Regulation of human apolipoprotein A-I gene expression by equine estrogens

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Abstract **Estrogen replacement therapies, such as conjugated equine estrogen (CEE, Premarin®), reduce the risk of coronary heart disease among postmenopausal women. In the present study, a HepG2 stable cell line (HepG2/S) that harbors a luciferase reporter gene cassette with the human apolipoprotein A-I (apoA-I) promoter region was used to examine the activity of CEE components in modulating human apoA-I promoter activity. A number of estrogens modulated apoA-I promoter activity, with equilenin (Eqn) being the most potent. Eqn produced a 3-fold increase in apoA-I promoter activity and a similar increase in apoA-I mRNA without affecting its degradation rate. Nuclear runoff assays indicated that the transcription rate of the apoA-I gene was increased 2.5-fold in Eqn-treated cells. When HepG2/S cells were exposed to Eqn, apoA-I protein secretion increased by 80%, whereas the level of secreted apoA-II remained unchanged. Transient transfection studies with human apoA-I promoter constructs derived from pGL3-luciferase reporter plasmid were used to identify the *cis*-acting element involved in Eqn-mediated induction. The results demonstrated that the apoA-I electrophile/antioxidant response element (EpRE/ARE) might be responsible for the increase in apoA-I promoter activity by Eqn. Cotransfection experiments using estrogen receptor (ERα and/or ERβ) expression vectors have indicated that neither receptor can potentiate the Eqn-mediated induction of apoA-I promoter activity. In addition, mobility shift analysis using antibody against either ERα or ERβ cannot detect the presence of these receptors in the DNA-protein complex. The data indicate that Eqn can induce the promoter activity of the human apoA-I gene, leading to an increase in apoA-I mRNA levels and apoA-I protein secretion through an ER-independent pathway involving apoA-I EpRE/ARE.**—Zhang, X., J-J. Jiao, B. R. Bhavnani, and S-P. Tam. **Regulation of human apolipoprotein A-I gene expression by equine estrogens.** ***J. Lipid Res.*** **2001.** 42: **1789–1800.**

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Coronary heart disease (CHD) is the major cause of morbidity and mortality in postmenopausal women (1, 2). Elevated levels of LDL cholesterol and low levels of HDL cholesterol and apolipoprotein A-I (apoA-I) are important risk factors for atherogenesis (3–5). Observational studies have found that estrogen replacement therapy in postmenopausal women significantly lowers the risk of CHD (6, 7). A large prospective randomized study, the Postmenopausal Estrogen/Progestin Intervention (PEPI) trial, has found that estrogen increased levels of HDL cholesterol and lowered levels of LDL cholesterol (8). In addition, the PEPI trial has indicated that inclusion of progesterone or medroxyprogesterone acetate in treatment regimens attenuated the beneficial effects of estrogens (8). The PEPI investigators have now extended these observations and reported that postmenopausal estrogen therapy, with or without concomitant progestin regimens, resulted in lower plasma lipoprotein [a] levels (9). In contrast to these findings, the Heart and Estrogen/Progestin Replacement Study (HERS), of postmenopausal women with existing CHD, found that although the lipid levels improved and lipoprotein [a] concentrations decreased, hormone treatment did not decrease the risk of CHD or CHD-related deaths at least during the first year of therapy (10). However, a beneficial effect (decrease of CHD events) was observed over time. These investigators further recommend that women receiving hormone replacement therapy continue to take it for primary prevention (10). Several mechanisms by which estrogens exert their protective effect have been proposed (7, 11, 12).

Some of the cardioprotective effects of estrogen replacement therapy appear to involve changes in lipids

Abbreviations: 17α-E_2, 17α-estradiol; 17β-E_2, 17β-estradiol; δ8-E_1, δ-8-estrone; apoA-I, apolipoprotein A-I; CEE, conjugated equine estrogen; CHD, coronary heart disease; CTFBS, charcoal-treated FBS; DES, diethylstilbestrol; EpRE/ARE, electrophile/antioxidant response element; E_1, estrone; Eq, equilin; Eqn, equilenin; ERE, estrogen response element; HEGO, human estrogen receptor α expression vector (wild type); HEO, human estrogen receptor α expression vector (variant); HepG2, human hepatoma cell line G2; HepG2/S, HepG2 stable cell line; MCF-7, human breast adenocarcinoma cell line; TAM, 4-hydroxytamoxifen; TOT, *trans*-hydroxytamoxifen.

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and lipoproteins, such as an increase in levels of HDL cholesterol and apoA-I, and a decrease in LDL cholesterol (7, 13, 14). HDL cholesterol and its major protein, apoA-I, have a number of antiatherogenic properties, such as reverse cholesterol transport (15, 16), inhibition of Cu^{2+} or endothelial cell-induced oxidation of LDL cholesterol (17), and inhibition of the proatherogenic activity of oxidized LDL (18), which is thought to play an important role in atherogenesis (19). Furthermore, transgenic mice expressing high amounts of human apoA-I are protected from the development of fatty streak lesions after being given high fat diets (20), indicating that apoA-I can play an important role in the prevention of atherogenesis.

Because the levels of plasma apoA-I are proportional to the level of apoA-I mRNA in vivo, the factors influencing levels of apoA-I may be mediated through the level of apoA-I gene expression. The *cis*- and *trans*-acting elements participating in apoA-I gene transcription have been extensively studied (21–25). The DNA region located between nucleotide base pair −256 and −41 upstream from the transcription initiation site of the human apoA-I gene contains the regulatory elements necessary for maximal expression in human hepatoma cell line G2 (HepG2) (23). The mechanisms by which estrogens exert their cardioprotective effects are not known. Exposure of HepG2 cells to 17β-estradiol (17β-E_2) results in changes in the apoA-I transcription rate (26); these changes are mediated by interaction of estrogen receptor α (ERα) and other transcription factors with the apoA-I promoter region (25). However, the human apoA-I promoter does not contain the classic estrogen response element (ERE), suggesting that the induction of apoA-I transcription may act through other *cis*-acting elements. Estrogens and some of their metabolites can also act as electrophile/redox-active factors that may possibly regulate genes through electrophile/antioxidant response elements (EpRE/ARE) (27–29). The presence of EpRE/ARE in the promoter region of human apoA-I gene has been demonstrated and compounds such as gramoxone and dimethyl sulfoxide appear to regulate the expression of human apoA-I gene via these response elements (24, 30).

Estrogens exhibit various effects (31–35) and although they are able to increase the levels of HDL cholesterol and apoA-I (7, 14), the mechanism by which estrogens influence the expression of apoA-I gene has not been fully described. In the present investigation, we examined the effects of various equine estrogens on apoA-I promoter activity, using a stable HepG2 cell line (HepG2/S) that permanently harbors a gene cassette containing 256 bp of the 5′-flanking region of a human apoA-I gene fused to the luciferase (luc) gene. We also studied the potential involvement of ER in the regulation of human apoA-I gene expression. The estrogens tested were the 10 biologically active components of conjugated equine estrogen (CEE) (Premarin®; Wyeth-Ayerst, Philadelphia, PA), the most commonly used estrogen for estrogen and hormone replacement therapy in postmenopausal women (34).

MATERIALS AND METHODS

Oligonucleotides

The following oligonucleotides and their complementary strands were synthesized [Beckman (Fullerton, CA) Oligo 1000 DNA synthesizer]: apoA-I(−41/+7), 5′-CTGAGATGGCTGCA GACATAAATAGGCCCTGCAAGAGCTGGCTGCTTAGAGACT AAGCTT-3′; apoA-I-EpRE/ARE, 5′-GAGCTCTTGCCCAGCCCC AGGGACAGAGCTGATCGCTAGCC-3′; apoA-I-mEpRE/mARE, 5′-AGCTCTTGCCCAGCCCCATTTGAGTGTATGATCGCTAG CC-3′; *Xenopus vitellogenin* A_2 ERE, 5′-GCTCCAAAGTCAGGTCA CAGTGACCTGATCGCTAGCC-3′.

Cell culture

The HepG2 cell line and the human breast adenocarcinoma cell line (MCF-7) were obtained from the American Type Culture Collection (Rockville, MD). HepG2 cells were cultured in MEM supplemented with 10% FBS as described previously (35). HepG2 cells that were stably transfected with the apoA-I promoter and luciferase reporter gene cassette (HepG2/S) were also grown in MEM supplemented with 10% FBS (36). Seven days before the experiments, cells were grown in phenol red-free and estrogen-depleted medium supplemented with 10% charcoal-treated FBS (CTFBS). MCF-7 cells were maintained in MEM plus phenol red supplemented with nonessential amino acids, 10% FBS, and 10 nM estradiol. Two weeks before the experiments, MCF-7 cells were grown for 2 days in MEM containing phenol red and 5% CTFBS. Cells were then grown in phenol red-free MEM supplemented with nonessential amino acids and 5% CTFBS for 7 days. Cells used in all experiments were in the log phase of growth and were at about 60% of confluence. Cell viability was routinely monitored by trypan blue exclusion and lactate dehydrogenase leakage as describe previously (37). In all experiments the number of dead cells never exceeded 5% of the total number of cells.

Plasmids

The pGL3-apoA-I(−41/+7)-luc construct was created by inserting a double-stranded oligonucleotide that contained *Xho*I and *Hin*dIII restriction sites at the 5′ end and 3′ end of the DNA element, respectively, and DNA sequence corresponding to base pairs −41 to +7 of the human apoA-I promoter (5′-TGGCTGCA GACATAAATAGGCCCTGCAAGAGCTGGCTGCTTAGAGACT-3′) into *Xho*I and *Hin*dIII sites in pGL3-Basic (Promega, Madison, WI). The pGL3-apoA-I-EpRE/ARE(−41/+7)-luc construct was generated by cloning a double-stranded oligonucleotide that had *Sac*I and *Nhe*I restriction sites at the 5′ end and 3′ end, respectively, and DNA sequence corresponding to base pairs −153 to −126 of the human apoA-I promoter (apoA-I-EpRE/ARE, 5′-TTGCCCAGCCCCAGGGACAGAGCTGATC-3′) into their respective sites in the pGL3-apoA-I(−41/+7)-luc construct. The pGL3-apoA-I-mEpRE/mARE(−41/+7) construct was created by inserting a double-stranded oligonucleotide that contained *Sac*I and *Nhe*I restriction sites at the 5′ end and 3′ end, respectively, and a mutated sequence related to DNA sequence corresponding to base pairs −153 to −126 of the human apoA-I promoter (apoA-I-mEpRE/mARE, 5′-TTGCCCAGCCCCATTTGAGTG TATGATC-3′) into their respective sites in the pGL3-apoA-I(−41/+7)-luc construct. The pGL3-apoA-I(−249/+7)-luc construct was generated by PCR by using the pGL2-apoA-I(−249/+1)-luc vector as described previously (37). Primer A (5′-GCTAGC CCGGGAGACCTGCAAGCCTGCA-3′) contained a sequence corresponding to the coding strand sequence of the apoA-I promoter from base pair −250 to base pair −228 and restriction sites of *Nhe*I and *Xma*I/*Sma*I. Primer B (5′-CGCAGATCTAGT CACTAAGCAGCCAGCTCTTGCAGGGCCT-3′) contained a sequence corresponding to the noncoding strand sequence of the

apoA-I promoter from base pair −24 to base pair +7 and the *Bgl*II restriction site. Amplification was carried out in an Eppendorf MicroCycler E for 30 cycles according to a procedure described previously (37). The PCR-generated fragment was digested with *Nhe*I and *Bgl*II, and the resulting DNA fragment was gel purified. The purified DNA fragment was cloned into the *Nhe*I and *Bgl*II sites of pGL3-Basic (Promega) and used to transform competent DH5α cells. The procedure for generating pGL3-apoA-I(−249/+7)mEpRE/mARE-luc was similar to the construction of pGL3-apoA-I(−249/+7), except that pGL2-apoA-I(−249/+1)mEpRE/mARE-luc was used as the PCR template instead. The pGL3-apoA-I(−117/+7)-luc construct was created by deleting a small DNA fragment from the pGL3-apoA-I(−249/+7)-luc construct, using restriction enzymes *Sma*I and *Afl*II followed by religation of the larger fragment. The pGL3-tk-luc construct was generated by cloning a DNA fragment that was digested from the pGL2-tk-luc construct at *Sma*I and *Hin*dIII sites into their respective sites in pGL3-Basic (Promega) (37). To generate pGL3-apoA-I-EpRE/ARE-tk-luc and pGL3-apoA-I-mEpRE/mARE-luc constructs, the double-stranded apoA-I-EpRE/ARE and apoA-I-mEpRE/mARE oligonucleotides that contained *Sac*I and *Nhe*I sites at the 5′ end and 3′ end of the DNA fragments, respectively, were cloned into their respective sites in the pGL3-tk-luc construct. pGL3-vitERE-luc was constructed by inserting double-stranded *X. vitellogenin* A_2 ERE oligonucleotides that contained *Sac*I and *Nhe*I sites at the 5′ end and 3′ end of the DNA fragments, respectively. These fragments were cloned into their respective sites in the pGL3-tk-luc construct. The human ERα expression vectors HEGO (wild-type ERα) and HEO (ERα variant), and the human ERβ expression vector, were kindly provided by T. S. Scanlan (Department of Pharmaceutical Chemistry, University of California, San Francisco, CA).

Construction of pEBV7-apoA-I(−249/+7)-luc expression vector and establishment of stable cell line HepG2/S

The pEBV7-apoA-I(−249/+7)-luc expression vector was created by using pEBV7 and pGL3-apoA-I(−249/+7)-luc constructs according to the procedure described previously (37). HepG2/S cell lines were prepared by transfecting the pEBV7-apoA-I(−249/+7)-luc expression vector that was previously linearized with *Sac*I into HepG2 cells by a standard calcium phosphate precipitation method. After overnight incubation with the calcium phosphate-DNA precipitate, the cells were washed twice with PBS and maintained in MEM plus 10% FBS for 48 h. Stable expression cells were selected by resistance to hygromycin B (300 μg/ml) (Boehringer Mannheim, Indianapolis, IN). After 2 weeks, the isolated colonies of surviving cells were selected and subcultured at low density and exposed to another round of clonal isolation. The HepG2/S cell line was used in these experiments and the cells were propagated over 8 months without losing luciferase activity or hygromycin resistance.

Screening procedures for various estrogens

The stably transfected HepG2/S cell line was routinely maintained as monolayers in 75-cm^2 flasks. When the cells reached 70% confluence, they were subcultured in 24-well tissue culture plates and grown as monolayer in phenol red-free MEM supplemented with 10% CTFBS. After 2 days, the cells were treated with either vehicle (ethanol) or estrogens at various concentrations and time periods (indicated in the figure legends). Treated cells were then washed with PBS three times; the cells were lysed with a passive-lysis buffer (Promega). Firefly luciferase activity was determined by using an EG&G Berthold (Salem, MA) microplate luminometer LB96V. The protein concentration of luciferase reaction solution was determined with a Bio-Rad (Hercules, CA) protein assay kit and the luciferase activity was normalized for protein concentration.

RNA isolation and Northern analysis

Total cellular RNA was isolated from HepG2/S cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method as described previously (36). For Northern analysis, 20 μg of total RNA was denatured by treatment with glyoxal and subjected to electrophoresis on a 1.5% agarose gel, and then transferred to Zeta-probe GT membrane (Bio-Rad) as described previously (36). The blots were then prehybridized and hybridized with ^{32}P-labeled human apoA-I cDNA. The relative intensities of the hybridized signals and ethidium bromide-stained 28S and 18S rRNA were quantitated by PhosphorImaging analysis software (Molecular Dynamics, Sunnyvale, CA). All results were then normalized to the 28S and 18 S rRNA signals.

Immunoprecipitation assays

Preswollen protein G-agarose (Boehringer Mannheim) was washed twice with PBS containing 0.5% Triton and 0.2% SDS and recovered by centrifugation for 20 s at 12,000 *g*. Monolayers of HepG2/S cells were washed twice with 10 ml of serum-free DMEM and incubated with 5 ml of methionine-free DMEM (without serum) supplemented with [^{35}S]methionine (500 μCi, 1,100 Ci/mmol) in the absence or presence of estrogens. At the indicated times, the medium was collected and transferred to a tube containing a protease inhibitor mixture and centrifuged at 1,500 *g* for 5 min to remove any dead cells and debris as described previously (30). Before immunoprecipitation, the supernatants were incubated with a protein G-agarose suspension overnight at 4°C. The mixtures were then centrifuged for 20 s at 12,000 *g* and the supernatants were transferred to a fresh tube. An aliquot (20 μl) of supernatant was used for determination of protein concentration. Another aliquot (100 μl) of the supernatant was used for immunoprecipitation of human apoA-I, apoE, and apoA-II. Specific polyclonal antibodies against these apolipoproteins (Boehringer Mannheim) were used for immunoprecipitation. The reaction mixtures were incubated overnight at 4°C and the immunocomplexes were precipitated by further incubation with prewashed protein G-agarose beads overnight at 4°C. Immunoprecipitates were collected by centrifugation and washed three times with 200 μl of PBS containing 0.5% Triton X-100 and 0.2% SDS. The final washed precipitates were resuspended in 100 μl of SDS-gel loading buffer and boiled for 5 min. After centrifugation, an aliquot of each supernatant was taken for scintillation counting and another portion was subjected to 12% SDS-polyacrylamide gel electrophoresis. To determine the radioactivity in the precipitated proteins, the relevant band was localized after fluorography, cut out of the dry gel, digested, and counted as described previously (30).

Preparation of nuclear extracts and gel mobility shift and supershift assays

Nuclear extract preparation and gel mobility shift assays have been described previously (37). For supershift assays, nuclear extracts were preincubated with specific antibody against human ERα or ERβ (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C from 45 min to overnight and then incubated with ^{32}P-labeled double-stranded oligonucleotides for 30 min. The resulting complex and free DNA were resolved on a 5% nondenaturing polyacrylamide gel. Gels were vacuum dried on 3MM Whatman (Clifton, NJ) paper and exposed to a PhosphorImage catching screen and then analyzed by a PhosphorImaging system (Molecular Dynamics).

Nuclear runoff transcription assay

Nuclei were prepared according to the method of Bartalena and co-workers (38). An in vitro nuclear runoff transcription assay was carried out as described (38) with minor modifications

(39). The procedure for nuclear runoff experiments has been described in detail previously (37).

Transient transfection and luciferase assays

HepG2 cells were transfected in 12-well plates by using a 1-μg/well concentration of reporter gene and 20 ng of ER expression vector. MCF-7 cells were transfected in six-well plates by using 2 μg of reporter gene per well. In all transfections, an internal control plasmid, pRL-TK or pRL-null, was included in order to correct for differences in transfection and harvesting efficiency. Transient DNA transfections were performed according to the FuGENE 6™ procedure (Boehringer Mannheim). The cells were then cultured in estrogen-depleted MEM in the presence of either vehicle (ethanol) or estrogens at various concentrations and periods of time, as indicated in the figure legends. Transfected cells were washed three times in PBS and then harvested with passive-lysis buffer. Both firefly luciferase and *Renilla* luciferase activities were measured with an EG&G Berthold microplate luminometer LB96V, according to the procedures of the dual-luciferase assay (Promega).

Data analysis

Each incubation was performed four times and the mean value for each of the estrogens on a plate was expressed as a percentage/fold of the change relative to the controls on the same plate. For each of the estrogens, six values were obtained in separate experiments on different days. Statistical analyses were performed with the SAS statistical package and GraphPad 2.01 on a PC computer. An unpaired *t*-test or one-way ANOVA was used to evaluate the effect of compounds on the percentage/fold changes from the control samples. Dunnett's test was used to compare each treatment group with the control group when the percentage/fold change was not monotonic across the treatments. The Student-Newman-Keuls test was used to compare pairs of all treatment groups.

RESULTS

Establishment of HepG2/S cell line

Changes of apoA-I gene transcription can be achieved in hepatoma cells by environmental stimuli such as with certain compounds; these changes can be determined by monitoring luciferase or chloramphenicol acetyltransferase reporter constructs with an upstream apoA-I promoter in these cells. However, all of these experiments were carried out under transient transfection conditions, in which the consideration of chromatin structure was ignored. Furthermore, the whole process was labor intensive and the results were influenced by the transfection efficiency among the experiments. To mimic in vivo conditions more closely and facilitate the process of screening various compounds, we established a HepG2 cell line stably transfected with a luciferase construct, which harbors a luciferase reporter cassette reporter with an upstream human apoA-I promoter region containing base pairs −249 to +7 of 5′-flanking sequence. The construct was linearized before being transfected into the parent cell line. Therefore, the construct had been integrated in the chromosome. This permanently transfected cell line was named HepG2/S.

Effects of estrogens on human apoA-I promoter activity

The effects of various estrogens on human apoA-I promoter activity were determined in HepG2/S cells. Although these estrogens are related structurally, differential effects on apoA-I gene transcription in HepG2/S cells were observed (**Fig. 1**). The estrogens equilin (Eq), 17β-equilin (17β-Eq), equilenin (Eqn), and 17β-equilenin (17β-Eqn) significantly ($P < 0.05$) increased the luciferase activity of this apoA-I promoter-containing cassette. Estrone (E_1), 17β-estradiol (17β-E_2), 17α-estradiol (17α-E_2), 17α-equilin (17α-Eq), 17α-equilenin (17α-Eqn), and δ-8-estrone (δ8-E_1) had no significant effect. Of the four active estrogens, Eqn was the most potent and it stimulated the apoA-I promoter activity 2- to 3-fold (Fig. 1) compared with the vehicle-treated control sample. The relative potencies had the following order: Eqn > 17β-Eqn > Eq = 17β-Eq.

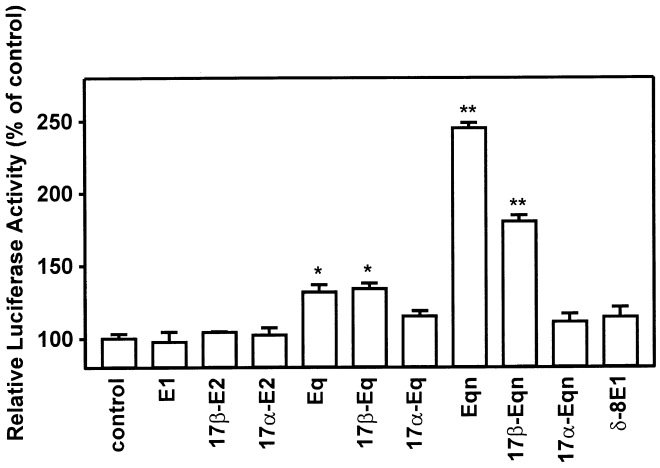

Fig. 1. Effects of various estrogens on the promoter activity of the human apoA-I gene in stably transfected HepG2/S cells. Cells were grown as a monolayer in a 24-well tissue culture plate in MEM without phenol red and supplemented with 10% CTFBS. When the cell density reached 60% confluency, cells were then treated with the indicated compounds at concentration of 5 μM for 24 h. E_1, Estrone; δ8-E_1, δ-8-estrone; 17β-E_2, 17β-estradiol; 17α-E_2, 17α-estradiol; Eq, equilin; Eqn, equilenin. Luciferase activity of the cells treated with the drug vehicle (ethanol) was arbitrarily set at 100%. Values represent the means ± SEM of six independent experiments. Each experiment was performed in quadruplicate. One-way ANOVA was used followed by Dunnett's test or the Student-Newman-Keuls test as post test for statistical analysis. * $P < 0.05$; ** $P < 0.01$.

Pharmacokinetics of stimulation of apoA-I promoter activity by Eqn

Because Eqn was the most potent stimulator of apoA-I promoter activity; all further work was carried out with this estrogen only. The effect of Eqn on the apoA-I transcription rate was determined (**Fig. 2**). apoA-I promoter activity increased with increasing concentration of Eqn and the maximum stimulatory effect of Eqn was observed at 10 μM (Fig. 2A). This effect was also time dependent and maximal activity at all concentrations was seen at approximately 24 h (Fig. 2B). After 2 h of exposure to Eqn, apoA-I promoter activity increased by 20% over the control value; between 16 and 24 h it increased by approximately 300%. After 24 h, the stimulatory effect gradually decreased but remained above the control value for up to 48 h.

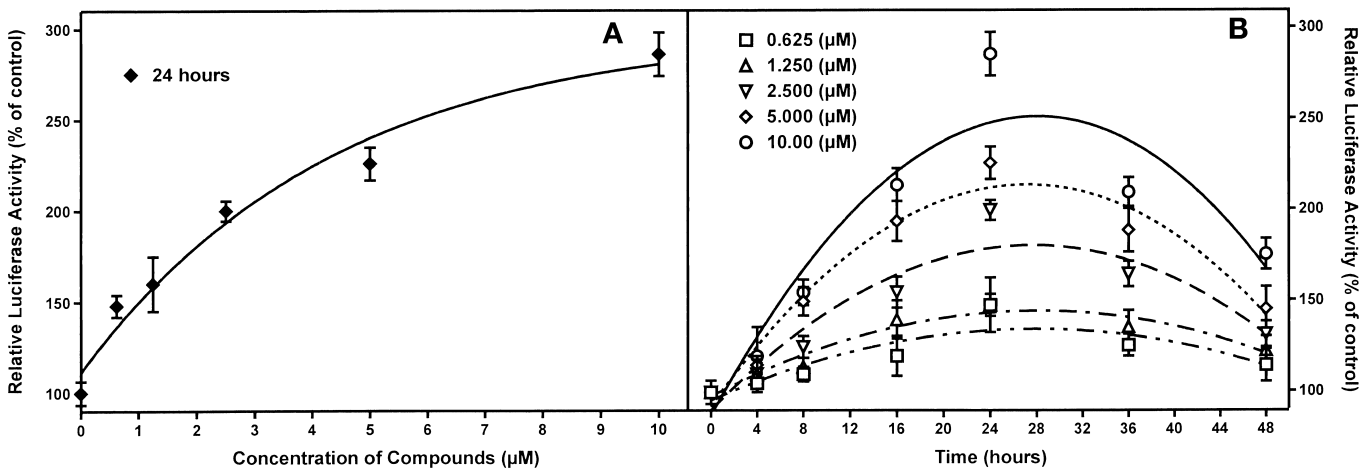

Fig. 2. Dose-response and kinetic studies of Eqn-mediated induction of luciferase activity in HepG2/S cells. Cells were treated with various concentrations of Eqn and harvested at the indicated time points. Cytosolic extracts were assayed for luciferase activities and protein concentrations. The normalized value of the cells treated with ethanol (65,893 ± 432 RLU/μg of protein) was arbitrarily set at 100%. Results represent the means ± SEM of six independent experiments. A: Concentration-dependent profile. B: Time-dependent profile.

Effect of Eqn on apoA-I mRNA levels and apolipoprotein levels

The apoA-I mRNA level after Eqn treatment was monitored by Northern blot analysis. Exposure of HepG2/S cells to Eqn resulted in a 2- to 3-fold increase in apoA-I mRNA levels (**Fig. 3**). To determine whether inhibition of RNA synthesis influences the induction of apoA-I mRNA levels, HepG2/S cells were treated both in the absence and presence of increasing amounts of actinomycin D for 30 min before the addition of Eqn (10 μM). Actinomycin D at concentrations ⩾1 μg/ml inhibited the increase in apoA-I mRNA levels when the cells were treated with Eqn (data not shown). To determine whether mRNA stabilization is responsible for the observed induction of apoA-I mRNA levels caused by Eqn, the half-lives for apoA-I mRNA were measured in the presence of actinomycin D (1 μg/ml) with and without Eqn treatment. HepG2/S cells were pretreated with Eqn (10 μM) or ethanol (drug vehicle) for 24 h. Cells were then cultured for additional periods ranging from 0 to 24 h in the presence of actinomycin D. The degradation rate of apoA-I mRNAs after the addition of actinomycin D with or without Eqn was approximately the same (Fig. 3). The apparent half-lives were 14.6 ± 0.3 and 14.8 ± 0.2 h, respectively; these results were not significantly different.

The de novo synthesis of apolipoproteins by HepG2/S cells after exposure of the cells to Eqn was determined by measuring the incorporation of radiolabeled methionine into apolipoproteins secreted into the medium. The rates of accumulation of secreted apoA-I, apoA-II, and apoE were determined at various time intervals after Eqn treatment. ApoA-I and apoE production increased by 80% and 50%, respectively, in Eqn-treated cells compared with vehicle-treated cells (**Fig. 4**). In contrast, no significant effect of Eqn on apoA-II secretion was observed. These results indicated that the effect of Eqn on production of apoA-I and apoE was quite specific. Eqn may not stimulate all major types of apolipoproteins found in HDL cholesterol.

Effect of Eqn on the rate of transcription of the human apoA-I gene

Nuclear runoff experiments were carried out to determine whether increased rates of transcription were responsible for the induction of apoA-I mRNA levels caused by Eqn treatment. The transcription rate of the apoA-I gene was measured by using isolated nuclei from HepG2/S cells cultured in the absence or presence of Eqn. The effect of Eqn on apoA-I transcription rates is illustrated in **Fig. 5**. The rate of transcription of the human apoA-I gene increased approximately 2.5-fold between 16 and 24 h after stimulation.

Characterization of the human apoA-I promoter region responsible for Eqn effect

The region involved in the transcriptional upregulation of the apoA-I gene by Eqn was further investigated by introducing various deletions and mutations in the base pair −249 to +7 region of the apoA-I promoter. These constructs were transiently transfected into HepG2 cells and the cells were then treated with vehicle or Eqn. The base pair −41 to +7 region of the human apoA-I gene, which contains minimal promoter activity for the human apoA-I gene, was used as the control vector. The luciferase activity of the pGL3-apoA-I(−41/+7)-luc construct in the cells treated with vehicle alone was arbitrarily set as 1 and the treatment with Eqn had no effect on this construct (**Fig. 6A**). In contrast, the basal promoter activity of the pGL3-apoA-I(−249/+7)-luc construct was about 70-fold higher than that of the pGL3-apoA-I(−41/+7)-luc construct. This activity was significantly induced 2.5-fold in the presence of Eqn ($P < 0.01$) (Fig. 6B). These results indicated that the region responsible for induction of Eqn was located between base pairs −249 and −41 of the apoA-I promoter. Although the basal promoter activity of pGL3-apoA-I(−117/+7)-luc (5′-deletion construct) was nearly 3-fold higher than that of the minimal promoter construct apoA-I(−41/+7) (Fig. 6C), Eqn had no effect on the promoter

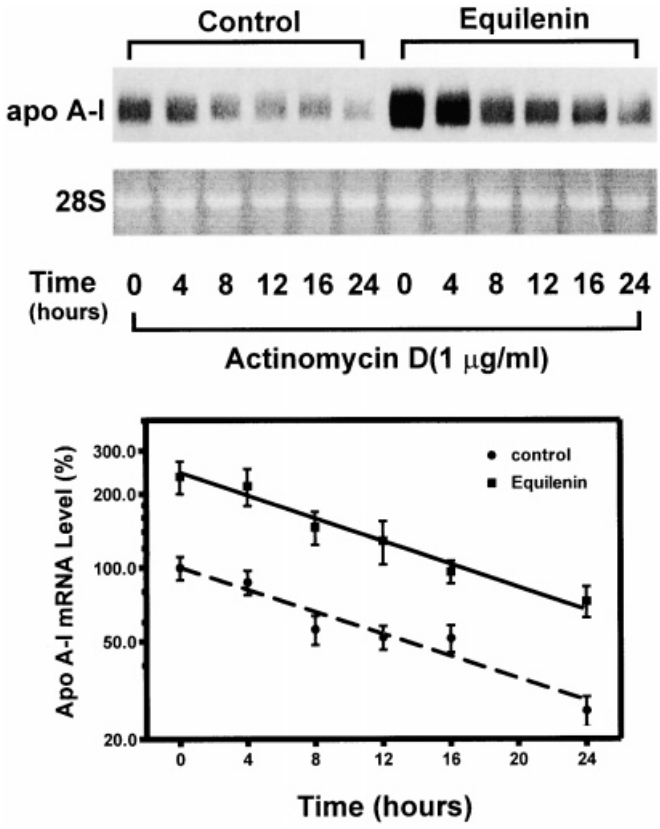

Fig. 3. Effect of Eqn on the level and half-life of apoA-I mRNA. Cells were cultured without or with Eqn (10 μM) for 24 h in MEM minus phenol red and supplemented with 10% CTFBS. Cells were then cultured for additional periods ranging from 0 to 24 h in the presence of actinomycin D (1 μg/ml). Total RNA was isolated at the indicated time points and then was separated by 1.5% agarose gel electrophoresis. ApoA-I mRNA was determined by Northern analysis (top). The relative intensities of the hybridized apoA-I mRNA signal and ethidium bromide-stained 28S rRNA were quantitated by PhosphorImager analyses. All results were normalized with ethidium bromide-stained 28S rRNA to correct for loading variations. The normalized apoA-I mRNA levels, in arbitrary units, are presented on a log scale. The half-lives for the apoA-I mRNAs were obtained from the first-order decay calculation. Solid circles and solid squares represent RNA samples isolated from cells treated without and with Eqn, respectively. Results represent means ± SEM of four experiments. An unpaired *t*-test was used to compare the half-life of the Eqn-treated group with that of the drug-vehicle-treated group.

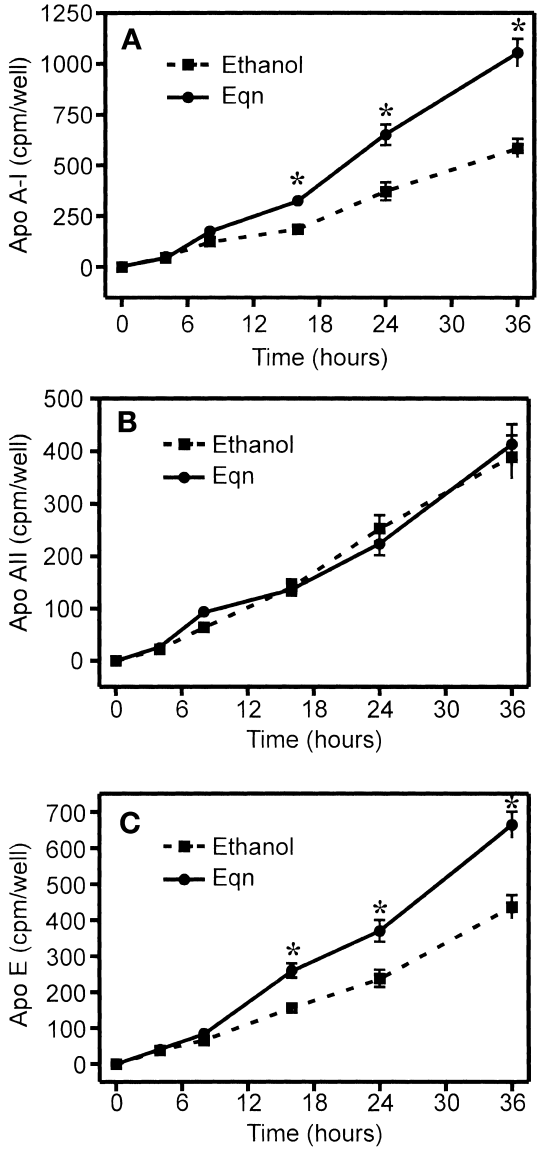

Fig. 4. Effect of Eqn on the kinetics of apoA-I, apoA-II, and apoE secretion by HepG2/S cells. HepG2/S cells were grown in 6-well tissue culture plates (approximately 10^6 cells/well). Cells were washed twice with 5 ml of PBS and cultured in serum- and methionine-free MEM plus [^{35}S]methionine (1,100 Ci/ml) in the presence of drug vehicle (control) or 10 μM Eqn for 36 h. At the time points indicated, medium was removed from the cells. ApoA-I (A), apoA-II (B), and apoE (C) were immunoprecipitated from the medium. The results represent means ± SEM of four experiments. An unpaired *t*-test was used to evaluate changes between the Eqn-treated group and the control group at each time point. * $P < 0.01$.

activity of this construct (Fig. 6C). These data suggested that the region downstream of base pair −117 of the apoA-I promoter contributed little to the basal promoter activity of the apoA-I gene in HepG2 cells.

Furthermore, the elements between base pairs −249 and −117 of the apoA-I promoter appear to play a crucial role in the response to Eqn-mediated transcriptional regulation of the apoA-I gene. This region contains several important *cis* elements essential for the regulation of the apoA-I gene, including the apoA-I antioxidant response element (apoA-I-EpRE/ARE) located between base pairs −153 and −126 upstream from a transcription initiation site of the human apoA-I gene. The role of apoA-I-EpRE/ARE in the regulation of the apoA-I gene by Eqn was investigated by using the apoA-I-EpRE/ARE mutation construct [pGL3-apoA-I(−249/+7)mEpRE/mARE-luc]. The luciferase activity of this construct was 7-fold lower compared with the pGL3-apoA-I(−249/+7)-luc construct (Fig. 6D). Treatment with Eqn had no effect on the luciferase activity of this mutant construct (Fig. 6D). A construct with one copy of apoA-I-EpRE/ARE inserted upstream of apoA-I(−41/+7)-luc displayed a 2- to 3-fold increase in luciferase activity relative to the pGL3-apoA-I(−41/+7)-luc construct; this activity was further increased 2-fold on treatment of Eqn (Fig. 6E).

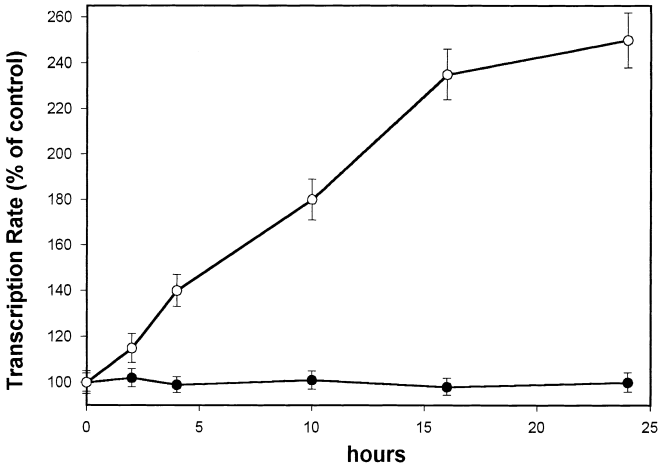

Fig. 5. Time course of transcription rates of the apoA-I gene in HepG2/S cells cultured in the absence (solid circles) or presence (open circles) of Eqn. Transcription rates were determined by nuclear runoff assays using nuclei isolated from HepG2/S cells cultured in the absence or presence of Eqn (10 μM) as described in Materials and Methods. Nuclei ($2–3 \times 10^7$) were isolated at 0, 2, 4, 10, 16, and 24 h. Hybridization of the newly synthesized [^{32}P]RNA to plasmids containing the apoA-I insert was carried out in triplicate. Nonspecific hybridization to each filter was measured by performing transcription with labeled pGEM 3Z control vector. Filters were washed extensively and radioactivity bound was determined by liquid scintillation counting. Relative transcription rates were calculated as parts per million (ppm) per filter = [(cpm/filter) − background cpm] divided by the amount of [^{32}P]RNA used in hybridization (input counts). These numbers were then normalized for hybridization efficiency as measured by binding of known amounts of ^{3}H-labeled riboprobes added to the hybridization mixture. This was then corrected for the size of the specific gene: ppm/gene = [(ppm/filter)/% hybridization] × (gene size/cDNA insert size). Sizes of the apoA-I gene and apoA-I cDNA fragments were 2.0 and 0.6 kb, respectively. Results represent the means ± SE expressed in percentage of the 0-h time point of four independent experiments.

However, the mutated version of this construct [pGL3-apoA-I-mEpRE/mARE(−41/+7)-luc] showed no change in luciferase activity relative to that of the pGL3-apoA-I(−41/+7)-luc constructs, nor did it respond to the addition of Eqn (Fig. 6F). Taken together, these data suggested that the integrity of the apoA-I-EpRE/ARE element not only plays an important role in the maintenance of basal promoter activity of the human apoA-I gene but also appears to be the key contributing factor responsible for Eqn-mediated transcriptional regulation of the human apoA-I gene.

Effect of estrogens and antiestrogens on the induction of apoA-I promoter activity by Eqn

To determine whether the Eqn-mediated induction of the apoA-I promoter can be further enhanced by estrogen (additive effect), or inhibited by antiestrogens, we tested the effect of 17β-E_2 and an antagonist, 4-hydroxytamoxifen (TAM), on apoA-I promoter activity in HepG2/S cells and HepG2 cells transfected with the apoA-I-EpRE/ARE-luc construct. Addition of these compounds together with Eqn did not further enhance or inhibit the Eqn-mediated induction of the apoA-I promoter, nor did either 17β-E_2 or TAM alone alter the apoA-I promoter activity (**Fig. 7**). These data indicated that the effect of Eqn on induction of the apoA-I promoter is specific and may not be mediated via the ER(s).

Regulation of apoA-I gene by Eqn is independent of ER

Electrophoretic mobility shift and supershift assays were performed to examine whether the Eqn-mediated transcriptional activation involved the binding of ER to the apoA-I-EpRE/ARE fragment. Nuclear proteins extracted from Eqn-treated HepG2 cells were incubated with radiolabeled apoA-I-EpRE/ARE fragment or vitellogenin estrogen response element (ERE) fragment. Using nuclear extract isolated from HepG2 cells, the DNA-binding activity interaction with radiolabeled apoA-I-EpRE/ARE was greater than that with radiolabeled ERE (**Fig. 8**). Although no supershift was observed with the addition of specific antibodies against ERα and ERβ, it appeared that addition of the antibodies resulted in decreased intensity of the retarded band (Fig. 8, lane 1 vs. lanes 2 and 3). These results strongly suggested that ERα and ERβ were not present in the DNA-protein complex. However, the possibility that the antibodies could bind specifically to ER and block the formation of the complex, without generating a supershift band, could not be ruled out completely. As shown in Fig. 8, nuclear proteins extracted from Eqn-treated MCF-7 cells bound efficiently to radiolabeled ERE to form a DNA-protein complex. This complex was supershifted by the specific antibodies against ERα and ERβ. In contrast, a weak complex resulted from the interaction of MCF-7 extracts with radiolabeled apoA-I-EpRE/ARE but did not supershift on addition of specific antibodies against ERα and ERβ (Fig. 8).

To rule out the involvement of ER in the regulation of apoA-I gene by Eqn, several transient transfection and cotransfection experiments were performed (**Fig. 9**). A significant increase in transcriptional activity of apoA-I(−249/+7)-luc and apoA-I(EpRE/ARE)tk-luc constructs was observed in HepG2/S (stable) cells and HepG2 cells, respectively, on treatment with Eqn (Fig. 9A). Cells transfected with ER expression vectors did not show an increase in luciferase activity of pGL3-apoA-I(−249/+7)-luc or apoA-I(EpRE/ARE)tk-luc constructs in the absence of Eqn (Fig. 9A). Cotransfection with both ERα and ERβ expression vectors did not change the response to Eqn treatment in both HepG2/S cells and HepG2 cells transfected with the apoA-I(EpRE/ARE)tk-luc construct (Fig. 9A). These results indicated that ER were not necessary in the regulation of the apoA-I gene by Eqn.

This was further evident by the transient transfection studies of ER-positive breast cancer cell line MCF-7. In contrast to the observations with HepG2/S and HepG2 cells, Eqn-mediated transcriptional activation was not observed with Eqn at 1 and 10 μM when the pGL3-apoA-I(−249+7)-luc construct or the pGL3-apoA-I(EpRE/ARE)tk-luc construct was transfected into MCF-7 cells (Fig. 9B). These data provided further evidence that ER were not required for Eqn-mediated transcriptional regulation.

Similarly, 17β-E_2- and DES-mediated transcriptional activation was not observed with either of these constructs,

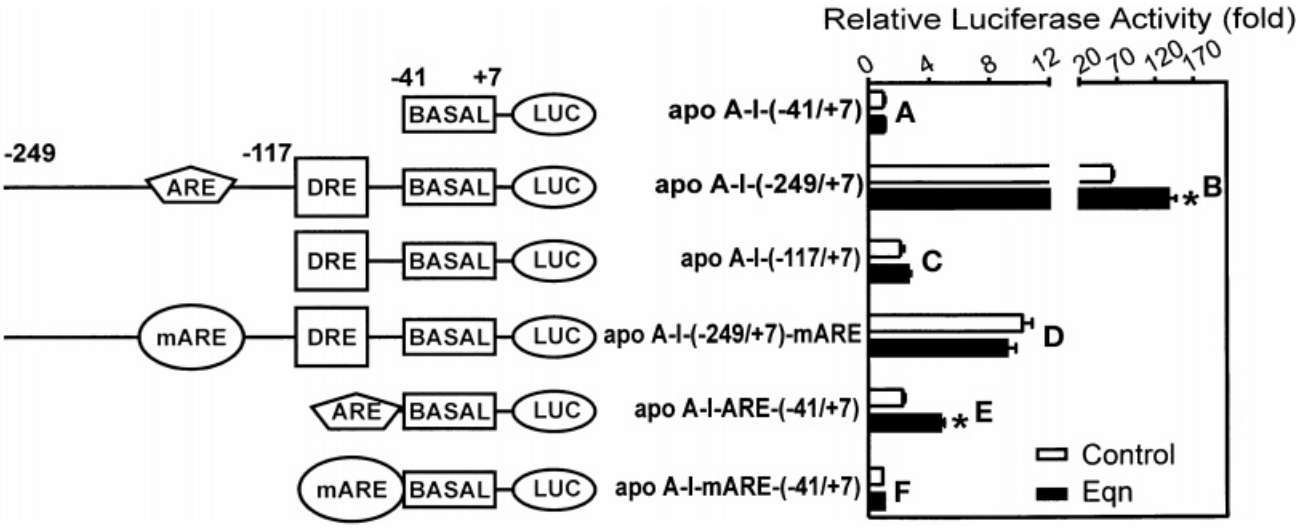

Fig. 6. Effect of Eqn on luciferase activity in various constructs of the apoA-I gene promoter in transfected HepG2 cells. Promoter constructs were prepared as described in Materials and Methods. Plasmid constructs contained the luciferase reporter gene under the control of the human apoA-I minimal basal promoter (−41 to +7 bp). Mutant constructs contained nucleotide substitutions that replace the apoA-I ARE. Freshly subcultured HepG2 cells on 24-well culture plates were transfected with each construct (0.5 μg/well) and with pRL-tk or pRL-null (10 ng/well), which was used to correct for variations in transfection efficiency. The dual-luciferase activities were assayed after cells were incubated with Eqn (10 μM) or drug vehicle (ethanol, <0.1%) for 24 h. Relative luciferase activity values represent the firefly luciferase activity versus the *Renilla* luciferase activity ratios relative to that of the pGL3-apoA-I(−41/+7) construct with drug vehicle incubation (arbitrarily defined as 1). The results represent means ± SEM of six independent experiments carried out in quadruplicate. One-way ANOVA was used followed by Dunnett's test or the Student-Newman-Keuls test as posttest for statistical analysis. * $P < 0.01$, unpaired *t*-test, versus control group for the same construct.

possibly because of the absence of ERE in the base pair −249 to +7 region upstream from the transcriptional initial site of the apoA-I promoter. These data suggested that other auxiliary transcriptional factor(s) might be involved in the regulation of the human apoA-I gene. In contrast, Eqn at a concentration of 1 μM was able to increase luciferase activities of both pGL3-vit(ERE)tk-luc and pS2 constructs in MCF-7 cells. However, the enhancing effect of Eqn was not as strong as compared with 17β-E_2 or DES (Fig. 9B).

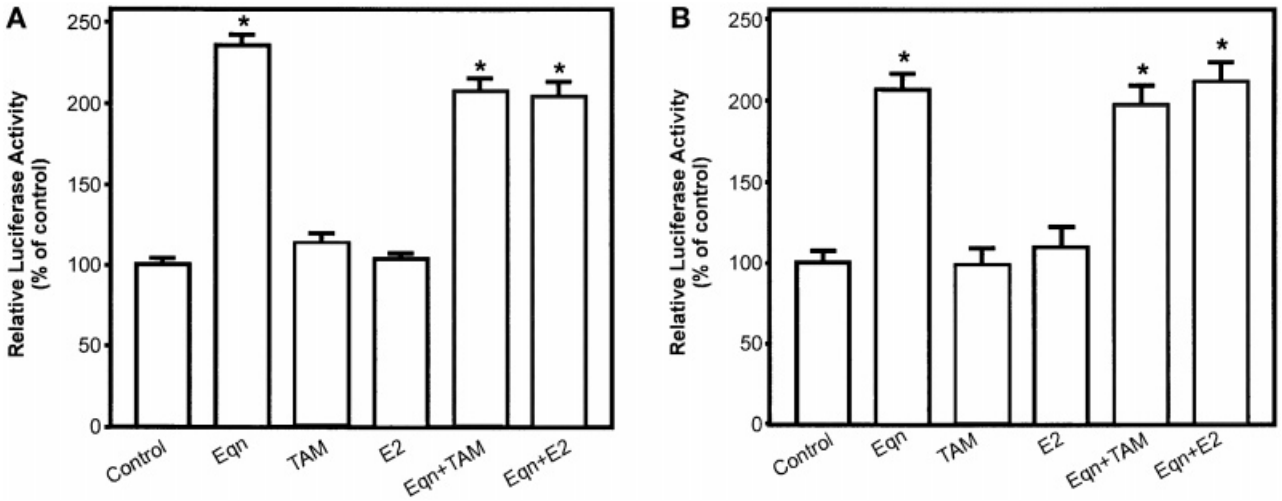

Fig. 7. Effect of estrogens and antiestrogens on Eqn-mediated transcriptional regulation of the apoA-I promoter. A: HepG2/S cells were treated with ethanol (control), Eqn (10 μM), 4-hydroxytamoxifen (TAM, 100 nM), estradiol (E_2, 10 nM), or a combination of Eqn with either tamoxifen or estradiol for 24 h. Firefly luciferase activity was measured and normalized with protein amounts. Values are expressed as a percentage of control. B: HepG2 cells were transfected with the apoA-I(EpRE/ARE)tk-luc construct. Sixteen hours after transfection, the medium was replaced with fresh medium containing ethanol (control), Eqn (10 μM), 4-hydroxytamoxifen (TAM, 100 nM), estradiol (E_2, 10 nM), Eqn plus TAM, or E_2. After 24 h of incubation, firefly luciferase and *Renilla* luciferase activities were determined. Results represent the means ± SEM of six independent experiments. One-way ANOVA was used followed by Dunnett's test or the Student-Newman-Keuls test as posttest for statistical analysis. * $P < 0.01$.

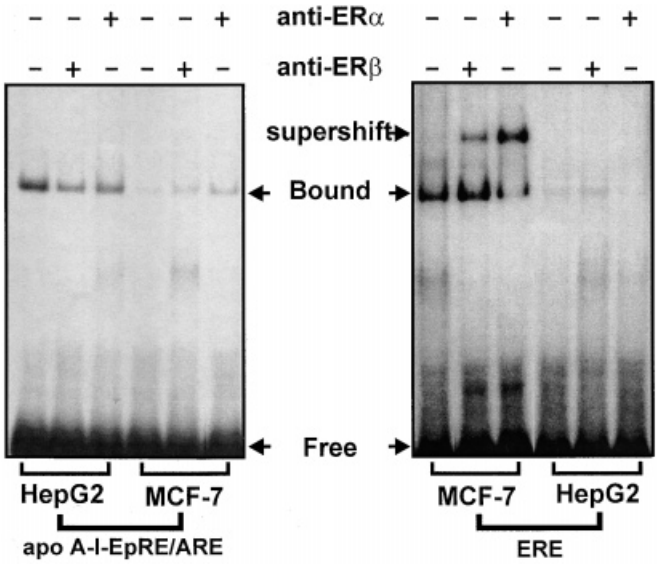

Fig. 8. Supershift experiments to determine whether ER interact with apoA-I-EpRE/ARE. Nuclear extracts isolated either from Eqn-treated HepG2 cells or Eqn-treated MCF-7 cells were incubated overnight with either nonspecific IgG or antibodies against human ERα or ERβ at 4°C. Reaction mixtures were added to double-stranded human apoA-I-EpRE/ARE or vitellogenin ERE oligonucleotides that were end labeled with [α-^{32}P]ATP. Incubation of either nonspecific IgG or antibodies against human ERα or ERβ with radiolabeled EpRE/ARE or ERE did not generate any DNA-protein complex (data not shown).

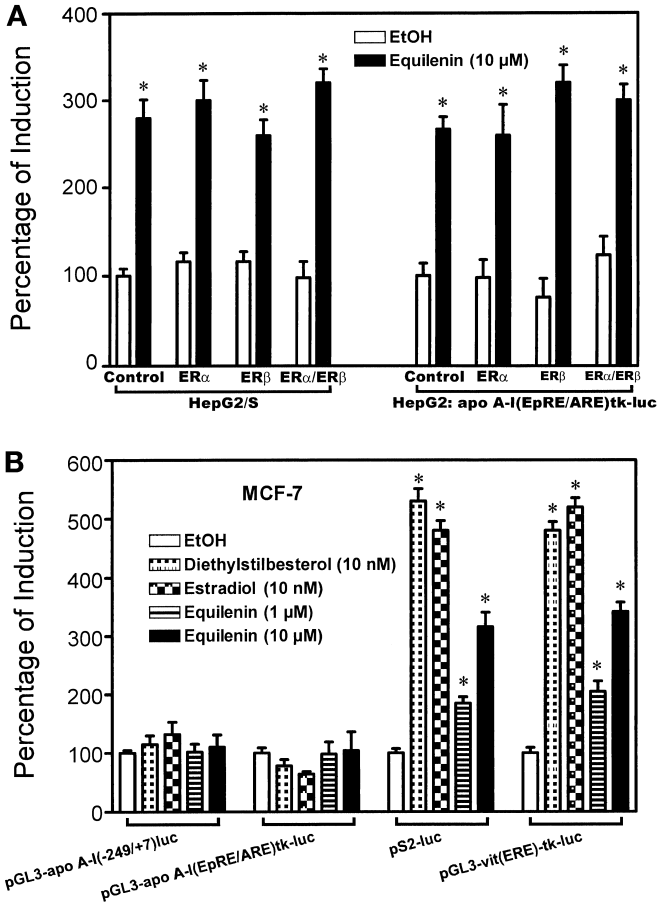

Fig. 9. The involvement of ER in Eqn-mediated transcriptional regulation of the apoA-I promoter. Eqn-mediated transcriptional regulation of the apoA-I promoter is independent of ER. A: HepG2/S cells were transfected with 0.2 μg of ER expression vectors or empty vector (control). HepG2 cells were transfected with 1 μg of pGL3-apoA-I (EpRE/ARE)tk-luc construct and either 0.2 μg of ER expression vectors or the same amount of empty vector (control). PRL-tk vector was used as an internal control to normalize transfection efficiencies in HepG2 cells. Cells were incubated with 10 μM Eqn or ethanol (EtOH). Firefly luciferase activity was measured for transfection experiments with HepG2/S cells and HepG2 cells. *Renilla* luciferase activity was measured in HepG2 cells. For experiments using HepG2/S cells, the values were obtained after normalization of firefly luciferase activity with protein amounts. Normalization for HepG2 cell experiments was made from the ratio of firefly luciferase activity to *Renilla* luciferase activity. Values are reported as a percentage of control. B: MCF-7 cells were transfected with four reporter gene constructs: pGL3-apoA-I (−249/+7)-luc, pGL3-apoA-I(EpRE/ARE) tk-luc, pS2-luc, and pGL3-vit(ERE)tk-luc, at concentrations of 2 μg/well, together with pRL-tk or pRL-null vector as internal control to normalize transfection efficiencies. After transfection, cells were treated with either estrogens or control (ethanol, <0.1%) for 24 h. Values represent percent induction. Results represent the means ± SEM of six independent experiments. * $P < 0.01$ versus ethanol-treated control.

DISCUSSION

Studies with transgenic mice and transient transfection analysis of the human apoA-I gene in cultured hepatoma cells have shown that liver-specific expression of the apoA-I gene is controlled by a strong hepatocyte-specific enhancer located in a 256-bp nucleotide region of a 5′-flanking sequence upstream from the apoA-I transcription start site (21, 23). Using functional DNA analysis of the human apoA-I gene promoter, the individual role of each estrogenic component of CEE that may play in the regulation of apoA-I gene expression was investigated.

The present data indicate that various estrogens present in CEE differentially regulate apoA-I promoter activity. Among the estrogens tested, the activities observed on the apoA-I promoter were Eqn > 17β-Eqn > 17β-Eq = Eq. CEE components such as E_1, 17β-E_2, 17α-E_2, 17α-Eq, 17α-Eqn, and δ8-E_1 did not significantly affect apoA-I promoter activity in this cell line. These observations are in keeping with previous observations that various estrogens have differential effects in terms of their antioxidant and neuroprotective properties (33, 40).

Other preparations (e.g., piperazine estrone sulfate, micronized estradiol) containing only one or two estrogen components may not be as active in the regulation of apoA-I promoter activity as CEE. Previous studies (34) have also demonstrated that ring B-unsaturated estrogens, such as Eq, equilin sulfate, and δ8-E_1, are activated in vivo in postmenopausal women to a greater extent than the estrogens E_1 and 17β-E_2. In the present study, the extent of metabolism under the culture conditions used was minimal (X. Zhang, J-J. Jiao, B. R. Bhavnani, and S-P. Tam, unpublished data); thus, the effects of various estrogens observed were most likely due to the estrogen tested rather than its metabolites. Because these in vitro experiments with HepG2 cells were carried out in the presence of 10%

CTFBS, the concentrations of estrogens needed to stimulate apoA-I transcription are high (micromolar). This is most likely due to lower concentrations of bioavailable or bioactive estrogen (estrogen unbound to serum proteins) that results from the binding of the estrogens to serum proteins such as albumin.

The 2.5- to 3-fold increase in apoA-I promoter activity caused by Eqn treatment was accompanied by a comparable increase in apoA-I mRNA. The half-life of apoA-I mRNA was not significantly affected by Eqn, suggesting that the induction of apoA-I mRNA level in response to Eqn is mediated at the transcriptional level. This observation was further confirmed by nuclear runoff assays, which indicated that the transcription rate of the apoA-I gene was also increased by 2.5-fold in Eqn-treated cells (Fig. 5). However, no significant correlation between apoA-I mRNA level and apoA-I protein secretion was observed. These data strongly suggest that apoA-I protein secretion is not only dependent on the amount of apoA-I mRNA available; both translational and/or posttranslational regulation may be involved. Previous studies (41, 42) of rodents have similarly shown that pharmacological doses of 17α-ethinyl estradiol and 17β-E_2 result in higher level of apoA-I mRNA and gene transcription, and an increase in apoA-I protein synthesis. Although Eqn also increased the level of secreted apoE protein, it did not affect apoA-II protein levels, indicating that the action of Eqn is selective and affects only some apolipoproteins. Previous studies have reported that 17β-E_2 increased apoE by regulation of the mRNA pool of apoE (43). It remains to be determined whether Eqn increases apoE by this mechanism.

Trans-activation of the human apoA-I promoter in response to Eqn was also observed in transient gene transfer experiments in cell cultures. The present study mapped the elements required to mediate the Eqn response by using several apoA-I promoter deletion and mutation luciferase reporter constructs. The element contains a core EpRE/ARE that had been previously identified in studies of the effect of gramoxone and dimethyl sulfoxide on apoA-I gene expression (24, 30). This EpRE/ARE was first identified in the regulation of the rat glutathione *S*-transferase Ya subunit gene, which acts in response to metabolites of pro-oxidant/antioxidant compounds (44). Although electrophilic metabolites, that is, electrophile/redox-active quinoids, can be generated in vitro from Eqn (28, 29), they were not formed under the culture conditions studied (data not shown). At moderately high concentrations, certain forms of reactive oxygen species may act as intracellular messengers in signal transduction that lead to the regulation of gene transcription through the EpRE/ARE (45). The data from the present study indicate that Eqn-mediated transcriptional regulation of human apoA-I gene does act through the apoA-I EpRE/ARE.

Estrogens have diverse physiological effects that are mediated via ER (ERα and ERβ) to a major extent. These receptors function as hormone-activated transcription factors and modulators of gene expression in target cells (27, 43, 46). Earlier studies have demonstrated that exposure of HepG2 cells to 17β-E_2 increases the levels of human apoA-I protein in a dose-dependent fashion (35, 47). The increase in protein levels correlated with the rise in apoA-I mRNA level (47). Studies using a cell line stably expressing ERα and transient transfection methods indicated that the action of 17β-E_2 on the apoA-I gene may occur through a transcription factor-sharing pathway involving ERα and other cofactors (25). A previous comparative study on the measurement of the relative binding affinities of the estrogenic components of CEE (in their unconjugated form) for ER in human endometrium and rat uterus demonstrated that all of these estrogens bind with high affinity to the ER, although Eqn, in comparison with others, had much lower receptor affinity (48). Thus, it was reasonable to speculate that the effect of Eqn on apoA-I promoter may function through an ER pathway. However, the present data raise questions regarding this hypothesis. First, as in previous studies, specific antibodies against both ERα and ERβ were unable to detect the presence of ER in HepG2/S cell by gel mobility supershift assays. Second, despite the lack of functional ER, Eqn still induced apoA-I gene promoter activity nearly 3-fold, which led to increased levels of apoA-I mRNA and apoA-I protein. Third, cotransfection with both ER into HepG2/S cells was unable to influence the action of Eqn on apoA-I gene promoter activity. Fourth, 17β-E_2 and E_1, and antagonists such as TAM, could not block the transcriptional induction of apoA-I promoter by Eqn. Finally, because the apoA-I EpRE/ARE has some resemblance to the consensus AP-1 response element, we also determined whether Fos-Jun binding to the apoA-I EpRE/ARE motif occurs. By performing electrophoretic mobility supershift assays, no DNA-protein complex was supershifted by the specific antibodies against c-Fos and c-Jun (X. Zhang, J-J. Jiao, B. R. Bhavnani, and S-P. Tam, unpublished results). This suggests that the alternative pathway of ER action via AP-1 sites is not responsible for Eqn action (46, 49). Taken together, these data indicate that ER are not directly involved in Eqn-mediated regulation of the human apoA-I gene, and that Eqn action is perhaps mediated via an ER-independent mechanism.

The observed activation of the apoA-I gene by Eqn via the apoA-I-EpRE/ARE differs from the data reported by Montano and Katzenellenbogen (50), which identified a unique estrogen response pathway in MCF-7 cells involved in the induction of the NAD(P)H:quinone oxidoreductase gene by antiestrogens such as *trans*-hydroxytamoxifen (TOT). This pathway was ER dependent and was mediated through an EpRE/ARE. The addition of 17β-E_2 had no effect on this pathway, but did antagonize the action of TOT, presumably through competition with ER. In the present transient transfection experiments using MCF-7 cells, 17β-E_2 also had no effect on the apoA-I-EpRE/ARE construct. However, in contrast to the HepG2 cell experiments, Eqn did not increase the luciferase activity of the construct, although the ERE construct could be induced by Eqn treatment. Thus the Eqn-mediated induction of apoA-I-EpRE/ARE was ER independent and required participation of hepatic factor(s) that have not yet been defined.

Both E_1 and 17β-E_2 had no effect on the activity of the apoA-I gene promoter in the present experiments. The failure of 17β-E_2-mediated transcriptional regulation of the apoA-I gene promoter may be due to the unsuitable combination of ERα and receptor-interacting protein 140 (RIP140), as demonstrated in one study (25). These investigators suggested that the appropriate ratio of ERα and RIP140 plays a crucial role in 17β-E_2-mediated transcriptional regulation of the human apoA-I gene. Whether Eqn-mediated transcriptional regulation of the apoA-I gene involves RIP140 in the absence of ER remains to be determined. Similarly, other transcriptional factors such as SP-1, hepatocyte nuclear factor 4 (HNF-4), and HNF-3β may also be involved in Eqn-mediated transcriptional regulation of human apoA-I promoter activity. This is currently being investigated. Furthermore, additional experiments will be required to further understand whether other *cis*-acting elements and distal enhancers in the apoA-I/apoC-III/apoA-IV gene cluster (51–53) are involved in the regulation of human apoA-I gene expression in response to Eqn treatment.

In conclusion, the present study identified Eqn as a strong modulator of transcriptional regulation of the human apoA-I gene. The activation of Eqn is mediated through an apoA-I-EpRE/ARE pathway that does not require the participation of ER but may need the involvement of liver-specific factor(s). This action of Eqn and other ring B-unsaturated estrogens is a novel finding that may explain the mechanism by which CEE administration reduces the risk of CHD in postmenopausal women.

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