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Ascorbic acid and α-tocopherol down-regulate apolipoprotein A-I gene expression in HepG2 and Caco-2 cell lines☆

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Abstract

HepG2 cells and Caco-2 cells were treated with various concentrations of select antioxidants to study some of the molecular pathways underlying antioxidant-related changes in apolipoprotein A-I (apoA-I) expression. Both α-tocopherol and ascorbate treatment over a time course of 72 hours caused a significant reduction in apoA-I messenger RNA and protein levels in a dose-dependent fashion. Albumin levels did not change with any treatment, suggesting that the effect is protein-specific. Similar changes were seen in Caco-2 cells. In contrast, apoA-I messenger RNA and protein levels significantly increased after 28 and 280 μmol/L dimethyl sulfoxide (DMSO) treatment. Cells were transfected with chloramphenicol acetyltransferase (CAT) reporter gene plasmid containing the full-length apoA-I promoter to determine if these changes occur at a transcriptional level, and after 24 hours, the HepG2 or Caco-2 cells were treated with varying concentrations of ascorbate or α-tocopherol. At the highest concentration of ascorbate and α-tocopherol used (5 mmol/L), there was a significant reduction in apoA-I promoter activity. DMSO treatment up-regulated apoA-I promoter activity, whereas increasing oxidative load with 50, 100, and 200 μmol/L hydrogen peroxide treatment did not significantly alter apoA-I promoter activity. Studies with deletional constructs of the promoter containing or lacking the antioxidant response element (ARE) indicated that the effect of ascorbate and α-tocopherol, unlike that of DMSO, was independent of this ARE. Using a series of apoA-I deletion constructs, it was shown that site A–containing segment of the promoter has a critical regulatory element. However, electromobility shift assays indicated that there was no significant change in nuclear factor binding activity to site A as a result of treatment with ascorbate or α-tocopherol. As expected, treatment with DMSO increased factor binding to the previously described ARE. It is concluded that the apoA-I promoter-stimulating effect of DMSO may be independent of its antioxidant activity and that some antioxidants at very high concentrations may have suppressive effect on the apoA-I gene expression. It appears that the inhibitory effect of ascorbate or α-tocopherol on the apoA-I promoter is either indirect or is the result of posttranslational modifications of the nuclear binding factors. The previously described ARE is not a response element for the ascorbate or α-tocopherol.

1. Introduction

Several epidemiological studies have shown that consumption of food rich in antioxidants may protect against atherosclerosis [1-3]. However, interventional trials using various antioxidant supplements have failed to show consistent benefits [1-3]. Even more disconcerting is that treatment with probucol, a potent antioxidant, was associated with a reduction in high-density lipoprotein (HDL) and its principle protein constituent, namely, apolipoprotein A-I (apoA-I) [4]. More recently, studies have shown that antioxidant supplementation was associated with blunting of HDL_2 cholesterol increase after treatment with a combination of simvastatin and niacin [5,6]. However, in these studies, antioxidant therapy alone did not significantly alter the plasma concentrations of HDL cholesterol or apoA-I [7].

Previously published studies have identified a positive regulatory element within the apoA-I promoter that is responsive to antioxidants [8]. Thus, apoA-I expression in HepG2 cells (a human hepatoma cell line) was found to increase in response to treatment with a potent antioxidant,

☆ Some of the data were previously presented in abstracts: Haas MJ, Wadud K, Wong NCW, Mooradian AD. Antioxidants suppress the apolipoprotein AI (apoAI) promoter in the cultured hepatoma cell line, HepG2. Endocrine Society 84th Annual Meetings 2002, p 262; and Haas MJ, Wadud K, Wong NCW, Mooradian AD. Differential effects of antioxidants on apolipoprotein AI gene expression in Hep G2 cells. The Annual Meetings of the Endocrine Society, New Orleans, LA, 2004, p 350.

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dimethyl sulfoxide (DMSO) [8]. This change was attributed to increased transcriptional activity of the apoA-I promoter where a putative antioxidant response element (ARE) was identified [8]. However, gramoxone, a compound capable of generating reactive oxygen species, was also shown to increase apoA-I promoter activity through interaction with the ARE [9]. Therefore, the effect of antioxidants and prooxidants on apoA-I expression is complex and may be related to peculiarities of the individual compounds used. It is not known whether other commonly used antioxidants are capable of positively modulating apoA-I gene expression in a fashion similar to DMSO. Given the conceptual importance of the problem and the potential clinical implications, we elected to study the effect of select antioxidants on apoA-I gene expression in cultured HepG2 cells. Because intestinal tissue is a significant source of apoA-I production [10], some additional confirmatory experiments were carried out in the human intestinal cell line, Caco-2.

2. Materials and methods

2.1. Materials

Acetyl-coenzyme A was purchased from Sigma (St. Louis, MO). Lipofectamine was purchased from Life Technologies (Gaithersburg, MD) and ^{14}C-chloramphenicol was from New England Nuclear (Boston, MA). Tissue culture media and fetal calf serum were purchased from BioWhittaker (Walkersville, MD). All other chemicals were of reagent grade and were purchased from either Sigma or Fisher Scientific (Pittsburg, PA).

2.2. Cell culture

HepG2 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively). Caco-2 cells were maintained in Earl modified essential medium supplemented with 15% fetal bovine serum, nonessential amino acids, 0.11 mg/mL sodium pyruvate, and penicillin and streptomycin. Cells were housed in a humidified incubator at 37°C with 5% CO_2 and 95% air. Cell viability after treatment with various agents was measured by trypan blue exclusion [11]. HepG2 cells are a transformed human hepatoma cell line that retains many of the functions of normal hepatocytes such as synthesis of albumin, lipoproteins, and other liver-specific proteins [12-15]. Caco-2 cells are a transformed human colonic carcinoma cell line that retains many of the functions of normal enterocytes, notably their ability to synthesize and secrete apoA-I.

2.3. ApoA-I Northern blot analysis

RNA was isolated as described by Chomczynski and Sacchi [16]. Recombinant plasmid pBR322 containing the rat apoA-I complementary DNA (cDNA) (NE-477) was kindly provided by Dr JI Gordon of Washington University (St. Louis, MO). Amplification and preparation of plasmid DNA were accomplished using established procedures [17,18]. The cleaved insert was separated from the vector DNA and labeled using random primers and (α-^{32}P) dCTP [19]. The isolated RNA was fractionated by electrophoresis in a 1.5% agarose gel containing 2.2 mol/L formaldehyde [20], transferred to the nylon membrane by diffusion blotting, and finally hybridized with the apoA-I cDNA insert [21]. The membrane was exposed to x-ray film for autoradiography. The blots were stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA to determine the specificity of changes in apoA-I messenger RNA (mRNA). ApoA-I mRNA levels (arbitrary integrator units [AIU]) were normalized to G3PDH mRNA levels as follows. The G3PDH signal densities were adjusted by dividing each value by an internal control included in each gel. Subsequently, the apoA-I mRNA signal densities were divided by their corresponding G3PDH mRNA values.

2.4. Western blot analysis

Conditioned media samples (25 μg protein) were fractionated by electrophoresis in a denaturing sodium dodecyl sulfate 10% polyacrylamide gel under reducing conditions [22]. Proteins in the gels were electrophoretically transferred to nitrocellulose membrane [23]. The membrane was incubated with either a human apoA-I antiserum (purchased from Calbiochem, San Diego, CA) at a final dilution of 1:1000 for 2 hours at room temperature or an anti-albumin antiserum (1:2500). Horseradish peroxidase–linked goat antirabbit immunoglobulin G was used at a final dilution of 1:10 000 for 1 hour at room temperature. Blots were developed using enhanced chemiluminescence Western blotting reagents as described by the manufacturer (Amersham-Pharmacia Biotech, Arlington Heights, IL). The apoA-I concentration was determined by densitometry using the personal densitometer from Molecular Dynamics (Sunnyvale, CA). The absorbance of bands was analyzed after background subtraction. The reproducibility of the apoA-I quantitation was established with gels loaded with different amounts of authentic apoA-I [24,25].

2.5. Plasmids and transient transfection analysis

The reporter plasmid pAI.474.CAT, containing the apoA-I gene promoter [26], was transfected into HepG2 or Caco-2 cells using Lipofectamine as described by the manufacturer to determine if the apoA-I promoter responds to select antioxidants or prooxidants. This reporter plasmid contains the *cis* elements critical for the regulation of apoA-I gene expression [26-28]. Cells cultured to 80% confluence were transfected with 1 μg of the apoA-I reporter plasmid DNA and 1 μg of the plasmid pCMV.SPORT-β-gal (Life Technologies). The latter plasmid, containing the β-galactosidase (β-gal) gene driven by the cytomegalovirus promoter, was used to normalize chloramphenicol acetyltransferase (CAT) gene activity to transfection efficiency. After 24 hours, the cells were treated

Table 1
Effect of antioxidant dose on apoA-I protein and mRNA levels

Treatment	ApoA-I protein (AIU)	Fold change	*P*	ApoA-I mRNA (AIU)	Fold change	*P*
DMSO (μmol/L)						
0	488.3 ± 7.8	–	–	634.0 ± 22.1	–	–
2.8	489.7 ± 7.5	1.00	NS	644.3 ± 7.9	1.02	NS
28	740.0 ± 8.7	1.52[a]	.0003	731.0 ± 17.9	1.15[a]	.03
280	1156 ± 50.9	2.37[a]	.0002	1051 ± 28.6	1.66[a]	.0003
α-Tocopherol (μmol/L)						
0	471.7 ± 13.3	–	–	676.0 ± 12.7	–	–
10	444.0 ± 10.7	0.94	NS	671.7 ± 6.4	0.99	NS
100	393.3 ± 3.52	0.83[b]	.005	596.7 ± 6.9	0.88[b]	.005
1000	316.3 ± 5.9	0.67[b]	.0004	493.0 ± 8.7	0.73[b]	.0002
Ascorbate (μmol/L)						
0	426.7 ± 13.5	–	–	443.0 ± 10.4	–	–
15	371.0 ± 6.7	0.87[c]	.02	388.3 ± 7.2	0.88[c]	.01
150	266.0 ± 7.6	0.62[c]	.0005	243.3 ± 10.1	0.55[c]	.00001
1500	193.0 ± 7.5	0.45[c]	.0001	190.3 ± 9.2	0.43[c]	.0000

HepG2 cells treated with increasing doses of α-tocopherol (0, 10, 100, and 1000 μmol/L), ascorbic acid (0, 15, 150, and 1,500 μmol/L), or DMSO (0, 2.8, 28, and 280 μmol/L). Culture media and total RNA were collected and prepared after 24 hours (n = 3 for each determination). NS indicates not significant.

[a] Significant relative to control cells in DMSO experiment.

[b] Significant relative to control cells in α-tocopherol experiment.

[c] Significant relative to control cells in ascorbic acid experiment.

with either α-tocopherol (50, 500, and 5000 μmol/L) or ascorbic acid (100, 500, and 5000 μmol/L) dissolved in culture medium or with DMSO (0, 2.8, 28, and 280 μmol/L) or hydrogen peroxide (H_2O_2, 0, 50, 100, and 200 μmol/L) and, after another 24 hours, harvested and assayed for CAT [29] and β-gal [30] activity. The H_2O_2 was used to study the effect of increased oxidative load on apoA-I expression.

HepG2 cells were transfected with the plasmids pAI.474-CAT, pAI.425CAT, pAI.375CAT, pAI.325CAT, pAI.186-CAT, pAI.170CAT, pAI.144CAT, and pAI.46CAT to determine if the effect of antioxidants is dependent on the previously described ARE within the promoter and, after 24 hours, treated with DMSO (0.28 mmol/L), α-tocopherol (1000 μmol/L), or ascorbic acid (1500 μmol/L). After 24 hours, CAT activity was measured. These constructs contain the apoA-I promoter region −474, −425, −375, −325, −186, −170, and −46 base pairs (from transcription start site), respectively. In addition, pAI.144CAT construct contains the ARE (−136), whereas the pAI.46CAT does not have the ARE sequences.

2.6. Nuclear extract preparation and gel shift analysis

HepG2 cells in 175-cm^2 flasks were either left untreated or were treated with DMSO (280 μmol/L), ascorbic acid (1500 μmol/L), or α-tocopherol (1000 μmol/L) for 24 hours in duplicate. The cells were washed 3 times in phosphate-buffered saline and suspended in 10 mL of nuclear wash buffer (10 mmol/L *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid [HEPES; pH 8.0], 15% sucrose, 1 mmol/L EDTA, 0.5% Triton X-100, 1 mmol/L dithiothreitol [DTT], 5 mmol/L $MgCl_2$, and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]), and incubated on ice for 10 minutes. The mixture was underlaid with nuclear wash buffer containing 30% sucrose, but no Triton X-100, and centrifuged at 3000*g* for 30 minutes at 5°C. The pellet was suspended in 1 mL of extraction buffer containing 10 mmol/L HEPES (pH 8.0), 500 mmol/L NaCl, 10 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, and 5 mmol/L spermidine and placed on ice for 1 hour. The supernatant fraction was obtained by centrifugation at 10 000*g* for 10 minutes and was dialyzed extensively against 20 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, and 20% glycerol. Protein concentration was determined using the Bradford [31] assay with bovine serum albumin as the standard. Protein-DNA binding was assessed using the electrophoretic mobility shift assay. The electrophoretic mobility shift assays contained 20 μg of nuclear protein extract and 15 000 cpm of a ^{32}P-labeled oligonucleotide probe containing site A from the apoA-I gene (sense, 5′-GGT GAA CCC TTG ATC CCA G-3′; antisense, 5′-AGA GCT GGG ATC AAG GGT T-3′) and the apoA-I "ARE" (sense, 5′-CAG CCC CAG GGA CAG A-3′; antisense, 5′-CAG CTC TGT CCC TGG G). The probes 5′ overhangs were filled in with [α-^{32}P]-dCTP using the Klenow fragment of DNA polymerase I and separated from the unincorporated nucleotides by chromatography on a Sephadex G50 spin column (Roche Applied Science, Indianapolis, IN). The protein extract was incubated with the probe in a solution containing 12% glycerol, 12 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 5 mmol/L $MgCl_2$, 4 mmol/L Tris-Cl (pH 7.9), 0.6 mmol/L EDTA, 0.6 mmol/L DTT, and 2 μg of poly(dI-dC) · poly(dI-dC), and placed on ice for 30 minutes. The mixture was loaded onto a 5% polyacrylamide gel and fractionated by electrophoresis in 0.25× TBE (1× TBE is 45 mmol/L Tris base, 32.3 mmol/L boric acid, and 1.25 mmol/L EDTA, pH 8.3) at 200 V for 60 minutes at 4°C. The gel was dried and exposed to film for autoradiography.

Binding specificity was assessed by adding increasing amounts (0.4, 4.0, and 40 ng) of the unlabeled site A, the

Table 2
The time course of the effect of antioxidants on apoA-I protein and mRNA levels

Treatment	ApoA-I protein (AIU)	Fold change	*P*	ApoA-I mRNA (AIU)	Fold change	*P*
DMSO (h)						
0	588.0 ± 13.1	–	–	441.3 ± 8.6	–	–
24	668.0 ± 8.9	1.14[a]	.007	649.3 ± 14.4	1.47[a]	.0002
48	1310 ± 57.4	2.23[a]	.0003	954.0 ± 33.2	2.16[a]	.0001
72	1763 ± 29.6	3.00[a]	.0000	1288 ± 11.7	2.84[a]	.00001
α-Tocopherol (h)						
0	435.3 ± 18.0	–	–	592.7 ± 9.7	–	–
24	418.7 ± 7.1	0.96	NS	594.0 ± 14.7	1.00	NS
48	403.3 ± 4.4	0.93	NS	554.7 ± 6.9	0.94[b]	.03
72	361.0 ± 9.5	0.83[b]	.02	476.3 ± 6.6	0.80[b]	.0006
Ascorbate (h)						
0	443.7 ± 11.5	–	–	530.0 ± 17.8	–	–
24	404.3 ± 3.5	0.91[c]	.03	450.3 ± 6.5	0.85[c]	.01
48	362.3 ± 8.2	0.82[c]	.004	324.0 ± 13.0	0.61[c]	.0007
72	248.0 ± 12.5	0.56[c]	.0003	219.7 ± 9.6	0.41[c]	.0001

HepG2 cells were exposed to DMSO (28 μmol/L), ascorbic acid (150 μmol/L), α-tocopherol (100 μmol/L), or no agent (control). Culture media and total RNA were collected and prepared at 0, 24, 48, and 72 hours (n = 3 for each determination).

[a] Significant relative to control cells in DMSO experiment.

[b] Significant relative to control cells in α-tocopherol experiment.

[c] Significant relative to control cells in ascorbic acid experiment.

apoA-I ARE, or the apoA-I insulin responsive core sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) oligonucleotides to 20 μg of nuclear protein extract from control HepG2 cells before addition of the ^{32}P-labeled site A or ARE probes. Gel electrophoresis and autoradiography were carried out as described above.

2.7. Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was carried out by Student *t* test for independent variables. Significance was defined as a 2-tailed $P < .05$. The number for each treatment was 3 to 6. The mean result from various experiments was calculated after normalizing the results from each experiment against an internal control, that is, cells treated with vehicle.

3. Results

3.1. Effects of antioxidants on apoA-I mRNA and protein levels

The dose-dependent changes in apoA-I mRNA of HepG2 cells and apoA-I protein in culture media after treatment of cells with various doses of DMSO, α-tocopherol, and ascorbate are summarized in Table 1. ApoA-I mRNA and protein levels were increased after DMSO treatment, whereas α-tocopherol and ascorbate treatment reduced these levels in a dose-dependent fashion. Significant changes were observed at α-tocopherol concentration as low as 100 μmol/L and ascorbate concentration of 15 μmol/L.

The time course of changes in apoA-I mRNA of HepG2 cells and apoA-I protein in culture media after treatment of cells with antioxidants are summarized in Table 2. Over time, apoA-I mRNA and protein levels were increased after DMSO treatment, whereas α-tocopherol and ascorbate treatment reduced these levels. Significant changes were evident as early as 24 hours and reached maximal change at 72 hours of incubation. The concentrations of the test substances in Table 2 were chosen based on dose-response curves. The chosen concentrations were high enough to see a statistically significant change,

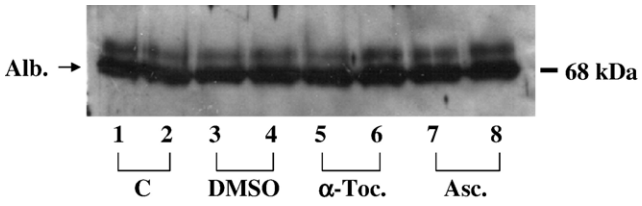


Fig. 1. Effect of antioxidants on albumin levels. HepG2 cells were treated with DMSO (28 μmol/L), ascorbic acid (Asc., 150 μmol/L), or α-tocopherol (α-Toc., 100 μmol/L) for 24 hours. The 68-kd albumin band is indicated (arrow). None of the antioxidants affected albumin accumulation in the media relative to control (C) cells.

Table 3
The effect of antioxidants on apoA-I promoter activity

Treatment	CAT activity (HepG2)	CAT activity (Caco-2)
Control	98.9 ± 5.3	97.9 ± 6.2
Ascorbate (μmol/L)		
100	102.7 ± 2.3	ND
500	86.5 ± 2.3	ND
5000	29.3 ± 13.4*	59.8 ± 7.6*
α-Tocopherol (μmol/L)		
50	97.6 ± 2.6	ND
500	53.7 ± 7.9*	ND
5000	60.4 ± 10.1*	46.6 ± 4.0*

HepG2 cells transfected with the apoA-I reporter gene were treated with the indicated concentrations of ascorbic acid and α-tocopherol for 24 hours. CAT activity (mean ± SEM) is expressed as percentage of an internal control and is normalized to β-gal activity. ND indicates no data at that concentration (n = 6).

* $P \le .001$ compared with controls.

Table 4
Effect of DMSO, α-tocopherol, and ascorbic acid on the apoA-I ARE

Plasmid/treatment	CAT activity (% acetylation)	Fold change	P
144CAT/control	8.7 ± 1.1	–	–
144CAT/DMSO	22.7 ± 0.5	2.6[a]	.0003
144CAT/α-tocopherol	8.2 ± 0.8	0.94	.72
144CAT/ascorbate	7.7 ± 0.5	0.89	.46
46CAT/control	8.3 ± 0.6	–	–
46CAT/DMSO	8.1 ± 0.5	0.98	.84
46CAT/α-tocopherol	8.3 ± 0.7	1.00	.94
46CAT/ascorbate	8.1 ± 0.5	0.98	.81

HepG2 cells were transfected with the plasmids pAI.144CAT (144CAT) and pAI.46CAT (46CAT), and after 24 hours, treated with DMSO (280 μmol/L), α-tocopherol (1000 μmol/L), or ascorbic acid (1500 μmol/L). After 24 hours, CAT activity was measured. DMSO induced CAT activity, whereas α-tocopherol and ascorbic acid had no effect on CAT activity from the 144CAT construct containing the ARE (−136). Removing the ARE (46CAT) inhibited the positive effect of DMSO (n = 6).

[a] Significant relative to 144CAT control samples.

but lower than the highest doses tested to avoid toxicity-related problems.

In Caco2 cells, apoA-I protein levels increased significantly in cells treated with 280 μmol/L DMSO for 24 hours (7555.0 ± 199.5 vs 5848 ± 85.0 arbitrary units in control cells; $P < .016$). In contrast, treating Caco-2 cells with either 1500 μmol/L α-tocopherol (5011 ± 40.5; $P < .012$) or 1000 μmol/L ascorbic acid (4891 ± 40.5; $P < .01$) significantly decreased apoA-I protein secretion.

Cell viability was monitored throughout these experiments and found to be greater than 95% in each treatment group. Treatment of cells with ascorbic acid, α-tocopherol, or DMSO was not associated with any significant changes in albumin concentrations in the culture media (5141 ± 210, 5211 ± 160, 5516 ± 138, and 5464 ± 144 AIU for control, DMSO, α-tocopherol, and ascorbate treatment, respectively) (Fig. 1).

3.2. Effects of antioxidants on apoA-I promoter activity

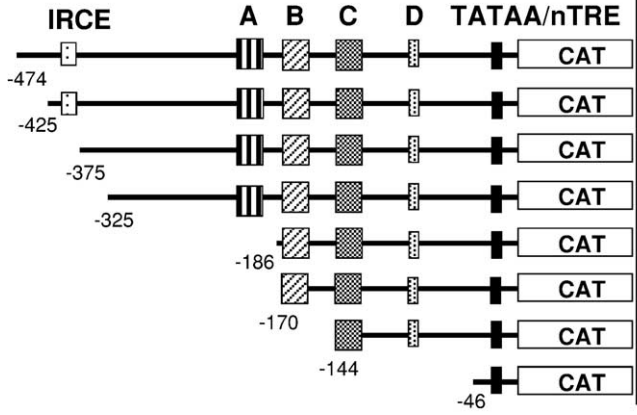
Treating cells with varying concentrations of α-tocopherol or ascorbate was associated with a significant reduction in apoA-I promoter activity at the highest concentrations used (Table 3). Similar results were seen in the human intestinal cell line Caco-2 cells treated with these antioxidants (Table 3).

As expected, DMSO treatment up-regulated apoA-I promoter activity (CAT activity expressed as percentage of acetylation and normalized to β-gal activity was 33.0% ± 1.41%, 36.5% ± 2.2%, 43.0% ± 2.0%, and 49.5% ± 3.5% in the presence of 0, 2.8, 28, and 280 μmol/L DMSO, respectively; $P < .01$). However, treating HepG2 cells with varying concentrations of an oxidant, H_2O_2, did not significantly alter apoA-I promoter activity. The CAT activity (as percentage of chloramphenicol acetylated) measured in transfected HepG2 cells in the presence of 0, 50, 100, and 200 μmol/L of H_2O_2 was 19.8% ± 1.1%, 18.8% ± 2.3%, 19.8% ± 1.9%, and 18.9% ± 2.3%, respectively. These differences were not statistically significant.

3.3. Effect of antioxidants on the apoA-I ARE

The effects of select antioxidants on the activity of apoA-I promoter deletional constructs are summarized in Table 4. In cells transfected with the pAI.144CAT construct containing the ARE (−136), DMSO induced CAT activity by 2.6-fold, whereas α-tocopherol and ascorbic acid had no effect on CAT activity. Deleting the ARE (pAI.46CAT construct) abolished the positive effect of DMSO on apoA-I promoter activity (Table 4).

The α-tocopherol– and ascorbic acid–related suppression of apoA-I promoter was observed in plasmids containing the full length of the promoter as well as with deletion constructs pAI.425CAT, pAI.375CAT, and pAI.325CAT



α-Tocopherol (% change from control)	P	Ascorbate (% change from control)	P
-37.1	0.002	-39.4	0.002
-38.0	0.016	-50.2	0.007
-27.5	0.012	-49.4	.0001
-41.9	0.001	-60.4	0.0004
-1.4	0.65	-6.2	0.31
-5.6	0.23	-14.3	0.1
4.8	0.83	-18.5	0.53
0.0	1.00	-2.9	0.95

Fig. 2. HepG2 cells were transfected with the indicated plasmids as well as with plasmid pCMV.SPORT-β-gal, and after 24 hours, treated with DMSO (0.28 mmol/L), α-tocopherol (1000 μmol/L), or ascorbic acid (1500 μmol/L). After 24 hours, CAT activity was measured. CAT (% acetylation) was normalized to β-gal activity. The percentage of decrease in CAT activity from control cells to cells treated with α-tocopherol or ascorbic acid is shown. There is a critical regulatory element in the −325 to −186 segment that contains site A of the promoter (n = 6 for each determination). A, B, C, and D indicate the previously identified critical regulatory sites within the apoA-I promoter.

(Fig. 2). However, there was no inhibitory effect observed with apoA-I deletion constructs pAI.186CAT, pAI.170CAT, pAI.144CAT, and pAI.46CAT, indicating that the region between −325 and −186 contains a critical regulatory element.

3.4. Effect of antioxidants on site A and ARE binding

Nuclear factor binding activity with site A and the ARE was examined in HepG2 cells exposed to DMSO, ascorbic acid, or α-tocopherol for 24 hours (Fig. 3). There was no change in site A oligonucleotide binding in cells treated with DMSO, ascorbate, or α-tocopherol. However, treatment with DMSO increased factor binding to the ARE oligonucleotide relative to control cells. No change in ARE binding was observed in cells treated with ascorbate or α-tocopherol.

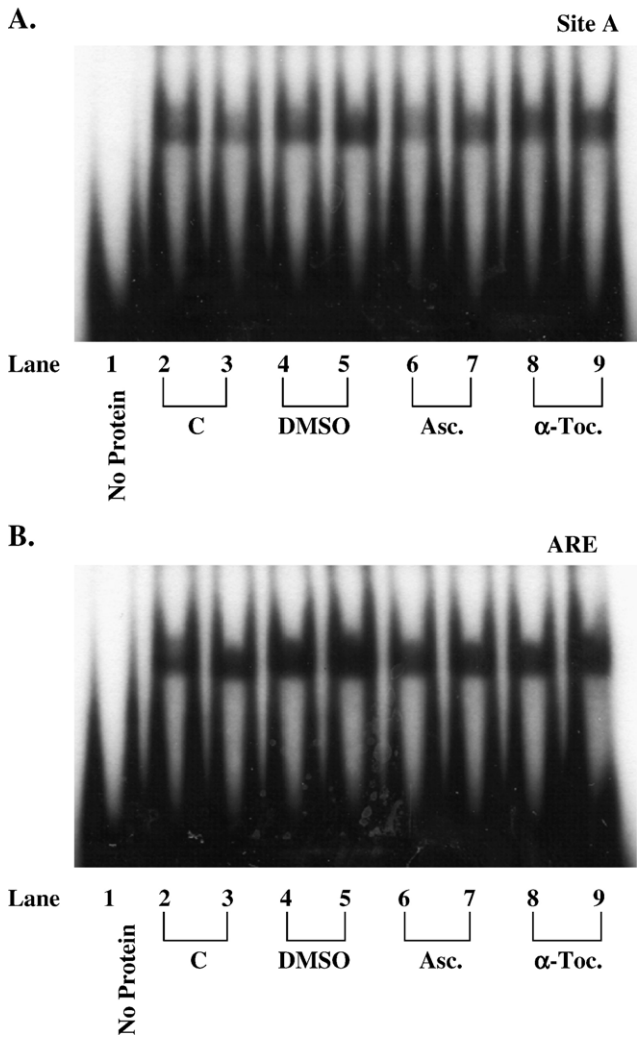


Fig. 3. Effect of antioxidants on site A and ARE binding. Nuclear factor binding activity with site A (A) and the apoA-I ARE (B) was examined in HepG2 cells exposed to DMSO (lanes 4 and 5), ascorbic acid (Asc., lanes 6 and 7), or α-tocopherol (α-Toc., lanes 8 and 9) for 24 hours. Control cells (C) received no treatment (lanes 2 and 3). No change in site A oligonucleotide binding was observed in C cells compared with cells treated with DMSO, Asc., or α-Toc. However, treatment with DMSO increased factor binding to the ARE oligonucleotide relative to C cells. No change in ARE binding was observed in cells treated with Asc. or α-Toc.

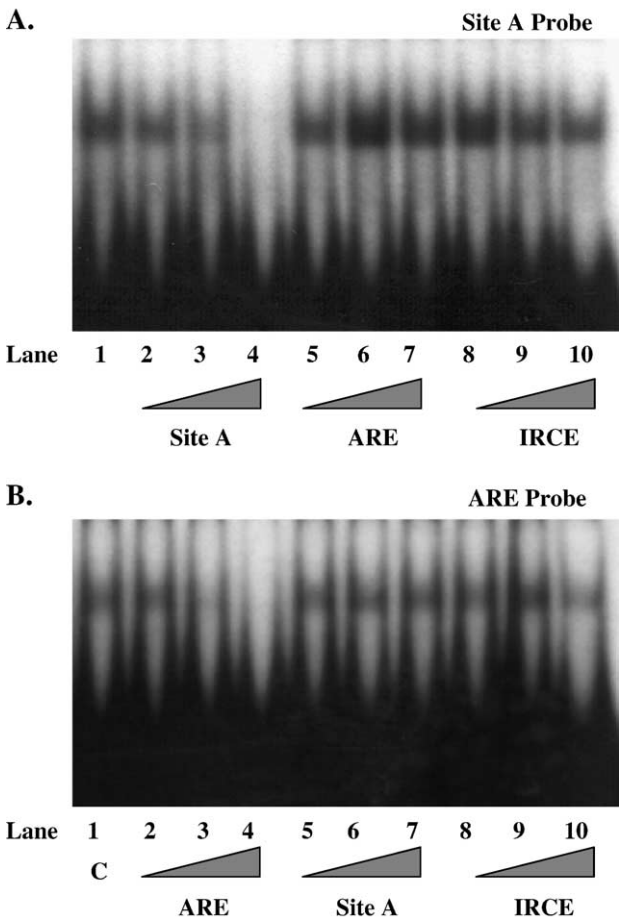


Fig. 4. Specificity of site A and ARE binding. Protein-DNA binding activity with site A (A) and apoA-I ARE probes (B) was examined in control HepG2 cells (C). Panel A, Competition with 0.1, 1, and 10 ng of unlabeled site A oligonucleotide competitor (lanes 2-4, respectively); 0.1, 1, and 10 ng of unlabeled apoA-I ARE (lanes 5-7); and 0.1, 1, and 10 ng of unlabeled IRCE (lanes 8-10). B, Competition with 0.1, 1, and 10 ng of unlabeled apoA-I ARE oligonucleotide competitor (lanes 2-4, respectively); 0.1, 1, and 10 ng of unlabeled site A (lanes 5-7); and 0.1, 1, and 10 ng of unlabeled IRCE (lanes 8-10). Only the site A competitor was able to compete for site A binding activity in panel A, whereas only the ARE competitor was able to compete for ARE binding activity in panel B.

Binding to site A and the ARE was specific because addition of only the site A–containing competitor oligonucleotide, but not the ARE, displaced binding to the site A probe. Binding to the ARE probe was displaced by the cold ARE oligonucleotide, but not the site A oligonucleotide, whereas the insulin-responsive core element did not displace binding to either probe (Fig. 4).

4. Discussion

The results from these studies clearly show that some commonly used antioxidants suppress apoA-I gene expression in HepG2 cells and Caco-2 cells. ApoA-I mRNA and protein levels decreased in HepG2 cells treated with either ascorbic acid or α-tocopherol (Tables 1 and 2). The effect of incubating cells with 100 μmol/L α-tocopherol for only

24 hours was marginal and not demonstrable in every experiment. Overall, the time course of changes in apoA-I mRNA of HepG2 cells and apoA-I protein in culture media after treatment of cells with antioxidants supports the notion that the effects seen are mostly pretranslational. The relatively slow maximal suppression of apoA-I mRNA and protein levels is consistent with previously determined slow turnover of apoA-I mRNA in this cell culture system [32]. As an internal positive control, HepG2 cells were also treated with DMSO, an antioxidant previously shown to up-regulate apoA-I gene expression [8]. As expected, apoA-I mRNA levels and apoA-I protein levels increased significantly after treatment with DMSO (Tables 1 and 2). Because previously a positive regulatory element in apoA-I promoter had been identified as an ARE [8], additional studies of the effect of antioxidants and an oxidant on apoA-I promoter activity was carried out. Treating cells with α-tocopherol or ascorbate was associated with a significant reduction in apoA-I promoter activity (Table 3). Similar results were seen in the human intestinal cell line Caco-2 cells treated with these antioxidants. Because intestinal production of apoA-I accounts for up to 55.7% of the plasma apoA-I [10], these observations suggest that the antioxidant-related changes in apoA-I promoter activity in both hepatic and intestinal tissues may have some effects on plasma apoA-I levels in vivo.

The dose required for showing a down-regulatory effect of α-tocopherol or ascorbate on the promoter activity was higher than the effective concentrations used to down-regulate the apoA-I mRNA and protein levels. It is possible that the dose-response characteristics of the endogenous apoA-I gene may differ from the transfected apoA-I constructs.

As expected from previously published studies [8], DMSO treatment up-regulated apoA-I promoter activity. However, treating HepG2 cells with varying concentrations of an oxidant (H_2O_2 at 0, 50, 100, and 200 μmol/L) did not significantly alter apoA-I promoter activity. These observations suggest that the apoA-I promoter-stimulating effect of DMSO may be independent of its antioxidant activity and that antioxidants do not uniformly modulate the ARE function. It is possible that there are hitherto unidentified negative AREs within the promoter region of the apoA-I gene. Alternatively, it is also possible that the suppressive effect of some antioxidants on apoA-I gene expression is indirect through intermediary cellular signaling pathways. Using a series of apoA-I deletion constructs, it was shown that site A–containing region between nucleotides −325 to −186 has a critical regulatory element that responds to α-tocopherol or ascorbate (Fig. 2). However, there was no significant change in nuclear factor binding activity to site A as a result of treatment with these antioxidants (Fig. 3). Thus, it appears that the inhibitory effect of α-tocopherol and ascorbate is either indirect or is the result of posttranslational modifications of the nuclear binding factors or occurs at a region distal to site A. As expected, treatment with DMSO increased factor binding to the previously described ARE oligonucleotide. The gel shift results also suggest that the mechanism of DMSO-related factor binding to the ARE is very different than the effect of ascorbate or α-tocopherol.

The effect of antioxidants on apoA-I promoter activity could not be attributed to nonspecific toxicity because cell viability was documented by trypan blue exclusion. Furthermore, β-gal activity in the transfected cells was not altered with antioxidant treatment. Finally, albumin content in the culture media showed no significant alterations associated with treating the cells with various antioxidants.

A potential limitation of the study is that the oxidative state of the cells was not documented. The doses of ascorbate and α-tocopherol used are high compared with physiological plasma levels (the reference range for plasma ascorbate is 0-114 μmol/L, and the reference range for serum α-tocopherol is 12-46 μmol/L) [33]. The precise physiological concentrations of these micronutrients within the appropriate target compartments are not known. However, it is noteworthy that significant reductions in apoA-I mRNA and protein levels were observed in HepG2 cells treated with as little as 15 μmol/L of ascorbate and 100 μmol/L of α-tocopherol. Nevertheless, despite the value of cell culture models such as those used here, there may well be fundamental changes in metabolism that differ from normal liver or intestinal cells. The effect of antioxidants in vivo has been demonstrated in human experiments. Treatment of human subjects with probucol, a potent antioxidant, was associated with a reduction in HDL and its principal protein constituent, namely, apoA-I [4]. On the other hand, antioxidant deficiency, notably deficiency of ascorbic acid, may be associated with low apoA-I mRNA [34]. In HepG2 cells, treatment with DMSO, an antioxidant, or gramoxone, a prooxidant, induces apoA-I promoter activity [8,9]. The effect of antioxidants on apoA-I expression may be dependent on peculiarities of each compound, and the effects may be biphasic, such that there is an optimal concentration required for apoA-I promoter activity, and concentrations that are below or above this optimal range would down-regulate apoA-I expression.

Another limitation in this study is that rat apoA-I promoter was used to study the effect of antioxidants in human cells. However, the region of the rat promoter that we used is 83% homologous to human promoter, and we have previously found that the responses of this promoter to various signals, with the exception of few cases, are similar to the responses of human promoter. The differences in dose response for apoA-I mRNA/protein and promoter (CAT) activity may be because of subtle differences between rat and human promoter elements. Alternatively, the differences may be because of different chromatin effects in antioxidant-treated cells with our transfected reporter gene compared with the endogenous apoA-I gene. This latter explanation is more likely because our data suggest that changes in site A binding are not observed in cells treated

with α-tocopherol or ascorbic acid, but that coactivators/corepressors may be involved. Coactivators and corepressors modulate gene expression in large part by remodeling chromatin. Coactivator/corepressor recruitment to the artificial CAT reporter gene may not be as efficient as recruitment to the endogenous apoA-I gene. Nevertheless, caution should be exercised in interpreting these results. In general, the use of deletion constructs of the apoA-I promoter points to a putative site, but is not a definitive evidence of the effects on native apoA-I mRNA.

The differences between the effect of DMSO and the other antioxidants tested on apoA-I expression are not readily explained. It is noteworthy that DMSO, although an antioxidant, also has multiple other biological properties. DMSO is known to be a modulator of gene expression [35,36] and a modulator of cell cycle [37,38]. In addition, DMSO selectively alters several components of protein kinase A pathways, but not protein kinase C pathways [39]. Thus, it is possible that some of the effects of DMSO, ascorbate, and α-tocopherol are independent of their antioxidant activity.

Despite the uncertainties related to the precise effects of antioxidants on apoA-I gene expression, the finding that commonly used antioxidants may decrease apoA-I gene transcription raises some concerns. Whether similar changes occur in vivo remains to be shown. However, these observations do not support the use of pharmacological doses of antioxidants to enhance cardioprotective lipoproteins.

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