographed (two-thirds of the TiA-extractable radioactivity was intracellular; data not shown). Three chromatographic systems were used in the analysis of TiA-extractable radioactivity: A, B, and C (described in Materials and Methods). When R_f values appear, they refer to mobilities using system A. These data are typical of replicate experiments.

	Concentration of metabolites (M × 10^9)										
	Nonpolar									Irreversibly bound	Highly polar
	Total	R_f 0.17	$20HEE_2$	EE_2	$20MeEE_2$						
(A) EE_2 (10^{-7} M)											
Female	36	≤0.4	5.6	18	10					2.1	46
Male	24	1.5	0.9	10	10					2.6	55
	Total	R_f 0.13	R_f 0.30	R_f 0.53	$20HE_1$	E_2	E_1	MeE_2	MeE_1		
(B) E_2 (10^{-7} M)											
Female	48	≤0.3	1.2	≤0.3	6.3	2.0	30	3.5	5.0	1.4	44
Male	37	3.8	10	3.5	2.5	1.0	10	2.9	2.1	2.0	54

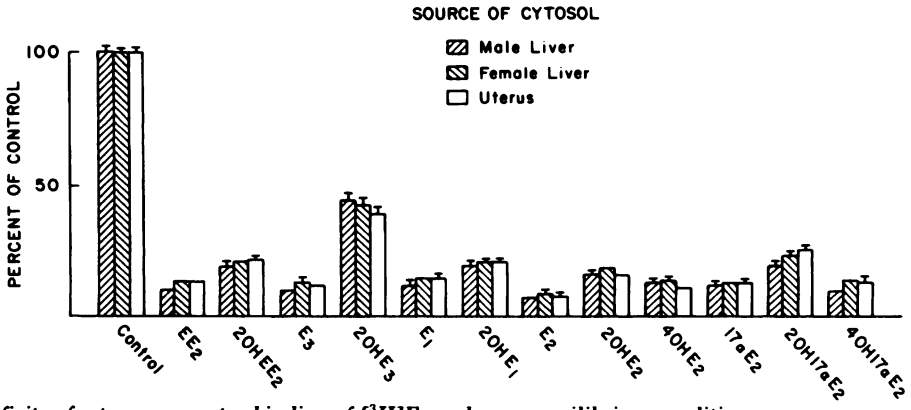

FIG. 2. *Steroid specificity of estrogen receptor binding of [^{3}H]E_2 under nonequilibrium conditions*

30% AS ppt cytosol from male or female rat livers or cytosol from rat uterus was incubated with 2 nM [^{3}H]E_2 and 10^{-7} M competitors for 1 h at 0°C. The control binding is 100%, and the competitors were EE_2, $20HEE_2$, estriol (E_3) $20HE_3$, estrone (E_1), $20HE_1$, E_2, $20HE_2$, $40HE_2$, 17α-estradiol (17α E_2), 20H17αE_2, and 40H17αE_2.

Identification of receptor-bound ^{3}H after incubations of hepatic parenchymal cells with [^{3}H]estrogen. When cells were incubated with [^{3}H]EE_2 and then fractionated into nuclei and partially purified cytosol fractions, nuclear receptor-bound ^{3}H cochromatographing with EE_2 and $20HEE_2$ was observed (Fig. 3). The level of $20HEE_2$ was higher in female than male liver cell nuclei. When the metabolite distribution was studied in partially purified cytosol fractions (Table 2), essentially the same results were observed. When an excess of nonradioactive DES was included in cellular incubations with [^{3}H]EE_2 to compete for ^{3}H-receptor binding, radioactivity in nuclear and cytosol fractions was reduced. The cellular metabolism of [^{3}H]EE_2 to nonpolar and polar metabolites was apparently unchanged in the presence of DES (data not shown). In the presence of DES, the amounts of bound EE_2 and $20HEE_2$ were both decreased, suggesting that EE_2 and $20HEE_2$ were both bound to receptors.

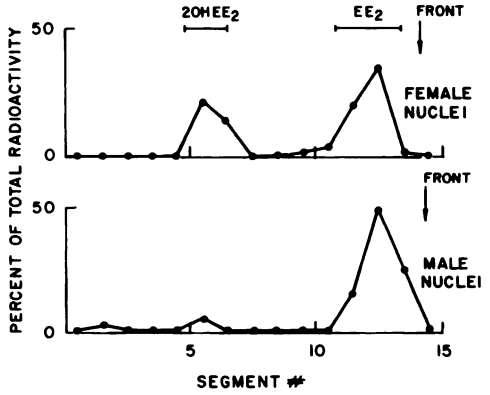

FIG. 3. *Receptor-bound estrogen after incubation with [^{3}H]EE_2 with male or female rat liver parenchymal cells*

Isolated liver parenchymal cells from male or female rat livers were incubated with 10^{-7} M [^{3}H]EE_2 with or without an excess of DES (10^{-5} M). Male cells were incubated for 30 min, while female cells were incubated for 15 min. The cells were then cooled on ice, pelleted, washed, homogenized, and fractionated. Finally, the solubilized nuclear fractions were gel filtered and TiA extracted. Nuclear receptor levels were 0.20 ± 0.04 pmol/g liver in the female and 0.24 ± 0.06 pmol/g liver in the male. The organic extracts of solubilized, gel-filtered nuclei from cells incubated with [^{3}H]EE_2 (without DES present) were then chromatographed on System A. These data are typical of two separate experiments.

When female liver cells were incubated with [^{3}H]EE_2 for 60 min instead of 15 min (Fig. 4), the nuclear level of estrogen-specific, bound ^{3}H was only one-third of the level at 15 min. When the estrogen-specific metabolite distribution of this nuclear ^{3}H was examined, $20HEE_2$ and EE_2 were present in approximately the same proportion after 15 min and 1 h.

[^{3}H]E_2 was incubated with liver cells, and then the cells were fractionated into nuclear and partially purified cytosol fractions. In female liver cell nuclei (Fig. 5), the major peak of ^{3}H cochromatographed with E_2, and there were smaller peaks comigrating with $20HE_1$, $20HE_2$, and

TABLE 2

Identity of receptor-bound estrogen in male or female rat liver parenchymal cell cytosol and nuclei after incubation with [^{3}H]EE_2

Male and female rat liver cells were incubated with 10^{-7} M [^{3}H]EE_2 ± 10^{-5} M DES, and then nuclear and 30% AS ppt cytosol fractions were prepared, gel filtered, extracted, and chromatographed (System A). The peak of EE_2 was then rechromatographed on System B, to separate it from any potentially contaminating methoxylated EE_2. The distribution of metabolites was determined. The dpm used for chromatography were as follows (first female, then male): 33,000 and 75,000 (for nuclei − DES), 7500 and 8300 (for nuclei + DES), 170,000 and 200,000 (for cytosol − DES), and 38,000 and 42,000 (for cytosol + DES). These data are typical of the results of two separate experiments.

	pmol/g liver in initial sample	Distribution of [^{3}H]EE_2 metabolites (percentage of total radioactivity)	
		$20HEE_2$	EE_2
(A) Female			
Purified nuclei			
− DES	0.27	35	60
+ DES	0.05	35	45
30% AS ppt cytosol			
− DES	0.61	28	64
+ DES	0.13	14	40
(B) Male			
Purified nuclei			
− DES	0.20	6	90
+ DES	0.03	3	75
30% AS ppt cytosol			
− DES	0.49	3	91
+ DES	0.11	2	50

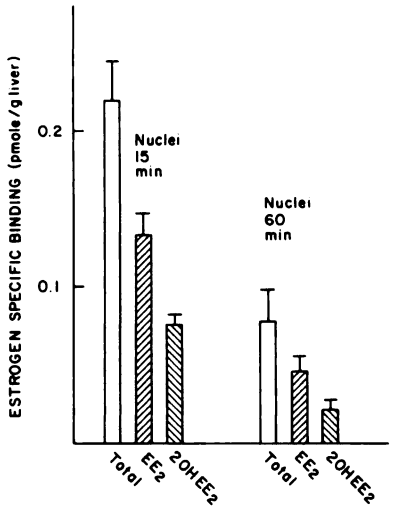

FIG. 4. *Radioactive estrogens attached to nuclear receptors of liver cells after 15 and 60 min of incubation*

Isolated female rat liver parenchymal cells were incubated with 10^{-7} M [^{3}H]EE_2 ± 10^{-5} M DES for either 15 min or 60 min. After cooling on ice to end the incubation, the cells were pelleted, washed, and homogenized. Purified nuclei were prepared, solubilized, gel filtered, and TiA extracted. $20HEE_2$ and EE_2 were then measured after chromatography of the extracts on System A.

E_1. In male liver cell nuclei, the major ^{3}H peak cochromatographed with $20HE_2$; smaller peaks comigrating with E_2 and E_1 were observed. When the identity of radioactivity bound to partially purified cytosol was studied in male and female liver cells (Table 3), a pattern of metabolites was observed which was similar to that found in the nuclei. When an excess of nonradioactive DES was included in cellular incubations with [^{3}H]E_2, levels of $20HE_2$, and $20HE_1$, E_2, and E_1 in nuclear and cytosol

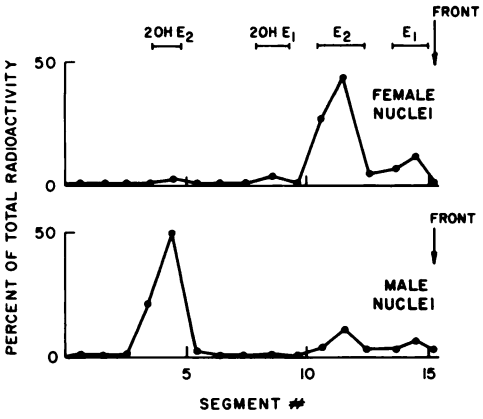

FIG. 5. *Nuclear receptor-bound ^{3}H after cellular incubations with [^{3}H]E_2*

Isolated liver parenchymal cells from female or male rat livers were incubated with 10^{-7} M [^{3}H]E_2 with or without an excess (10^{-5} M) of DES. Male cells were incubated at 37°C for 30 min, while female cells were incubated for 15 min. Following incubation, the cells were cooled on ice, pelleted, washed, homogenized, and fractionated. Solubilized nuclear fractions were gel filtered and TiA extracted. Nuclear receptor levels were 0.09 ± 0.02 pmol/g liver in the male and 0.10 ± 0.02 pmol/g liver in the female. The organic extracts of nuclei from cells incubated with [^{3}H]E_2 (without DES present) were chromatographed on System A to separate the metabolites. These data are typical of two separate experiments.

TABLE 3

Identity of receptor-bound estrogen after incubation with [^{3}H]E_2

Male and female rat liver cells were incubated with 10^{-7} M [^{3}H]E_2 ± 10^{-5} M DES, and then nuclear and 30% AS ppt cytosol fractions were prepared, gel filtered, extracted, and chromatographed (System A). The distribution of metabolites was determined. System B was used to separate E_2 and E_1 from any potentially contaminating methoxylated E_2 and E_1. The dpm used for chromatography were as follows (first female, then male): 13,800 and 3600 (for nuclei −DES), 3600 and 2000 (for nuclei + DES), 90,000 and 36,000 (for cytosol −DES), and 20,000 and 12,000 (for cytosol + DES). These data are typical of the results from two separate experiments.

	pmol/g liver in initial sample	Distribution of [^{3}H]E_2 metabolites (percentage of total radioactivity)			
		E_2	R_f 0.30	E_1	$20HE_1$
(A) Female					
Purified nuclei					
− DES	0.13	75	3	19	3
+ DES	0.04	60	3	16	3
30% AS ppt cytosol					
− DES	0.33	65	4	26	3
+ DES	0.08	40	4	20	4
(B) Male					
Purified nuclei					
− DES	0.14	12	70	11	≤1
+ DES	0.05	17	45	16	≤1
30% AS ppt cytosol					
− DES	0.17	8	68	6	≤1
+ DES	0.04	6	54	10	≤1

fractions were reduced. This suggests receptor binding by all of these species. In the presence of DES, the cellular metabolism of [^{3}H]E_2 to other nonpolar and polar metabolites was apparently unchanged (data not shown). If an excess of nonradioactive E_2 (10^{-5} M) was included during cell washes and homogenization at 4°C, the levels of binding and the metabolite pattern in male and female nuclei and cytosol were unchanged (data not shown). This suggests that receptor binding occurred before homogenization [as previously shown with [^{3}H]EE_2 (18, 19)].

The identity of chromatographically separated peaks, comigrating with E_2, E_1, $20HE_2$, and $20HE_1$ (after incubation with [^{3}H]E_2) and EE_2 and $20HEE_2$ (after incubation with [^{3}H]EE_2), was further studied by chemical modification. Radioactive estrogens and their metabolites were methylated using catechol-*O*-methyl transferase (specific for catechols) or dimethyl sulfate. The chromatographic mobilities of the methylated derivatives of the peaks of radioactivity from cell incubations, cytosol receptors, and nuclear receptors were in accord with their tentative identification (with one exception) (Tables 4 and 5). The exception was that with male liver cell incubations, cytosol receptor, and nuclear receptor, most of the radioactivity comigrating with $20HE_2$ (R_f 0.30 on System A) did not methylate with catechol-*O*-methyl transferase. Its identity is unknown.

Figure 6 compares the levels of estrogens and their metabolites in cellular incubations (after 5 min) and in cytosol and nuclear receptor fractions (after 15 min). Female liver cells were used. E_2, EE_2, $20HE_2$, and $20HEE_2$ were all apparently capable of receptor translocation, but E_1 and $20HE_1$ were much less effective (Fig. 6).

TABLE 4

[^{3}H]EE$_2$ and its metabolites: Further identification of chromatographic fractions by methylation

Cells were incubated with 10^{-7} M [^{3}H]EE$_2$, the metabolite distribution was studied in the incubation mixture after 5 min, and the receptor-bound metabolites were studied after 15 min (female) or 30 min (male). Receptor-bound metabolites were prepared using chromatography System A, and incubation mixture metabolites by Systems A and B used sequentially. Chromatographically separated radioactive peaks were then chemically modified by methylation. Methylation with rat liver COMT was used to identify catechol estrogens, and methylation with dimethyl sulfate was used to identify other estrogens. The percentage methylation is the percentage of the radioactivity comigrating with methylated standards. Data are typical of determinations from replicate experiments. The levels of metabolites (from Tables 1 and 2) were multiplied by the fraction methylated to obtain the concentrations listed.

	Incubation mixture		Cytosol receptor		Nuclear receptor	
	Methylated	Concentration	Methylated	Bound	Methylated	Bound
	%	M × 10^9	%	*pmol/g*	%	*pmol/g*
Female						
EE_2	91	16	93	0.36	95	0.15
$20HEE_2$	90	5.0	86	0.15	90	0.09
Male						
EE_2	92	9.2	93	0.41	92	0.17
$20HEE_2$	98	0.70	80	0.01	85	0.01

TABLE 5

[^{3}H]E$_2$ and its metabolites: Further identification of chromatographic fractions by methylation

Cells were incubated with 10^{-7} M [^{3}H]E$_2$, the metabolite distribution in the incubation mixture was studied after 5 min, and the receptor-bound metabolites were studied after 15 min (female) or 30 min (male). Metabolites were chromatographically separated and methylated (as in Table 4). The levels of metabolites (from Tables 1 and 3) were then multiplied by the fraction methylated to obtain the concentrations listed.

	Incubation mixture		Cytosol receptor		Nuclear receptor	
	Methylated	Concentration	Methylated	Bound	Methylated	Bound
	%	M × 10^9	%	*pmol/g*	%	*pmol/g*
Female						
E_2	90	1.8	95	0.24	90	0.088
E_1	85	25	90	0.077	42	0.010
$20HE_2$	99	1.2	98	0.013	98	0.004
$20HE_1$	69	4	84	0.008	21	0.001
Male						
E_2	63	0.63	60	0.0082	60	0.010
E_1	80	8.0	85	0.0088	75	0.012
$20HE_2$	33	3.3[a]	5	0.0058[b]	27	0.027[c]
$20HE_1$	60	1.3	ND[d]	ND	ND	ND

[a] 6.7×10^{-9} M unidentified.
[b] 0.11 pmol/g unidentified.
[c] 0.071 pmol/g unidentified.
[d] ND = no data; insufficient radioactivity for characterization.

DISCUSSION

The data in the present study demonstrate that 10^{-7} M [^{3}H]EE$_2$ was capable of promoting higher levels of nuclear translocation of the estrogen receptor than 10^{-7} M [^{3}H]E$_2$. These data are in agreement with a previous study which utilized a ^{3}H-exchange assay for receptor quantitation.[2] One possible explanation for this phenomenon was that [^{3}H]E$_2$ was metabolized more rapidly than [^{3}H]EE$_2$, limiting its availability for interaction with receptors. The data were consistent with this hypothesis. When the degree of metabolism of each estrogen was studied after only 5 min of incubation with the cells, 10-fold higher levels of [^{3}H]EE$_2$ than [^{3}H]E$_2$ remained.

It has been shown that after incubation of 10^{-7} M nonradioactive EE$_2$ with female liver cells, one-half-maximal depletion of cytosol receptors rapidly occurs (5 min) in conjunction with the appearance of nuclear receptors after 15 min (19). The level of [^{3}H]EE$_2$ remaining after 5 min in the present study was 20×10^{-9} and 10×10^{-9} M in incubations of female and male liver cells, respectively. The concentration of [^{3}H]EE$_2$ remaining after 5 min of incubation with 10^{-7} M EE$_2$ is in close agreement with the concentration of E$_2$ required for one-half-maximal depletion of cytosol receptors in uterine organ culture (29). In contrast, the concentrations of [^{3}H]E$_2$ remaining in the incubations of the present study after 5 min were 2×10^{-9} and 1×10^{-9} M in female and male liver cells, respectively. These concentrations of E$_2$ were capable of promoting only a small amount of depletion of cytosol receptor in uterine organ culture (29).

The level of unmetabolized [^{3}H]E$_2$ was low after 5 min of incubation with liver cells, but the level of its potentially estrogenic metabolites was high in comparison. In particular, levels of E_1 were 30×10^{-9} M, 15-fold higher than E$_2$ in the female liver cell incubations and 10-fold higher than E$_2$ in male liver cell incubations. In addition, there were metabolites cochromatographing with $20HE_2$

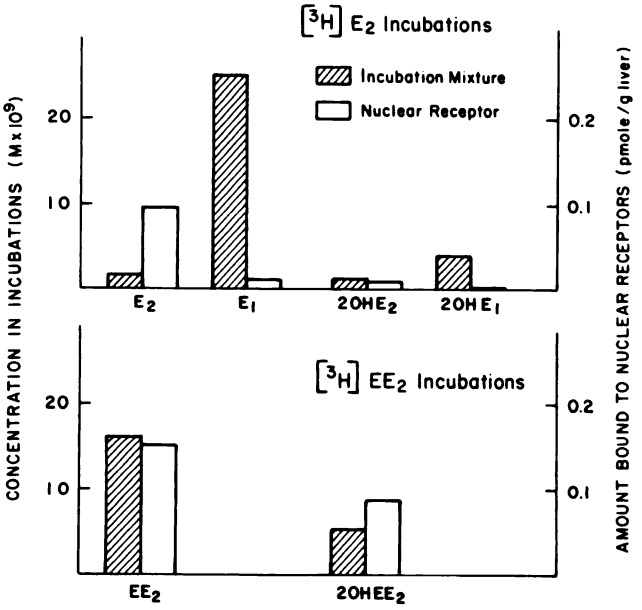

FIG. 6. *Relative efficiency of estrogens and their metabolites for receptor translocation in female liver cells*

Data from Tables 4 and 5 (incubation mixture after 5 min and nuclei after 15 min) are presented graphically to demonstrate differences in the efficiency of receptor translocation between various estrogens.

[2] Dickson, R. B., and A. J. Eisenfeld, unpublished observations.

and $20HE_1$. After incubation of [^{3}H]EE_2 with liver cells, $20HEE_2$ was identified as a metabolite. The low levels of E_2 and the higher levels of its potentially estrogenic metabolites may be less capable of binding to receptors and promoting receptor translocation than EE_2 (present in higher levels than E_2) and its metabolite $20HEE_2$.

The ability of a variety of potentially estrogenic metabolites to bind to the estrogen receptor *in vitro* was then studied. E_1, $20HE_1$, $20HE_2$, E_2, $20HEE_2$, and EE_2 could all compete for binding by [^{3}H]E_2 to cytosol receptors prepared from male or female rat liver or rat uterus. Therefore these estrogens could all be considered to have the potential for receptor binding and translocation after *in situ* production by isolated rat liver cells exposed to [^{3}H]E_2 or [^{3}H]EE_2. This is the first demonstration that catechol estrogens are capable of binding to isolated receptors from the liver. This is also the initial report that $20HEE_2$, a principle metabolite of EE_2, an estrogen of the oral contraceptive, is capable of binding to estrogen receptors from any target tissue for estrogens.

When male or female liver cells were incubated with [^{3}H]EE_2, only $20HEE_2$ and unchanged EE_2 were found attached to nuclear and cytoplasmic receptors fractions from male or female liver cells. In female liver cells, $20HEE_2$ comprised one-third of the receptor-bound radioactivity, but less than one-tenth in male liver cells. There was a similar difference in the levels of $20HEE_2$ in cell incubation extracts comparing male and female. It has been shown (19) that after 1-h incubation of female [but not male (18)] liver cells with 10^{-7} M nonradioactive EE_2, the occupied nuclear receptor level (determined by exchange with [^{3}H]E_2) is substantially lower than after 15 min. The present study confirms this finding in the female using [^{3}H]EE_2. After 1 h, the levels of nuclear receptor-bound $20HEE_2$ and EE_2 were both reduced in female liver cells. This suggests that the more rapid disappearance of occupied nuclear receptors observed in female liver cells *in vitro* (19) and in whole liver *in vivo* (17) is not due to different levels of a receptor-bound, rapidly dissociating metabolite of EE_2. The reason for the sex difference in retention of the nuclear receptor is unknown, but could involve differences in "processing" of nuclear receptors of differences in nuclear acceptors. Another possibility is that receptor translocation is a process which continues for at least 1 h and that the lower levels of nuclear receptor in the female liver cells after 1 h are a consequence of lower levels of unmetabolized estrogen available for receptor interactions. It is also possible that the recently described, male-specific sex steroid binder (11) could retain high intracellular levels of EE_2 in the cytosol compartment. Thus, more EE_2 could be available for receptor interactions in the male after 1 h, even though estrogen metabolism is generally more extensive in male compared with female rat liver.

When female liver cells were incubated with [^{3}H]E_2, unchanged E_2 was the principle estrogen bound to receptors. Low levels of $20HE_2$, E_1, and $20HE_1$ were also observed. When male liver cells were incubated with [^{3}H]E_2, the principle receptor-bound estrogen cochromatographed with $20HE_2$; smaller peaks comigrated with E_2 and E_1. The peak of radioactivity cochromatographing with $20HE_2$ was only partially $20HE_2$. In male liver cell incubation extracts, as well as in nuclei, $20HE_2$ comprised one-third of the radioactivity in the chromatographic fraction and almost none of the radioactivity in the cytosol. Other metabolites of E_2 which cochromatograph with $20HE_2$ in the separation system used here (chlorobenzene:ethyl acetate, 3:1) are 16-epiestriol (16,β-hydroxy-17,β-estradiol) and 6- and 7-hydroxylated derivatives of E_1 (30). 16-epiestriol may be a likely candidate since it binds well to estrogen receptors (31) and has a high biologic activity (32). However, 6- and 7-hydroxylated metabolites are also possibilities since some of them are also biologically active (32). High levels of $20HE_2$ and another metabolite with a similar chromatographic mobility have also been described using [^{3}H]E_2 incubations with male (but not female) rat liver homogenates (10).

The biologic activities of the various metabolites of estrogen when bound to the nuclear estrogen receptor are not known. The levels of nuclear-bound catechol estrogens after cellular incubations with [^{3}H]EE_2 were higher than after [^{3}H]E_2. These metabolites are potentially toxic (5) and may result in covalent binding to protein (21) and even DNA (33). It is possible that their proximity to chromatin may contribute to hepatoma formation in women taking the oral contraceptive (20).

E_2 was less effective than EE_2 in the promotion of receptor translocation. This was apparently due to conversion of E_2 to less active metabolites (primarily E_1). In another recent study we have shown that inhibition of the conversion of E_2 to E_1 in female liver cells results in increased levels of unchanged E_2 and increased nuclear translocation of the estrogen receptor.[2] E_1 was also less potent than E_2 for receptor translocation in superfused uterine slices (34) and in uterine organ culture (29). In addition, E_1 was less effective than E_2 in increasing levels of plasma renin substrate (35), an effect thought to be mediated by the liver (15).

The greater ability of EE_2 compared to E_2 for promoting translocation of the hepatic estrogen receptor may be of interest in interpreting some reports comparing the biologic activities of these two estrogens. Although E_2 and EE_2 are both very active in the female reproductive tract (1), E_2 is less active than EE_2 in inducing liver-synthesized, serum proteins and lipids (35–37). Some of these serum components may contribute to the development of various side effects to the oral contraceptive (20). The isolated liver cells may be useful in screening for other estrogens which may have utility as contraceptives. Such useful estrogens should be active in sites of antifertility action but rapidly metabolized in the liver, limiting their interaction with receptors in this organ which may be involved in undesirable side effects.

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