Cyclooxygenase Inhibition Is Associated With Downregulation of Apolipoprotein AI Promoter Activity in Cultured Hepatoma Cell Line HepG2

Mohamad H. Horani, Freij Gobal, Michael J. Haas, Norman C.W. Wong, and Arshag D. Mooradian

Prostanoids have been implicated in the transcriptional control of several genes. Since prostanoid synthesis inhibitors are commonly used in subjects with coronary heart disease we studied the effect of cyclooxygenase (COX) inhibition on apolipoprotein AI (apoAI) expression in a human hepatoma cell line (HepG2) transfected with full-length apoAI promoter attached to the chloramphenicol acetyl transferase (CAT) reporter gene. To control for transfection efficiency, the cells were cotransfected with the plasmid pCMV.SPORT-β-gal containing the β-galactosidase gene driven by the cytomegalovirus promoter. Treatment of these cells with varying concentrations of indomethacin (INDO, 0, 50, 100, and 300 μmol/L) resulted in a dose-dependent decrease in apoAI promoter activity (% acetylation corrected for β-galactosidase activity: were 46.1 ± 2.6, 29.9 ± 1.2, 25.2 ± 2.9, and 17.2 ± 2.8, respectively, $P < .001$). INDO treatment did not cause significant changes in β-galactosidase activity. A similar reduction in apoAI promoter activity was found after treating the cells with 50 μmol/L acetylsalicylic acid (ASA) (31.8 ± 1.8%, $P < .001$), suggesting that the effect of INDO is related to COX inhibition rather than a peculiar effect of INDO. Nuclear run-off assays indicated that treatment of cells with 50 μmol/L INDO resulted in 31.4% reduction in apo A1 transcription rate ($P < .0002$). Northern blot analysis of RNA from HepG2 cells treated with 50 μmol/L of INDO for 72 hours showed that the apoAI mRNA concentration relative to G3PDH mRNA was 4,043.0 ± 84.6 and 3,064.0 ± 49.8 in control and INDO-treated cells, respectively ($P < .0006$). Kinetic studies of apoAI mRNA in HepG2 cells indicated that the half-life of apoAI mRNA was not significantly altered with 50 μmol/L INDO treatment. Apo AI mRNA half-life was 25.3 hours in control cells and 26.9 hours in INDO-treated cells. Western blot analysis of culture media of HepG2 cells treated with 50 μmol/L of INDO for 72 hours showed a significant reduction in apoAI protein (6,760.0 ± 318.1 *v* 4,773.0 ± 112.0 arbitrary units, $P < .004$). Treatment of cells with either arachidonic acid (COX substrate) or various prostanoids including prostaglandin I_2, thromboxane B_2, (±)5-HETE, or (±)12-HETE did not significantly alter apoAI promoter activity. However, prostaglandin E_1 and E_2 at the highest concentration tested (50 nmol/L) significantly repressed apoAI promoter activity. COX activity measurements in HepG2 cells verified the efficacy of COX inhibition by INDO. It is concluded that COX inhibition with INDO or ASA downregulates apoAI expression at the transcriptional level. This effect could not be attributed to either arachidonic acid excess or to a deficiency in various prostanoids tested.

APOLIPOPROTEIN AI (apoAI) is the major apoprotein of the high-density lipoprotein (HDL) particle and has been credited for several anti-atherogenic properties.[1-4] Several hormonal and nutritional factors modulate the expression of apoAI.[5-15] However, the effect of prostanoids on apoAI expression has not been well studied.

Previously published studies have shown an important role of prostanoids in the transcriptional control of several genes.[16-21] Some but not all of these effects are mediated through peroxisome proliferator activator (PPAR) receptors.[22,23] Since PPARs are also implicated in the regulation of apoAI expression,[24,25] it is possible that prostanoids may also have a role in modulating apoAI expression. Such a role would have clinical implications since prostanoid synthesis inhibitors, such as aspirin, are commonly used in subjects with coronary heart disease. In addition, prostanoids are implicated in insulin signaling in the liver.[26,27] Since insulin is an important upregulator of apoAI expression,[5] it is possible that interference with prostanoid production within the liver may alter insulin effects on apoAI expression.

To test the hypothesis that prostanoids alter apoAI expression, we studied the effect of cyclooxygenase (COX) inhibition on apoAI expression in a human hepatoma cell line (HepG2). The results show that COX inhibition with indomethacin (INDO) or acetylsalicylic acid (ASA) downregulates apoAI protein and mRNA expression at the transcriptional level. This effect could not be attributed to either arachidonic acid excess or to a deficiency in various prostanoids tested.

MATERIALS AND METHODS

Materials

Acetyl-coenzyme A, ASA, arachidonic acid, and INDO were purchased from Sigma Chemical Co (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 BioWittaker (Walkersville, MD). Prostanoids were purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals were of reagent grade and were purchased from either Sigma Chemical Co or Fisher Scientific (Pittsburg, PA).

Cell Culture

HepG2 cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and

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penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively). Caco-2 cells were grown in Earl's modified essential medium (EMEM) supplemented with 15% FBS, nonessential amino acids, 0.11 mg/mLl sodium pyruvate, and penicillin and streptomycin. Cells were housed in a humidified incubator at 37°C with 5% CO_2 and 95% air.

Plasmids and Transient Transfection

HepG2 cells were transfected with the plasmids pAI.474.CAT and pCMV.SPORT-β-gal (Life Technologies) The plasmid pAI.474.CAT contains the full-length rat apoAI promoter in front of the chloramphenicol acetyl transferase (CAT) reporter gene. This promoter fragment contains all the *cis*-elements necessary for apoAI expression.[10,12,28] The latter plasmid expresses β-galactosidase under the control of the cytomegalovirus (CMV) immediate-early promoter and was used to normalize for transfection efficiency. One microgram of each plasmid was transfected into HepG2 cells using 5 μL of Lipofectamine as described by the manufacturers, and after 24 hours, treated with varying concentrations of INDO (0, 50, 100, and 300 μmol/L) or 50 μmol/L ASA. After 24 hours, the cells were harvested and assayed for CAT[29] and β-galactosidase activity,[30] as previously described. To test for the effects of various metabolites of prostanoid synthesis on apoAI promoter activity, the cells were also treated with arachidonic acid (COX substrate) or various prostanoids including prostaglandin I_2, thromboxane B_2, (±) 5-HETE or (±)12-HETE, and prostaglandin E_1 and E_2.

Nuclear Run-Off Transcription Assays

HepG2 cells in 75-cm^2 flasks were exposed in triplicate either to INDO (50 μmol/L) or an equivalent volume of dimethyl sulfoxide (DMSO) solvent for 24 hours. The culture medium was then removed and the cells were washed twice with phosphate-buffered saline (PBS), removed by scraping, and collected by centrifugation at 500 × *g* for 5 minutes at 4°C. While vortexing, 4 mL of NP-40 lysis buffer (10 mmol/L Tris-Cl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.5% Nonidet P-40) was added, and continued for another 10 seconds. After a 5-minute incubation on ice, the nuclei were collected by centrifugation at 500 × *g* for 5 minutes at 4°C. The nuclear pellet was resuspended in another 4 mL of NP-40 lysis buffer while vortexing as described above, and the nuclei collected by centrifugation at 500 × *g* for 5 minutes at 4°C. The pellet was then resuspended in 200 mL of glycerol storage buffer (50 mmol/L Tris-Cl, pH 8.3, 40% glycerol, 5 mmol/L $MgCl_2$, and 0.1 mmol/L EDTA) and stored in liquid nitrogen.

To perform the run-off reaction, 200 μL of nuclei were thawed and incubated with 200 mL of 2X reaction buffer (10 mmol/L Tris-Cl, pH 8.0, 2 mmol/L $MgCl_2$, 0.3 mol/L KCl, 1 mmol/L adenosine triphosphate [ATP], 1 mmol/L guanosine triphosphate [GTP], 1 mmol/L cytosine triphosphate [CTP], 5 mmol/L diothiothreitol [DTT]) and 10 ml of [α-^{32}P] uridine triphosphate (UTP; 760 Ci/mmol, 10 mCi/mL) and incubated at 30°C for 30 minutes. Each reaction was then incubated in 0.6 mL of high salt buffer (HSB) buffer containing RNase-free DNaseI (40 μL of a 1-mg/mL solution), and incubated at 30°C for another 5 minutes before addition of 200 μL of 0.5% sodium dodecyl sulfate (SDS), 0.5 mol/L Tris-Cl, pH 7.4, 0.125 mol/L EDTA, and 10 μL of 20-mg/mL proteinase K. The samples were incubated for 30 minutes at 42°C, and then extracted with phenol/chloroform/isoamylalcohol (25:24:1). Two milliliters of dH_2O and 10 μL of 10 mg/mL *Escherichia coli* tRNA were added to the samples before addition of 3 mL of 10% tricholoracetic acid (TCA) containing 60 mmol/L phyrophosphate. Samples were incubated on ice for 30 minutes, then the precipitate collected onto Whatman GF/A glass fiber filters (Clifton, NJ), and washed 3 times with 10 mL of 5% TCA, 30 mmol/L pyrophosphate. The filters were transferred to glass scintillation vials and incubated in RNase-free DNase I (37.5 μL of a 10-mg/mL solution per sample) in 20 mmol/L HEPES, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, for 2 minutes at 37°C. The reaction was stopped by addition of 45 μL of 0.5-mol/L EDTA and 68 μL of 20% SDS, and heated to 65°C for 10 minutes. The RNA was transferred to another tube while the filter was soaked in 1.5 mL elution buffer (1% SDS, 10 mmol/L Tris-Cl, pH 7.5, 5 mmol/L EDTA), incubated for 10 minutes at 65°C, and combined with the previous tube. The samples were then digested with proteinase K (4.5 μL of a 20-mg/mL solution) for 30 minutes at 37°C, extracted with phenol/chloroform, isoamyl alcohol, and transferred to a 30-mL Corex tube (Corning, NY). To the RNA, 0.75 mL of 1-mol/L NaOH was added, incubated on ice 10 minutes, then the pH adjusted by the addition of 1.5 mL of 1-mol/L HEPES (free acid). The RNA was precipitated by addition of 0.53 mL of 3-mol/L sodium acetate, 14.5 mL absolute ethanol, and incubation at −20°C overnight, and collected by centrifugation at 10,000 × *g* for 30 minutes at 4°C. One milliliter of TES solution (10 mmol/L N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid [TES], pH 7.4, 10 mmol/L EDTA, 0.2% SDS) was added to the RNA and when dissolved, 5 μL was counted in a scintillation counter. Equivalent CPM were prepared in a total volume of 1 mL TES solution, to which 1 mL of TES/NaCl (10 mmol/L TES, pH 7.4, 10 mmol/L EDTA, 0.2% SDS, 0.6 mol/L NaCl) was added then allowed to hybridize to immobilized probes on strips of nylon (Hybond, Amersham, Arlington Heights, IL) for 24 hours at 65°C. The membranes were then washed 2 times in 2X SSC for 1 hour each at 65°C. They were then incubated in 8 mL of 2X SSC containing 8 μL of 10-mg/mL Rnase A at 37°C for 30 minutes, washed again in 2X SSC for 1 hour at 37°C, and exposed to film for autoradiography.

Northern Blot Analysis

RNA was isolated using phenol-guanidium isothiocyanate as previously described.[31] Recombinant plasmid pBR322 containing the rat apoAI cDNA (NE-477) was kindly provided by Dr J.I. Gordon of Washington University (St Louis, MO). Amplification and preparation of plasmid DNA was accomplished using established procedures.[32] The 412-bp NE-477 insert was cleaved from the purified recombinant plasmid DNA with *Eco*RI and *Sac*I using conditions specified by the supplier of the restriction endonucleases. The cleaved insert was separated from the vector DNA and random prime-labeled with [α-32P] dCTP (33) The isolated RNA was electrophoresed in an 1.5% agarose gel containing 2.2 mol/L formaldehyde,[34] transferred to a nylon membrane by diffusion blotting, and finally hybridized with the apoAI cDNA insert.[35] After exposing the membrane to x-ray film for autoradiography, the blots were stripped and reprobed with a glyceraldehyde-3 phosphate dehydrogenase (G3PDH) cDNA to normalize the changes in apoAI mRNA.

Measurements of apoAI mRNA Turnover

ApoAI mRNA half-life was measured in HepG2 cells treated either with INDO (50 μmol/L) or an equivalent volume of DMSO solvent for 0, 3, 6, 24, and 48 hours, and 1 μg/mL actinomycin D as previously described.[36,37] Total RNA was isolated as described above and subjected to Northern blot analysis. Best-fit linear curves were determined using Statistica (StatSoft, Tulsa, OK). Under these conditions, no toxicity was apparent.

Western Blot Analysis

Serum free culture media from HepG2 cells treated with COX inhibitors were collected and protein concentrations determined using the Bradford assay.[38] Protein samples (20 μg) were dissolved in Laemmli sample buffer,[39] fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose,[40] and incubated with the primary anti-apoAI antiserum (1:500) for 16 hours. To detect primary antibody binding, horseradish peroxidase–linked goat anti-rabbit IgG was used at a final dilution of 1:10,000 for 1 hour at

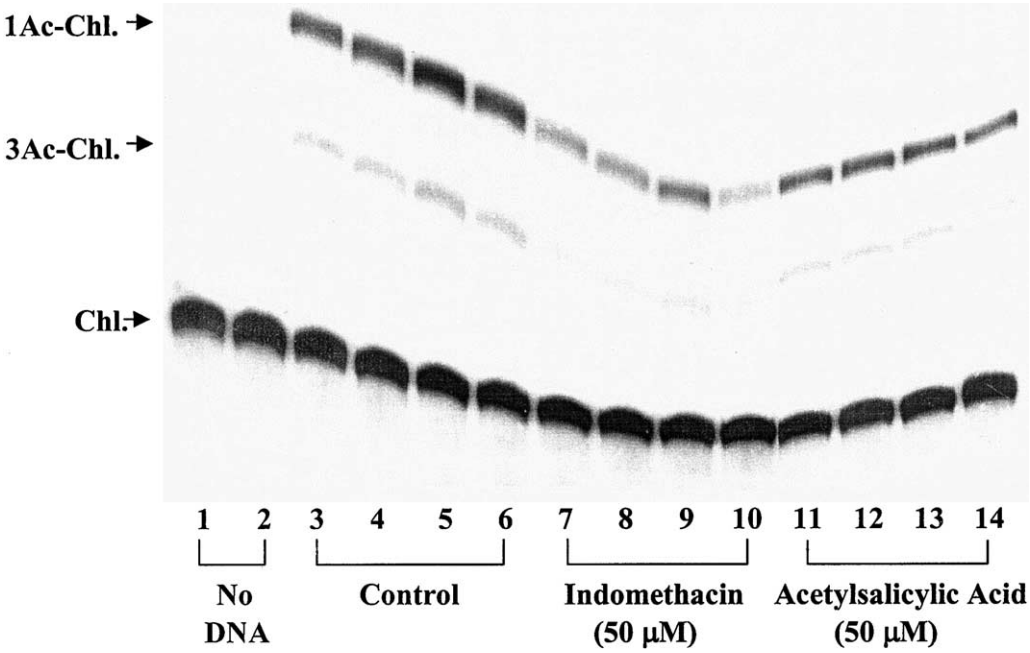

Fig 1. Effect of COX inhibition on apoAI promoter activity. HepG2 cells were transfected with pAI.474.CAT and pCMV.SPORT-β-gal, and after 24 hours, treated with various concentrations of INDO or ASA. After 24 hours of treatment the cells were harvested and assayed for CAT and β-galactosidase activity. The positions of the acetylated chloramphenicol derivatives, 1-acetyl chloramphenicol (1-Ac-Chl.) and 3-acetyl chloramphenicol (3-Ac-Chl.), as well as the unacetylated chloramphenicol substrate (Chl.), are indicated. CAT activity, normalized to β-galactosidase activity, was significantly decreased with all treatments. See text for details.

room temperature. Blots were developed using enhanced chemiluminescence (ECL) Western blotting reagents as described by the manufacturer (Amersham-Biosciences, Arlington Heights, IL). The apoAI concentration was determined by densitometry using the personal densitometer from Molecular Dynamics (Sunnyvale, CA). Absorbance was analyzed after background subtraction. The reproducibility of the apoAI quantitation was established with gels loaded with different amounts of authentic apoAI.[13,14] The correlation coefficient between the amount of apoAI applied to the gel and the optical density of the band found on immunoblot was 0.99.

COX 2 mRNA Expression by Reverse-Transcription Polymerase Chain Reaction

COX 2 mRNA expression was examined in HepG2 and Caco-2 cells by reverse- transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from HepG2 and Caco-2 cells treated with 50 μmol/L INDO or an equivalent volume of solvent (DMSO) for 24 hours as previously described.[31] One microgram of RNA was reverse-transcribed in a reaction mixture containing 10 mmol/L tris-(hydroxymethyl) aminoethane hydrochloride (Tris-Cl) (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 250 μmol/L deoxynucleotide triphosphates, 10 pmol each primer, 2.5 U Taq polymerase (Fisher Biotech, Pittsburgh, PA), and 200 U M-MLV reverse transcriptase (Promega, Madison, WI), and incubated at 45°C for 60 minutes, 95°C for 2 minutes. COX 2 mRNA was amplified as follows: 95°C, 1 minute; 55°C, 1 minute, 15 seconds; and 72°C, 5 minutes, for 40 cycles with the primers COX-2S, 5′-TCC TGT TGC GGA GAA AGG AG-3′ and COX-2AS, 5′-ATT GGA AAC ATC GAC AGT GT-3′. A 10-μL aliquot of each sample was fractionated by electrophoresis on a 1% agarose gel containing 1 μg/mL ethidium bromide in 1x TBE buffer, and photographed. The reverse-transcriptase reaction was performed with the same primers as the PCR.

Measurement of COX Activity

COX activity was measured in Caco-2 and HepG2 cells treated with 50 μmol/L INDO or an equivalent volume of solvent for 24 hours using a commercially available kit (Cox Activity Assay, Cayman Chemical, Ann Arbor, MI). Briefly, cells were scraped from 6-well cluster dishes and sonicated briefly in 0.1 mol/L Tris-Cl (pH 7.8), 1 mmol/L EDTA, 250 mmol/L mannitol, and 0.3 mol/L diethyldithiocarbamic acid. Supernatant fractions were obtained by centrifugation at 10,000 × *g* for 15 minutes at 4°C. Aliquots were assayed for COX peroxidase activity colorimetrically by measuring the accumulation of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine at 590 nm.[41]

Statistical Analysis

All results are expressed as mean ± SEM. Analysis of variance (ANOVA) followed by the Neuman-Keuls procedure for subgroup analysis was performed using the statistical package Statistica for Windows (Statsoft). Significance was defined as a 2-tailed $P < .05$.

RESULTS

A representative example of the experiments on apoAI promoter activity in HepG2 cells treated with either INDO or ASA is shown in Fig 1. The cell viability determined with trypan blue exclusion was confirmed in each experiment to be greater than 95 %.[42] Treatment of HepG2 cells with varying concentrations of INDO (0, 50, 100, and 300 μmol/L) resulted in a dose-dependent decrease in apoAI promoter activity (% acetylation corrected for β-galactosidase activity: 46.1 ± 2.6, 29.9 ± 1.2, 25.2 ± 2.9, and 17.2 ± 2.8, respectively, $P < .001$). INDO treatment did not cause significant changes in β-galactosidase activity. A similar reduction in apoAI promoter

Table 1. Effect of Arachidonic Acid and Various Prostaglandins, HETE's, and Thromboxane B_2 on apoAI Promoter Activity in HepG2 cells

Treatment	Control	Low	Middle	High
AA	27.1 ± 1.1	26.3 ± 0.7	26.0 ± 0.1	26.8 ± 0.3
PGI_2	39.2 ± 0.1	37.8 ± 1.2	37.5 ± 1.1	40.8 ± 0.8
PGE_1	41.4 ± 1.0	37.4 ± 0.7	34.6 ± 1.2	29.7 ± 1.2*
PGE_2	42.0 ± 1.5	39.5 ± 1.3	37.8 ± 1.8	29.6 ± 1.8*
(±)5-HETE	35.6 ± 0.7	34.7 ± 1.0	34.7 ± 0.5	36.0 ± 0.7
(±)12-HETE	37.5 ± 0.5	36.7 ± 1.3	36.7 ± 1.1	36.0 ± 1.0
TXB_2	40.9 ± 0.7	40.6 ± 1.2	39.9 ± 0.7	39.9 ± 1.2

NOTE. HepG2 cells were transfected with the apoAI reporter construct pAI 474.CAT and pCMV.SPORT-β-gal, and after 24 hours, treated with the indicated compounds at 3 different doses. Arachidonic acid (AA) was added to final concentration of 2 (low), 10 (middle), and 50 μmol/L (high). Prosteglendin (PG) I_2, PGE_1, and PGE_2 were added to final concentrations of 2 (low), 10 (middle), and 50 nmol/L (high). (±)5-HETE was added to final concentrations of 100 (low), 200 (middle), and 400 nmol/L (high), while (±)12-HETE was added to final concentrations of 50 (low), 100 (middle), and 200 nmol/L (high). Thromboxane $(TX)B_2$ was added to final concentrations of 250 (low), 750 (middle), and 1,500 pg/mL (high). After another 24 hours, CAT and β-galactosidase activity were measured. PGE_1 and PGE_2 significantly suppressed apoAI promoter activity, but only at the highest doses used (50 nmol/L). N = 6.

*$P < .002$.

activity was found after treating the cells with 50 μmol/L ASA (31.8% ± 1.8%, $P < .001$), suggesting that the effect of INDO is related to COX inhibition rather than a peculiar effect of INDO. These experiments were performed twice independently with n = 3 in each experiment.

The results of studies on the effect of arachidonic acid and select prostanoids on apoAI promoter activity are summarized in Table 1. Treatment of cells with either arachidonic acid (COX substrate) or various prostanoids including prostaglandin I_2, thromboxane B_2, (±)5-HETE, or (±)12-HETE did not significantly alter apoAI promoter activity. However, prostaglandin E_1 and E_2 at the highest concentration tested (50 nmol/L) significantly repressed apoAI promoter activity (Table 1).

Nuclear run-off assays indicated that treatment of cells with 50 μmol/L INDO resulted in a 31.4% reduction in apoA1 transcription rate ($P < .0002$) (Fig 2). ApoAI mRNA levels in control cells and INDO treated cells were 4,307 ± 29.7 and 2,956 ± 52.8 arbitrary units, respectively, while the G3PDH mRNA levels in control cells and INDO-treated cells were 6,180 ± 31.2 and 6,128 ± 15.1, respectively. Background levels for pBR322 hybridization were negligible (Fig 2). These experiments were repeated 3 times in duplicates.

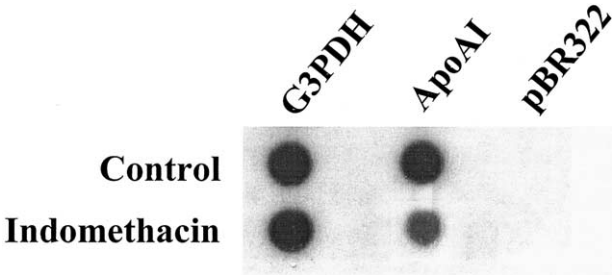

Fig 2. Effect of INDO on apoAI transcription rate. Nuclear run-off assays were performed using nuclei isolated from HepG2 cells treated with either 50 μmol/L INDO or an equivalent volume of solvent (control). The labeled RNA was hybridized to immobilized probes for G3PDH, apoAI, and the plasmid pBR322. Treatment with INDO significantly suppressed *apoAI* gene transcription rate.

To determine if changes in apoAI promoter activity correlate with the changes in apoAI mRNA or protein levels, Northern and Western blot analyses were performed. Samples were prepared from cells exposed to 50 μmol/L INDO for 72 hours. A representative Northern blot of apoAI mRNA from HepG2 cells is shown in Fig 3. The apoAI mRNA concentration relative to G3PDH mRNA was 4,043.0 ± 84.6 and 3,064.0 ± 49.8 in control and INDO-treated cells respectively (n = 9; $P < .0006$).

Kinetic studies of apoAI mRNA in HepG2 cells indicated that the half-life of apoAI mRNA was not significantly altered with 50 μmol/L INDO treatment (Fig 4). ApoAI mRNA half-life was 25.3 hours in control cells, while in INDO-treated cells apoAI mRNA half-life was 26.9 hours.

A representative Western blot of apoAI secreted into the culture media after 72 hours of treatment with 50 μmol/L INDO is shown in Fig 5. The apoAI concentration in the media was significantly reduced in the presence of INDO. The apoAI content of the culture media (in arbitrary units adjusted for internal controls) was 6,760.0 ± 318.1 versus 4,773.0 ± 112.0 in controls and INDO-treated cells, respectively (n=9; $P < .004$). Gels stained with Coomassie Blue showed that treatment of HepG2 cells with 50 μmol/L INDO did not result in a significant change in overall profile of proteins secreted by HepG2 cells in the culture media (data not shown).

Finally, to determine if HepG2 cells express INDO-suppressible COX activity, cells were cultured in 50 μmol/L INDO or an equivalent volume of DMSO for 24 hours. COX activity was also measured in Caco-2 intestinal epithelial cells as an internal control since they have been shown to express COX activity.[43] In Caco-2 cells, COX activity decreased 53.9% from 93.0 ± 2.5 to 42.9 ± 0.9 nmol/min/mL (n = 6; $P < .00001$). COX activity in solvent-treated HepG2 cells decreased 41.2% from 64.1 ± 2.3 to 37.7 ± 0.5 nmol/min/mL (n = 6; $P < .00003$).

In order to verify that HepG2 cells express COX 2, COX 2 mRNA expression was examined by RT-PCR. Caco-2 cells expressed COX 2 mRNA as shown by others[43] (Fig 6, lane 2). HepG2 cells also expressed COX 2 mRNA (Fig 6, lane 4). No transcript was detected when reverse transcriptase was omitted from the reaction (Fig 6, lanes 1 and 3).

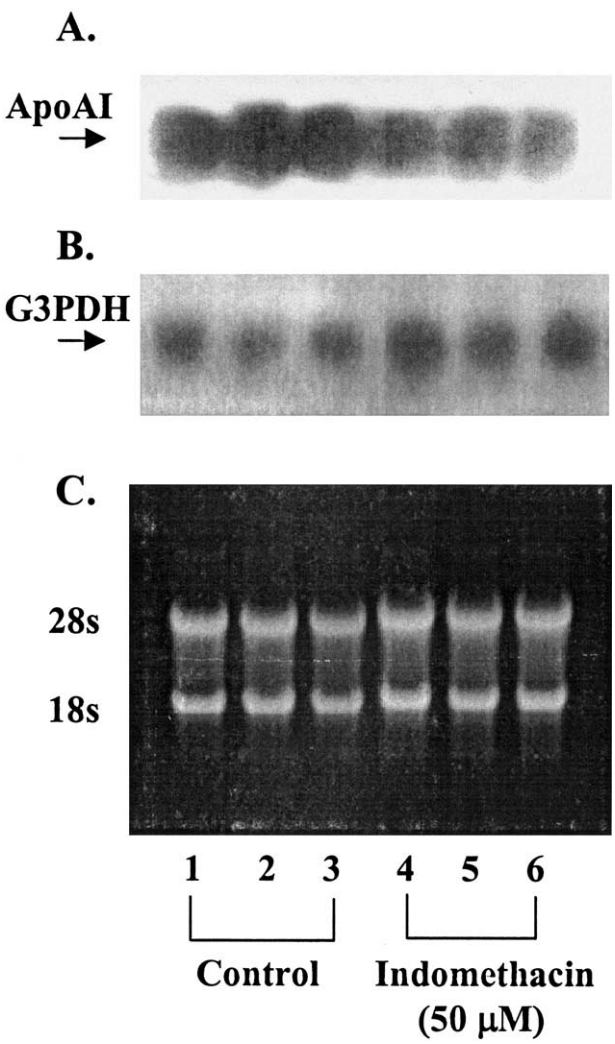

Fig 3. A representative Northern blot of RNA isolated from HepG2 cells treated with 50 μmol/L of INDO for 72 hours (lanes 4-6) or control culture media (lanes 1-3). The blots were hybridized with the apoAI cDNA. The expressed 0.9-kb apoAI band (A), G3PDH band (B), and ethidium bromide stained gel (C) are shown. INDO treatment repressed apoAI mRNA levels.

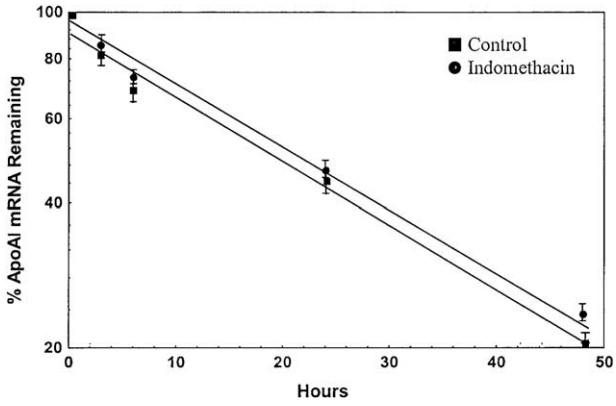

Fig 4. Effect of INDO on apoAI mRNA half-life. HepG2 cells were treated with either DMSO or 50 μmol/L INDO in the presence of actinomycin D for up to 48 hours. ApoAI mRNA levels were measured by Northern blot analysis and percent apoAI mRNA remaining during the course of treatment determined and plotted as percent remaining. INDO had no effect on apoAI mRNA half-life. ApoAI mRNA half-life was 25.3 hours in control cells, while in INDO-treated cells apoAI mRNA half-life was 26.9 hours.

DISCUSSION

Although a multitude of prostaglandin-initiated signals arise at cell surface receptors, some prostaglandins may act directly at nuclear signaling sites.[44] One such notable nuclear site is the activation of PPAR receptors.[23] Since apoAI expression is modulated by PPAR receptors,[24,25] we evaluated the effect of prostaglandin synthesis inhibitors on apoAI expression in a human hepatoma cell line known to express apoAI. This cell line retains multiple characteristics of primary hepatocytes including production of albumin and apoAI and has been extensively used as a model system to study apoAI gene expression.[45-48] The results of these experiments show that treating these cells with prostaglandin synthesis inhibitors such as INDO and ASA significantly downregulates apoAI promoter activity. In addition, apoAI mRNA and protein levels were also significantly reduced with INDO treatment. To determine if this effect could be attributed to accumulation of arachidonic acid or to one of the prostanoids, additional experiments were performed. None of the prostanoids tested could account for the suppressive effect of INDO on apoAI expression. The adipogenic prostanoid, 15-deoxy-delta 12, 14-prostaglandin J_2,[49] was not tested since it targets PPAR gamma receptor, and we have previously found that PPAR gamma activation with troglitazone in this model does not alter apoAI promoter activity.[50] Since only limited number of prostanoids were tested it is still possible that the inhibition of a critical prostanoid with apoAI-inducing activity may have accounted for the changes observed with COX activity inhibition. Alternatively, the ef-

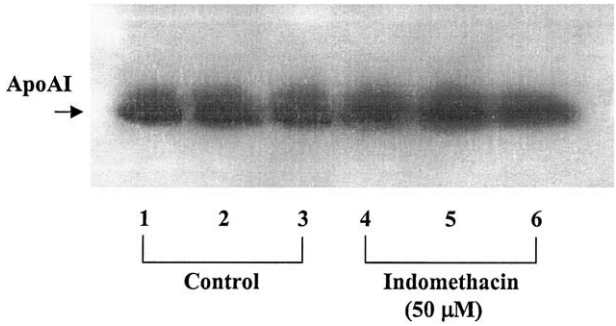

Fig 5. Representative Western blots of protein samples of culture media (20 μL) from HepG2 cells treated with 50 μmol/L of INDO for 72 hours (lanes 4-6) or control culture media (lanes 1-3). The blots were probed with a rabbit anti-human apoAI antiserum at a 1:1,000 dilution for 2 hours and developed with ECL. The expected single 28-kd apoAI band is apparent. INDO treatment significantly reduced apoAI protein levels in the culture media. The experiments were repeated twice in triplicate.

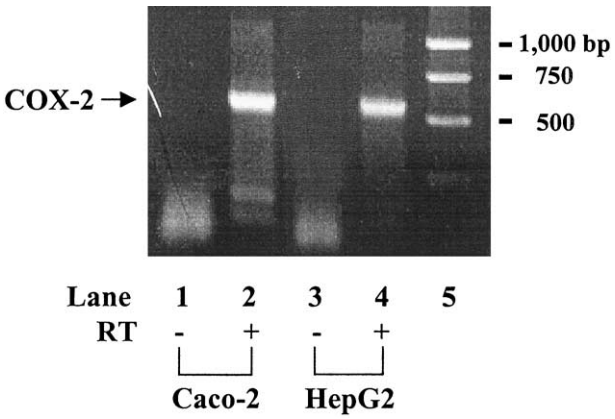

Fig 6. COX 2 mRNA expression in HepG2 (lanes 3,4) and Caco-2 cells (lanes 1,2) by RT-PCR. No band is present in samples when reverse transcriptase was omitted. The 550-bp COX 2 band is the size predicted from the primers.

fects of INDO and inhibition of COX activity may have been independent of prostaglandin synthesis. This speculation is based on previously published observations indicating that COX-induced cell cycle arrest does not require the synthesis of prostaglandins.[51] The latter is an unlikely hypothesis since the apoAI downregulation was also documented after treatment with ASA, another known COX inhibitor.

The precise underlying mechanism of INDO-related downregulation of apoAI expression is not known. The observed changes could be related to COX inhibition or could be independent of COX inhibition. Although normal hepatocytes do not express COX, it is possible that inhibition of COX activity in nonparenchymal hepatic cells, such as Kupfer cells, would alter paracrine signaling. Alternatively, the observed effects could be independent of COX activity. Indeed several COX-independent effects of INDO have been previously described.[52,53] In addition, INDO, as well as some other nonsteroidal anti-inflammaorty drugs, behave as mitochondrial uncouplers,[54,55] and some also alter H^+–adensoine triphosphotase (ATPase) activity,[54] as well as intracellular pH, membrane potential, and Cl^- channels.[56] Alterations in intracellular pH and in plasma membrane ionic channels have been found to alter apoA1 expression through specific pH-responsive elements in apoAI promoter.[8] Finally, aspirin is found to inhibit nulear factor kappaB (NF-κB)[57] and inhibit the activity of activated protein-1 (AP-1) by altering intracellular H^+ concentrations[58] or by blocking activation of mitogen-activated protein kinases (MAPKs).[59] the apoAI gene contains binding sites for several transcriptional factors, including an AP-1 binding site,[60] and previous studies have shown that insulin action on apoAI gene transcription may occur through MAPK activation.[61] Thus, the latter pathways may also be implicated in the INDO- or aspirin-related downregulation of apoAI gene transcription.

It is noteworthy that aspirin but not INDO has been shown to decrease the expression of another lipoprotein, apolipoprotein (a) [apo (a)] in human hepatocytes by suppressing the transcriptional activity.[62] This effect was independent of COX inhibition. It is possible that both apoAI and apo (a) genes have aspirin-responsive regions in the promoter. Large clinical studies are needed to address the clinical relevance of the effects of aspirin on apoAI and apo (a) expression.

Previously published studies have failed to demonstrate COX activity in isolated hepatocytes and the hepatic COX activity has been attributed to the nonparanchymal cells of the liver.[19] Therefore, it was paramount that we demonstrate COX activity and its inhibition with INDO in HepG2 cells. The results from both COX activity measurements and the presence of COX mRNA indicate that HepG2 cells, unlike isolated hepatocytes, express COX mRNA and have COX enzymatic activity. It is likely that when hepatocytes undergo neoplastic transformation, COX activity is expressed. Indeed, COX activity can be induced in a variety of hyperplastic pathological conditions.[51]

It is noteworthy that prostaglandin E_2 or E_1 treatment of cells was associated with significant reduction in apoAI promoter activity at the highest doses tested (Table 1). Since tumor necrosis factor alpha (TNFα) increases prostaglandin E_2 synthesis,[63,64] it is possible that the reduced expression of apoAI following treatment with TNFα[65-68] may be mediated by prostaglandin E_2.

The observed effect of COX inhibitors on apoAI promoter activity could not be attributed to nonspecific toxicity since cell viability, documented by trypan blue exclusion, was unchanged. Furthermore, the β-galactosidase activity in the transfected cells was not altered following INDO treatment. Finally, the examination of the proteins secreted in the culture media by Coomassie blue staining showed no significant alterations associated with treating the cells with 50 μmol/L INDO.

A potential limitation in these studies is that the range of prostanoids used was limited. Since it is not practical to screen all the available prostanoids, only a handful of representative compounds were tested. Thus it is likely that downregulation of apoAI expression with COX inhibition may well be mediated by a product of COX that has stimulatory effects on apoAI expression. The identification of such product may have important clinical implications, as it may be the basis for developing a therapeutic agent to increase high-density lipoprotein levels in people with hypoalphalipoproteinemia.

These results taken together suggest that COX inhibition with INDO or aspirin downregulates apoAI expression at the transcriptional level. This effect could not be attributed to either arachidonic acid excess or to a deficiency in various prostanoids tested. Despite our failure of attributing the observed changes in apoAI to a specific prostanoid deficiency following COX inhibition, the present results highlight an important role for some prostanoids in regulating apoAI gene expression and suggest potential adverse effects of COX inhibition on apoAI expression. However, caution should be exercised when extrapolating the results of this study in a transformed cell line to potential changes in the intact liver. Because of a lack of definitive establishment of mechanism, a direct role of COX in modulating *apoAI* gene expression remains unproven. The physiologic implications of the experiments from the standpoint of human apoAI metabolism are not clear at the present time.

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