Estradiol Stimulates Apolipoprotein A-I– but Not A-II–Containing Particle Synthesis and Secretion by Stimulating mRNA Transcription Rate in Hep G2 Cells

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Abstract—Estrogen therapy increases plasma HDL levels, which may reduce cardiovascular risk in postmenopausal women. The mechanism of action of estrogen in influencing various steps in hepatic HDL and apolipoprotein (apo) A-I synthesis and secretion is not fully understood. In this study, we have used the human hepatoblastoma cell line (Hep G2) as an in vitro model system to delineate the effect of estradiol on multiple regulatory steps involved in hepatic HDL metabolism. Incubation of Hep G2 cells with estradiol resulted in the following statistically significant findings: (1) increased accumulation of apoA-I in the medium without affecting uptake/removal of radiolabeled HDL-protein; (2) accelerated incorporation of [3H]leucine into apoA-I; (3) selective increase in [3H]leucine incorporation into lipoprotein (LP) A-I but not LP A-I+A-II HDL particles (HDL particles without and with apoA-II, respectively); (4) increased ability of apoA-I–containing particles to efflux cholesterol from fibroblasts; (5) stimulated steady state apoA-I but not apoA-II mRNA expression; and (6) increased newly transcribed apoA-I mRNA message without effect on apoA-I mRNA half-life. The data indicate that estradiol stimulates newly transcribed hepatic apoA-I mRNA, resulting in a selective increase in LP A-I, a subfraction of HDL that is associated with decreased atherosclerotic cardiovascular disease, especially in premenopausal women. (Arterioscler Thromb Vasc Biol. 1998;18:999-1006.)

Key Words: estrogen ▪ high density lipoproteins ▪ apolipoprotein A-I ▪ cardiovascular disease

Atherosclerotic cardiovascular disease is the leading cause of mortality among postmenopausal women. Abnormalities in lipid and lipoprotein metabolism (eg, increased LDL and decreased HDL levels) commonly seen in postmenopausal women have been attributed to the increased coronary heart disease–related mortality in these individuals. Because the decline in estrogen levels is the primary metabolic alteration observed in postmenopausal women, it has been thought that endogenous concentrations of estrogen may have fundamental roles in lipoprotein-mediated development of atherosclerotic coronary heart disease. Clinical studies have indicated that estrogen therapy significantly elevated plasma HDL levels and decreased LDL concentrations, suggesting a favorable effect on the plasma lipoprotein profile. Recent comparative studies by meta-analysis showed that postmenopausal women on estrogen therapy have a lower relative risk of coronary events than postmenopausal women who are not on estrogen therapy. Immunoaffinity techniques have revealed that HDL particles exist in 2 major classes: LP A-I and LP A-I+A-II (ie, without and with apoA-II, respectively). ApoA-I and apoA-II are major proteins of HDL. There is considerable evidence to suggest that LP A-I is more importantly linked to decreased atherosclerosis risk. Premenopausal women have higher levels of LP A-I than age-matched men. Oral estrogen replacement therapy in postmenopausal women was shown to increase LP A-I levels. Mice made transgenic with LP A-I are dramatically protected against diet-induced atherosclerosis compared with mice transgenic for LP A-I+A-II. LP A-I is also associated negatively with extent of arteriographically defined coronary disease. However, recent studies indicated decreased levels of both LP A-I and LP A-I+A-II particles in patients with hypertriglyceridemia, familial combined hyperlipidemia, and a history of coronary heart disease. In spite of several biological mechanisms proposed to support a beneficial role for estrogen, the favorable alterations in HDL levels appear to be a well-established effect of estrogen in preventing atherosclerotic cardiovascular disease. However, the mechanism by which estrogen raises HDL levels is not clearly understood. Plasma turnover studies (kinetic) have indicated that the effect of estrogen on increasing HDL level was solely due to increased production rate of HDL-protein and apoA-I without altering the FCR. Contrary to these observations, it was shown that the treatment of premenopausal women with estradiol resulted in decreased hepatic lipase activity and suggested that estrogen may increase HDL level by decreasing the rate of HDL catabolism, which has been thought (but not proven) to be mediated via this enzyme. In a recent turnover study, it was...
found that estradiol selectively increased LP A-I transport rate and not that of LP A-I+A-II. In this study, hepatic lipase activity was reduced, but the catabolism of HDL subfractions was not changed, indicating an unclear role for hepatic lipase.

Although these in vivo studies provided valuable clues to understanding the effect of estrogen on HDL metabolism, additional studies are required to fully understand the cellular and molecular processes involved in various steps of transcriptional and posttranslational processing of HDL. In this regard, using Hep G2 cells, early studies have shown that estrogen significantly increased the steady state mRNA level and media accumulation of apoA-I in association with increased high-affinity nuclear estrogen binding sites. Nevertheless, the role of estrogen in multiple hepatic cellular steps that regulate apoA-I and HDL mass (including effect on transcription or transcript degradation, and uptake of HDL-apoA-I). Additional studies were performed to examine the effect of estradiol on the synthesis of HDL particles containing apoA-I with and without apoA-II particles and their ability to efflux cholesterol from human fibroblasts. The data to be presented indicate that estradiol stimulates the transcription rate of apoA-I mRNA, resulting in a selective increase in functional LP A-I particles by cultured Hep G2 cells.

Methods

Materials

Tissue-culture materials, media, and estradiol were obtained from Sigma Chemical Company unless otherwise noted. FBS was obtained from HyClone Laboratories. [4,5-3H]-Leucine and [3P] nucleotides were purchased from Amersham Corporation. Human hepatoblastoma cell line (Hep G2), human fibroblast cells, and human apoA-I DNA probe were obtained from American Type Culture Collection. Polyclonal antibody for human apoA-I was obtained from Boehringer Mannheim Biochemicals. All other chemicals used were of analytical grade.

Studies on Secretion of ApoA-I by Hep G2 Cells

Hep G2 cells were plated in 60-mm Petri dishes at a concentration of 4 x 105 cells per dish in 4 mL high-glucose DMEM (containing 10% FBS, 1% glucose- penicillin-streptomycin and 1% fungizone) and grown for 3 to 4 days until they attained 75% to 80% confluence. The studies examining the dose response of estradiol on apoA-I secretion were performed by incubating Hep G2 cells with various amounts of estradiol (0 to 20 μmol/L) at 37°C for 24 to 48 hours. After the incubation, culture medium from each dish was collected for apoA-I measurement and the cell monolayer was washed with PBS, digested in 1 N NaOH, and used for cellular protein measurement. A 50-μL sample of culture medium was assayed for apoA-I, as described earlier, by an ELISA using a human apoA-I–specific monoclonal antibody (HB-22) developed and characterized in our laboratory. The concentration of apoA-I was expressed as micrograms per milligram of cellular protein.

De Novo Synthesis of ApoA-I

Studies examining the effect of various doses of estradiol on the de novo synthesis of apoA-I by Hep G2 cells were performed by measuring the incorporation of radiolabeled leucine into apoproteins secreted into the medium. Hep G2 cells (4 x 105) were incubated with varying concentrations of estradiol (0 to 20 μmol/L) in DMEM containing 10% FBS for 48 hours at 37°C. After incubation, the medium was replaced with leucine-poor DMEM (5% leucine of normal medium) without FBS containing the corresponding amounts of estradiol and [3H]leucine (5 μCi/mL) and incubated for an additional 18 hours at 37°C. At the end of the incubation, the medium was collected and used for immunoprecipitation of apoA-I. The cell monolayer was washed with PBS and collected for protein measurement. The effect of incubation time on apoA-I synthesis was examined by incubating Hep G2 cells with estradiol (2 μmol/L) at 37°C in a humidified incubator. The medium was replaced with leucine-poor DMEM without FBS, containing corresponding amounts of estradiol and [3H]leucine (5 μCi/mL), and incubated at 37°C for varying periods (2 to 32 hours). After the incubation, the media and cells were collected for immunoprecipitation and cellular protein measurement, respectively. The incorporation of radiolabeled leucine into apoA-I was measured by immunoprecipitation using nonspecific antibodies for apoA-I as described earlier. The incorporation of [3H]leucine into albumin was also measured by immunoprecipitation using anti-human albumin antibody. The incorporation of [3H]leucine into apoA-I or albumin was expressed as counts per minute per milligram cellular protein.

Separation of LP A-I and LP A-I+A-II Particles

Experimental protocols for these studies were exactly the same as described for de novo synthesis of apoA-I. After the incubation of Hep G2 cells with estradiol and [3H]leucine, the medium was collected and used to isolate LP A-I and LP A-I+A-II particles by immunofinity column chromatography as described earlier. In brief, affinity columns specific for apoA-I or apoA-II were prepared by coupling polyclonal antibodies for human apoA-I or apoA-II to CNBr-activated Sepharose 4B (Pharmacia) according to the proce-
dure described in the instruction manual. Aliquots of culture medium (250 μL) were loaded onto the apoA-I-affinity column and incubated at 4°C for 16 to 18 hours to allow binding of apolipoprotein particles to specific antibody. The affinity column was then washed with 0.5 mol/L NaCl, and retained apoA-I-containing particles were eluted with 3 mol/L NaSCN, pH 6.0. An aliquot of eluted fraction was counted for radioactivity and represents LP A-I with and without A-II particles. Similarly, another aliquot (250 μL) of culture medium was subjected to apoA-II-specific immunoaffinity column chromatography, and an aliquot of NaSCN-eluted retained fraction was counted for radioactivity. This retained fraction on apoA-II-specific affinity column represents the contribution of apoA-II in LP A-I-A-II particles. Quantitative analysis of the incorporation of [3H]cholesterol into LP A-I particles (without A-II) was attained by the difference in radioactivity between retained fractions on apoA-I-affinity column and apoA-II-affinity column.

Measurement of Cholesterol Efflux

Experimental protocols for these studies were exactly the same as described for secretion of apoA-I. After the incubation of Hep G2 cells with estradiol, the medium was collected and used for cholesterol efflux measurement. An aliquot of culture medium (5 mL) was concentrated to 1 mL by lyophilization and dialyzed against DMEM to remove excess salt present in the concentrated sample. The ability of these concentrated samples to efflux free cholesterol was measured by a previously described modified procedure25 of Fielding and Fielding26 and Rothblatt et al27 using [3H]cholesterol-labeled human fibroblasts. In this method, the cytoplasmic pool of fibroblasts was labeled with [3H]cholesterol. Additionally, to ensure specific and homogeneous incorporation of radioabeled cholesterol throughout the cytoplasmic matrix of fibroblasts, we incubated fibroblasts with [3H]cholesterol for 72 hours at 37°C, washed with PBS, and then incubated with fresh DMEM containing 1% fetal bovine albumin for 16 hours. Cholesterol efflux assay was initiated by incubating concentrated culture medium with [3H]cholesterol-labeled fibroblasts for 20 hours at 37°C in a humidified incubator as described earlier.25,30,31 Quantitative analysis of the ability of Hep G2 cell-culture medium (in presence or absence of estradiol) to efflux cholesterol was performed by measuring the [3H]cholesterol radioactivity appearing in the medium per milliliter of incubation medium per milligram of fibroblast cellular protein.

Northern Blot Analysis

Experimental protocols for these studies were exactly the same as described for secretion of apoA-I. Total RNA was isolated from Hep G2 cells using the protocol of Chomczynski and Sacchi,28 and steady state mRNA expression of apoA-I and apoA-II was performed by Northern blot analysis, using human apoA-I and apoA-II cDNA probes as described earlier.25 Quantification of mRNA signal was performed by densitometric scanning of autoradiographic bands and normalized with GAPDH mRNA signals, using the LKB laser densitometer (Pharmacia, LKB Biotech).

Measurement of ApoA-I mRNA Half-Life

Hep G2 cells were incubated in the presence of actinomycin D (1 μmol/L) at 37°C for 0, 2, 4, 8, 16, 24, and 48 hours. For experimental sets, Hep G2 cells were preincubated with estradiol (2.0 μmol/L) for 24 hours at 37°C. After the preincubation, the medium was replaced with fresh DMEM containing estradiol (2.0 μmol/L) and actinomycin D (1 μmol/L) and incubated for the same time intervals as for control Hep G2 cells. After the incubation, cells were washed and collected for RNA isolation. Northern blot analysis was performed as described earlier, and the half-life for apoA-I transcript was calculated from the density of the autoradiographic bands at each time point of incubation of cells in the presence of actinomycin D.25,35

Measurement of Newly Transcribed ApoA-I mRNA

Hep G2 cells were grown in DMEM +10% FBS for 4 to 5 days in 175-cm² flasks. For experiments, cells were incubated with

![Figure 1. Effect of estradiol on apoA-I secretion by Hep G2 cells.](http://atvb.ahajournals.org/)

Data presented are the mean ± SE of 3 separate experiments. All incubations were performed in triplicate. Statistical significance was calculated by using the Student’s t test, and a value of P<0.05 was considered significant.

**Results**

The incubation of various amounts of estradiol (0 to 20.0 μmol/L) for 48 hours with Hep G2 cells showed a dose-dependent increase in apoA-I accumulation in the medium, as measured by ELISA (Figure 1). A significant increase in apoA-I secretion by Hep G2 cells was noted at 1.0 μmol/L estradiol, and the maximum effect was observed at 10.0 μmol/L (51% increase compared with control). Incubation of Hep G2 cells with estradiol at 0.01 to 20.0 μmol/L concentration for 48 hours did not alter the morphology or the viability of cells.
Additional experiments were designed to examine the de novo synthesis of apoA-I by measuring the incorporation of 
$[^3$H]leucine into newly synthesized apoA-I secreted into the medium. Data from these studies show that the incorporation of radiolabeled leucine into apoA-I increased in a dose-dependent manner by Hep G2 cells incubated with estradiol (Figure 2). A significant increase in the incorporation of radiolabeled leucine into immunoprecipitable apoA-I was noted in the presence of as low as 0.1 μmol/L estradiol. At higher concentrations of estradiol (10.0 to 20.0 μmol/L), the elevation in the incorporation of radiolabeled leucine into apoA-I was persistent, with a maximum effect noted at 10.0 μmol/L estradiol compared with control. Similar studies examining the de novo synthesis of albumin showed that the incorporation of estradiol (0 to 20.0 μmol/L) with Hep G2 cells did not alter the de novo synthesis of albumin, as measured by the incorporation of radiolabeled leucine into immunoprecipitable albumin in the culture medium (data not shown). Similarly, the treatment of Hep G2 cells with estradiol did not alter the incorporation of radiolabeled leucine into the total trichloroacetic acid–precipitable protein (data not shown).

To examine the effect of estradiol on the de novo synthesis of LP A-I– and LP A-I+ A-II– containing HDL particles in Hep G2 cells, aliquots of medium from de novo synthesis experiments were fractionated into LP A-I and LP A-I+ A-II particles by immunoadfinity chromatography. Results from these experiments revealed that the incorporation of estradiol with Hep G2 cells increased in a dose-dependent manner the secretion of newly synthesized LP A-I HDL particles into the medium, but not LP A-I+ A-II HDL (Figure 3).

The ability of estradiol-induced apoA-I– containing lipoprotein particles to efflux cholesterol was examined by using $[^3$H]cholesterol-labeled fibroblasts. Cholesterol efflux studies using conditioned medium obtained from Hep G2 cells treated with varying amounts of estradiol showed a dose-dependent increase in cholesterol efflux, as measured by the release of $[^3$H]cholesterol from fibroblasts into the culture medium (Figure 4). Experiments were then performed to assess the ability of LP A-I and LP A-I+ A-II particles isolated from conditioned medium to efflux fibroblast cholesterol. ApoA-I mass was measured (by ELISA) in both particle preparations, and efflux was normalized to reflect radioactivity in the medium per unit of apoA-I mass. In control medium, both particles showed similar efflux per unit mass of apoA-I (LP-A-I, 27.7±0.7 cpm/μg apoA-I; LP-A-I+ A-II, 26.8±0.5 cpm/μg apoA-I); the efflux property per unit apoA-I remained unchanged with particles isolated from estradiol (1 μmol/L)-incubated Hep G2 cells (LP-A-I, 26.4±1.1 cpm/μg apoA-I; LP-A-I+ A-II, 25.1±0.5 cpm/μg apoA-I).

Results from Northern blot analysis showed that the incubation of varying amounts of estradiol with Hep G2 cells induced dose-dependently the steady state mRNA transcripts (0.9 kb) for apoA-I (Figure 5). Quantitative analysis of apoA-I mRNA message, as measured by densitometric scanning of blots (after normalizing with GAPDH mRNA message as an internal standard), indicated that the treatment of Hep G2 cells with estradiol as low as 0.02 μmol/L concentration stimulated steady state apoA-I mRNA levels (147% compared with control), the maximal effect noted at 10 to 20 μmol/L (188% compared with control).
contrast to apoA-I mRNA message, estradiol had no effect on the steady state mRNA expression of apoA-II. Densitometric scanning of apoA-II Northern blots and normalization with GAPDH mRNA message gave the following quantitative arbitrary values for control and estradiol doses of 0.01, 0.1, 1.0, 10, and 20 μmol/L: 4.36, 4.20, 4.34, 4.23, 4.27, and 4.29, respectively.

Because steady state mRNA expression reflects both transcription and transcript degradation, we examined the effect of estradiol on the newly transcribed message and transcript degradation of apoA-I in Hep G2 cells. The incubation of estradiol (2.0 μmol/L) with Hep G2 cells for 48 hours significantly increased apoA-I mRNA transcription rate, as measured by nuclear runoff assays (Figure 6). Quantitative analysis of newly transcribed apoA-I message by densitometric scanning indicated arbitrary values for control and estradiol-treated Hep G2 cells of 1.67±0.08 and 2.77±0.18, respectively (Figure 6). The mRNA half-life studies in which new RNA production was inhibited by actinomycin D showed that the treatment of Hep G2 cells with estradiol had no effect on degradation of apoA-I mRNA transcripts (Figure 7). Based on the quantitative scanning of autoradiographic apoA-I mRNA bands, we constructed a curve to calculate apoA-I transcript degradation rate and half-life according to the procedure described earlier. Data from these studies indicated that the calculated apoA-I mRNA half-life in control cells and cells treated with estradiol was approximately 14.3 hours and 14.7 hours, respectively (Figure 7).

Discussion

Epidemiological and case-controlled studies have shown an inverse correlation between the plasma concentrations of HDL and the severity of the development of atherosclerotic cardiovascular disease. Although in vivo mechanisms for this association are not clearly understood, the cardioprotective effects of HDL have been largely attributed to the ability of apoA-I, the major protein of HDL, to initiate cholesterol efflux, thereby facilitating the removal of excess cholesterol from peripheral tissues and delivering it to the liver for degradation through the reverse cholesterol transport pathway. Furthermore, direct in vivo evidence to support the antiatherogenic properties of HDL has been derived from studies demonstrating that the direct infusion of high doses of HDL to cholesterol-fed rabbits could regress atherosclerotic lesion formation in these animals and that cholesterol-fed...
transgenic mice engineered to produce high concentrations of apoA-I failed to develop atherosclerotic lesions.14

In this study, using a Hep G2 cell system, we have delineated the effect of estradiol on various cellular processes involved in HDL metabolism that in turn determine the overall concentration of apoA-I/HDL mass. The data indicate that estradiol significantly increased the synthesis and accumulation of apoA-I in the culture medium. Since estradiol did not alter the incorporation of [3H]leucine into albumin or total trichloroacetic acid–precipitable protein, the stimulatory effect of estradiol on apoA-I synthesis was specific. This observation on the effect of estradiol on apoA-I production is consistent with in vivo studies demonstrating that the increased HDL levels in postmenopausal women on estrogen therapy were the result of increased HDL production rate rather than altering its catabolic rate.19,20 Our results regarding increased apoA-I synthesis and secretion in estradiol-treated Hep G2 cells are also consistent with previous studies in Hep G2 cells.22–24 In these studies, investigators showed that estradiol as low as 20 nmol/L can induce apoA-I accumulation in the medium by approximately 50% compared with control, and a 50-fold higher concentration of estradiol showed only an additional 10% increase in apoA-I message in Hep G2 cells.22–24 However, we could not find a significant effect at such a low dose of estradiol to induce apoA-I production. By comparing data from multiple experiments, we observed that approximately 1 to 10 μmol/L estradiol was required to induce a similar degree of apoA-I production, as reported by previous investigators using 20 nmol/L estradiol.22–24 The mechanisms of this dose-response variation in the action of estradiol to induce apoA-I in our and previous reports are not known. Since a considerable amount of estradiol has been shown to bind to circulating proteins, the availability of free unbound estradiol in various experimental designs may play an important role in cellular responses, including the effect on apoA-I production.

Because the liver produces HDL particles containing apoA-I both with and without apoA-II (LP A-I+A-II and LP A-I particles, respectively)9,10 and since LP A-I has been suggested to be more antiatherogenic than LP A-I+A-II, we assessed the effect of estradiol on the production of these particles. Incorporation of [3H]leucine into both types of particles separated by immunoaffinity techniques showed selective increase in LP A-I but not LP A-I+A-II particles in Hep G2 cells treated with estradiol. Congruent with these in vitro observations, Brinton11 recently showed that oral estrogen replacement therapy in postmenopausal women selectively raised LP A-I–containing HDL particles without significant change in LP A-I+A-II. HDL kinetic studies showed that estrogen therapy significantly increased the production rate of LP A-I but not LP A-I+A-II particles.11 Furthermore, no change in FCR of either LP A-I or LP A-I+A-II was observed in these patients undergoing estrogen therapy.11

Additionally, we have shown that the HDL particles accumulated in the culture medium of Hep G2 cells treated with estradiol were able to significantly increase cholesterol efflux from human fibroblasts, suggesting that these particles are biologically active in initiating reverse cholesterol transport. However, the ability of either LP A-I or LP A-I+A-II to efflux cholesterol per unit mass of apoA-I remained identical. Using adipocytes, it was shown that the ability of LP A-I particles to efflux cholesterol was greater than that of LP A-I+A-II particles.26 However, Cheung et al42 and Johnson et al25 have reported no significant differences in cholesterol efflux by LP A-I or LP A-I+A-II in various cell types, including rat hepatoma cells, human skin fibroblasts, and rabbit aortic smooth muscle cells. Our results are in line with the latter report. Other evidence unrelated to cellular choles-
terol efflux indicated that LP A-I is associated with decreased atherosclerosis compared with LP A-I+A-II. For example, transgenic mice producing human apoA-I have significantly less atherosclerosis than those producing LP A-I+A-II when fed an atherogenic diet. Patients with specific increases in LP A-I are less prone to atherosclerosis than those with LP A-I+A-II reduction. Considering these observations, our data indicate that estradiol selectively increased LP A-I production, which may, at least in part, contribute to its cardioprotective properties.

Parallel to de novo synthesis of apoA-I data, estradiol increased the steady state mRNA expression of apoA-I. Steady state mRNA expression is the result of new message transcription and transcript degradation. Therefore, we assessed whether the effect of estradiol to increase steady state apoA-I mRNA expression is mediated by transcription or mRNA degradation. Significantly increased transcription rate of apoA-I without altering its degradation rate was found. Previous studies have demonstrated a similar transcriptional initiation site for the apoA-I gene in Hep G2 cells and in human liver, further strengthening the plausibility that the primary function of estradiol in postmenopausal women on estrogen therapy is to increase apoA-I mRNA transcription. In contrast to apoA-I mRNA expression, estradiol did not alter the steady state mRNA expression of apoA-II. Considering the stimulatory effects of estradiol on apoA-I but not on apoA-II mRNA expression, it is possible that the synthesis of LP A-I particles may be regulated by the apoA-I gene, whereas the apoA-II gene may affect LP A-I+A-II particle production or apoA-II production, with subsequent processing to produce LP A-I+A-II particles. However, further studies are required to identify these putative regulatory mechanisms for the production of LP A-I and LP A-I+A-II particles.

In summary, these data indicate that estradiol increases hepatic apoA-I but not apoA-II mRNA transcription, thereby selectively increasing the production of physiologically active apoA-I-containing HDL particles (LP A-I), which mediate reverse cholesterol transport. The data thus further define the primary mechanism by which estradiol prevents atherosclerotic cardiovascular complications.

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