Effects of Estrogen on Apolipoprotein Secretion by the Human Hepatocarcinoma Cell Line, HepG2*

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We have examined the effect of estrogen on the rate of accumulation of apolipoproteins secreted by the human hepatocarcinoma cell line, HepG2. Prior to exposure to hormone, we detected less than 300 high-affinity, nuclear, estrogen-binding sites/cell. Within 48 h of growth in the presence of 20 nM 17β-estradiol this number rose to 3000-3500 sites/cell. Rates of accumulation of two of the major apolipoproteins, apo-C-II and apo-A-I increased 2.5- and 2.0-fold, respectively, in response to estrogen treatment. Other major apolipoproteins were not affected at this concentration of hormone. Induction of both proteins was completely antagonized by 20 nM testosterone. The density distribution of apolipoproteins secreted by the hepatocytes was similar to that reported using perfused liver systems. The consequences of estrogen treatment were to increase the apo-C-II/apo-C-III ratio in very low density lipoproteins as well as to decrease the overall very low density lipoprotein:high density lipoprotein ratio.

Numerous studies have demonstrated that estrogenic steroids influence apolipoprotein levels in a wide variety of species (1-4). However, in mammals the precise mechanisms involved remain poorly delineated. Epidemiological studies indicate that premenopausal women have lower VLDL and apo-A-I and apo-C-II and apo-A-I increased 2.5- and 2.0-fold, respectively, in response to estrogen treatment. Other major apolipoproteins were not affected at this concentration of hormone. Induction of both proteins was completely antagonized by 20 nM testosterone. The density distribution of apolipoproteins secreted by the hepatocytes was similar to that reported using perfused liver systems. The consequences of estrogen treatment were to increase the apo-C-II/apo-C-III ratio in very low density lipoproteins as well as to decrease the overall very low density lipoprotein:high density lipoprotein ratio.

Experimental Procedures

RESULTS

Growth and maintenance of HepG2 cells in medium supplemented with 17β-estradiol (20 nM) or medium containing charcoal-treated serum had no detectable effects on their doubling time or viability when compared with control cultures. The levels of high-affinity estrogen-binding proteins in nuclei isolated from cells cultured in the presence and absence of estrogen and in medium containing complete or charcoal-treated serum were estimated by saturation exchange assay at 37 °C (Fig. 1) as described under “Methods.”

When cells were cultured for 2 days in medium containing complete fetal calf serum and 17β-estradiol (2 × 10⁻⁶ M), the levels of nuclear estrogen-specific binding increased 6- to 7-fold relative to control cultures. Exposure of the cells to 2 × 10⁻¹ M and 2 × 10⁻⁶ 17β-estradiol resulted in an additional 15

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1 The abbreviations used are: VLDL, very low density lipoprotein(s); HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); apo, apolipoprotein.

2 Portions of this paper (including “Experimental Procedures,” part of “Results,” and Fig. 2B) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9850 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2403, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Response of HepG2 Cells to Estrogen

and 25% in nuclear binding levels, respectively. Maintenance of the cells in charcoal-treated serum for a period of 2 days resulted in approximately a 2-fold reduction in specific nuclear binding capacity, relative to control cultures. Addition of 17β-estradiol to the medium under these conditions induced a 13- to 14-fold increase in nuclear binding sites during the subsequent 24–48 h, so that saturation levels were similar in estradiol-treated cells cultured in either complete or charcoal-treated serum (Fig. 1A).

Affinities of the nuclear binding sites were examined in both control cells and cells treated with 20 nM 17β-estradiol. A typical titration curve for hormonally treated cells is shown in Fig. 1B. The shape of the curve, especially in the case of nuclei from estrogen-treated cultures, suggested the presence of at least two binding components of different affinities. A Scatchard plot of the binding data obtained with nuclei from treated cells is shown in Fig. 1C. The plot was resolved into two components as described by Rosenthal (28). One of these had a $K_D$ of $9.5 \times 10^{-10}$ M, the other a $K_D$ of $7.25 \times 10^{-9}$ M. In estrogen-treated cells, the high-affinity component was estimated to comprise approximately 25% of the total binding, which, based upon the specific activity of the ligand and the number of cells used per assay, suggests the presence of approximately 3000–3500 sites/nucleus.

The low levels of high-affinity binding in nuclei from control cultures precluded obtaining data of sufficient accuracy to warrant estimation, by Scatchard analysis, of the proportion of binding sites which were of high affinity. In order to obtain such an estimate, we took advantage of the observation that exchange with high-affinity nuclear receptors does not occur at 0 °C (29). Saturation assays of both control and estrogen-treated nuclei were carried out at 0 and 37 °C, and the fraction of sites that were of high affinity was calculated from the difference between the two curves. This method also indicated that in both cases the high-affinity sites accounted for approximately 25% of the total estrogen-specific nuclear binding.

**Effect of Estrogen on Levels of Secreted Apolipoproteins**—The relative levels of apolipoproteins secreted into the culture medium in the presence and absence of 20 nM 17β-estradiol were estimated by immunoprecipitation with 125I-labeled monospecific antibodies that have been characterized previously (Fig. 2A). The protocol of hormone additions and medium changes is described under “Methods.” Estradiol treatment for a period of 24 or 48 h resulted in a 2.0-fold increase in the level of apo-A-I and a 2.5-fold increase in the level of apo-C-II present in the medium, relative to those found in control cultures of similar cell density. No significant alterations in apo-C-III, apo-E, apo-B, or serum albumin levels were detected. The increases in apo-C-II and apo-A-I levels were obtained when estradiol was added either to dividing cells or cultures that had been confluent for up to 24 h prior to addition of estrogen. However, a decreased response (data not shown) was observed in cells that had been confluent for longer periods prior to hormone treatment.

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**FIG. 1. Nuclear estrogen-specific binding sites.** A, effect of estradiol on the level of nuclear estrogen-specific binding sites of HepG2 cells. Nuclei were isolated from cells cultured for 2 days in medium containing complete or charcoal-treated (C.T.) fetal calf serum in the absence (Le. control) or presence of $2 \times 10^{-4}$, $2 \times 10^{-7}$, and $2 \times 10^{-4}$ M 17β-estradiol. Specific binding of [3H]estradiol in the nuclear extracts was measured by saturation exchange assay at 37 °C using 120 nM 17β-[3H]estradiol in the presence and absence of excess unlabeled diethylstilbestrol as described under “Methods.” B, saturation analysis of specific nuclear binding in 17β-estradiol-treated HepG2 cells. Nuclei were isolated from cells cultured for 2 days in the presence of 20 nM 17β-estradiol. Nuclear exchange assay was carried out at 37 °C in final concentrations of 0.3–120 nM 17β-[3H] estradiol ± diethylstilbestrol as described under “Methods.” Results represent the mean of duplicate experiments. C, Scatchard plot of exchange assays of nuclei from HepG2 cells treated with 17β-estradiol (20 nM). Data analyzed are the same as those presented in Fig. 2A. Dissociation constants were determined by the graphical procedure of Rosenthal (28).
induction of the other apolipoproteins. However, in both the presence and absence of estrogen the levels of several of the apolipoproteins (apo-B, -C-III, and -E) were 15–20% higher in cultures supplemented with lipoprotein depleted as opposed to complete serum (data not shown).

Density fractions corresponding to VLDL (d < 1.006 g/ml), LDL (d = 1.006–1.063 g/ml), HDL (d = 1.063–1.21 g/ml), and free apolipoproteins were isolated from 30-ml aliquots of culture media, and the distribution of individual apolipoproteins in each fraction was determined (Fig. 3). The results obtained with lipoprotein-depleted serum prepared by both methods were in good agreement, exhibiting less than a 10% variation in all cases. In control and estrogen-treated cultures, less than 8% of any of the apolipoproteins were found in a density fraction (d > 1.21) in which the free proteins would be expected to reside. In contrast, 97% of serum albumin was recovered in this fraction. The majority of apo-B (68.5 ± 1.3%, n = 4) was present in a VLDL density range, the remainder being in the LDL density fraction. The VLDL, LDL, and the HDL fractions contained 29.5 ± 1.3, 10.5 ± 2.1, and 55.3 ± 1.5% of the apo-C-III and 39.8 ± 2.2, 13.3 ± 1.7, and 40 ± 1.7% of the apo-E, respectively. Estrogen had no effect on the distribution of these apolipoproteins but did significantly alter the distribution of apo-C-II.

In control cultures, 42 ± 2, 6.5 ± 0.7, and 44 ± 2.8% (n = 4 in all cases) of apo-C-II were recovered in VLDL, LDL, and HDL fractions, respectively, while the distribution following estrogen treatment was 29.5 ± 2.1, 8 ± 0.5, and 66.5 ± 5.0%. This alteration in distribution reflects a 55 ± 11% increase in the amount of apo-C-II recovered in the VLDL fraction as opposed to a 182 ± 11.3% increase in the HDL fraction. Thus, approximately 65% of the additional apo-C-II found after estrogen treatment remains associated with the HDL fraction.

Although estrogen induced a 2-fold increase in the rate of accumulation of secreted apo-A-I, it did not alter the density distribution of the apolipoprotein. In both control and hormonally treated cultures 91.0 ± 1.4% of the apo-A-I was present in the HDL density fraction, the remainder being divided more or less equally between the LDL and free protein fractions.

**DISCUSSION**

Estrogen administration is known to alter several hepatic functions in humans, ranging from increased secretion of specific plasma proteins to alterations in drug metabolism (30). High-affinity, low-capacity, estrogen-specific binding proteins have been identified in livers of several mammals including man, suggesting that the hormone may exert a direct effect on the tissue (30, 31). The few studies that have been carried out on heman liver have examined only cytosolic fractions. No data have been published on the levels of nuclear high-affinity binding proteins in human liver. Thus, prior to examining the effects of estrogen on apolipoprotein production in the HepG2 cell line we carried out a series of experiments to determine the number of high-affinity, nuclear, estrogen-binding sites in these cells.

The level of nuclear binding sites found in control cultures was extremely low and decreased still further when the cells were cultured in the presence of charcoal-treated rather than complete serum. However, when the cells were maintained in estradiol and complete serum for 36-48 h prior to assay we obtained a 6- to 7-fold increase in total estrogen-specific binding compared with control cultures. From analyses of these data we have attributed the binding to two components.

The high-affinity component has a $K_D = 9.5 \times 10^{-11}$ M and appears not to undergo steroid exchange at 4°C. In these
respects it is similar to high-affinity mammalian receptors characterized in other estrogen target tissues such as uterus (29). The number of high-affinity binding sites detected after estradiol pretreatment of the cells is 3000–3500/cell. While this is low when compared to levels found in uterus, it is in the same range as that found in avian liver following induction by estrogen (32). The lower affinity component we detected has an apparent \( K_d = 7.25 \times 10^{-8} \) M. Rat uterus has been shown to contain a nuclear, lower affinity, estrogen-binding protein, designated Type II receptor by Clark (33), that has a \( K_d \) of \( 3.3 \times 10^{-8} \) M. However, this protein exhibits cooperative binding of estradiol, while the lower affinity component that we identify apparently does not. Its dissociation constant is also comparable to cytosolic “high capacity-lower affinity” binding proteins that have been identified in livers from several species, raising the possibility that some of the lower affinity binding we detect may be attributable to contamination with cytosolic binding proteins (34).

Maintenance of the cells in a wide range of estradiol concentrations indicated that adjustment of the medium to 20 nM in hormone every 12 h was adequate to maintain nuclear binding at 90% of the maximal levels obtainable. This regimen of addition was followed for all experiments. The initial level of hormone used is approximately 20-fold higher than peak circulating concentrations of estradiol found in premenopausal nonpregnant women, but since hepatocytes metabolize estradiol extremely rapidly the effective steady-state concentrations of the hormone in the culture system are likely to be considerably lower. Consequently, it is not possible to equate the nominal concentrations in the cultures with circulating \textit{in vivo} levels. Under the conditions examined, estradiol was found to increase the rate of accumulation of secreted apo-C-II and apo-A-I, 2.5- and 2-fold, respectively, while having no effect on apo-C-III, apo-E, and apo-B.

Apo-C-II and apo-A-I are important regulators of triglyceride and cholesterol metabolism. The former is an activator of lipoprotein lipase (35) and the latter an activator of lecithin-cholesterol acyltransferase (36). At the moment, the compositions of nascent VLDL and HDL secreted by human liver are not known. Based upon studies with animal models, both apoproteins are believed to be secreted from the liver associated in a nascent HDL particle, from which apo-C-II subsequently transfers to VLDL and chylomicrons (37, 38). If this assumption is correct the presence of apo-C-II and apo-C-III in the VLDL fraction we have examined suggests that redistribution of apoproteins may occur in the culture medium in a manner similar to that observed \textit{in vivo}.

The effects of estrogen on apolipoprotein density distribution were to elevate the apo-C-II:apo-C-III ratio in both VLDL and HDL by increasing the total amount of apo-C-II in those fractions and to double the apo-A-I content of HDL. Since apo-A-I is the major protein constituent of HDL this results in a significant decrease in the overall VLDL:HDL ratio in the medium. In this respect the response we observe is consistent with epidemiological data indicating that premenopausal women maintain lower VLDL:HDL ratios than age-matched males and with studies indicating that transient elevations in HDL levels can be correlated with ovulation when estradiol levels are at a maximum (7). One of the most recent studies on the effects of estrogen administration on the synthesis and turnover of apolipoproteins in premenopausal women also concluded that the hormone significantly increased the rate of synthesis of apo-A-I, but apo-C-II was not examined (39). In addition, these authors reported that they observed a 1.8-fold increase in the rate of VLDL apo-B synthesis, calculated on the basis of steady-state levels and

![Fig. 3. Effect of estrogen on the distribution of apolipoproteins between various density fractions.](image-url)

Various density classes of lipoproteins were isolated from cells grown in medium supplemented with lipoprotein-deficient calf serum in the absence (−) or presence (+) of 17β-estradiol (20 nM), respectively. VLDL was isolated at \( d < 1.006 \) g/ml; LDL was isolated from \( d = 1.006 \) to \( 1.063 \) g/ml; HDL was isolated from \( d = 1.063 \) to \( 1.21 \) g/ml, and free proteins were isolated in the \( d > 1.21 \) g/ml infranatant. An aliquot of each fraction was analyzed by immunoprecipitation using various \( ^{125}\text{I} \)-labeled monospecific antibodies as described under “Methods.” Results presented are the mean of two experiments from cells grown in lipoprotein-deficient serum prepared by ultracentrifuge (——) and Cab-O-Sil treatment (−−).
turnover rates, in subjects given 0.1 mg of ethinyl estradiol/day. This observation is consistent with the results of several studies documenting increases in VLDL levels during estrogen therapy (7, 40, 41). Recent experiments using significantly higher levels of estradiol indicate that estradiol levels at least 25-fold higher than those required to increase apo-C-II and apo-A-I production are necessary to increase the levels of apo-B secreted by the HepG2 cells. Further experiments with slowly metabolized estrogen analogues will be required before we can place more accurate estimates on the threshold concentrations necessary to induce these two different effects.

In contrast to the results obtained with estrogen, testosterone had no discernible effect on the secreted levels of any of the apolipoproteins examined. However, at an initial concentration of 20 nM it completely prevented the estrogen-induced increase in apo-C-II and apo-A-I. At the moment, the mechanism involved is not known. In several species, androgens increase in apo-C-I1 and apo-A-I. At the moment, the mechanism involved is not known. In several species, androgens increase in apo-C-I1 and apo-A-I. However, at an initial concentration of 20 nM it completely prevented the estrogen-induced increase in apo-C-II and apo-A-I. At the moment, the mechanism involved is not known.

Data from primary hepatocytes indicate that little if any turnover of newly synthesized apolipoproteins occurs suggesting that rates of accumulation in the medium do provide an acceptable indication of synthetic rates. This occurs suggesting that rates of accumulation in the medium do provide an acceptable indication of synthetic rates. This has yet to be confirmed for HepG2 cells.

Despite the possibility that some metabolism may be taking place, it does appear, at least in the case of apo-C-II (15), that the major effect of estrogen is to increase the rate of synthesis of the apolipoprotein and that it does so by elevating the levels of apo-C-II mRNA. Similar studies are in progress to determine if this also applies to the induction of apo-A-I, apo-E, and apo-B.

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Supplemental Material to
Effects of Estrogen as Apolipoprotein Secretion by the Human Hepatocarcinoma Cell Line, Hep G2
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Experimental Procedures

Materials
All chemicals used were reagent grade. Minimal essential medium with Earle's salts, fetal calf serum, penicillin, streptomycin, trypsin-EDTA solution, trypsin were purchased from竺el Laboratories, Inc. Sephadex G-50 was purchased from Pharmacia Fine Chemicals, Inc. Radioisotope Standard was from Amersham Pharmaceuticals, Inc. 

Methods
Cell Culture. The cell line HepG2 was grown in 11.5% flask containing 9.0 ml of Earle's minimal essential medium supplemented with: fetal calf serum (10% vol/vol), penicillin (100 U/ml), streptomycin (100 μg/ml) and l-glutamine (2mM). The cells were plated at 8.0 × 10^5 cells per flask and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2. After 2 days, the cells were grown to 50% confluence and the culture medium was removed. The cells were washed three times with Dulbecco's phosphate-buffered saline (pH 7.2) to remove the non-adherent cells and then lysed by the trypan blue dye exclusion method and cell number was determined by counting with a hemocytometer. In all experiments the number of dead cells exceeding 5% of the total number of cells was determined by trypan blue dye exclusion. The culture medium was kept in 96-well plates in Nunc culture plates. The cell lines were treated by adding the radioisotope to the medium at the desired concentration. 

Preparation of Monospecific Antibodies to Human Apolipoproteins and Antibody. Preparations of monospecific antibodies to human apo A-I, apo A-II, apo A-III and apo B-100 have been described previously (12). Monospecific antibodies to human apo A-I, apo A-II, apo A-III and apo B-100 were prepared by injecting rabbits with purified human apo A-I, apo A-II, apo A-III and apo B-100. 

Preparation of Cell Cultures Samples for Immunoprecipitation. After incubation for 24 h, the conditioned supernatant was collected, centrifuged at 900 g for 10 min, and the supernatant was removed. 

Immunoprecipitation. The supernatant was added to 100 μl of a mixture of cold and intact monospecific antibodies with a specific activity of 3.5 × 10^6 cpm/μg protein and incubated at room temperature overnight. The immunoprecipitates were collected by centrifugation for 10 min in an Eppendorf microcentrifuge and washed twice with 1.0 ml of cold PBS containing 0.05% (v/v) Tween 20. The immunoprecipitates were dissolved either in 1.0 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) or 0.1 ml of 1% SDS containing 20 μg/ml proteinase K. The samples were incubated at 55°C for 30 min. The immunoprecipitates were then extracted with acetone.

Preparation of Nuclear and Extracts of Labeled Estrogen-Inducible Activity. Cells were grown to confluence in 100-mm dishes and then labeled with [3H]-estradiol-17β. 

Electrophoretic Protein Blotting Procedure. Lysates were isolated from cells grown in medium supplemented with labeled deoxycholate serum. 10% (v/v) of lysates were electrophoresed in a 10% SDS-polyacrylamide gel containing 1% (w/v) 2-mercaptoethanol.

Immunological Detection of Proteins on Nitrocellulose. The electro- 
phoretic blots were cut out of the nitrocellulose filter for autoradiography. The blots were immersed twice with PBS containing 0.05% Tween-20 and incubated with 100 μl of 1:50,000 dilution of goat anti-human apo A-I, apo A-II, apo A-III, apo B-100 and apo C-III antibodies for 1 h at 37°C and then washed twice with PBS containing 0.05% Tween-20 and incubated in 200 μl of 1:10,000 dilution of goat anti-rabbit IgG for 1 h at room temperature. The electrophoretic blots were subsequently washed three times with PBS containing 0.05% Tween-20 and then thoroughly dried. The blots were exposed to Kodak X-Omat X-ray film for 2 days. An example of such an experiment is shown (Fig. 3B).