Estrogen Increases Apolipoprotein (Apo) A-I Secretion in Hep G2 Cells by Modulating Transcription of the Apo A-I Gene Promoter

Stefania Lamon-Fava, Jose M. Ordovas, Ernst J. Schaefer

Abstract—Estrogen administration to postmenopausal women has been shown to increase plasma levels of apolipoprotein (apo) A-I. A human hepatoma cell line, Hep G2, was used to test the hypothesis that estrogen increases the hepatic production of apo A-I by modulating gene expression. When Hep G2 cells were treated for 24 hours with E₂, the apo A-I content in the medium increased 4.3±1.0-fold at 10 μmol/L E₂ and 1.8±0.4-fold at 1 μmol/L E₂ compared with untreated cells. A time-course experiment indicated that there was no E₁-dependent (10 μmol/L) increase in apo A-I medium content at 1 hour and 2 hours and that apo A-I was 165% of controls at 6 hours and 440% at 24 hours. Hep G2 cells were transfected, by the cationic lipid method, with constructs containing serial deletions of the 5′ region of the apo A-I gene (−41/+397, −256/+397, and −2500/+397) cloned in front of the luciferase gene and with or without a 7-kb region spanning the apo C-III/A-IV intergenic region, which has been shown to contain regulatory elements for the expression of the apo A-I gene. With the exception of the construct containing only the basal promoter (−41/+397), the expression of all constructs was 2- to 3-fold greater in the presence of E₂. The smallest construct that maintained E₂ responsiveness, the −256/+397 construct, does not contain a typical estrogen-responsive element. In the same transfection experiments, the 4-fold increase in apo A-I in the culture medium was preserved. However, when the same set of transfections was performed by the calcium phosphate precipitation method, the E₂ effect on the apo A-I content in the culture medium and on transcription activation was nearly abolished. This effect was probably mediated by Ca²⁺, because incubation of cells with 20 mmol/L CaCl₂ abolished the E₂ response. In conclusion, E₂ increases apo A-I production in hepatic cells by increasing the transcription of the apo A-I gene. (Arterioscler Thromb Vasc Biol. 1999;19:2960-2965.)

Key Words: 17β-estradiol ■ estrogen receptor ■ apolipoprotein A-I

Epidemiological studies have shown that HDL cholesterol levels are inversely correlated with the risk of developing coronary heart disease.¹–³ This inverse association is present in both men and women and is due to the important role played by HDL in the reverse cholesterol transfer pathway, in which HDL functions as an acceptor of excess cholesterol from peripheral cells and transports it back to the liver for removal. Apolipoprotein A-I (apo A-I) is the major protein constituent of HDL and is required for HDL formation, as indicated by the presence of very low levels of HDL cholesterol in subjects with apo A-I gene deletions or rearrangements.⁴–⁶ Also, apo A-I is sufficient for HDL formation, as shown in fibroblasts transfected with an expression vector containing the apo A-I gene.⁷

Epidemiological studies have shown that postmenopausal women on estrogen replacement therapy have a lower risk of heart disease than women who do not take estrogen.⁸ Estrogen has been shown to significantly affect plasma lipid levels by lowering LDL cholesterol and apo B levels and by increasing HDL cholesterol and apo A-I levels in both premenopausal and postmenopausal women.⁹–¹¹ The estrogen-mediated increase in plasma HDL cholesterol and apo A-I levels is one of several mechanisms responsible for the protection exerted by estrogen against cardiovascular disease in women. Metabolic studies have demonstrated that the increase in apo A-I levels by estrogen is due to an increase in apo A-I production rate, with nonsignificant effects on apo A-I catabolism.⁹,¹²,¹³ In humans, the apo A-I gene is expressed in both liver and intestine, and studies in Hep G2 cells, a human hepatoma cell line, have demonstrated that estrogen can increase apo A-I concentration in the medium of these cells in a dose-dependent manner.¹⁴,¹⁵ This effect is paralleled by an increase in apo A-I mRNA steady-state levels.¹⁴,¹⁵ In most genes, the mRNA levels are modulated by estrogen by 2 major mechanisms. The most common mechanism is the direct regulation of the transcription activity of the gene by interaction of the estrogen/estrogen-receptor complex with a specific DNA sequence called the estrogen response element (ERE).¹⁶ In some genes, estrogen has been shown to promote transcription through the AP-1 site, a
binding site for Fox and Jun, possibly by an estrogen receptor (ER)–independent mechanism. Alternatively, estrogen can modulate levels of a specific mRNA by affecting its degradation.

The 5′ region of the apo A-I gene between nucleotides −222 and −110 contains regulatory elements that are necessary for transcription of this gene in hepatic cells. This region, known as the apo A-I hepatic enhancer, contains 3 different DNA binding elements, A (−214 to −192), B (−169 to −146), and C (−134 to −119), which have been shown to bind several nuclear transcription factors.

In addition, the intergenic region between the apo C-III and apo A-IV genes (located in a cluster with the apo A-I gene on the same chromosome within a 15-kb DNA fragment) contains additional DNA elements that enhance and regulate the expression of the apo A-I gene governed by the apo A-I hepatic enhancer.

In this study, we examined the 5′ and 3′ DNA regions of the apo A-I gene to understand the mechanism responsible for the estrogen responsiveness of apo A-I gene expression.

**Methods**

**Cell Culture**

Hep G2 cells were grown in high-glucose DMEM (BioWhittaker) supplemented with 10% FBS (HyClone), and 100 U/ml penicillin. Hep G2 cells were grown in high-glucose DMEM (BioWhittaker) supplemented with 10% FBS (Hyclone) and 100 U/mL penicillin, 10% charcoal/dextran-treated FBS (HyClone); after 24 hours, cells were washed and incubated in serum-free medium; on day 3, fresh serum-free media were added to cells, together with E2 dissolved in 95% ethanol (control cells received ethanol only). On day 4, cells were collected by scraping the bottom of culture dishes. Cell extracts were stored at −70°C until β-galactosidase and luciferase activities were assayed. The activities of these enzymes were measured with commercially available kits from Promega. Media were also collected for the measurement of apo A-I concentrations.

**Cell Transfection**

Transfection of Hep G2 cells was carried out with the Lipofectamine reagent (Life Technologies). Briefly, cells were plated on 60-mm dishes, as described above, and on day 2 were transfected with 2 μg of RSV–β-galactosidase plasmid and 3 μg (or the molar equivalent) of apo A-I promoter/luciferase constructs in the presence of 20 μL of Lipofectamine for 14 hours in serum-free and antibiotic-free conditions; on day 3, cells were washed and incubated in fresh serum-free medium, and estrogen was added at a final concentration of 10 μmol/L; on day 4, cells were collected by scraping the bottom of culture dishes. Cell extracts were stored at −70°C until β-galactosidase and luciferase activities were assayed. The activities of these enzymes were measured with commercially available kits from Promega. Media were also collected for the measurement of apo A-I concentrations.

**Plasmid Construction**

Plasmid −2500A-I(C-III/A-IV).Luc (a gift from Dr S. Karathanasis, Wyeth-Ayerst Laboratories, St. Davids, PA) contains the cDNA from the HEO plasmid into the pMT2 expression vector.

**Statistical Analysis**

Experiments were conducted in duplicate. Data presented are the mean±SD of ≥2 separate experiments. Statistical significance was determined with Student’s t test. Differences were considered significant at a value of P<0.05.

**Results**

Incubation of Hep G2 cells with different concentrations of E2 for 24 hours resulted in a dose-dependent increase in apo A-I concentration in the medium (Figure 1). The apo A-I concentration, relative to total protein concentration, in cells exposed to 10 μmol/L E2 was 4.3±1.0-fold greater than in control cells (n=6, P<0.001), and in cells exposed to 1 μmol/L E2, it was 1.8±0.4-fold greater than in control cells (n=4, P=0.09). E2 at 10 μmol/L and 100 μmol/L concentration did not significantly affect apo A-I secretion by Hep G2. A time-course experiment indicated that when cells were incubated for 0, 1, 2, 6, and 24 hours in 10 μmol/L E2, apo A-I increased in cells treated with estrogen compared with control cells at −6 hours, when a 65% increase in apo A-I was observed, and the increase in apo A-I reached 4.4-fold at 24 hours (Figure 2).

As shown in the Table, the estrogen-related increase in apo A-I concentration in the medium was associated with an increase in specific apo A-I mRNA steady-state levels, relative to β-actin mRNA levels, in the cells. β-Actin was chosen as a reference gene because it is a housekeeping gene and its expression is not regulated by estrogen.
The increase in apo A-I mRNA steady-state levels suggested either an increase in transcriptional activation of the apo A-I gene or a decrease in mRNA degradation. The transcriptional activity of the apo A-I gene in the presence of estrogen was tested in a series of transfection experiments. Because it has been shown that the apo C-III/apo A-IV intergenic region contains elements that are important in the activation of transcription of the apo A-I gene, 2 series of constructs were used: constructs containing serial deletions of the 5' region of the apo A-I gene, with or without the apo C-III/apo A-IV intergenic region. As shown in Figure 3, the plasmid constructs containing only 41 bp of the 5' region of the apo A-I gene did not respond to estrogen administration. The plasmids containing 256 bp of the 5' region of the apo A-I gene were the shortest constructs to maintain estrogen responsiveness. The presence of the apo C-III/A-IV intergenic region did not affect estrogen responsiveness. In these transfection experiments, where a reagent consisting of cationic liposomes was used, cells treated with estrogen also showed the characteristic 4-fold increase in apo A-I concentration in the medium. However, when the same transfection experiments were performed by the calcium phosphate precipitation method, the estrogen responsiveness was nearly abolished both at the level of the transcriptional activity of the apo A-I gene and of the apo A-I concentration in the medium (data not shown): only a 10% increase ($P=\text{NS}$) in apo A-I in the medium was observed under these conditions, with no change in the transcriptional activity. To explore the possible role of calcium in the lack of response to estrogen under the calcium phosphate method, cells were incubated with estrogen and with 0, 1, 4, 20, and 40 mmol/L CaCl$_2$, and apo A-I was measured in the medium. Whereas calcium at concentrations of 1 mmol/L and 4 mmol/L did not affect apo A-I concentrations in the medium, calcium at concentrations of 20 and 40 mmol/L abolished the estrogen response (Figure 4).

**Figure 1.** E$_2$ increases apo A-I concentrations in the media of Hep G2 cells. Hep G2 cells were grown as described in the Methods section. After exposure for 24 hours to the indicated E$_2$ concentrations, media were collected and apo A-I was measured. The apo A-I concentration is calculated as the ratio between apo A-I ($\mu$g/mL) and total protein ($\mu$g/mL) in the medium and is expressed as percentage of control cells.

**Figure 2.** Time course of estrogen effect on apo A-I accumulations in the media of Hep G2 cells. Media from Hep G2 cells treated with 10 $\mu$mol/L E$_2$ were collected at different time points after the addition of E$_2$, as indicated. Apo A-I concentration in the medium was calculated as the ratio between apo A-I ($\mu$g/mL) and total protein ($\mu$g/mL) and is expressed as percentage of control cells.

**Figure 3.** Effect of estrogen on transcriptional activity of the apo A-I gene. Hep G2 cells were transfected, by the cationic lipid method, with 2 $\mu$g of $\beta$-galactosidase plasmid and 3 $\mu$g of the $-41A-I-Luc$ construct or the molar equivalent of the other A-I constructs. After 30 hours' incubation with (+) or without (−) 10 $\mu$mol/L E$_2$, cells were collected and the luciferase and $\beta$-galactosidase activities were measured from cell extracts.

<table>
<thead>
<tr>
<th>E$_2$, $\mu$mol/L</th>
<th>Apo A-I/$\beta$-Actin, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>138±13</td>
</tr>
<tr>
<td>10</td>
<td>191±43</td>
</tr>
</tbody>
</table>

Results are means of 2 experiments.
indicating a role of calcium in the modulation of apo A-I transcription by estrogen.

We investigated whether ER plays a role in the increased production of apo A-I in Hep G2 cells by estrogen. Plasmid −41A-Luc and −256A-Luc were cotransfected with increasing amounts of an ER-α expression vector. As indicated in Figure 5, increasing amounts of ER-α were associated with decreasing activity of the apo A-I gene promoter in the presence of estrogen, so that at 0.5 μg of ER-α plasmid, the transcriptional activity of the apo A-I promoter was approximately half that of control. A plasmid construct containing the luciferase gene under the control of the tk promoter and 2 ERE (5'-GGTCANNNTGACC-3') was also used in transfection experiments in the presence or absence of E2. As shown in Figure 6, the transcription of this construct failed to be activated by E2 alone but was activated severalfold in the presence of both E2 and ER-α.

**Discussion**

Because of the inverse relationship between HDL and apo A-I levels and the risk of coronary heart disease in the general population, factors that regulate and, in particular, increase plasma levels of these parameters are of great interest for their potential health benefits. It has been projected that for each 0.026-mmol/L increase in HDL cholesterol levels, the risk of coronary heart disease decreases by 2% to 3%. Estrogen has clearly been shown to fall in the category of HDL cholesterol-raising drugs. However, clinical studies have shown that the route of administration of estrogen influences its effects on HDL cholesterol levels. It has been shown that transdermal administration of estrogen does not significantly increase HDL cholesterol levels in postmenopausal women; even though serum estradiol levels in these women are raised to premenopausal levels. Oral administration of estrogen results in a 15% to 36% increase in HDL cholesterol levels. This suggests that exposure of hepatic cells to higher levels of estrogen, as obtained in the oral administration, may be necessary for estrogen to exert its effects on plasma lipids. The present study indicates that E2, at the superphysiological concentrations of 1 and 10 μmol/L, increases the synthesis of apo A-I by hepatic cells. These results are in agreement with those of 2 previous studies in Hep G2 cells using E2 concentrations of 10 μmol/L and 10 μmol/L. We found that the increase in apo A-I synthesis by hepatic cells is accompanied by an increase in apo A-I mRNA levels, as also shown in previous studies. However, Harnish et al. recently found that apo A-I mRNA levels were not affected by treatment with 1 μmol/L E2 in Hep G2 cells. The reason for this discrepancy in results is not clear.

As indicated by the time-course experiment, the rate at which apo A-I levels increase in the medium of Hep G2 cells treated with E2 is slow, starting at ~6 hours after the addition of estrogen in the medium. This was also observed by Archer et al. and suggests that the mechanism responsible for the increase in apo A-I production by hepatic cells does not involve an immediate response but rather requires induction.

Apo A-I mRNA levels may be regulated either at the transcriptional level or by posttranscriptional mechanisms. It has been shown that estrogen does not affect the degradation of apo A-I mRNA. We have tested the hypothesis that the E2-related increase in apo A-I mRNA is due to increased transcriptional activity of the gene. Transfection experiments with plasmids containing serial deletions of the 5' region of the apo A-I gene, with or without the apo C-III/A-IV intergenic region, indicated that the estrogen responsiveness
is maintained in the plasmid constructs containing 256 bp of the 5′ region of the apo A-I gene and is not affected by the apo C-III/A-IV intergenic region. The DNA region that extends to 256 bp upstream of the start site of the apo A-I gene contains the apo A-I hepatic enhancer, which has been shown to be necessary and sufficient for expression of this gene in liver cells. The hepatic enhancer is composed of 3 different regions, A, B, and C, which bind to different nuclear transcription factors. This region does not contain a canonical ERE binding site, but site A contains half of the canonical ERE sequence (TGACC) separated by 3 nucleotides by a tandem imperfect repetition (TGAACT). There are numerous examples in the literature of estrogen-responsive genes containing noncanonical ERE in their 5′ flanking region: it has been shown that the rabbit uteroglobulin gene, the mouse lactoferrin gene, and the human pS2 gene contain an imperfect ERE. Also, it has been shown that the chicken ovalbumin gene contains several half-palindromic motifs that confer estrogen inducibility to the gene by acting synergistically. The lower affinity for the ER of these DNA sequences may be responsible for the lower induction of transcription of these genes by estrogen compared with the classic ERE-containing genes. However, we have conducted preliminary experiments with plasmid constructs containing mutations of site A of the human apo A-I gene, and our results indicate that mutations in either of the putative ERE half-sites do not affect estrogen responsiveness (data not shown).

Two isoforms of ER, ER-α and ER-β, have been described. Until recently, only ER-α was known, but in 1996 ER-β was isolated by Kuiper et al. Hep G2 cells have been shown to contain ER-β, specifically isoform 5, in low amounts. Even though it has been shown that ER-β can bind to the same DNA sequence as ER-α, it has been speculated that ER-β may also bind to different and as yet uncharacterized sequences. The observation that cotransfection of the estrogen-responsive apo A-I plasmid with increasing amounts of an expression vector for ER-α progressively decreases the estrogen-related expression of apo A-I and that at the higher dose ER-β actually inhibits the transcription of the apo A-I gene seems to indicate that ER-α, when present in high concentration in the cell nucleus, competes with other transcription factors for binding to the enhancer region or, as suggested previously, competes for binding to a coactivator common to the factors binding to the enhancer. In both cases, partitioning of the relevant regulatory protein to the ER may lead to decreased activation of the enhancer. Our transfection experiments with the −256A-I.Luc construct and with an ERE construct with or without ER-α suggest that the mechanism of transcription activation of the apo A-I gene by estrogen is different from the classic ER-α-mediated regulation of transcription. It is not known whether apo A-I gene transcription is regulated by the ER-β differently than by ER-α.

We have observed that Hep G2 cells, when transfected with the cationic lipid methods, maintained their estrogen responsiveness by increasing the amount of apo A-I secreted in the medium after exposure to E2. However, it was interesting to observe that in similar transfection experiments using the classic calcium phosphate precipitation method, cells ceased to respond to estrogen by showing a lack of increase in apo A-I in the medium after exposure to estrogen. This effect was paralleled by a lack of transcription activation of the apo A-I gene by estrogen. Harnish et al recently published data indicating that the apo A-I promoter does not respond to estrogen. In their study, transfection experiments were carried out with the classic calcium phosphate method, and therefore, the discrepancy in results between our study and the study by Harnish et al may be explained by the methodology used. In an attempt to define the problem related to the calcium phosphate technique, we have exposed Hep G2 cells grown in the presence of E2 to different calcium concentrations. The estrogen-related increase in apo A-I in the medium was nearly abolished when cells were exposed to calcium concentrations similar to those used in the precipitation method. The mechanism responsible for this effect of calcium is unclear, but it has been shown that calcium can modulate the interaction of the ER with its ligand.

Our results indicate that the apo A-I gene is regulated by estrogen at the transcriptional level. The estrogen-related activation in transcription leads to increased levels of mRNA for apo A-I and increased synthesis of apo A-I by hepatic cells. The DNA region of the apo A-I gene involved in the activation of transcription is located in the first 256 bp 5′ of the gene. Because this region does not contain a classic ERE, it is possible that estrogen can activate transcription indirectly through induction of other transcription factors.

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References

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