Effects of Dehydroepiandrosterone on Rat Apolipoprotein AI Gene Expression in the Human Hepatoma Cell Line, HepG2

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Serum apolipoprotein AI (apoAI) levels correlate with the risk of developing atherosclerosis. Previous studies have suggested that dehydroepiandrosterone (DHEA) lowers high-denstity lipoprotein (HDL)-cholesterol levels. We investigated whether or not DHEA may lower HDL-cholesterol levels by suppressing apoAI gene transcription in hepatocytes. ApoAI mRNA levels, assessed by Northern blotting, were suppressed in HepG2 cells treated with DHEA (34%) (10 μg/mL) or testosterone (36%) (T, 1 μg/mL). Estradiol alone (E_2, 1 μg/mL) had relatively little effect on apoAI mRNA levels, while E_2 in combination with DHEA prevented a decrease in apoAI mRNA levels compared to DHEA alone. To determine whether these effects were due to changes in apoAI gene transcription, HepG2 cells were transfected with a plasmid carrying the full-length promoter of the rat apoAI gene ligated into a chloramphenicol acetyltransferase (CAT) reporter construct. The plasmid pCMV.SPORT-β-gal was included in each transfection to normalize the data to transfection efficiency. Cells were then cultured in the presence or absence of DHEA (10 μg/mL), T (1 μg/mL), 17α-methyltestosterone (MTT, 1 μg/mL), 5α-dihydrotestosterone (DHT, 1 μg/mL), E_2 (1 μg/mL), or a combination of DHEA plus E_2, T plus E_2, MTT plus E_2, and DHT plus E_2, for 24 hours. CAT activity, relative to β-galactosidase activity, was reduced by 19.6%, 57.6%, 38.6%, and 54.6% with DHEA, T, DHT, and MTT addition, respectively. E_2 increased CAT activity by 43.8%. When the androgens (ie, DHEA, T, DHT, or MTT) were combined with E_2, apoAI promoter activity was suppressed. We conclude, therefore, that androgens downregulate apoAI promoter activity in the presence or absence of E_2. However, the changes in mRNA levels do not always reflect changes in promoter activity, suggesting that these steroids may have additional post-transcriptional effects on steady-state apoAI mRNA levels. It remains to be established if the transcriptional effects we observed are mediated through an androgen response element.

DEHYDROEPIANDROSTERONE (DHEA) is an adrenal androgen without a well-defined function. It is, however, a substrate for the synthesis of testosterone and estrogen.[1] Although its precise function is not known, DHEA has become a popular supplement for enhancing athletic performance. Presumably, it also has favorable effects on a host of other biological parameters, including the immune system[2] and the central nervous system.[3] Although the putative beneficial effects of DHEA are highly controversial, it is well established that its use as a supplement is associated with reduced high-density lipoprotein (HDL)-cholesterol levels.[4-6] This latter effect could be the result of a direct effect of DHEA on apolipoprotein AI (apoAI) expression or could be secondary to the cumulative effects of the more potent hormones derived from DHEA. Since HDL-cholesterol is a well-established predictor of coronary artery disease,[7,8] it is important to study the effects of various hormones and nutritional factors on apoAI gene expression.

The effect of estrogens on lipoprotein metabolism has been studied extensively, especially with regard to HDL-cholesterol and/or apoAI expression.[9-11] Androgens on the other hand, have been examined to a lesser extent with regard to apoAI expression. However, in vivo studies have failed to demonstrate a direct role for androgens in modulating HDL levels.[12,13]

The molecular mechanisms responsible for the effect of DHEA on HDL-cholesterol reduction are unknown. DHEA may alter HDL metabolism through various physiological pathways. We hypothesized that one such pathway may include downregulation of apoAI, the main protein moiety of the HDL particle. To test this hypothesis, we studied the effect of DHEA and its metabolic end-products, testosterone (T) and estradiol (E_2), on apoAI mRNA expression and promoter activity in the human hepatoma cell line, HepG2.

MATERIALS AND METHODS

Materials

Acetyl-coenzyme A, E_2, DHEA, 5α-dihydrotestosterone (DHT), 17α-methyltestosterone (MTT), and T were purchased from Sigma Chemical Co (St Louis, MO). Lipofectamine was purchased from Life Technologies, Inc (Gaithersburg, MD). New England Nuclear (Boston, MA) was the supplier for [^{14}C]chloramphenicol, while tissue culture media and fetal calf serum were purchased from BioWhittaker (Walkersville, MD). Hybridization membrane, [α-^{32}P]-dCTP, film, and Rapid Hyb were purchased from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade and were purchased from either Sigma Chemical Co or Fisher Scientific Co (Pittsburgh, PA).

Cell Culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). Experiments were performed on cells between passages 8 and 14. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), and penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively). Cells were housed in a humidified incubator at 37°C with 5% CO_2, 95% air.

To conduct the experiments, HepG2 cells were switched to DMEM containing 5% charcoal-stripped FBS,[9] for 48 hours prior to transfection. The hormones were dissolved in dimethyl sulfoxide (DMSO) at a

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concentration 100× the final concentration in the culture media, and were made up fresh prior to each experiment. Cells were fed again 4 hours post-transfection with fresh media containing charcoal-stripped FBS, then fed again 24 hours later with fresh medium containing charcoal-stripped FBS. Hormones were added immediately after the second feeding. T, DHEA, DHT, and MTT were added to a final concentration of 1 μg/mL, 10 μg/mL, 1 μg/mL, and 1 μg/mL, respectively. E_2 was added to a final concentration of 1 μg/mL. Before leaving in the evening, the media was changed and another aliquot of hormone was added. This regimen was necessary to maintain elevated hormone levels in the metabolically active cells.

Plasmids and Transient Transfection Analysis

The reporter plasmid pAI.474.CAT, containing the rat apoAI gene promoter 5' to the chloramphenicol acetyltransferase (CAT) coding region,[14] was transfected into the HepG2 cells. This promoter fragment contains most of the *cis*-elements necessary for regulation of apoAI gene expression.[15-17] Cells cultured to 80% confluence were transfected with 1 μg of the apoAI reporter plasmid and 1 μg of the plasmid pCMV.SPORT-β-gal (Life Technologies) using Lipofectamine. The latter plasmid, containing the β-galactosidase gene driven by the cytomegalovirus immediate-early (CMV_{ie}) promoter, was used to normalize reporter gene activity to transfection efficiency. Cells subjected to mock transfection (no plasmid DNA) were included as internal controls. After 24 hours, the cells were treated with hormones as described above, and after an additional 24 hours, the cells were harvested and assayed for CAT activity.[18] A portion of the protein-extract was used for determination of β-galactosidase activity as previously described[19]

RNA Isolation and Northern Blotting

RNA was isolated from HepG2 cells as described previously[20] after treating the cells with hormones as indicated for 24 hours. Fifteen micrograms of RNA was fractionated by electrophoresis through a formaldehyde-agarose gel, and transferred to a nylon hybridization membrane. A cDNA probe specific for the apoAI mRNA was labeled with ^{32}P by random priming, and allowed to hybridize to the immobilized RNA in Rapid Hyb (Amersham, Piscataway, NJ), then washed under high-stringency conditions (final washes: 0.1× sodium chloride/sodium citrate solution [SSC], 0.1% sodium dodecyl sulfate [SDS], 2 times, 30 minutes each at 65°C). The membrane was exposed to hyperfilm (Amersham) for 2 to 3 days, then stripped and hybridized with a ^{32}P-labeled cDNA probe specific for the glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH). The amount of signal was determined with a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA). The signal present in the 0.9-kb apoAI band was normalized to that in the 1.2-kb G3PDH band.

Statistics

Means and standard errors were calculated using Statistica (Tulsa, OK). Significance between values reported for CAT assays and Northern blotting was determined using Student's *t* test for independent variables. *P* values less than .05 were considered significant.

RESULTS

Effect of Androgens and E2 on apoAI mRNA Levels

To determine if androgens as well as E_2 effect apoAI mRNA levels in hepatocytes, HepG2 cells were treated with T, DHEA, and E_2 (1, 10, and 1 μg/mL, respectively), and E_2 + DHEA (1 and 10 μg/mL, respectively). After 24 h, RNA was isolated and apoAI mRNA levels were determined by Northern blot analysis (Fig 1A). The results were quantified and are shown in Fig 1B.

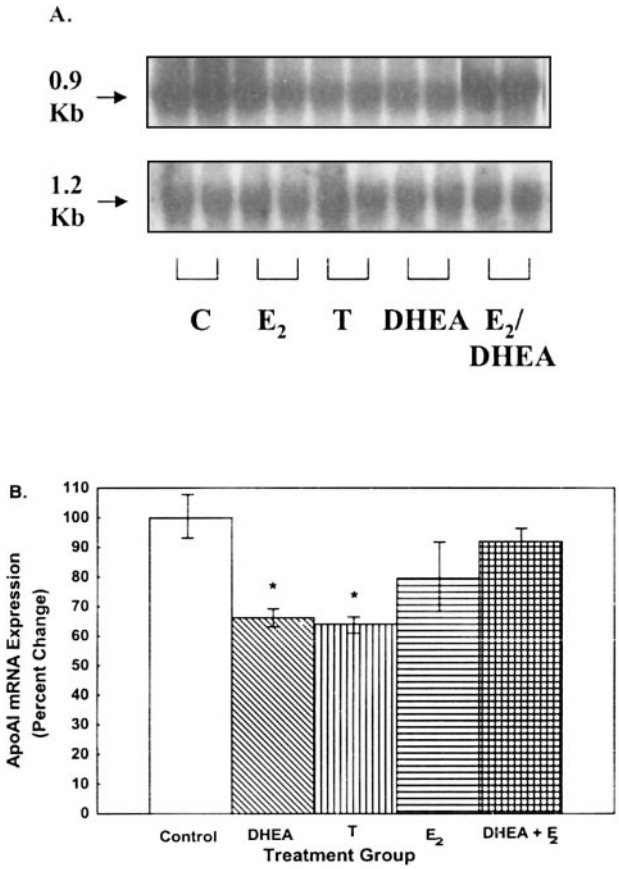

Fig 1. ApoAI mRNA expression in HepG2 cells treated with DHEA, T, E_2, and DHEA + E_2. HepG2 cells were incubated with DHEA (10 μg/mL), T (1 μg/mL), E_2 (1 μg/mL), or DHEA + E_2 (10 and 1 μg/mL, respectively) for 24 hours in charcoal-stripped medium prior to RNA preparation. (A) representative Northern blot shows the expected 0.9-kb apoAI band. The same blot was stripped and hybridized to the G3PDH probe, showing the expected 1.2-kb band. No significant changes in G3PDH expression were observed with hormone treatment. ApoAI mRNA expression was significantly reduced with T and DHEA treatment. Addition of E_2 had no significant effect on apoAI mRNA levels. However, when added with DHEA, E_2 normalized apoAI mRNA levels. (B) Results from various Northern blots were quantified and expressed as percent change, relative to control cells (set at 100%). Mean ± SEM apoAI mRNA levels relative to G3PDH mRNA levels are indicated. n = 5. *P < .05 relative to solvent-treated controls.

T and DHEA suppressed apoAI mRNA levels 36% and 34%, respectively, relative to control cells ($P < .05$) (Fig 1B). E_2 addition appeared to reduce apoAI mRNA levels slightly; however, this was not statistically significant from apoAI mRNA levels found in control cells (Fig 1B). When E_2 was combined with DHEA, apoAI mRNA levels were nearly identical to apoAI mRNA levels in control cells (Fig 1B). These results suggest that in HepG2 cells, E_2 may have little or no effect on apoAI mRNA levels. However, E_2 reverses the repressive effect of DHEA on apoAI mRNA levels.

Effect of Androgens and E_2 on apoAI Promoter Activity

To determine if the changes in apoAI mRNA levels reported above are due to alterations in apoAI promoter activity, tran-

sient transfection assays with the rat apoAI promoter were performed. HepG2 cells were transfected with the plasmid, pAI.474.CAT, and after 24 hours, DHEA, E_2, and DHEA + E_2 were added to the cells. Protein extracts were prepared from cells after 24 hours, and CAT activity was determined. E_2 stimulated apoAI promoter activity, while DHEA, either alone or in combination with E_2, suppressed apoAI promoter activity (Fig 2).

Since DHEA is a weak androgen, we performed several transient transfection experiments to further examine the ability of other more potent androgens such as T and DHT to repress the apoAI promoter. We also examined the effect of the nonmetabolizable T-analog MTT on apoAI promoter activity. HepG2 cells were transfected with pAI.474.CAT, and after 24 hours, treated with DHEA, T, DHT, MTT, or E_2. Control cells were treated with the solvent, DMSO. After 24 hours, the cells were harvested and assayed for CAT activity. The results (Table 1) show that the weak androgen DHEA at 10 μg/mL suppressed CAT activity 19.6% ($P < .05$) in this experiment. However, the more potent androgens T, DHT, and MTT, each at 1 μg/mL, suppressed CAT activity 57.6%, 38.6%, and 54.6%, respectively ($P < .05$). In contrast, the addition of E_2 (1 μg/mL) elevated CAT activity driven by the apoAI promoter by 43.8% ($P < .05$) (Table 1).

Table 1. Effect of Androgens and Estradiol on apoAI Promoter Activity

Treatment	CAT Activity	% Change
Control	38.3 ± 0.6	—
DHEA	30.7 ± 3.0	−19.6*
T	16.3 ± 5.2	−57.6*
DHT	23.5 ± 4.1	−38.6*
MTT	17.4 ± 0.2	−54.6*
E_2	55.1 ± 2.6	43.8*
E_2 + DHEA	11.6 ± 4.8	−69.7*
E_2 + T	8.8 ± 3.3	−77.0*
E_2 + DHT	17.1 ± 4.2	−55.3*
E_2 + MTT	17.5 ± 0.4	−54.3*

NOTE. HepG2 cells were transfected with the plasmids pAI.474.CAT and pCMV.SPORT-β-gal, and after 24 hours, treated with either the indicated hormone(s) or with the solvent, DMSO (control). After 24 hours, cells were harvested and assayed for CAT and β-galactosidase activity. CAT activity was normalized to β-galactosidase activity. DHEA, T, DHT, and MTT suppressed apoAI promoter activity relative to control cells, while E_2 enhanced apoAI promoter activity. When E_2 was combined with any of the androgens, apoAI promoter activity was suppressed. CAT activity is expressed as percent conversion of its substrate chloramphenicol to its acetylated derivatives. n = 3, in duplicate.

*$P < .05$, relative to DMSO-treated control cells.

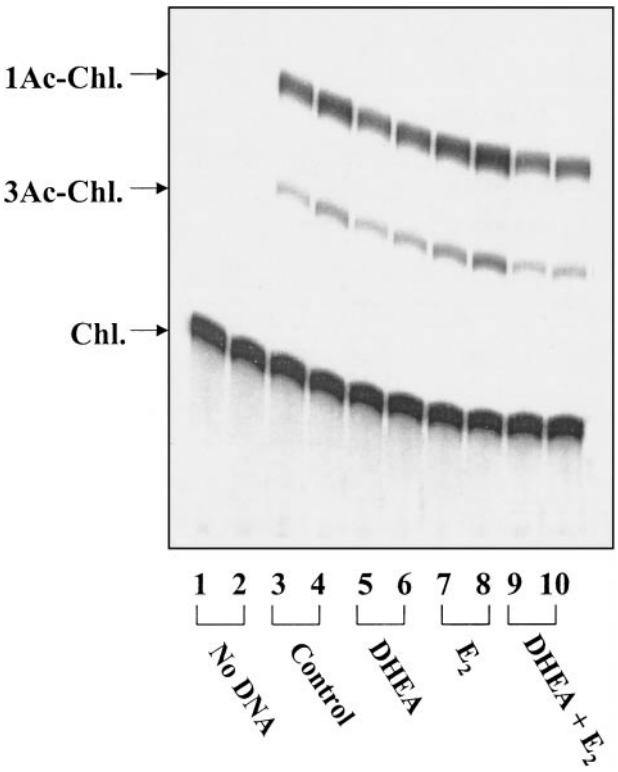

Fig 2. Effect of DHEA, E_2, and DHEA + E_2 on apoAI promoter activity. HepG2 cells were transfected with the plasmids pAI.474.CAT and pCMV.SPORT-β-gal, and after 24 hours, treated with DHEA (10 μg/mL), E_2 (1 μg/mL), or DHEA + E_2 (10 and 1 μg/mL, respectively) for 24 hours in charcoal-stripped medium prior to CAT and β-galactosidase activity measurements. Cells subjected to mock transfection (No DNA) were included as internal controls. The positions of the acetylated-chloramphenicol derivatives 1-acetyl-chloramphenicol (1Ac-Chl.) and 3Ac-Chl., as well as the nonacetylated-chloramphenicol substrate (Chl.) are indicated. These experiments were repeated 3 times, in duplicates.

Since E_2 reversed the effect of DHEA on apoAI mRNA levels, we examined the effect of exposing HepG2 cells to E_2 as well as T, DHT, and MTT concomitantly by transient transfection analysis. In the presence of both E_2 and DHEA, apoAI promoter activity was repressed 69.7% ($P < .05$) (Table 1). Furthermore, addition of T, DHT, or MTT, in combination with E_2, significantly repressed apoAI promoter activity to a similar extent (77.0%, 55.3%, and 54.3%, respectively, Table 1) ($P < .05$).

DISCUSSION

These results clearly show that DHEA downregulates apoAI mRNA expression in HepG2 cells (Fig 1). Furthermore, this effect could be attributed to decreased apoAI promoter activity (Table 1). Similar decreases in apoAI promoter activity were observed in the presence of other more potent androgens such as DHT and MTT at nearly 10-fold lower doses (Table 1). Because dose-response studies were not carried out, the relative potency of these androgens cannot be ascertained in this in vitro system. The effects of E_2 on apoAI mRNA and promoter activity were not congruent. Whereas E_2 treatment had only a modest effect on apoAI mRNA levels in HepG2 cells (Fig 1), E_2 stimulated apoAI promoter activity significantly (Table 1). This inconsistency in the effect of E_2 on apoAI expression in hepatoma cells has been reported previously.[9,17,21] The discrepancy is probably related to the E_2 receptor content of the cell lines used, which varies according to the number of passages in culture.[22] Despite the inconsistencies observed with E_2, the addition of any of the androgens, namely, DHEA, T, DHT, and

MTT, combined with E_2, reduced apoAI promoter activity (Table 1).

It is noteworthy that the changes in apoAI mRNA levels did not always reflect the changes in promoter activity, suggesting that these steroids may have additional post-transcriptional effects on apoAI gene expression. Measurements of apoAI mRNA turnover kinetics should be performed in the future. Of note is that rat apoAI construct was used in HepG2 cell transfection experiments while the Northern blot analysis was used to measure the endogenous human apoAI mRNA response to hormones. Estrogen administration to male rats causes a decrease in hepatic apoAI mRNA levels,[10] while in HepG2 human cell line estrogen may increase apoAI mRNA levels.[9] Therefore, data from human and rat experiments may not be compatible.

A previously published study suggested that T lacks a real effect on apoAI gene expression, and that the primary role of androgens is in antagonizing E_2's stimulatory effects on apoAI gene transcription.[9] This latter study measured apoAI transcription rate using nuclear run-off assays. In the present study, using the more sensitive reporter gene assay, it is apparent that DHEA, as well as other aromatizable and nonaromatizable androgens, in the presence or absence of E_2, can downregulate apoAI promoter activity. These observations suggest that androgens have an independent effect on apoAI transcription.

It is intriguing that the androgen suppression of CAT activity was enhanced in E_2-treated cells. The precise mechanisms underlying these observations are not known. Conceptually there are at least 2 general mechanisms that may explain these observations. The first possibility is that E_2 alters the metabolism of androgens in the HepG2 cells. The other potential explanation is that E_2 and androgens effect apoAI transcription through independent signaling pathways that may under certain circumstances augment androgenic effects. These speculations should be subjected to experimental testing in future studies.

In conclusion, DHEA readily suppressed apoAI promoter activity in HepG2 cells, which may contribute to the decreased HDL-cholesterol levels in subjects taking DHEA supplements.

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