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PRELIMINARY COMMUNICATION

ESTROGEN RECEPTOR IN THE MAMMALIAN LIVER: EFFECT OF METABOLISM ON THE AMOUNT AND IDENTITY OF RECEPTOR-BOUND ESTROGEN

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Direct effects of estrogen on the liver may be mediated by an estrogen receptor previously observed in mammalian liver (1). In the liver, as in other target tissues, estrogen interacts initially with receptors in the cytoplasm followed by translocation of the complexes to nuclei and attachment to chromatin (1,2). It may be possible to minimize the estrogen-liver interaction and design safer oral contraceptives by exploiting differences in estrogen receptor function between the liver and sites of anti-fertility action (hypothalamic-pituitary axis).

We have found using intact tissue slices that the estrogen receptor system in liver may have unusual features. Extensive metabolism of the estrogen limits its availability for binding to the receptor (3). We have also observed that particular estrogen metabolites are bound non-covalently to the estrogen receptor translocated from the cytoplasm to the hepatic nucleus. We have the first evidence that catechol estrogens comprise a substantial fraction of the receptor-bound radioactivity observed in purified nuclei after incubation of liver slices with ^{3}H-estradiol (^{3}H-E_2).

Liver slices (0.5 mm thick, prepared with a Stadie-Riggs microtome) from ovariectomized adult female rats (CD strain, Charles River Labs.) were incubated with either 2,4,6,7-^{3}H-E_2 (specific activity 100 Ci/mmole, 220 dpm/fmole, results in Figure 1), or with 6,7-^{3}H-E_2 (47 Ci/mmole, 100 dpm/fmole, results in Figure 2) in Krebs-Ringer Henseleit phosphate buffer (pH 7.4). Tissue slices were homogenized in 6 volumes (w/v) of 0.25 M sucrose, 0.01 M

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$MgCl_2$, 0.025 M KCl in 0.05 M Tris-HCl, pH 7.55. The high speed cytosol (prepared by recentrifugation of the 1000g supernate of the crude homogenate at 105,000g for 1 hour) was purified partially (AS cytosol) by precipitation using a neutralized solution of saturated ammonium sulfate to 30% of saturation with respect to cytosol (v/v) as previously described (4). Highly purified nuclei were prepared by recentrifugation of the 1000g pellet of the crude homogenate through dense sucrose (2.2 M sucrose, 0.005 M $MgCl_2$, 0.015 M KCl in 0.05 M Tris-HCl, pH 7.55) containing 0.2% (v/v) Triton X-100 (5). Macromolecular binding in aliquots of AS cytosol or in 5M urea, 2M NaCl extracts of nuclei was determined by gel filtration on columns (1.1 x 12 cm) of Biogel P-10 (polyacrylamide beads, exclusion MW, 20,000), equilibrated with 0.01 M Tris-HCl, pH 7.4 for AS Cytosol and 1M NaCl in 0.01 M Tris-HCl, pH 7.4 for nuclear extracts. Macromolecular-bound radioactivity identified by co-elution with blue dextran-2000 was extracted into toluene:isoamyl alcohol (TiA), 19:1 (v/v) and measured by scintillation spectrometry with an efficiency of 50%.

After incubation of liver slices with ^3H-E_2 (5 x 10^{-9}M) at 25°, macromolecule-bound radioactivity can be observed in AS Cytosol and in purified nuclear fractions (Figure 1A). Addition of a 100 fold excess of diethylstilbestrol (DES) to the incubation medium

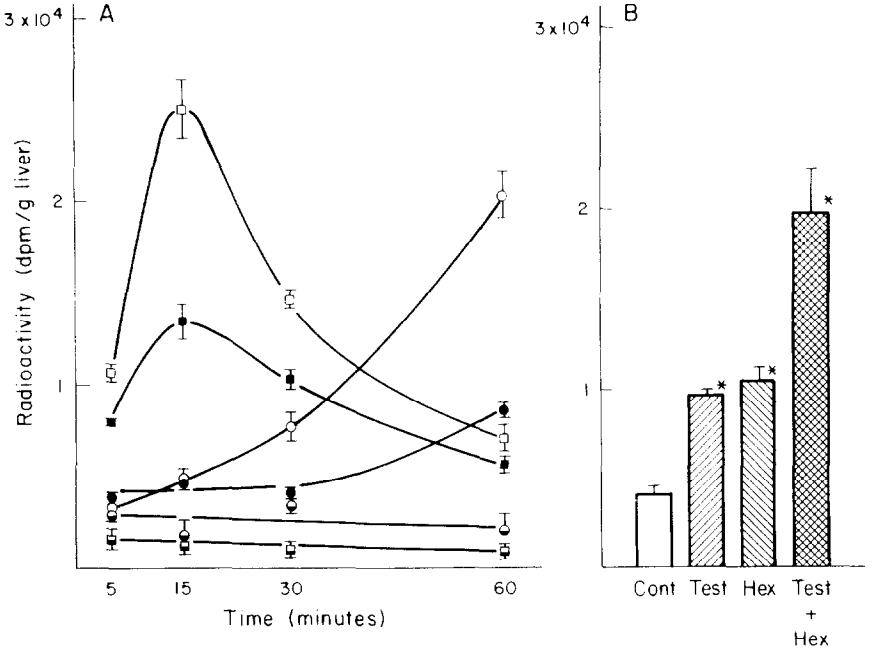

Figure 1. Macromolecular Binding of Radioactivity in Partially Purified Cytosol (AS Cytosol) and Highly Purified Nuclear Fractions from Liver Slices Incubated with ^3H-E_2.

A. Time Course. Liver slices were incubated with ^3H-E_2 (5 x 10^{-9}M) at 25° at a tissue: medium ratio of about 100 mg tissue/ml medium. An atmosphere of 95%-O_2, 5% CO_2 was maintained. Replicate incubations contained DES (5 x 10^{-7}M) or testosterone (5 x 10^{-6}M). Nuclear binding has been corrected for recovery of DNA in the purified nuclei which averaged 40% of the DNA in the crude homogenate. Nuclear Binding: ^3H-estradiol only, ●--●; ^3H-estradiol + DES, ◒--◒; ^3H-estradiol + testosterone, ◡--◡. AS Cytosol Binding: ^3H-estradiol only, ■--■; ^3H-estradiol + DES, ◪--◪; ^3H-estradiol + testosterone, □--□.

B. Effect of Testosterone and Hexobarbital on Nuclear Macromolecular Binding. Liver slices were incubated for 60 minutes at 25° with ^3H-estradiol (5 x 10^{-9}M) in the absence (Cont) or in the presence of either testosterone (Test, 5 x 10^{-6}M), hexobarbital (Hex, 1 x 10^{-3}M) or both. The asterisk (*) indicates significantly different than control ($P < 0.05$).

substantially reduced macromolecular binding in these fractions. DES is known to have high affinity for estrogen receptors and the difference in binding in the absence and presence of DES is interpreted as specific receptor binding. Macromolecular binding of radioactivity in liver cytosol and nuclear fractions after incubation of liver slices with $^{3}H\text{-}E_2$ exhibits other characteristics observed in classical estrogen target tissues including dependence of nuclear binding on incubation temperature, limited binding capacity and sensitivity to proteolytic enzymes (3).

High concentrations of $^{3}H\text{-}E_2$ (5×10^{-8}M) are necessary in order to observe maximum AS cytosol and nuclear binding in liver slices. This high estrogen concentration requirement for receptor binding in liver slices is unexpected. In isolated liver cytosol, the equilibrium dissociation constant for binding estradiol is 10^{-10}M (1) and is comparable to that determined for estradiol binding in isolated uterine cytosol preparations (2).

The liver is known to be the major organ responsible for metabolism of steroid hormones including estrogens. We observe that, after incubation of liver slices at 25°, 97% of the $^{3}H\text{-}E_2$ originally present in the incubation medium is metabolized in less than 15 minutes. The extent of the effect of this metabolism on receptor binding has not been recognized previously. It may limit estrogen-receptor interaction in the liver by reducing free cytoplasmic levels of potent estrogens. If estrogen metabolism is a contributing factor to the high estrogen concentration requirement observed for receptor binding then inhibition of estrogen metabolism might result in enhanced levels of cytosol and nuclear macromolecule-bound radioactivity following incubation of liver slices with $^{3}H\text{-}E_2$. Liver slices were incubated with a submaximal concentration of $^{3}H\text{-}E_2$ in the presence of testosterone (5×10^{-6}M). Tesotsterone can partially inhibit $^{3}H\text{-}E_2$ metabolism by competition with substrate for enzymes catalyzing steroid hydroxylation (6) and 17β-oxidation (7). In the presence of testosterone (Figure 1a) enhanced binding of radioactivity is observed in the AS cytosol at 5, 15 and 30 minutes of incubation. Increased binding in the nuclear fraction is then observed at 30 and 60 minutes of incubation. Additional results (Figure 1b) demonstrate that hexobarbital (1×10^{-3}M) is as effective as testosterone in enhancing the levels of nuclear macromolecule-bound radioactivity observed after 1 hour of incubation. Hexobarbital also competes with $^{3}H\text{-}E_2$ for liver metabolizing enzymes responsible for steroid hydroxylation (6). Following incubation of liver slices with both testosterone and hexobarbital there is a five-fold increase in nuclear macromolecule-bound radioactivity. These effects are probably due to enhanced levels of free potent estrogens such as $^{3}H\text{-}E_2$ which we observed in the medium and which also might occur in the cytoplasm as well.

Hepatic metabolism of estradiol includes hydroxylation at positions 2, 4, 6, 7 and 16 as well as conjugation with glucuronic acid, sulfate and glutathione (8). Some of these

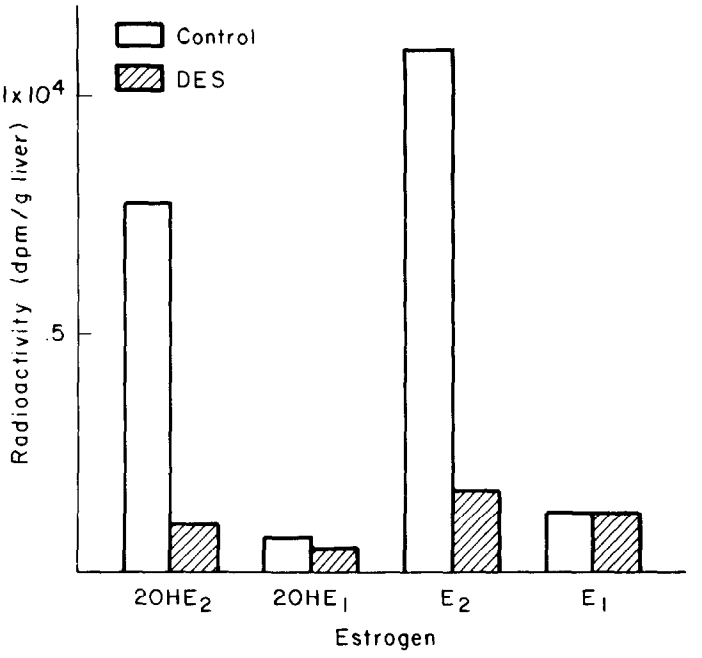

Figure 2. Distribution of Estrogen Metabolites in Nuclear Macromolecule-Bound Fractions from Liver Slices Incubated with ^{3}H-E_2.
Liver slices were incubated for 1 hour at 37° with 10^{-7}M ^{3}H-E_2 without (Control) and with DES (10^{-5}M). Descending paper chromatography under reducing conditions was carried out as previously described (9). The chromatogram was developed with a formamide and ascorbic acid saturated mobile phase solvent of mono-chlorobenzen:ethyl acetate (3:1 v/v). R_f's were 2-hydroxyestradiol, 0.16; 2-hydroxyestrone, 0.35; estradiol, 0.55; estrone, 0.83. Control nuclear binding was 21,000 dpm/g liver and in the presence of DES, 5,000 dpm/g liver.

metabolites might bind to the receptor. The identity of the radioactivity present in organic extracts of nuclear macromolecular binding fractions was analyzed by paper chromatography under reducing conditions. This precaution was necessary to prevent oxidative decomposition of catechol estrogens (9). About 94% of the organic soluble radioactivity co-chromatographs with four authentic estrogen standards: estradiol (E_2), estrone (E_1), 2-hydroxyestradiol (2-OHE_2) and 2-hydroxyestrone (2-OHE_1). Further support for the identity of these metabolites has been obtained by recrystallization to constant specific activity and enzymatic derivatization with catechol-O-methyl transferase. This latter method has been shown to specifically methylate estrogens which are catechols and has been used to assay for endogenous catechol estrogens in various tissues of the rat (10).

Figure 2 indicates the identity of the radioactive estrogens in the nuclear receptor fractions after incubation of liver slices with 10^{-7}M ^{3}H-E_2 at 37° for 1 hour. Estradiol represents 52% and 2-hydroxyestradiol 36% of the bound radioactivity. Addition of DES to the liver slice medium reduces nuclear macromolecular binding from 21,000 to 5,000 dpm/g liver. The reduction in binding observed in the presence of DES can be accounted for almost

completely by a reduction in binding of the two major metabolites: 2-OHE_2 and E_2. The reduction in macromolecular binding of E_2 and of 2-OHE_2 by DES strongly suggests a specific interaction between the receptor and these estrogens.

Endogenous catechol estrogens have been detected in brain regions in addition to liver (10). Results from several experiments have been interpreted to suggest that catechol estrogens may exhibit both estrogenic and anti-estrogenic activities (11). 2-Hydroxyestradiol has also been shown to stimulate dry and wet weight gains of the uterus of the rat but was not as potent as E_2 (12). The nature of the biologic activity of catechol estrogens in the liver is unknown but conceivably could encompass both forms of estrogenic activity. In addition, in liver, catechol estrogens may have other effects. They can be converted by hepatic microsomal enzymes into highly reactive, electrophilic compounds which will bind covalently to hepatic microsomal proteins (13). Incubation with mushroom tyrosinase will also lead to the formation of reactive intermediates capable of binding to DNA (14). These data suggest that a relationship between the receptor-mediated localization of catechol estrogens in the nucleus and the occurrence of liver tumors associated with long term use of oral contraceptives (15) should be thoroughly investigated.

Other rare, but serious side effects of oral contraceptive drugs which may be mediated, in part, by an estrogen-liver receptor interaction include gallstones and cardiovascular disease. A high cholesterol/bile acid ratio in hepatic bile may be responsible for the occurrence of cholesterol gallstones (16). Changes in the synthesis of plasma proteins of hepatic origin may contribute to cardiovascular disease. Increases in clotting factors, decreases in antithrombin III and increases in renin substrate may be particularly critical changes (1).

We suggest that dissociation of estrogen effects on the liver from desired effects on other target organs (hypothalamus, pituitary, uterus) might be accomplished by choosing potent estrogens that are metabolized preferentially to inactive derivatives in the liver thus minimizing the estrogen-receptor interaction in the liver.

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