REGULATION OF APOLIPOPROTEIN A-I GENE EXPRESSION: MECHANISM OF
ACTION OF ESTROGEN AND GENISTEIN

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ABSTRACT

We have previously shown that 17-β-estradiol (E₂) and genistein increase the secretion of apolipoprotein (apo) A-I, the major protein component of high-density lipoproteins, in Hep G 2 cells by increasing the transcription of the apo A-I gene. The aim of this study was to elucidate the mechanism mediating the increase in apo A-I gene expression by these compounds. A series of plasmid constructs containing serial deletions of the apo A-I promoter region was generated. The −220 to −148 region of the apo A-I promoter was the smallest region maintaining response to E₂ and genistein, and the estrogen antagonist ICI 182,780 completely inhibited the E₂ and genistein effect. This region contains a binding site for transcription factors belonging to the steroid/thyroid nuclear receptor superfamily (site A), a binding site for HNF-3β (site B), and two binding sites for Egr-1. Nuclear extracts from cells treated with E₂ and genistein showed increased binding to site B and nuclear extracts from genistein-treated cells showed increased binding to Egr-1 oligonucleotide, compared to control cells. An increase in the concentrations of Egr-1 and HNF-3β was observed in nuclear extracts of cells treated with E₂ and genistein, compared to control cells. Treatment of Hep G2 cells with a specific inhibitor of MAP kinase, but not with other inhibitors, abolished the stimulation of apo A-I gene expression by E₂ and genistein. These results indicate that the MAP kinase pathway is involved in the regulation of apo A-I gene expression by genistein and E₂, possibly through downstream regulation of
transcription factors binding to the promoter region.

Key words: estrogen, genistein, liver, gene expression, MAP kinase, Egr-1, HNF-3β.
INTRODUCTION

Apolipoprotein (apo) A-I, the major protein component of HDL, is synthesized by the liver and intestine and is both necessary and sufficient for the formation of HDL (1;2). Apo A-I plays an important role in the reverse cholesterol pathway by: a) interacting with the ATP binding cassette A1 (ABCA1) cell membrane receptor and promoting cell cholesterol efflux via this receptor, b) activating LCAT, the enzyme involved in cholesterol esterification, and c) carrying the excess esterified cholesterol back to the liver for excretion (3). Several studies have indicated that plasma levels of apo A-I and HDL cholesterol are significant predictors of coronary heart disease (4-6). Treatment with estrogen is associated with an increase in plasma apo A-I levels (7), and metabolic studies, conducted in women using either radio-labeled or endogenously labeled apo A-I, have indicated that an increased production rate of apo A-I is responsible for the increase in its plasma levels during estrogen treatment (8-10). Only oral administration of estrogen has been shown to increase both apo A-I concentration and production rate, since postmenopausal transdermal estrogen delivery, while effective in restoring serum estradiol levels to premenopausal levels, is not associated with changes in protein levels or production rates (9). The differential effect of oral and transdermal estradiol implicates a first pass through the liver as a necessary step for the increased production of apo A-I.

Genistein, an isoflavone phytoestrogen present in high concentration in soy, has partial estrogen agonist activity (11). Phytoestrogens from soy have been shown to increase plasma HDL cholesterol levels in some (12;13) but not all (14;15) studies.
We (16) and others (17) have previously shown that estrogen increases apo A-I gene transcription in liver cells, in accordance with the results of clinical metabolic studies. We have also shown that genistein increases apo A-I transcription in liver cells (18). The increase in apo A-I gene expression by estrogen and genistein is mediated by the −256 to −41 region of the apo A-I promoter (16;18). This region acts as a liver-specific enhancer and contains three transcription factors binding sites, site A (−214 to −192), site B (−169 to −146), and site C (−134 to −119) (19). Site A is involved in apo A-I gene expression regulation by members of the steroid/thyroid nuclear receptor superfamily, such as hepatocyte nuclear factor 4 (HNF-4) and retinoid X receptor α (RXRα) (20). Site B has been shown to bind HNF-3β (21). Site C is structurally similar to site A and binds transcription factors similar to those recognizing site A. Two binding sites for the transcription factor early growth response factor 1 (Egr-1) (−221 to −213 and −189 to −181) are also found in this region (22). An antioxidant response element (ARE) has been described in the apo A-I promoter (23). The apo A-I promoter region does not contain a classic estrogen response element (ERE). However, two ERE half-palidromic sites are located within site A, but previous work has shown that the estrogen receptor (ER) does not bind to this sequence, suggesting that other transcription factors may be involved in the estrogen-mediated increase in apo A-I expression (24).

The current report shows that the mechanism responsible for the transcription activation of the apo A-I gene by estrogen and genistein in liver cells is mediated by a short sequence in the promoter region which binds the Egr-1 and HNF-3β transcription factors. Nuclear concentration of these two transcription factors is increased by treatment of cells with these estrogenic
compounds. Activation of the MAP kinase pathway mediates, at least in part, the increase in apo A-I gene expression by estrogen and genistein.

MATERIALS AND METHODS

Genistein and 17-β-estradiol (E2) were purchased from Sigma (St Louis, MO). The MAP kinase inhibitor PD 98059 was purchased from Cell Signaling (Beverly, MA). The protein kinase A (PKA) inhibitor myristoylated 14-22 amide and the protein kinase C (PKC) inhibitor bisindolylmaleimide I were obtained from Calbiochem (San Diego, CA). ICI 182,780 was purchased from Tocris (Balwin, MO). The Egr-1 (rabbit polyclonal) and HNF-3β (goat polyclonal) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). γ-32P-ATP was obtained from Perkin-Elmer Life Sciences (Boston, MA).

Cell culture experiments

Hep G2 cells were maintained in DMEM (BioWhittaker, Walkesville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone Logan, UT), and 1% Glutamax, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, Chicago, IL) as previously reported (16;18). All experiments were carried out in phenol red-free DMEM/F12 medium containing 5% charcoal/dextran-treated FBS. An enzyme-linked immunosorbent assay was used for the
determination of apo A-I in the culture media, as previously described (16). Transfection experiments were carried out in 6-well dishes with 1.5 µg of the test plasmid and 0.25 µg of Renilla luciferase plasmid (Promega, Madison, WI) as an internal control reporter and using the Lipofectamine reagent (Invitrogen, Chicago, IL). Six hours after transfection, genistein and E₂ were added at the concentration of 10 µM. When indicated, ICI 182,780 was added to the culture medium 1 hr before genistein and E₂ at the concentration of 10 µM. Similarly, inhibitors of MAP kinase (10 µM), PKA (10 µM), and PKC (1 µM) were added to the culture medium 30 min before genistein and E₂. Cells were collected for luciferase measurements after 36 hours.

Results are presented as the mean±SD of 3 to 6 independent experiments conducted in duplicate.

Plasmid constructs

The −256A-I.Luc and −41A-I.Luc plasmids, containing the −256 to +396 and the −41 to +396 region of the apo A-I gene, respectively, have been previously described (16). To generate the −192A-I.Luc plasmid, the −192 to −42 Pst I-Pst I fragment was isolated form the −256A-I.Luc plasmid and inserted in the Pst I site of the −41A-I.Luc plasmid, and correct orientation was documented by sequencing. The −133A-I.Luc construct was created by cloning the Sau 3A-Hind III fragment of −256A-I.Luc into the Bgl II-Hind III site of the pGL2 basic vector (Promega, Madison, WI). The −220[Δ-110/-42]A-I.Luc plasmid, which contains the −220 to −110 region of the apo A-I promoter inserted in front to nucleotide −41 of the −41A-I.Luc
plasmid, was obtained by cloning the Hind III-Hind III fragment of plasmid –220[(Δ-110/-42)]A-I.CAT previously described and a gift from Dr. S. Karathanasis (19), into the Hind III site of the pGL2 basic vector. To generate the –256[Δ-132/-42]A-I.Luc construct the –256 to –133 region of the apo A-I gene was amplified by PCR with the forward primer containing an Sst I overhanging site, and the reverse primer containing an Xho I overhanging site; the PCR product was then cut with Sst I and Xho I and cloned in the Sst I-Xho I site of –41A-I.Luc. A similar approach was used for the generation of both the –256[Δ-148/-42]A-I.Luc and the –256[Δ-185/-42]A-I.Luc plasmid constructs. A plasmid containing 2 sites A (2siteAA-I.Luc) was constructed by cloning a double-stranded synthetic oligonucleotide into the Sst I-Xho I site of the –41A-I.Luc plasmid. The 2siteBA-I.Luc and 2AREA-I.Luc plasmids were generated following the same procedure. The –256A-I.Luc plasmid was used as template for site-directed mutagenesis of the two ERE half-palindromic sequences in site A. Mutagenesis was performed by the Quick-Change method (Stratagene, La Jolla, CA) using the following primers (and their complementary oligonucleotides): 5’-

CCGCCCCACTGGGCCCTTGACCCTGCTGAG-3’ and 5’-CCGCCCCACTGAACCTTGGGC

(mutated sequences are underlined). Correct mutations were confirmed by plasmid sequencing.

**Electrophoresis mobility shift assay (EMSA)**

Double-stranded oligonucleotides were labeled with 32P using T4 polynucleotide kinase and γ32P-ATP. Nuclear extracts from Hep G2 cells that had been treated with 10 μM E2 or 10 μM
genistein, or control cells, were prepared as previously described (25). EMSA were performed with 7 µg of nuclear protein extracts in a 20 µl reaction mixture containing 10 mM Tris HCl pH 7.5, 4% glycerol, 1mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 5 µg of poly(dI-dC). When indicated, specific non-labeled oligonucleotide competitors were used at 100x molar excess concentration. The binding reactions were incubated at 4 °C for 10 min, and then with 2 pmol of a specific double-stranded ³²P-labeled oligonucleotide for 20 min. For supershift assays, 1 µg of specific antibody was added to the reaction mixture at the end of the binding reaction and incubated for additional 40 min at room temperature before electrophoresis.

Reactions were subjected to electrophoresis in a 5% non-denaturing polyacrylamide gel in 0.5% TBE buffer. The labeled oligonucleotides were: site B (5’- CTTGCTGTTGCCCACTCTATTTGCCCAGCCC-3’), and Egr-1 (5’- GGATCCAGCGGGGGCGAGCGGGGGCGA-3’).

Western blots

Twenty µg of nuclear extracts were electrophoresed in a 10% polyacrylamide gel and transferred to Hybond ECL nitrocellulose membranes. Non-specific protein binding sites were blocked using blocking solution B (1x TBS, 1% non-fat dry milk, 1% bovine serum albumin, 0.05% Tween 20) for 1 hour at room temperature. Incubation with primary antibody (1:200 dilution) was performed at room temperature for 1 hour in blocking solution B. The membrane was then rinsed 3x with the washing solution (1x TBS, 0.05% Tween 20), incubated with
horseradish peroxidase-conjugated secondary antibody in blocking solution for 1 hour at room temperature, and rinsed again 3x with washing solution. Finally, membranes were incubated with developing reagent (WesternBreeze, Invitrogen, Chicago, IL), and exposed to X-ray films.

RESULTS

Characterization of the promoter region of the apo A-I gene mediating the increased expression by estrogen and genistein

We have previously shown that treatment of Hep G2 cells with E2 or genistein is associated with a dose-dependent increase in apo A-I concentration in the culture media (16;18). The maximum effect (4- to 5-fold) is observed at 10 µM concentration of both E2 and genistein (16). The need for super-physiologic concentrations of E2 is consistent with the clinical observations of increased production of apo A-I during oral, but not transdermal, administration of estrogen (9). Therefore, all experiments were performed with 10 µM E2 and genistein.

Transfection experiments previously conducted in our laboratory have indicated that the −256 to −41 region of the apo A-I gene is responsive to treatment with either E2 of genistein (16;18). To further characterize the region of the apo A-I promoter involved in the E2- and genistein-mediated activation of transcription, plasmids containing serial deletions of the −256 to −41 region of the apo A-I gene promoter were constructed. As indicated in Figure 1A, two plasmid
constructs, the plasmid containing the –220 to –110 sequence and the plasmid containing the –256 to –148 sequence, maintained full estrogenic response when transfected into Hep G2 cells. Further deletion of this region, as in plasmids containing the –192 to –41 sequence or the –256 to –185 region, partially abolished the estrogenic response. These results indicate that the –220 to –148 region is sufficient for estrogenic response. This region contains the following well-characterized transcription binding sites: site A (–214 to –192), site B (–169 to –146), and two Egr-1 sites (–221 to –213 and –189 to –181) (Figure 1B). To explore the individual role of site A and site B on the estrogenic response of the apo A-I gene, plasmid constructs containing two sites A or two sites B were transfected into Hep G2 cells; however neither of these two plasmids did show an increased expression following treatment with E2 or genistein (Figure 1A). In addition, mutations of each of the two ERE half-sites in site A did not affect estrogen responsiveness, indicating that these sequences are not relevant for the response to estrogen. A plasmid containing two apo A-I ARE sites did not respond to treatment (Figure 1A), and a plasmid containing the –133 to –41 region failed to respond to treatment (data not shown).

The effect of ICI 182,780 on the modulation of apo A-I gene expression by E2 and genistein was tested in transient transfection experiments conducted with the plasmid containing the –256 to –148 region of the apo A-I promoter (–256[Δ-148/-42]A-I.Luc). While E2 and genistein significantly increased expression of this plasmid construct compared to control (P< 0.02), both compounds failed to increase expression when cells were pre-treated for 1 hour with ICI
ICI 182,780 alone had no effect on the expression of the –256[Δ-148/-42]A-I.Luc plasmid.

**Effect of estrogen and genistein on nuclear Egr-1 and HNF-3β concentrations**

EMSA experiments were conducted with site B and Egr-1 oligonucleotides. As shown in Figure 3A, an increase in the DNA-protein complex was observed when nuclear extracts of cells treated with genistein were incubated with the Egr-1 probe, as compared to control cells. The binding was specific and abolished by 100-molar excess of cold Egr-1 competitor. Incubation with the Egr-1 antibody resulted in a weakening of the band (Figure 3A). However, a supershift band is not visible, probably due to the light signal. When the site B oligonucleotide was used, nuclear extracts of cells treated with E2 and genistein showed greater intensity of two bands compared to control cells (two upper bands on Figure 3B). Incubation of DNA-protein complexes with the antibody against HNF-3β resulted in the disappearance of one of these two bands (second band from the top), with resulting supershift clearly evident with extracts from cells treated with E2 and genistein.

To assess the effect of these estrogenic compounds on the nuclear concentration of Egr-1 and HNF-3β, western blotting experiments were performed. Egr-1 levels were increased 2-fold in E2-treated cells, and 4-fold in genistein-treated cells, as assessed by densitometry scanning (Figure 4). A 4-fold increase in HNF-3β protein concentrations in cells treated with E2 and genistein, as compared to control cells, was observed as well.
Role of MAP kinase in the activation of apo A-I gene expression by estrogen and genistein.

Because Egr-1 is induced via the MAP kinase pathway in other cells (26), we tested whether this second messenger pathway is important in apo A-I induction by E2 and genistein in liver cells. After transfection, cells were pre-treated for 30 min with the MAP kinase inhibitor PD 98059 (10 µM) and then with E2 or genistein. PD 98059 abolished the estrogen- and genistein-mediated increase in expression of the −256[A-148/-42]A-I.Luc plasmid (Figure 5). Inhibitors of PKA (10 µM) and PKC (1 µM) did not have a significant effect on the estrogentic upregulation of this plasmid (Figure 5).

DISCUSSION

Oral estrogen treatment has been shown to increase plasma levels of HDL cholesterol and apo A-I (7). While this effect has been known for almost 50 years (27), the underlying mechanism has never been fully elucidated. Our study shows that both estrogen and genistein activate the expression of the apo A-I gene in liver cells, leading to increased production of apo A-I protein. However, the classical genomic ER ligand-activated pathway does not directly mediate the transcription activation of the apo A-I gene by estrogen and genistein. While site A on the promoter region of the apo A-I gene contains two ERE half-palindromic sequences, these do not play a role on the estrogen regulation of apo A-I gene expression, as indicated by a similar
estrogenic response in the wild-type plasmid and the site A mutation plasmids. Instead, under control conditions, the expression of the site A mutated plasmids was reduced compared to the wild-type plasmid (data not shown), in agreement with previous observations (19), indicating that binding of transcription factors to this site is important for basal apo A-I expression. Our finding that reducing the binding of transcription factors to site A did not change the estrogen response indicates that site A may not be relevant in the activation of apo A-I gene expression by estrogen and genistein. This is also suggested by the lack of estrogenic response of the plasmid containing two sites A. The estrogen antagonist ICI 182,780 completely abolished the estrogen- and genistein-mediated increased expression of the plasmid containing the −256 to −148 region of the apo A-I promoter, indicating that ER activation through ligand binding indirectly regulates apo A-I gene expression. Over the last few years, it has become evident that estrogen, and steroid hormones in general, may regulate gene expression via alternative pathways that do not require a classical genomic action. Non-genomic effects of estrogen, via the activation of the MAP kinase, PKC, and PKA pathways, have been described (28). In most cases, this mode of non-genomic regulation of gene expression requires a cell membrane-bound ER that, when ligand-activated, elicits a specific response via second messengers.

Our results show that the estrogen-responsive region of the apo A-I promoter is contained in the −220 to −148 sequence. This region contains 2 binding sites for Egr-1, and sites A and B. The abundance of Egr-1 protein was increased in nuclear extracts of liver cells treated with estrogen and genistein, suggesting a role of Egr-1 in the activation of apo A-I expression by
these compounds. Both western blotting and EMSA experiments indicated a greater effect of genistein on nuclear Egr-1 content, compared to estrogen. The molecular basis for this difference is not known. In cardiac myocytes, estrogen causes an increase in Egr-1 expression leading to increased Egr-1 protein (26). The regulation of Egr-1 by estrogen in cardiomyocytes is mediated by serum response elements located in the promoter region of Egr-1, and ICI 182,780 completely abolishes this effect (26). The activation of Egr-1 expression by estrogen in cardiomyocytes is also abolished by the MAP kinase inhibitor PD 98059, implicating the MAP kinase pathway in the activation of Egr-1 expression by estrogen (26). Egr-1 is a transcription factor that plays an important role in several tissues and has been also implicated in the basal apo A-I transcription (29). Our study provides evidence that estrogen and genistein are important modulators of apo A-I expression through Egr-1. Egr-1 has been shown to be involved in the increase in apo A-I gene expression in human apo A-I transgenic mice with nephrotic syndrome (29), and also to play an important role in the reduced apo A-I expression in zinc-deficient status (30). It has been shown that MAP kinase activation leads to increased apo A-I expression also by a different pathway, not involving the −220 to −148 promoter region, but an upstream region (−425 to −376), and requiring the participation of the transcription factor Sp1 (31). Epidermal growth factor and insulin mediate an increase in apo A-I expression via this mechanism (31). Our analysis of the signaling pathway involved in apo A-I gene expression in liver cells by estrogen and genistein has indicated that MAP kinase is specifically involved. Transfection experiments with the −256 to −148 apo A-I plasmid construct have shown that cells grown in the presence of the MAP
kinase inhibitor PD 98059 do not respond to either estrogen or genistein. These results are consistent with the concept that upregulation of apo A-I expression is effected by estrogen-mediated activation of the MAP kinase pathway. PKC has been implicated in the regulation of MAP kinase activation (32). However, in our experiments, inhibition of PKC did not have an effect on apo A-I activation by estrogen and genistein.

We also showed an increase in HNF-3β levels in nuclear extracts of liver cells treated with estrogen or genistein. EMSA analysis of the site B in the apo A-I promoter, which binds with high affinity this transcription factor, indicated a specific increase in the binding of nuclear transcription factors to site B in cells treated with estrogenic compounds, and in particular a specific increase in HNF-3β binding, as indicated by the specific supershift of a DNA-protein complex by the anti-HNF-3β antibody. The HNF-3β gene has been cloned (33) but little is known about its regulation by estrogen. Therefore, we do not know if HNF-3β levels are directly or indirectly regulated by estrogen. Our transfection experiments with serial deletion constructs of the apo A-I promoter region have shown that, as for site A, the isolated site B does not respond to estrogen, even when present in duplicate copy. These results, taken together, indicated that a synergism of different transcription factors may be necessary for the activation of apo A-I expression by estrogenic compounds.

To our knowledge, there is only one previous study in the literature that has examined the modulation of the apo A-I promoter activity by estrogenic compounds: Zhang et al (23) showed that equilenin (a compound of conjugated equine estrogen, the most commonly prescribed
hormonal replacement therapy), but not estradiol, increases apo A-I media concentration and promoter activity in Hep G2 cells via interaction with the ARE site (23). Our results show that the ARE site does not play a role in the modulation of apo A-I gene expression by estrogen and genistein, as indicated by a lack or response of the plasmid construct containing 2 ARE sites and the positive estrogenic response of plasmids not containing the ARE site (−256[Δ-148/-42]A-I.Luc). In addition, our results of a significant increase in apo A-I concentration in the media and in the A-I transcription activation following treatment with estradiol, while in contrast to the findings of Zhang et al (23), are clearly in agreement with previous studies (16,17, 34).

In summary, we have demonstrated that; 1) estrogen and genistein increase the expression of the apo A-I gene in liver cells and that this effect is abolished by the estrogen antagonist ICI 182,780; 2) estrogen and genistein increase Egr-1 and HNF-3β concentration in nuclear extracts of liver cells; and 3) this process requires the MAP kinase signaling pathway.
REFERENCES


**FIGURE LEGENDS**

**Figure 1. Characterization of the estrogenic response sequence in the apo A-I gene promoter.**

*Panel A.* Hep G2 cells were transiently transfected with serial deletion constructs of the apo A-I promoter (1.5 µg) and a Renilla luciferase plasmid (0.25 µg) for 6 hours, followed by treatment with vehicle, E2 (10 µM), or genistein (10 µM) for 36 hours. The last plasmid construct contains a mutation on the half ERE on site A, as indicated in the Methods section (only one mutant is shown, but similar results were obtained with both mutants of the two half ERE). Fold activation represents the mean±SD of 3 or more independent transfection experiments conducted in duplicate. *Panel B* shows the nucleotide sequence of the −220 to −148 region of the apo A-I promoter and indicates the transcription factor binding regions.
Figure 2. Effect of ICI 182,780 on the activation of apo A-I promoter by estrogen and genistein.

Cells were transiently transfected with the –256 to –148 apo A-I promoter construct and then grown for 36 hours in vehicle, E2 (10 μM), or genistein (10 μM), with or without pretreatment for 1 hour with ICI 182,780 (10 μM). Bars represent the mean (±SD) of 6 independent experiments. * P<0.02 compared to control.

Figure 3. EMSA. Seven μg of nuclear protein extracts from control cells (C) and from cells treated with 10 μM E2 (E) or with 10 μM genistein (G) were incubated with 32P-labeled Egr-1 (Panel A) and site B (Panel B) double-stranded oligonucleotides as described in the Methods. Specific Egr-1 and HNF-3β antibodies were added to the reaction mixture where indicated. The protein/DNA complexes were run on a 5% non-denaturing polyacrylamide gel. The specific protein/DNA complexes are indicated by arrows on the left.

Figure 4. Modulation of Egr-1 and HNF-3β nuclear concentrations by estrogen and genistein.

Twenty μg of nuclear protein extracts from control (C) cells and from cells treated with E2 (E) and genistein (G) were electrophoresed in a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane, followed by blotting with specific antibodies against Egr-1 and HNF-3β.

Figure 5. Effect of MAP kinase inhibition on the estrogen and genistein activation of apo A-I gene expression. Hep G2 cells were transiently transfected with the plasmid containing the –256 to –148 region of the apo A-I promoter. Following transfection cells were pretreated for 30
min with inhibitors of the MAP kinase (PD 98059, 10 µM), PKA (myristoylated PKI 14-22 amide, 10 µM), and PKC (bisindolylmaleimide I, 1 µM) pathways. E₂ and genistein were then added and cell extracts were collected after 36 hours for the determination of luciferase activity (expressed as % of control untreated cells). Bars represent the mean (±SD) of 4 independent experiments with MAP kinase inhibitors and 2 independent experiments (in duplicate) with PKA and PKC inhibitors. * P<0.05 compared to respective control.