Effects of dimethyl sulfoxide on apolipoprotein A-I in the human hepatoma cell line, HepG2

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Abstract Exposure of HepG2 cells to 1% (v/v) dimethyl sulfoxide (DMSO), an effective free radical scavenger, for 24 h resulted in a 2-fold increase in the levels of apolipoprotein (apo) A-I mRNA and secreted protein, with no significant change in apoA-II, apoB, and apoE mRNA and protein levels. The induction of apoA-I was accompanied by a 50% increase in secreted HDL. Nuclear run-off assays indicated that the transcription rate of the apoA-I gene was also increased 2-fold in DMSO-treated cells. Consistent with nuclear run-off assays, transient transfection experiments, using a series of pGL2-derived luciferase reporter constructs containing the human apoA-I proximal promoter, demonstrated that DMSO treatment increased apoA-I promoter activity 2-fold. We have identified a potential 'antioxidant response element' (ARE) in the apoA-I promoter that may be responsible for the increase in apoA-I transcriptional activity by DMSO. Gel mobility shift assays with an apoA-I–ARE revealed increased levels of a specific protein-DNA complex that formed with nuclear extracts from DMSO-treated cells. The formation of this complex is sequence specific as determined by DNA competition studies. When a copy of the ARE was inserted upstream of the SV40 promoter in a luciferase reporter plasmid, a significant 2-fold induction in luciferase activity was observed in HepG2 cells in the presence of DMSO. In contrast, a plasmid containing a mutated apoA-I–ARE did not confer responsiveness to DMSO treatment. Furthermore, pGL2 (apoA-I–250 mutant ARE), in which point mutations eliminated the ARE in the apoA-I promoter, showed no increase in luciferase activity in response to DMSO. These results implicate protein-DNA interactions at the antioxidant response element region in the transcriptional induction of human apoA-I gene expression by DMSO.—**Tam, S-P., X. Zhang, C. Cuthbert, Z. Wang, and T. Ellis.** Effects of dimethylsulfoxide on apolipoprotein A-I in the human hepatoma cell line, HepG2. *J. Lipid Res.* 1997. **38:** 2090–2102.

Supplementary key words apolipoproteins • gene expression • atherosclerosis

Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent and an effective scavenger of hydroxyl radicals (1). It has great permeability which allows it to penetrate all membranes impenetrable to other antioxidants, particularly enzymatic antioxidants. DMSO has biologically diverse effects on mammalian cells. Addition of 2% (v/v) DMSO to the culture medium of primary cultures of rat hepatocytes maintained biochemical and differentiated functions for extended periods (2). Results of Isom and co-workers (3) suggested that DMSO promoted the persistence of some liver-specific mRNAs in cultured hepatocytes. DMSO also partially protected the loss of total cytochrome P450 content in cultured rat hepatocytes (4, 5), and induced the alcohol-inducible P450IIE1 in vivo (6). In addition, increase in haem synthesis in HepG2 cells in response to DMSO has been shown to be a transcriptionally activated event (7).

Several reports have suggested that DMSO may also be effective in inhibiting cholesterol-induced atherosclerosis in experimental animals (8–10). However, the mechanisms by which DMSO inhibits cholesterol-induced atherosclerosis are still not clear. Studies have indicated that DMSO slows the development of dietary cholesterol-induced atherosclerosis in rabbits and reduces the accumulation of cholesterol in tissues despite severe hypercholesterolemia. By far the most prominent effect of DMSO in rabbits on an atherogenic diet was the marked decrease in both the number of foam cells and their content of lipid droplets (10). The inhibition of atherosclerosis in the presence of high plasma cholesterol levels would suggest that DMSO may act via mechanisms that prevent the uptake of cholesterol by macrophages or endothelial cells of the aorta. The "oxidation hypothesis of atherosclerosis" proposes that oxidative modification of low density lipoprotein (Ox-

Abbreviations: apoA-I, apolipoprotein A-I; ARE, antioxidant response element; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FBS, fetal bovine serum; GST, glutathione-S-transferase; HDL, high density lipoprotein; MEM, minimal essential medium; Na_2 EDTA, disodium ethylene-diamine tetraacetate; Ox-LDL, oxidized low density lipoprotein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; XRE, xenobiotic response element.

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LDL) is crucial in the pathogenesis of the atherosclerotic lesion (11). Ox-LDL is a prerequisite for macrophage uptake and cellular accumulation of cholesterol (12, 13). However, oxidized high density lipoprotein is not avidly taken up by macrophages and does not lead to foam cell formation (14). Furthermore, HDL has been reported to inhibit endothelial cell-mediated LDL modification (14, 15) and to reduce cellular uptake and degradation of native and Ox-LDL (14, 16). HDL is also capable of protecting against LDL peroxidation in vitro (14, 17) and in vivo (18). We hypothesize that DMSO would prevent the formation of Ox-LDL either directly or indirectly through the modulation of HDL production. These two possibilities, though not mutually exclusive, are supported by the studies of Layman, Alam, and Newcomb (9), and De La Vega and Mendoza-Figueroa (19). The first study indicated that the addition of 2% DMSO to human skin fibroblasts resulted in a greater than 35% reduction in uptake and binding of ^{125}I-labeled LDL and a 26% decrease in its degradation (9). Two simple explanations for this observation are *i*) DMSO treatment causes a reduction in the number of LDL receptors on the fibroblasts, or *ii*) DMSO slows the oxidation process of LDL. The second study, which supports the latter hypothesis, has shown that adult rat hepatocytes cultured on a feeder layer of lethally treated 3T3 cells increases the de novo synthesis and secretion of total lipids by 2- to 3-fold after 14 days of 2% DMSO treatment (19). Although the secretion of various lipoprotein classes by 3T3 hepatocytes was not determined, the investigators suggested that an increase in VLDL and HDL would be expected (19). To test this hypothesis, we have examined the effects of DMSO on the synthesis of apoA-I by using the human hepatoma cell line, HepG2, as an in vitro model. This cell line is highly differentiated and retains many of the specialized functions normally lost by hepatocytes in culture (20, 21).

The current study explores some of the molecular mechanisms by which DMSO modulates human apoA-I production. We have demonstrated that exposure of HepG2 cells to 1% DMSO resulted in a 2-fold increase in apoA-I transcription rate and a corresponding increase in both apoA-I mRNA and secreted apoA-I. Evidence summarized below suggest that the expression of the human apoA-I gene may be modulated by DMSO-induced nuclear factor. We have identified a putative antioxidant response element (ARE) located in the apoA-I promoter between nucleotides −142 and −132 relative to the transcription start site of the gene. This element was first identified by Rushmore, Morton, and Pickett (22) during short-term transfection studies on the regulation of the rat glutathione-S-transferase (GST) Ya subunit gene in response to metabolites of planar aromatic compounds and phenolic antioxidants. Here, we describe experiments showing that the putative ARE is necessary and sufficient for DMSO-mediated induction of human apoA-I gene expression.

METHODS

Materials

Complementary pairs of oligonucleotides were synthesized using the Beckman Oligo 1000 DNA Synthesizer according to the manufacturers instructions. The following oligonucleotides (and their complementary strands) were prepared:

apoA-I–ARE 5′–CAGCCCCAGGGACAGAGCTG–3′

Mutated ARE 5′–CAGCCCCATTTGAGTGTATG–3′

GST-ARE 5′–CTAATGGTGACAAAGCAG–3′

Xenobiotic response element (XRE) 5′–AGTGCTGT CACGCTAG–3′

SPI consensus binding element 5′–CTGCGGGGCGG GGCAGA–3′

Cell culture

The human hepatoma cell line, HepG2, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in T75 flasks containing 20 ml of Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) as described previously (23). Confluent monolayers were washed twice with MEM and then incubated with fresh serum-free MEM for 0–24 h in the absence or presence of 1% or 2% (v/v) DMSO. In some experiments, DMSO and cycloheximide were added to cells to give final concentrations of 1% and 10 μg/ml, respectively. Cell viability was routinely monitored by trypan blue exclusion and lactate dehydrogenase leakage as described previously (24). In all experiments, the number of dead cells never exceeded 5% of the total number of cells.

RNA isolation and detection

Total cellular RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform extraction method described by Chomczynski and Sacchi (25). RNA detection and quantitation were determined by slot blot analyses. For slot blots, multiple RNA samples (0.5–5.0 μg) from cells cultured under a variety of conditions were denatured with formaldehyde and applied

to wells of a slot blot apparatus (Bio-Rad) onto zeta-probe GT membranes. Blots were prehybridized and hybridized with nick-translated apoA-I, apoA-II, apoB, and apoE cDNA probes as described (26). The apolipoprotein cDNAs were obtained from the American Type Culture Collection. All results were normalized using densitometric scans of slot blots probed with radiolabeled oligo-dT to correct for loading variations as described previously (26).

Detection of the apolipoprotein and lipid components of various lipoprotein fractions

Metabolic labeling and immunoprecipitation of various apolipoproteins were performed by methods as described previously (26) with minor modifications. Briefly, monolayers of HepG2 cells were washed twice with 10 ml of serum-free MEM and incubated with 5 ml of methionine-free MEM (without serum) supplemented with [^{35}S]methionine (500 μCi, 1100 Ci/mmol) in the absence or presence of 1% DMSO. At the indicated time periods, the medium was removed from cells, transferred to a tube containing a protease inhibitor mixture (final concentrations: 1 mM benzamide, 100 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 100 units/ml aprotinin, 5 mM EDTA and 50 mM Tris-HCl, pH 8.0), and centrifuged at 1,500 *g* for 5 min to remove any dead cells and debris. A portion (20 μl) of supernatant was adjusted with trichloroacetic acid to give a final concentration of 10% in order to determine total trichloroacetic acid precipitable counts. Another aliquot (50 μl) of the supernatant was used for immunoprecipitation of human apoA-I, apoA-II, apoB, and apoE. Immunoprecipitations were carried out using specific polyclonal antibodies against these apolipoproteins obtained from Boehringer Mannheim Canada and protein G-Sepharose was used to bind the immune complexes. The immunoprecipitates were washed three times with phosphate-buffered saline containing 0.5% Triton X-100 and 0.2% SDS. The final washed pellets were resuspended in 200 μl of 50 mM Tris-HCl, pH 6.8, containing 1% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue, and 5% glycerol, followed by incubation in a boiling water bath 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 (27).

The synthesis of various classes of lipids was monitored by incubating HepG2 cells with 10 μCi [^{3}H]glycerol (specific activity = 40 mCi/mmol) and 10 μCi [^{14}C]acetate (specific activity = 56.5 mCi/mmol) as described previously (24). Confluent monolayers of HepG2 cells in T75 flasks were washed twice with MEM and then incubated for 24 h at 37°C in 5 ml serum-free MEM in the absence or presence of 1% (v/v) DMSO. Various density classes of lipoproteins were isolated from the media by the modification (28) of the method of Havel, Eder, and Bragdon (29) in a Beckman L-80 ultracentrifuge using a SW41 rotor. VLDL ($d < 1.006$ g/ml), LDL (d 1.006–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated by sequential ultracentrifugation using NaBr for density adjustments. Cellular and secreted lipoprotein lipid were determined by the method of Folch, Lees, and Sloane Stanley (30). Lipids were separated by thin-layer chromatography with hexane–diethylether–acetic acid 80:20:1 as developing solvent. By using lipid standards, the appropriate spots on the silica gels were scraped into vials and assayed for radioactivity by scintillation counting. Cellular protein content was determined by the method of Lowry and co-workers (31).

Polyacrylamide gradient gel electrophoresis

HepG2 cells were cultured as described above. Confluent monolayers were washed twice with phosphate-buffered saline and incubated with fresh serum-free MEM for 24 h in the absence or presence of 1% DMSO. After incubation, the medium was collected into a tube containing Na_2 EDTA to give a final concentration of 1 mg/ml. HDL fraction was isolated by ultracentrifugation as described above and concentrated by an ultrafree MC filter unit (Millipore, Bedford, MA). The HDL fractions were then separated by electrophoresis in a linear polyacrylamide gradient gel ranging from 3% to 18% as described by Gambert and co-workers (32). Briefly, lipoproteins were prestained by mixing equal volumes of HDL fractions and 0.5% solution of Sudan black B in ethylene glycol. The running buffer was Tris/glycine at a concentration of 14:100 mM (pH 8.3) solution. After pre-electrophoresis at 50 V for 30 min, an aliquot (10 μl) of each mixture was applied to the gel. The electrophoresis was carried out in a Bio-Rad Mini-Protean II apparatus at 4°C for 1 h at 20 V and 18 h at 100 V. Human plasma HDL was obtained from Sigma-Aldrich as reference.

Nuclear run-off transcription assay and preparation of nuclear extracts

Nuclei were prepared according to the procedure of Bartalena and co-workers (33). An in vitro nuclear run-off transcription assay was carried out as described (33) with minor modifications (34). Procedures for nuclear run-off experiments and preparation of nuclear extracts from HepG2 cells have been described in detail previously (35).

Bandshift assays

For bandshift assays, nuclear extracts (1.0 μg) were incubated with 100 μg of poly (dI-dC) in binding buffer containing 5 mM DTT and 5 μM $ZnCl_2$, on ice for 30 min. Then 2 fmol (10,000 cpm) 5′ end labeled synthetic oligonucleotide corresponding to the apoA-I ARE was added to the reaction mixtures and incubated on ice for another 30 min. Procedures for bandshift assays have been described in detail previously (35). Competition assays were performed by adding the unlabeled competitor DNA 15 min prior to the addition of labeled apoA-I–ARE as indicated in the figure legends.

Preparation of luciferase constructs

Two Genelight™ vectors (Promega, Life Technologies) were used: pGL2-Basic (pGL2-B) and pGL2-Promoter (pGL-2-P). A 491 bp DNA fragment of the human apoA-I promoter between nucleotides −491 to +1 was generated by polymerase chain reaction (PCR) amplification described in detail previously (35). The sequence of this DNA fragment was confirmed by DNA sequencing. The fragment was inserted into the Xho site of the pGL2 Basic vector, upstream of the luciferase gene. This plasmid is hereafter referred to as pGL2 (apoA-I −491) luc. Plasmid pGL2 (apoA-I −250) luc was constructed by releasing a DNA fragment (−491 to −251 of the apoA-I promoter) from pGL2 (apoA-I −491) using Sma I. The vector was gel purified and religated.

Plasmid pGL2 (apoA-I − 250 mutant ARE) luc was prepared by a PCR-based protocol as described by Morrison and Desrosiers (36). To generate this mutated ARE plasmid, two sets of primers were used. One set of primers, designated GL and LUC, hybridized to specific regions of the plasmid pGL2. The other set of primers which contained the mutagenic ARE residues were named primers FOR and REV. The primers GL (5′-TGTATCTTATGGTACTGTAACTG-3′) and REV (5′-GATCATACACTCAAATGGGGCTGGG-3′) were complementary to the non-coding strand of DNA while primers FOR (5′-CCCATTTGAGTGTATGATCCTTGA AC-3′) and LUC (5′-GGCGTCTTCCATTTTACC-3′) were complementary to the coding strand of DNA. The plasmid pGL2 (apoA-I −250) luc was used as the PCR template. Amplification was carried out as described previously (33). The DNA fragment generated was then digested with Sma I and Hind III and the resulting DNA fragment was purified from an agarose gel. The purified DNA fragment was cloned into the Sma I and Hind III sites of the pGL2-basic (Promega Inc.) and used to transform competent *E. coli* cells. DNA was prepared from individual clones by the alkaline lysis miniprep procedure and the entire DNA insert including the area of mutagenesis was sequenced using the sequenase version 2.0 system (United States Biochemical).

Two pGL2-P vectors containing apoA-I–ARE and mutated ARE were also constructed. pGL2-P was digested with the restriction enzymes Kpn I and Nhe I. The restriction digest was electrophoresed and the digested plasmid was gel purified. Synthetic oligomers corresponding to the putative ARE from the human apoA-I promoter and mutated ARE were inserted individually into the Kpn I and Nhe I of the linearized pGL2-P vectors. The inserts in these two plasmids have the same 5′ → 3′ orientation as found in the natural human apoA-I promoter. The sequence of all pGL2-P constructs was confirmed by DNA sequencing.

Transfection and luciferase assay

The human hepatoma cell line, HepG2, was maintained as monolayers on 100-mm plates in MEM supplemented with 10% FBS. Transient DNA transfections were performed by the calcium phosphate precipitation procedure described by Gorman, Moffat, and Howard (37) and detailed previously (35). The cells were then cultured in the absence or presence of 1% (v/v) DMSO for 24 h, unless indicated otherwise.

Transfected HepG2 cells were harvested by washing three times in PBS and assayed for luciferase activity as described previously (35). In all transfections, 5 μg of an internal control plasmid (pSG Δ LacZ) containing the *E. coli* LacZ gene under the control of the SV40 early promoter and enhancer, was included in order to correct for differences in transfection and harvesting efficiency. Transfected cells were harvested as described (35) and β-galactosidase activities in the cell lysates were determined (35). The pGL2-promoter vector, which contains an SV40 promoter, was used as a reference for both transfection and luciferase assays. All luciferase activities are reported as mean ± SEM. Significance of group difference was determined by Student's *t*-test, using two group *t* test or by matched *t* test where appropriate.

RESULTS

Cell viability

The amount of cellular protein obtained from T-75 flasks during the first 24 h of culture was used as an indicator of cell attachment and was not found to be significantly altered by the addition of either 1% or 2% (v/v) DMSO in the culture medium (**Fig. 1A**).

Cell viability was also monitored by trypan blue exclusion and lactate dehydrogenase leakage experiments as

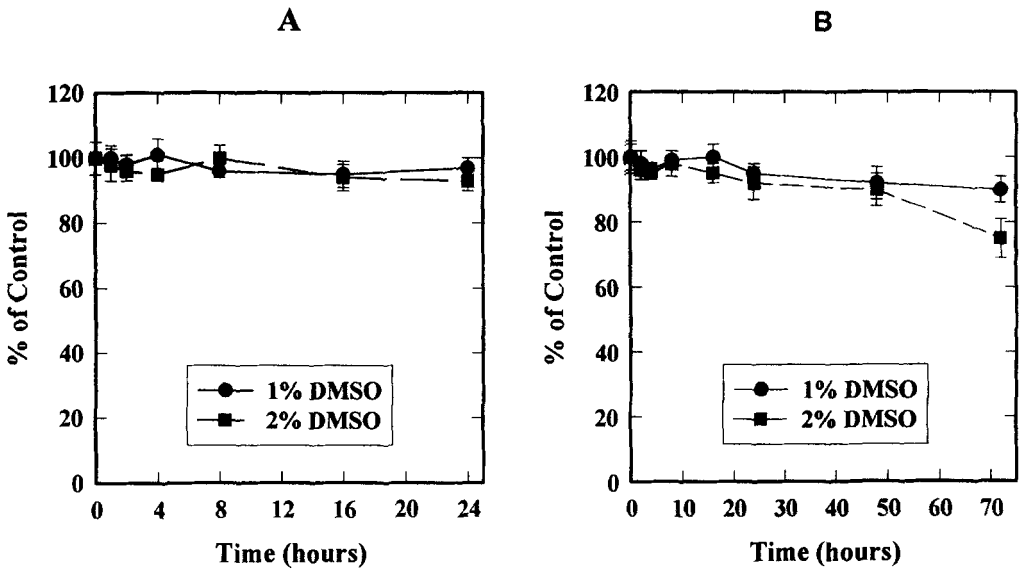

Fig. 1. Effects of DMSO on cell damage. Newly confluent HepG2 cells (30 million cells/flask) were exposed to 1% and 2% (v/v) DMSO for up to 72 h. At various times, cellular protein content (A) and cell viability assays (B) were determined. Protein content was expressed as percent of control (untreated cells) at the corresponding time points indicated. The mean ± SEM of total cellular protein content per flask is 2.9 ± 0.1 mg. Cell viability was monitored by lactate dehydrogenase leakage assay (B). Similar results were also observed by using trypan blue exclusion studies (data not shown). Results are mean ± SEM of four experiments.

described in Methods. The loss of hepatocytes from culture flasks was negligible (<5%) when HepG2 cells were exposed to either 1% or 2% of DMSO for 48 h. However, after addition of 2% of DMSO to the medium for more than 48 h, cell survival dropped significantly (Fig. 1B). Therefore, in all further experiments, 1% (v/v) of DMSO was used.

Effect of DMSO on secreted lipoprotein lipid in HepG2 cells

Table 1 summarizes the effect of DMSO on lipoprotein lipid secretion in HepG2 cells. Secreted VLDL cholesterol and triglyceride levels were not affected by incu-

TABLE 1. Effect of DMSO on various lipids in different lipoproteins classes in HepG2 cells

	DMSO % (v/v)	
Lipoprotein Lipid	0	1.0
μg/g cell protein/h		
VLDL cholesterol	12.1 ± 2.5	13.8 ± 2.4
VLDL triglyceride	425 ± 96	448 ± 108
LDL cholesterol	37.5 ± 4.2	42 ± 5.1
LDL triglyceride	329 ± 65	376 ± 68
HDL cholesterol	104 ± 18	158 ± 16[a]
HDL phospholipid	304 ± 29	472 ± 58[a]

Different lipoprotein classes were isolated by ultracentrifugation as described in the text. Results are mean ± SEM of three experiments. Lipids were determined by the procedure described in Methods.

[a]Significantly different from control, $P < 0.01$ by matched t test.

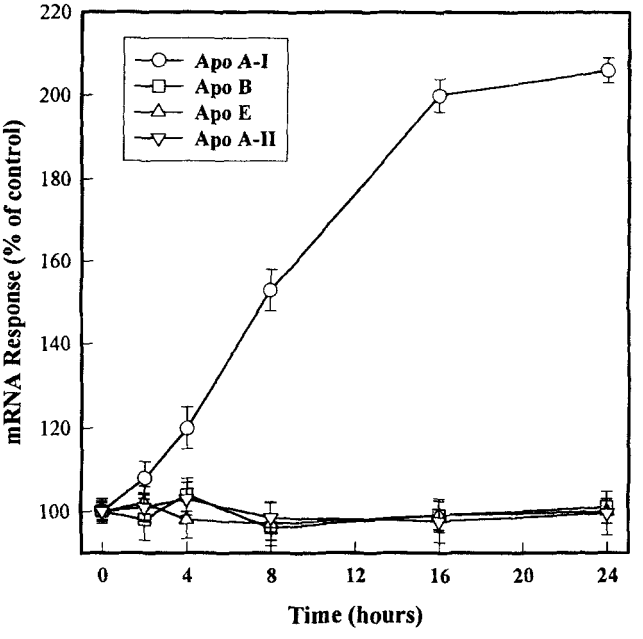

Fig. 2. Effect of DMSO on apolipoprotein mRNA levels in HepG2 cells. Newly confluent HepG2 cells were grown in media containing 10% FBS. Cells were then cultured in the presence of 1% (v/v) DMSO for various time periods. Total RNA was isolated at 0, 2, 4, 8, 16, and 24 h. The levels of apoA-I, apoA-II, apoB, and apoE mRNAs were determined by slot blot analysis as described in Methods. All results were normalized using densitometric scans of slot blots probed with radiolabeled oligo-dT cDNA to correct for loading variations. Steady state levels of apoA-I, apoA-II, apoB, and apoE mRNAs at zero time were arbitrarily set at 100%. Results are mean ± SEM of four experiments..

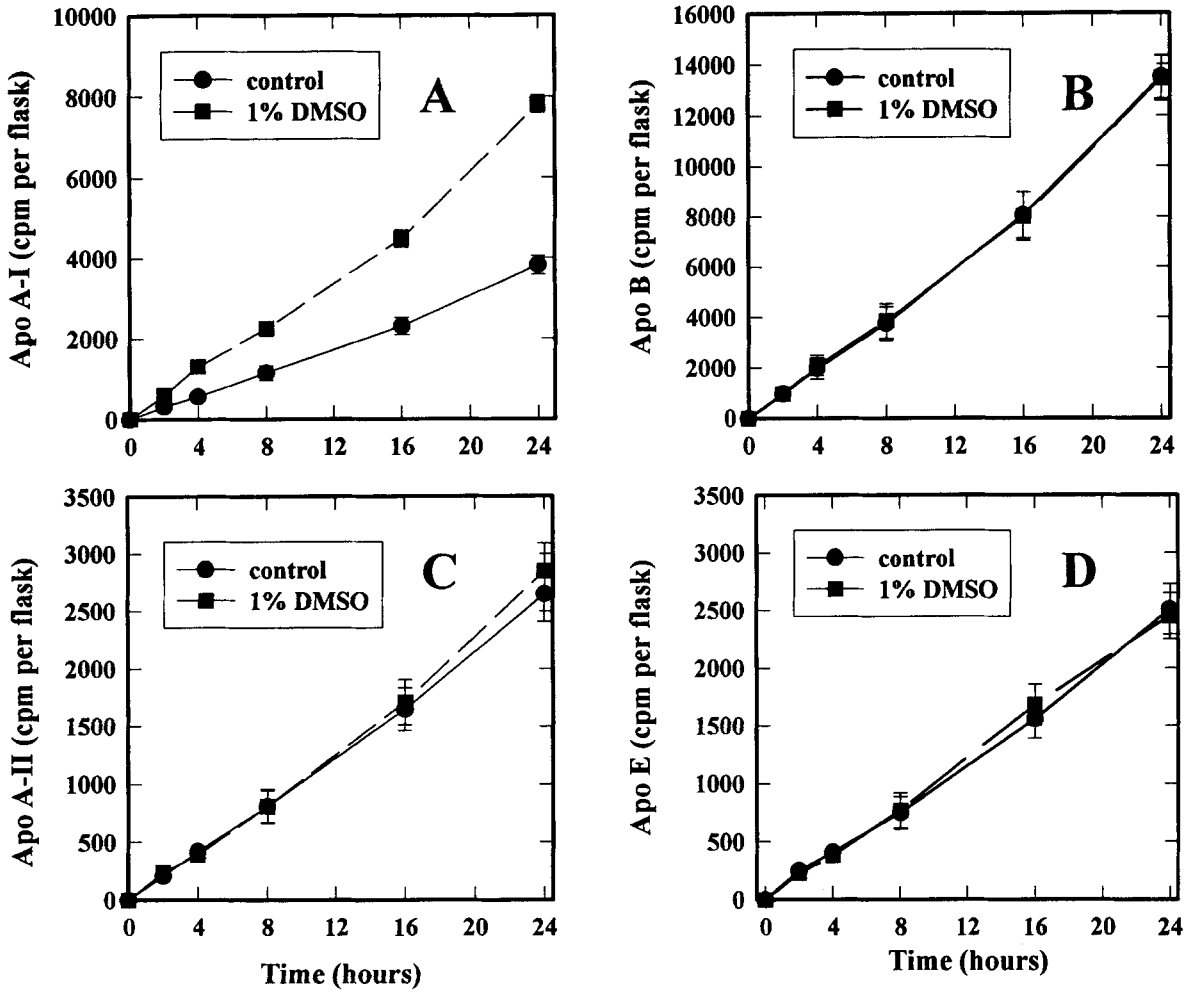

Fig. 3. Effect of DMSO on apolipoprotein production in HepG2 cells. Newly confluent HepG2 cells were washed twice with 10 ml of phosphate-buffered saline and maintained in serum-free MEM and [^{35}S]methionine in the absence (control) or presence of 1% (v/v) DMSO for 24 h as described in Methods. At the times indicated, the medium was removed from cells. ApoA-I (panel A), apoB (panel B), apoA-II (panel C), and apoE (panel D) were immunoprecipitated from the medium as described under Methods. Results are ± SEM of 4 experiments.

bation of the cells with 1% (v/v) DMSO for 24 h. LDL cholesterol and triglyceride levels were slightly increased, but the change was not significant. By contrast, HDL cholesterol and phospholipid was increased by 50% in the medium ($P < 0.01$). DMSO treatment did not affect cellular levels of total cholesterol, triglyceride and phospholipid (data not shown).

Modulation of steady state apolipoprotein mRNA levels by DMSO

The effects of DMSO on the levels of apoA-I, apoA-II, apoB, and apoE mRNAs in HepG2 cells were determined by slot blot analysis using the levels of poly A + RNA determined by oligo-dT hybridization to control for variation in RNA loading (**Fig. 2**). Exposure of HepG2 cells to 1% (v/v) DMSO resulted in a 2-fold increase in apoA-I mRNA levels. However, there was no significant change in the levels of apoA-II, apoB, and apoE mRNA in HepG2 cells after DMSO treatment. Figure 2 shows that maximal induction of the levels of apoA-I mRNAs peaked between 16 h and 24 h.

Effects of DMSO on secreted apolipoprotein levels and HDL fraction

Studies examining the effects of DMSO and time of incubation on the de novo synthesis of apolipoproteins by HepG2 cells were performed by measuring the incorporation of radiolabeled methionine into apolipoproteins secreted into the medium. At various times, the rates of accumulation of secreted apoA-I, apoA-II, apoB, and apoE in culture media were determined. A 2-fold increase in apoA-I production was observed in the DMSO-treated cells compared to control cells (**Fig. 3A**). No significant effect on apoA-II, apoB, and apoE

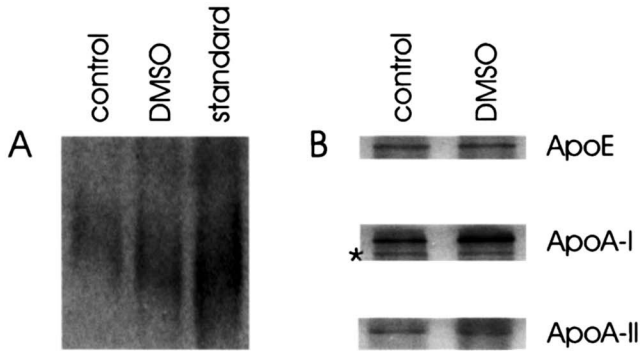

Fig. 4. Effects of DMSO on HDL and its major protein constituents in HepG2 cells. Panel A: Confluent monolayers were washed twice with phosphate-buffered saline and incubated with fresh serum-free MEM for 24 h in the absence or presence of 1% DMSO. The HDL fractions were then isolated and separated by linear polyacrylamide gradient gel ranging from 3% to 18% as described in Methods. HDL subfractions were quantified by densitometric scanning of the gradient gels. Human plasma HDL was used as a standard reference. Panel B: An illustration of the specificity of the immunoprecipitation technique was shown. *The identity of the lower protein band that coprecipitated with apoA-I is not known.

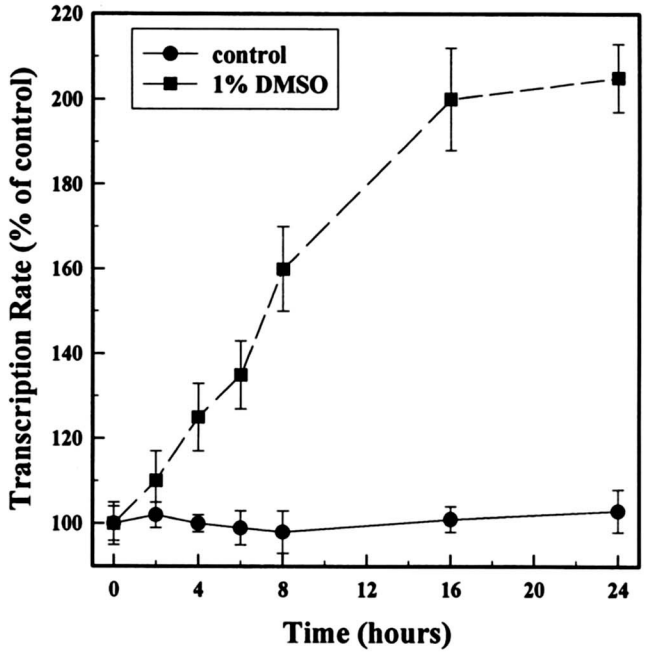

Fig. 5. Time course of transcription rates of apoA-I gene in HepG2 cells cultured in the absence of presence of 1% DMSO. Rate of transcription of apoA-I gene was determined by nuclear run-off assay using nuclei isolated from HepG2 cells-cultured in the absence or presence of 1% DMSO as described in Methods. Nuclei ($2–3 \times 10^7$) were isolated at 0, 2, 4, 6, 8, 16, and 24 h. Hybridization of the newly synthesized [^{32}P]RNA to plasmids containing the apoA-I insert were carried out in triplicate. Nonspecific hybridization to each filter was determined by performing transcription with labeled pGEM 3Z control vector. Filters were washed extensively and radioactivity bound was measured by a liquid scintillation counter. Relative transcription rates were calculated as part per million (ppm)/filter = (counts/min per filter counts/min background) ÷ amount of [^{32}P]-RNA used in hybridization (input count). These numbers were then normalized for hybridization efficiency as measured by binding of known amounts of [^{3}H]riboprobes added to the hybridization mixture. This was then corrected for the size to 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 kilobases, respectively. Results are mean ± SEM expressed in percentage of the 0 h time point of three independent experiments.

secretion was observed with DMSO treatment, indicating that the effect of DMSO on production of apoA-I was not a general phenomenon applied to apolipoproteins (Fig. 3B,C,D). Furthermore, no significant change in the rate of total protein synthesis was detected when HepG2 cells were cultured in the presence of 1% DMSO (data not shown). The specificity of this immunoprecipitation technique was examined by analyzing the immune complexes by SDS-polyacrylamide gel electrophoresis (**Fig. 4B**). These studies indicated that the apoA-I antibody precipitated two protein bands. The upper band corresponds to the size of human apoA-I. The identity of the lower, distinct protein band is not known. On the basis of quantitative densitometric scanning of these two protein bands, the intensity of the upper band increased 2-fold upon DMSO treatment while the lower band gave a similar signal on the X-ray autoradiograph in both control and DMSO-treated cells. Thus, the lower protein band may be used as an internal control for protein loading.

In order to examine the effects of DMSO on HDL subfraction electrophoretic behavior, we performed linear gradient gel electrophoresis. By densitometric analysis, we found that the HDL fraction increased by 50% when HepG2 cells were treated with 1% DMSO for 24 h (Fig. 4A). The HDL electrophoretic patterns of both control and DMSO-treated cells were similar to that of human plasma HDL prepared by ultracentrifugation prestained and then submitted to the same conditions of electrophoresis (Fig. 4A). However, the majority of the HDL particles isolated from the DMSO-treated cells appeared to be in the HDL_3 size range.

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

Nuclear run-off assays were performed to determine whether increased rates of transcription were responsible for the observed induction of apoA-I mRNA levels by DMSO treatment. The transcription rates of the apoA-I gene were determined using isolated nuclei from HepG2 cells cultured in the absence or presence of DMSO. The effect of DMSO on apoA-I transcription rates at various times is shown in **Fig. 5.** The rate of transcription of the apoA-I gene increased approxi-

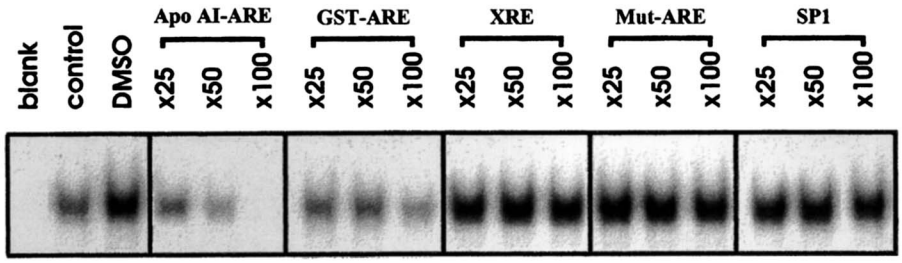

Fig. 6. Gel mobility shift assay of the apoA-I ARE in response to 1% DMSO. Synthetic double-stranded oligonucleotides corresponding to the DNA sequence between nucleotides −149 and −130 upstream from the transcription start site (+1) of the human apoA-I gene, designated apoA-I–ARE, were used as probes to study protein–DNA interactions. In addition, synthetic double-stranded GST-ARE, XRE, and mutated ARE (Mut–ARE) were also used as competitors. ^{32}P-labeled oligo-apoA-I–ARE (10,000 cpm) was incubated with no protein (blank) or with nuclear extracts isolated form untreated cells (control) or cells treated with 1% DMSO for 24 h as described in Methods. Competition studies were performed using gramoxone-treated nuclear extract and ^{32}P-labeled apoA-I–ARE together with 25-, 50-, and 100-fold molar excesses of unlabeled apoA-I–ARE, GST–ARE, XRE, Mut–ARE, and SP1 oligomers and shown. Similar results were observed in three independent experiments.

mately 2-fold between 16 h and 24 h after DMSO treatment.

Protein-DNA interactions at the ARE region of the human apoA-I gene

The possibility that induction of apoA-I transcription by DMSO involved protein-DNA interactions at the putative ARE, was examined by bandshift assays using a 5′ end labeled double-stranded apoA-I–ARE, corresponding to the apoA-I promoter region between nucleotides −149 to −130. Extracts prepared from control HepG2 cells contain a factor that will bind to apoA-I–ARE (**Fig. 6**). However, treatment with DMSO for 24 h resulted in an induction of a retarded complex. The induction of this complex was not blocked by inhibiting protein synthesis with cycloheximide (data not shown). In nuclear extracts from DMSO-treated HepG2 cells, binding to the labeled apoAI–ARE probe was efficiently blocked by competition with 50- to 100-fold molar excess of unlabelled apoA-I–ARE or GST-ARE. However, no competition was observed with up to 100-fold molar excess of the xenobiotic response element (XRE), or a mutated ARE oligomer or the SP1 consensus sequence.

The effect of DMSO on the formation of the protein–ARE complex at various times is shown in **Fig. 7.** Densitometric analyses of the autoradiograph indicated maximum induction (7-fold) of the protein-DNA complex occurred at 2 h. The induction was maintained, albeit at a lower level (3.5 to 5.0-fold) for at least 24 h.

Transient transfection studies

To analyze the role of apoA-I–ARE in the regulation of human apoA-I gene expression in response to DMSO, we carried out transient transfection experi-

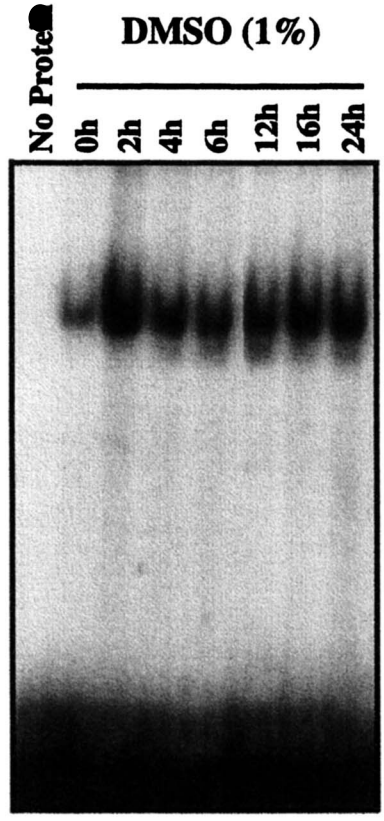

Fig. 7. Time course analysis of the effect of DMSO on the formation of the protein–ARE complex. Gel mobility shift assay was carried out as described in the legend to Fig. 6. The assays were performed using ^{32}P-labeled double-stranded apoA-I ARE oligomer and nuclear extracts isolated from HepG2 cells treated with 1% DMSO for various times as indicated. Similar results were observed in three independent experiments.

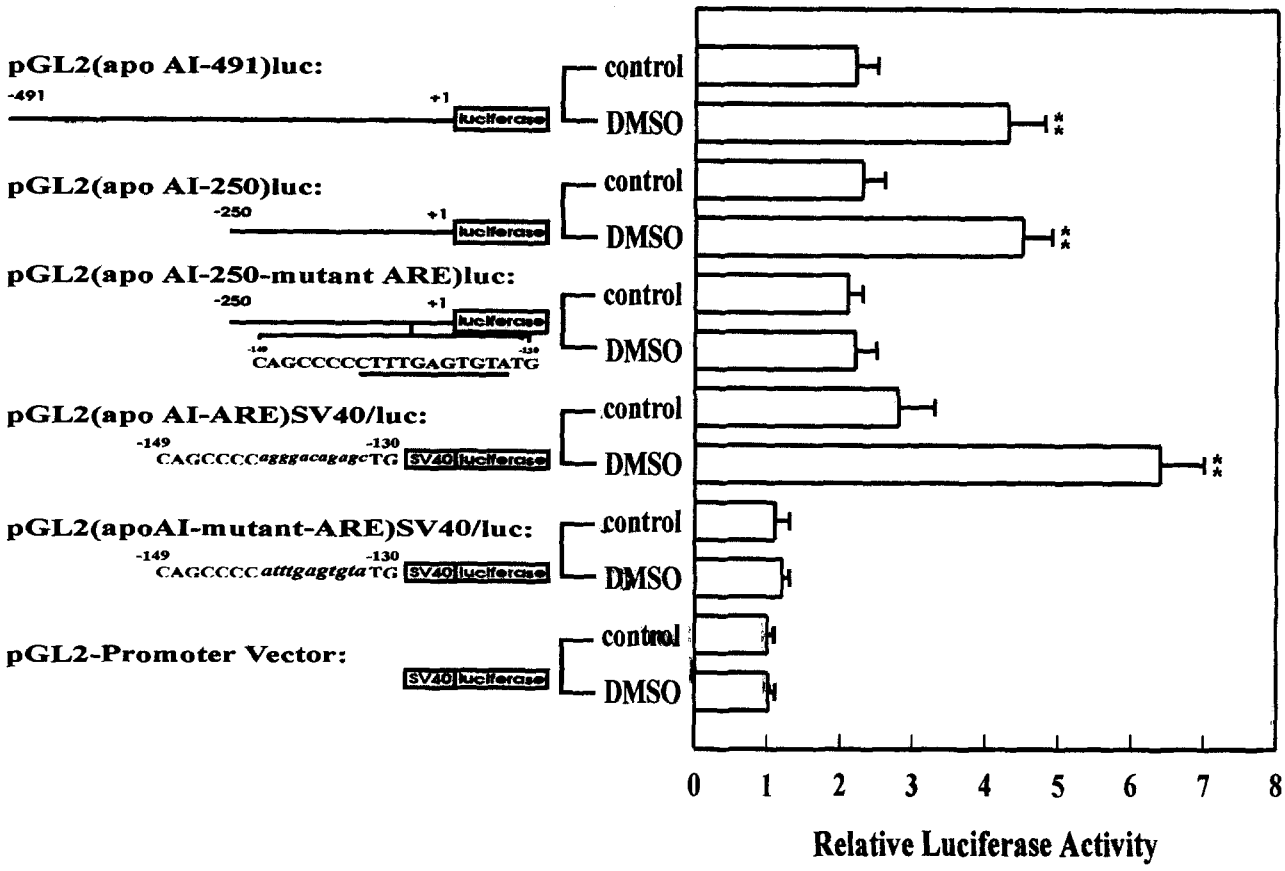

Fig. 8. Schematic representation of pGL2–apoA-I and pGL2–ARE SV40/luciferase reporter gene constructs and analysis of luciferase activity in transfected HepG2 cells. Diagrams at left represent the pGL2–apoA-I/luc construct series that contain sequences −491 to + 1 and −250 to +1 of the human apoA-I proximal promoter region. The pGL2 (apoA-I −250 mutant ARE) contains a DNA sequence in which point mutations eliminated the apoA-I–ARE in the human apoA-I promoter. The pGL2/SV40/luc series are constructed by placing a synthetic apoA-I–ARE or mutated ARE in front of the SV40 promoter. Construction of these plasmids has been described in Methods. Freshly seeded HepG2 cells were transfected with the reporter plasmid and β-galactosidase as internal control to normalize for differences in transfection efficiency. Cells were then cultured in the absence or presence of 1% (v/v) DMSO for 24 h prior to harvesting. The graph at right represents relative luciferase activity in transfected cells cultured in the absence (control) or presence of DMSO. Luciferase activities of the constructs are expressed relative to that of pGL2 promoter vector (arbitrarily set at 1). Results are mean ± SEM for five independent experiments carried out in duplicate. **Significantly different from control ($P < 0.01$, two-tailed t test).

ments using a series of pGL2-derived luciferase reporter plasmids. Plasmids were transfected into HepG2 cells in the absence or presence of 1% (v/v) DMSO, and luciferase activity was measured. As shown in **Fig. 8,** both pGL2 (apoA-I −491)luc and pGL2 (apoA-I −250) luc constructs showed a significant 2-fold induction of luciferase activity in the presence of DMSO. By contrast, pGL2 (apoA-I −250 mutant ARE) in which the wild-type ARE sequence was replaced by a mutant ARE (G → T, C → A, T → G, A → C), showed no increase in luciferase activity in response to DMSO treatment.

In order to further demonstrate that the ARE was able to confer responsiveness to DMSO, transient transfection experiments were carried out using a series of pGL2 P/luc constructs. The control vector pGL2 P/luc showed no change in luciferase activity in response to DMSO treatment (Fig. 8). To determine whether binding of nuclear factors to ARE is necessary for DMSO-mediated induction of apoA-I gene expression, one copy of the ARE was inserted upstream of the SV40 promoter, and the construct was tested for inducibility by DMSO. After transient transfection of these constructs into HepG2 cells, pGL2 (apoA-I–ARE) SV40/luc showed luciferase activity approximately 3-fold higher than that observed by the control vector pGL2 P/luc. This suggests that the ARE may enhance the basal rate of transcription of the reporter gene. Furthermore, the luciferase activity of pGL2 (apoA-I–ARE) SV40/luc was significantly increased by an additional 2-fold in the presence of DMSO. However, a plasmid containing one copy of the mutated apoA-I–ARE, pGL2 (apoA-I–mu-

tant ARE) SV40/luc, had a basal rate of expression similar to the control vector and displayed no responsiveness to DMSO treatment.

DISCUSSION

Although oxygen is essential for aerobic survival, it can also be lethal to respiring organisms through the production of various toxic reactive metabolites. Healthy cells are constantly challenged by reactive oxygen species (ROS) as a result of normal biological processes, exposure to xenobiotics or pollutants, or as a consequence of disease pathology (38, 39). Recent evidence suggests that at moderately high concentrations, certain forms of ROS such as H_2O_2 may act as intracellular messengers in signal transduction (40). There is growing interest in understanding the role and mechanisms of antioxidants as inhibitors of deleterious oxidative processes, particularly cancer and atherosclerosis (41). DMSO, a compound that has intrinsic antioxidant properties, has been shown to decrease the extent of lesion development in cholesterol-induced atherosclerosis in animals despite severe hypercholesterolemia (8–10). However, the reasons for this observation are still not clear. In the present study, we have utilized HepG2 cells as an in vitro model to examine the effects of DMSO on apoA-I and HDL production. We have demonstrated that the observed anti-atherogenic property of DMSO could be explained, in part, by the increased rate of synthesis of apoA-I and HDL. We have also showed that DMSO treatment resulted in a 2-fold increase in secreted apoA-I level without affecting the level of apoA-II. Elevation in secreted apoA-I levels was accompanied by a significant increase (50%) in HDL lipid content during DMSO treatment. This result was confirmed by densitometric analysis of HDL subfractions after gradient gel electrophoresis. Our studies also indicated that the majority of the HDL particles produced by the DMSO-treated cells were in the HDL_3 size range. Recently, Castro and co-workers (42) have demonstrated that HDL containing human apoA-I is a more effective agent in efflux of cellular cholesterol than HDL containing both apoA-I and apoA-II. Klimov and co-workers (17) have reported a protective effect of HDL against oxidation induced by iron and postulated that this protection is related to the presence of LCAT in the HDL_3 fraction. It has also been suggested that HDL may play a protective role in atherogenesis by preventing the formation of Ox-LDL (14, 17, 18). Taken together, the apoA-I-enriched HDL_3 fraction produced by HepG2 cells after DMSO treatment could have a dual role: *i*) to promote the reverse cholesterol transport and *ii*) to protect LDL against oxidation.

The results from our studies demonstrate for the first time that DMSO selectively increases apoA-I mRNA levels in HepG2 cells. Although the molecular mechanisms by which DMSO modulate apoA-I expression are not fully understood, we have provided evidence to indicate that the 2-fold increase in apoA-I mRNA levels is primarily due to a 2-fold increase in apoA-I transcription rate. Both in vitro nuclear run-off transcription assays (Fig. 5) and transient transfection experiments (Fig. 8) indicated a significant 2-fold increase in apoA-I gene expression by DMSO. Previously, our laboratory (34, 35, 43) and other investigators (44–47) demonstrated that apoA-I gene expression could be regulated at both transcriptional and post-transcriptional levels. The mechanisms by which DMSO induces various responses in diverse cell types are not understood. There are suggestions, however, that transcriptional regulation events are involved (3, 7, 48).

Recently, both positive- and negative-acting factors that influence apoA-I gene expression have been identified in human liver cells (49–51). The negative-acting factor appears to belong to the steroid receptor superfamily (51). Furthermore, different *cis*-acting 5′ sequence elements control expression of the mammalian apoA-I gene in liver and intestine (52, 53), supporting the suggestion that multiple regulatory pathways, acting independently or in combination, direct the complex pattern of apoA-I gene expression. Elucidation of the mechanism by which DMSO modulates apoA-I gene expression requires the identification of promoter elements and transcription factors responsible for mediating the biological response to DMSO. We have identified one copy of the putative ARE with one mismatched nucleotide in the apoA-I promoter that may be responsible for the increase in apoA-I transcriptional activity by DMSO. Gel mobility shift assays have demonstrated that both untreated and DMSO-treated HepG2 nuclear extracts contain factors that bind specifically to the ARE oligomer, suggesting that these factors are constitutively expressed. However, the binding activity was induced significantly upon DMSO treatment. The induction of this protein–DNA complex was observed after 2 h of DMSO exposure. No induction was observed when several time points were taken between 0 to 2 h (data not shown). Furthermore, the induction of the protein–apoA-I–ARE complex was not blocked by cyclohemixide, suggesting that the inducible binding activity might be due to modification of a pre-existing factor and does not require de novo protein synthesis.

At least two well-defined transcription factors, nuclear factor (NF) kappa B and activator protein (AP)-1

have been identified to be regulated by the intracellular reduction-oxidation state (40). To examine the possible interaction of these transcription factors with apoA-I–ARE, we have performed supershift experiments using polyclonal antibodies raised against c-Jun, c-Fos, and NF kappa B (Santa Cruz Biotechnology, CA). For supershift assays, the nuclear extracts isolated from DMSO-treated cells were preincubated with each individual antibody mentioned above at 4°C for 18 h prior to the standard gel mobility shift assays. These experiments showed no effect on the binding activity of apoA-I–ARE to the DMSO-treated nuclear extracts when either anti-c-Jun, anti-c-Fos, or anti-NF kappa B were used (data not shown). Very recently, we have examined the effect of oxidative stress on the regulation of human apoA-I gene expression (43). To induce oxidative stress, HepG2 were exposed to gramoxone, a compound that undergoes a one electron reduction to form a stable free radical that is capable of generating ROS. UV cross-linking experiments were carried out by using labeled apoA-I–ARE as a probe. These studies indicated binding of two polypeptides of apparent molecular masses of approximately 115 and 100 kDa to the apoA-I–ARE in both untreated and gramoxone-treated nuclear extracts (43). However, gramoxone treatment increased labeling of these two proteins by approximately 2-fold. We are currently investigating whether or not DMSO-treated nuclear extracts contain similar amounts and sizes of proteins that interact with apoA-I–ARE.

In order to establish a functional role for ARE in apoA-I gene transcription in response to DMSO, we have performed transient transfection experiments using pGL2-derived luciferase reporter plasmids. The involvement of the ARE in DMSO-mediated induction of apoA-I gene expression was established by using the pGL2 (apoA-I −250 mutant ARE) as well as pGL2 (apoA-I–mutant ARE) SV40/luc. Taken together, the data indicated that the ARE located in the apoA-I promoter region functions independently as a bona fide regulatory element that is responsive to DMSO.

In conclusion, we have identified DMSO-inducible nuclear proteins that bind specifically to the ARE region of the human apoA-I gene. This protein–DNA interaction may provide some insights into a novel mechanism by which DMSO-inducible *trans*-acting factors modulate apoA-I gene expression. Incubation of HepG2 cells with DMSO resulted in increased apoA-I transcription, steady state apoA-I mRNA, secreted apoA-I, and HDL production. Increasing HDL synthesis may provide at least one of the mechanisms by which DMSO prevents cholesterol-induced atherosclerosis in experimental animals.

The authors wish to thank Sandra Caine for her excellent secretarial skills. This work was supported by grant #B2703 from the Heart and Stroke Foundation of Ontario (HSFO). S-P. Tam also holds a Career Investigator Award from the HSFO.

Manuscript received 28 January 1997 and in revised form 8 July 1997.

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