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Hepatic High-Density Lipoprotein Secretion Regulates the Mobilization of Cell-Surface Hepatic Lipase†

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ABSTRACT: HDL acts much like heparin to liberate hepatic lipase (HL) from cell surface proteoglycans and stimulate triglyceride clearance. Experiments were undertaken to evaluate the effects of factors that stimulate the secretion of HDL from the liver on the release of HL. Treatment of HepG2 cells with linoleic acid phospholipids (LAPL) (12 μM) promotes a similar increase in the accumulation of both HDL and HL in the cell media. LAPL also induce both apoA-I and HL release from primary human hepatocytes. Dilinoleoylphosphatidylcholine has a greater effect on both apoA-I secretion and HL release than palmitoyllinoleoylphosphatidylcholine. HL released from HepG2 cells is inactive and associated with a large HDL complex containing both apoA-I and apoA-II. Inclusion of the PPARα inhibitor, MK-886, or MAPK inhibitor, U0126, completely blocks the LAPL-induced apoA-I and HL accumulation in the media. LAPL-treated cell lysates, however, showed no change in HL protein expression nor HL mRNA. LAPL-induced HL release appears to be a consequence of the displacement ability of newly secreted HDL. Overexpression of pre-pro-apoA-I in HepG2 cells increased HL release, while siRNA inhibition of the apoA-I gene reduced HL in the media. The data show that factors that stimulate HDL secretion in hepatocytes act to also increase the release of HL. This may partly explain why HDL therapeutics often impact plasma triglyceride levels.

Human hepatic lipase (HL)[1] is a 66 kDa protein that is synthesized and secreted by the liver and hydrolyzes triglycerides (TG) and phospholipids in plasma lipoproteins (*1*). HL is primarily found associated with heparan sulfate proteoglycans (HSPG) on the hepatocyte cell surface, and studies have shown that HL is catalytically inactive when associated with HSPG (*2*, *3*). Heparin is able to release HL from cell surface HSPG and stimulate the hydrolysis and clearance of TG from the blood (*4*, *5*). Heparin is thought to displace HL by competing for binding sites on the HSPG (*5*) or through a more complex route involving protein kinase signaling (*6*). HDL can act similarly to heparin and can also displace HL from the cell surface HSPG (*2*, *3*). A high post-heparin HL activity therefore appears to be a measure of increased inactive liver-bound HL. Elevated post-heparin HL activity is common in patients with low HDL levels (*7*, *8*) and may be related to an inefficient displacement and activation of HL (*3*).

Linoleic acid phospholipids (LAPL), such as dilinoleoylphosphatidylcholine (DLPC), stimulate apoA-I and HDL secretion from hepatocytes (*9*). Linoleic acid itself has no hepatic HDL secretory activity. LAPL-induced HDL secretion was shown to involve a phospholipase C/protein kinase C activation of mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor-α (PPARα) signaling pathways through upregulation of PPARα protein expression (*9*). HDL secretion and the accumulation of apoA-I in the hepatocyte media are the net result of both apoA-I synthesis and the reuptake and degradation of apoA-I. However, apoA-I synthetic pathways do not appear to be upregulated by LAPL, and instead, increased HDL secretion appears to be the consequence of a decrease in the reuptake and degradation of apoA-I (*10*, *11*).

Addition of purified HDL or apoA-I to the media of HepG2 cells stimulates the release of HL into the media (*2*, *3*). We therefore hypothesized that an increased secretion of HDL by liver cells would stimulate the release of HL from the cell surface and increase HL accumulation in the cell media. As expected, we show that factors that stimulate hepatic apoA-I secretion act to promote HL release into the media, similar to exogenously added apoA-I (*2*, *3*). LAPL act through MAPK and PPARα pathways to stimulate the secretion of apoA-I and apoA-II and the release of HL. Evidence suggests that HL accumulation in the media may be a product of cell surface displacement, resulting from HDL and HL interactions at the cell surface. The data show that HDL and triglyceride metabolism may be coregulated.

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[1]Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; DLPC, dilinoleoylphosphatidylcholine; HDL, high-density lipoprotein; HL, hepatic lipase; HSPG, heparan sulfate proteoglycans; LA, linoleic acid; LPL, lipoprotein lipase; LAPL, linoleic acid phospholipids; PHH, primary human hepatocytes; PKC, protein kinase C; PLPC, palmitoyllinoleoylphosphatidylcholine; PPARα, peroxisome proliferator-activator receptor-α; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; TG, triglyceride.

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MATERIALS AND METHODS

Chemicals. The phospholipids, palmitoyllinoleoylphosphatidylcholine (PLPC) and dilinoleoylphosphatidylcholine (DLPC), were purchased from Avanti Polar Lipids Inc., Alabaster, AL. Linoleic acid sodium salt was acquired from Sigma, Oakville, Ontario, Canada. MK-886, a noncompetitive PPARα inhibitor, and clofibrate (PPARα agonist), were from Cayman Chemicals, Ann Arbor, MI. The selective MEK 1/2 inhibitor, U0126, was acquired from Cell Signaling Technology, Beverly, MA, along with the mouse monoclonal anti-human β-actin antibody. Antibodies for apoA-I used for ELISA were obtained from Biodesign, Saco, ME, and the mouse monoclonal anti-human apoA-I antibodies (4H1 and 5F6) were obtained from Dr. Marcel in the Lipoprotein and Atherosclerosis Research Group at the University of Ottawa Heart Institute. The goat polyclonal anti-human apolipoprotein A-II antibody was from Chemicon International, Billerica, MA. The mouse monoclonal anti-human HL (XHL3-6a) antibody was from Dr. Bensadoun, Cornell University. Unless otherwise stated, drugs and inhibitors were of analytical grade and solubilized in DMSO.

Cell Culture and Treatment. HepG2 cells were cultured in normal glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Almost confluent cells were subjected to stimulation with drugs for 24 h under serum-starved conditions, as indicated. Briefly, cells were treated as indicated, with or without various inhibitors/agonist, namely, 10 μM MK-886 (PPARα inhibitor), U0126 (MEK1/2 inhibitor), or clofibrate (PPARα agonist), in serum-free DMEM for 30 min prior to the addition of 12 μM DLPC and then incubated for 24 h. For time-course experiments, cells were incubated with or without 12 μM DLPC for the indicated time points. Concentrations up to 24 μM DLPC had no cytotoxic effects. All inhibitors were used at the recommended IC_{50} concentration for particular targets and have been previously reported from our laboratory (*9*, *11*).

Primary Human Hepatocytes. Collagen-coated, and HIVI, hepatitis B and C, mycoplasma, bacteria, yeast, and fungi test-negative primary human hepatocytes (PHH) were obtained from freshly donated livers supplied by Lonza Walkersville (Walkersville, MD). PHH were incubated overnight in hepatocyte medium supplemented with transferrin, insulin, and recombinant human epidermal growth factor (rhEGF). The hepatocytes were incubated for 24 h with or without drugs as for HepG2 cells, and then conditioned medium and cell protein were collected for analysis.

Preparation of Phospholipids. One milligram of palmitoyllinoleoylphosphatidylcholine (PLPC) or dilinoleoylphosphatidylcholine (DLPC) (in chloroform) was dried under N_2 gas, and 1 mL of DMSO was added and then vortexed for 1 min. The mixture was then sonicated in a Branson 5200 water bath sonicator for 3 × 10 min pulses with brief vortexing between pulses.

ApoA-I ELISA. ApoA-I in conditioned medium, from each treatment, was analyzed by ELISA on a 96-well plate as previously described (*9*). Briefly, the Nunc Immuno-maxisorp 96-well plates were coated overnight with a mouse anti-human apoA-I monoclonal antibody. Samples and standards were incubated in the wells for 2 h, followed by a 1 h incubation with a horseradish peroxidase-linked goat anti-human apoA-I antibody. Both antibodies were purchased from Biodesign. K-blue Max TMB substrate (Neogen Corp., Lexington, KY) was added to each well, the reaction was stopped with a 1 M HCl solution, and the absorbance was recorded at 470 nm on a microplate reader. The ELISA assay was shown to not be sensitive to apoA-I conformation interference.

HL Immunoanalysis. After incubation with the inhibitors/agonists and/or phospholipids for the indicated times and doses, the conditioned media were removed and briefly centrifuged to remove any cells. The cells were washed twice with ice-cold PBS on ice and then lysed with NP40 lysis buffer (Biosource, Camarillo, CA) (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% NP40, and 0.02% NaN_3) supplemented with 1 mM PMSF and 1× protease inhibitor cocktail (Sigma, St. Louis, MO) (AEBSF, aprotinin, bestatin hydrochloride, E-64, EDTA, and leupeptin hemisulfate salt). Total protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL), and 30 μg of cell lysate or 30 μL of a 1:1 dilution of conditioned media in Laemmli sample buffer containing β-mercaptoethanol was separated by 8% SDS−PAGE. The proteins were transferred onto a PVDF membrane and probed using a 1:5000 dilution of the mouse monoclonal anti-human HL antibody and a 1:20000 dilution of the goat anti-mouse IgG-linked HRP secondary antibody (KPL, Gaithersburg, MD) in 1% BSA/TBST. Blots were developed using the West Femto maximum sensitivity substrate (Pierce, Rockford, IL) on the Fluorochem AlphaImager (Alpha Innotech Corp., San Leandro, CA). Band intensities were analyzed using the Spot-densitometer application of the AlphaEaseFC software. Cell lysate blots were then stripped and probed for β-actin for normalization, while the band intensities of conditioned media blots were normalized to total cell protein.

Immunoanalysis of Nondenaturing Gradient Gel Electrophoresis. Conditioned media from HepG2 cells stimulated with DLPC were electrophoresed in triplicate on a 4−20% Tris−glycine Novex gel (Invitrogen, Carlsbad, CA) under nondenaturing conditions for 19 h at 100 V alongside high molecular weight native markers (Amersham, Piscataway, NJ). The gel was then soaked in 0.1% SDS for 15 min to give the proteins a slight negative charge in order for unidirectional transfer onto a PVDF membrane for 4 h at 125 V in Tris−glycine transfer buffer containing 20% methanol. The membrane was allowed to dry, at which point the molecular weight markers were outlined and the membrane was cut in three to probe for apoA-I, apoA-II, and HL. The apoA-I membrane was blocked with 5% milk/TBST and then probed for apoA-I using a 1:2500 dilution of the mouse monoclonal anti-human apoA-I antibodies (4H1 and 5F6) and a 1:20000 dilution of the goat anti-mouse IgG-linked HRP secondary antibody (KPL, Gaithersburg, MD) in 1% milk/TBST. ApoA-II was probed by blocking in 1% BSA/TBST followed by incubation with a 1:1000 dilution of the goat polyclonal anti-human apolipoprotein A-II antibody in 1% BSA/TBST and a 1:10000 dilution of the donkey anti-goat IgG-HRP secondary antibody in 1% BSA/TBST (Santa Cruz Biotechnology, Santa Cruz, CA). HL was probed as described in Western blot analysis for HL. Blots were developed using the West Femto maximum sensitivity substrate (Pierce, Rockford, IL) on the Fluorochem AlphaImager. Densitometry profiles were obtained using the 1D-Multi application of the AlphaEaseFC software.

Quantitative RT-PCR. Total RNA was isolated using TRI reagent (Sigma, Oakville, Ontario, Canada) and treated with DNase using the DNA-free DNase treatment kit (Ambion, Inc., Austin, TX) according to manufacturer's specifications. RNA purity and integrity were assessed, and first strand cDNA synthesis was performed with 2 μg of RNA using the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA) according

to manufacturer's instructions and then purified using the Wizard PCR Preps DNA purification system (Promega, Madison, WI). cDNA was quantified using the NanoDrop ND-1000 spectrophotometer, and 2 ng of purified cDNA was then subjected to 30 rounds of amplification using the Eppendorf Mastercycler thermal cycler using human HL RT-PCR primer sequences and conditions obtained from Gonzalez-Navarro et al. (*13*) and normalized to GAPDH. PCR products were run on 1.5% high-resolution agarose gel and visualized under UV using the Bio-Rad Gel-Doc system using Quantity One software. Real-time quantitative RT-PCR was performed using human HL forward, 5′-GGAGGAATCTGTTCAACTCTCTCG-3′, HL reverse, 5′-AGAAAGACGATTGCTGGGGG-3′, GAPDH forward, 5′-GACATCAAGAAGGTGGTGAA-3′, and GAPDH reverse, 5′-CCACATACCAGGAAATGAGC-3′, primer sequences, reagents, and conditions according to Sirvent et al. (*14*) with the minor change of conducting 45 cycles of amplification on a Roche Lightcycler 480.

ApoA-I Overexpression in HepG2 Cells. The pCMV5 vector containing the full-length pre-pro-apoA-I cDNA subcloned into the *Pst*I and *Bam*HI was a kind gift from Dr. Mary Sorci-Thomas (Wake Forest University). The pre-pro-apoA-I-pCMV5 and the control pCMV5 plasmids were both grown and purified using the Plasmid Maxi kit (Qiagen Inc., Mississauga, Ontario, Canada) according to the manufacturer's specifications. HepG2 cells were transiently transfected with pCMV5 plasmid alone or the pre-pro-apoA-I-pCMV5 plasmid by reverse (fast forward) transfection (where the cells were seeded and transfected on the same day at the same time) using FuGENE HD (Roche Applied Science, Laval, Quebec, Canada). In brief, complexes were prepared per manufacturer's instructions with a FuGENE HD-to-DNA volume-to-mass ratio of 6:2 (μL to μg) in 100 μL of Opti-MEM I reduced serum media (Invitrogen, Carlsbad, CA). HepG2 cells were trypsinized and seeded in 12-well plates at a density of 500000 cells/well in a volume of 1 mL in normal growth media containing 10% FBS in the absence of penicillin/streptomycin, and then 50 μL of the transfection complexes were immediately added to the suspended cells. The cells were incubated with the complexes for 24 h and then switched to serum-free DMEM for another 24 h, at which point the cells were harvested and the media collected for analysis for apoA-I by ELISA and HL by immunoblotting as described previously. Protein in the conditioned media was normalized to total cell protein. Transfection of the pCMV5 plasmid alone showed no adverse cytotoxic effects compared to nontransfected cells.

siRNA Knockdown of AapoA-I in HepG2 Cells. HepG2 cells were transiently transfected with All Stars Negative siRNA or four different apoA-I siRNA sequences (separately) from the Flexitube Gene Solution siRNA kit (Qiagen Inc., Mississauga, Ontario, Canada) by reverse transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In brief, complexes were prepared per manufacturer's specifications with a Lipofectamine 2000-to-siRNA volume-to-mole ratio of 2:40 (μL to ρmol) in 200 μL of Opti-MEM I reduced serum media (Invitrogen, Carlsbad, CA). HepG2 cells were seeded, transfected, and harvested as described in the apoA-I overexpression studies. Protein in the conditioned media was normalized to total cell protein. Transfection of the All Stars Negative siRNA alone showed no adverse cytotoxic effects compared to nontransfected cells.

Statistical Analysis. Values are shown as mean ± SD, and $P < 0.05$ was considered significant. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) on ranks by a pairwise multiple comparison using the Student−Newman−Keuls posthoc test (SigmaStat; Systat Software, Inc., San Jose, CA).

RESULTS

Effect of LAPL on ApoA-I Secretion and HL Release from Human Hepatocytes. Treatment of HepG2 cells with different linoleic acid phospholipids (LAPL) has shown that a stimulation of apoA-I secretion is associated with increased HL accumulation in the media (Figure 1). Linoleic acid alone (24 μM) had no effect on apoA-I secretion or HL release, relative to control DMSO incubations. PLPC (12 μM), which contains one linoleoyl acyl chain, stimulated a 2-fold increase in apoA-I (panel A) and a 1.5-fold increase in HL release/accumulation (panel B) at 24 h. DLPC (12 μM), which contains two linoleoyl groups, stimulated a 3-fold increase in apoA-I secretion and 2.2-fold increase in HL mass in the media. Thus, HL release into the media parallels apoA-I secretion, and the extent of both apoA-I secretion and HL accumulation in the media is dependent upon the number of linoleic acid groups in the phospholipid.

Experiments with primary human hepatocytes were undertaken to confirm observations in HepG2 cells. Figure 1, panel A inset, shows that DLPC was able to induce a 2-fold increase in apoA-I secretion from primary hepatocytes and a 1.75-fold increase in HL release (Figure 1, panel B inset) at 24 h. LAPL therefore appear able to stimulate apoA-I secretion and HL accumulation to a similar extent in both HepG2 cells and primary human hepatocytes.

Effect of LAPL on Cellular HL in HepG2 Cells. To determine whether the increase in HL accumulation in the media, after treatment with LAPL, is associated with increased cellular levels of HL, HepG2 cells were treated with DLPC for 24 h, and then HL mass was quantified. To compare media to lysate HL levels, equivalent volumes of cell lysate and media ($^1/_{30}$th total volume) were probed on the same gel. Figure 2 shows that treatment of HepG2 cells with DLPC increased media HL levels but had no effect on cellular HL. When cellular HL protein levels were normalized to β-actin, cellular HL mass was shown to be similar between control and treated cells (data not shown). When media and lysate HL mass levels were compared, DLPC significantly increased media HL levels, from 60% that of control cellular levels, to ~120% (Figure 2B).

Experiments were also undertaken to quantify HL levels on the plasma membrane. Cell surface biotinylation and reisolation experiments were conducted to determine cell surface HL levels after DLPC treatment. There was no significant difference in plasma membrane HL levels 24 h after DLPC treatment compared to control cells (Supporting Information Figure 1, panel A). Displacement experiments were also performed with heparinase I and heparin to determine the amount of HL that could be released into the media from the HepG2 cell surface after DLPC treatment. Similar to the cell surface biotinylation experiments, there was no significant difference in the amount of HL displaced from the DLPC-treated HepG2 cells compared to control cells (Supporting Information Figure 1, panel B).

Effect of Time and Dose on DLPC-Induced ApoA-I Secretion and HL Release. Figure 3 shows that apoA-I secretion parallels HL release into the media over the 24 h incubation. A low level of secretion of apoA-I and release of HL was observed prior to the 12 h time point. A similar secretion profile was observed for control incubations. The net accumulation

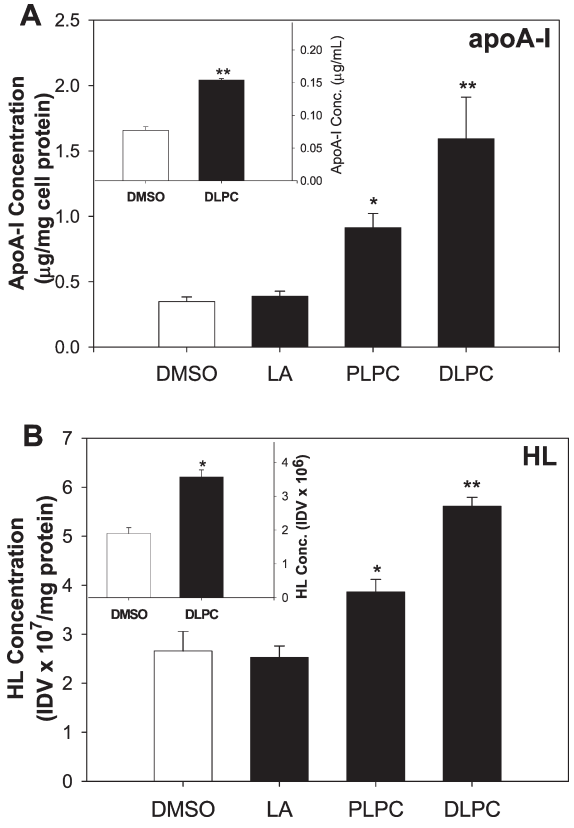

FIGURE 1: Linoleic acid phospholipids (LAPL) stimulate apoA-I secretion and HL release from human hepatocytes. HepG2 cells were treated with LA (24 μM) and PLPC or DLPC (12 μM) for 24 h, conditioned media were collected, and apoA-I secretion (panel A) and HL (panel B) release into the media were quantified by immunoblotting. Primary human hepatocytes (PHH) were treated with DLPC (12 μM) for 24 h, and apoA-I (inset A) and HL (inset B) levels in the media were quantified. Integrated density values (IDV) from immunoanalysis are expressed as mean ± SD. At least three independent experiments were performed with HepG2 cells, and two independent triplicate experiments were performed with primary human hepatocytes. *, $P < 0.05$, and **, $P < 0.001$, vs DMSO.

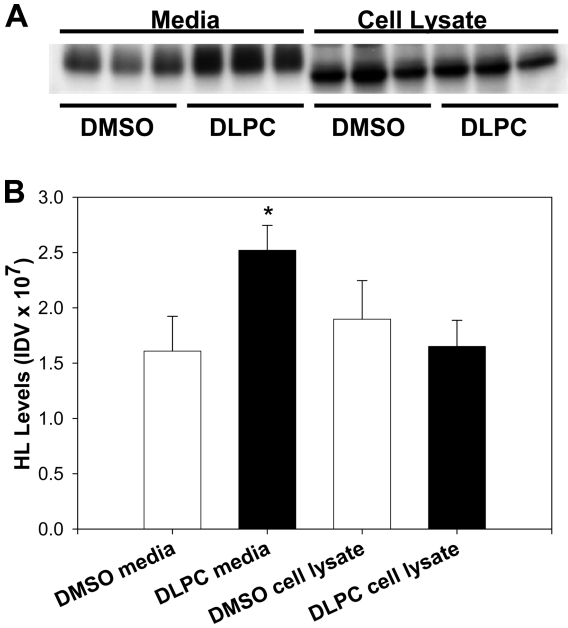

FIGURE 2: HL cellular protein levels are unaffected by LAPL. HepG2 cells were treated with 12 μM DLPC for 24 h. HL mass in equivalent volumes of cell lysate and media ($^1/_{30}$th total volume) was measured by immunoblotting for HL (panel A). Cellular and media HL levels were then quantified and graphically illustrated (panel B). Values are expressed as mean ± SD and are representative of three independent experiments. *, $P < 0.05$ vs DMSO media.

of apoA-I and HL increased between the 12 and 24 h time points with DLPC treatment. The effect of DLPC concentration on apoA-I and HL was also evaluated. HepG2 cells were treated with 0, 6, 12, and 24 μM DLPC for 24 h, and media were probed for apoA-I and HL. Both apoA-I and HL accumulation increased with DLPC dose and reached maximum levels in the media at 12 μM DLPC (data not shown). Treatment of HepG2 cells with DLPC was also shown to increase apoA-II secretion (data not shown).

HL Association and Activity. Figure 4 illustrates densitometry profiles of immunoblots of conditioned DLPC media samples that were electrophoresed on nondenaturing gradient gels and probed for HL, apoA-I, and apoA-II. HL is associated with HDL particles containing both apoA-I and apoA-II and ranging in size from 9 to 14 nm. Previous studies have also shown that HL is primarily associated with an 11 nm HDL complex and that HL and apoA-I nondenaturing gradient profiles are very similar after a 45 min displacement experiment (*15*). HL activity measurements in the treated media samples showed that the secreted HDL-associated HL was inactive (data not shown). This is consistent with our earlier work, which showed that while apoA-II stimulates HL displacement (*15*), it directly inhibits HL activity (*16*).

Effect of Signaling Inhibitors on DLPC-Induced ApoA-I Secretion and HL Release. LAPL act via the Ras, MAPK, and PPARα signaling pathways to stimulate apoA-I secretion (*9*, *11*). Experiments were conducted to examine the effects of the MEK 1/2 inhibitor, U0126, and the PPARα inhibitor, MK-886, on HL release. Figure 5 shows that, similar to apoA-I secretion (panel A), the stimulation of HL release by DLPC (panel B) was completely blocked when HepG2 cells were pretreated with 10 μM U0126 for 30 min. Pretreatment of the HepG2 cells with 10 μM PPARα inhibitor, MK-886, also inhibited the DLPC-induced release of HL (Figure 5, panel B).

Effect of Clofibrate and DLPC on HL Release. Experiments were undertaken to examine the effects of clofibrate on HL release. Figure 6 shows that clofibrate modestly stimulates the accumulation of apoA-I and HL in HepG2 cell media (panel A and panel B, respectively). Clofibrate, however, augments the apoA-I secretion effects of DLPC and significantly increases the effects of DLPC on HL release.

Effect of LA Phospholipids on HL RNA Levels. Experiments were performed to determine whether the increase in HL release seen in hepatocytes treated with LAPL was related to a transcriptional stimulation resulting in increased cellular HL mRNA levels. HepG2 cells were treated with DLPC for 24 h, and cell lysates were collected for isolation of total RNA. RT-PCR experiments were initially conducted to determine if LAPL treatment affected steady-state HL mRNA levels. Figure 7, panel A, shows that DLPC treatment did not result in any change in HL mRNA levels by RT-PCR. This result was then confirmed by real-time quantitative PCR, which also showed that HL RNA levels were unaffected by DLPC treatment (normalized to GAPDH), as compared to DMSO controls (Figure 7, panel B).

Effect of ApoA-I Expression on HL Release. HepG2 cells were transiently transfected with pre-pro-apoA-I to

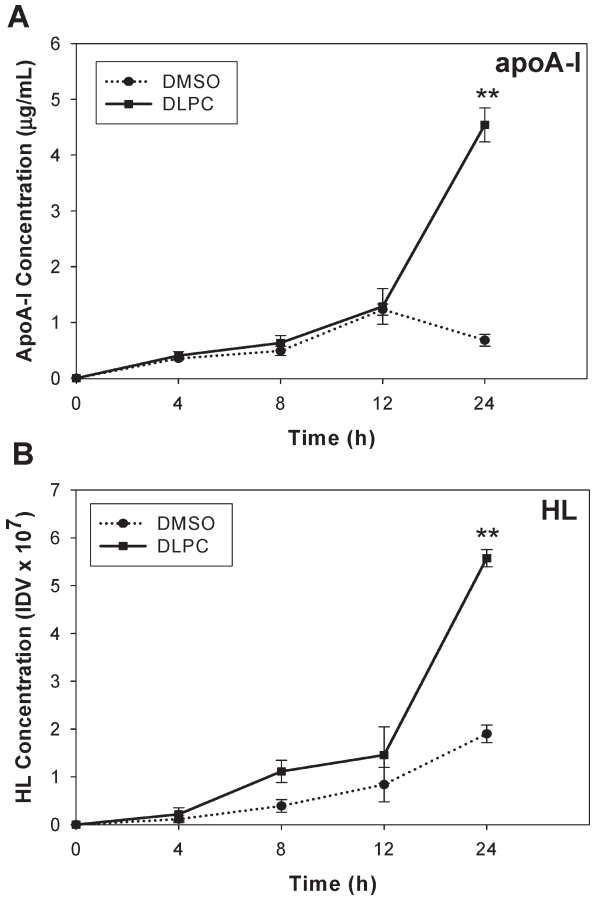

FIGURE 3: Effects of time on DLPC-induced apoA-I and HL release. HepG2 cells were treated with 12 μM DLPC for the indicated time points (4, 8, 12, and 24 h). The conditioned media were collected, and an ELISA was performed to determine the amount of apoA-I that was secreted (panel A). Immunoblotting was conducted to quantify HL in the media (panel B). Integrated density values (IDV) from immunoanalysis are expressed as mean ± SD. At least three independent experiments were performed. **, $P < 0.001$ vs DMSO at 24 h.

determine whether increased apoA-I secretion was directly associated with increased HL accumulation in the media. Figure 8, panel A, shows that the transient transfection with apoA-I-pCMV5 construct resulted in a 2.2-fold increase in apoA-I secretion, as compared to pCMV5 alone or nontransfected cells. This increase in apoA-I secretion was associated with a 1.3-fold increase in HL in the cell media (Figure 8, panel B). ApoA-I siRNA knockdown experiments showed that a 40% knockdown of apoA-I secretion from HepG2 cells was associated with a 30% decrease in HL accumulation in the media (Figure 9). These experiments confirm that apoA-I secretion is linked to the release and accumulation of HL in the HepG2 cell media.

DISCUSSION

Ramsamy et al. previously showed that apoA-I and HDL can act similarly to heparin and liberate HL from cell surface HSPG (*2*). Previous reports showed that HSPG-bound HL has reduced lipolytic activity and that its liberation from HSPG by heparin or HDL increases TG hydrolysis (*2*, *5*). Elevated HDL levels would therefore be expected to decrease liver-bound HL and result in enhanced TG clearance (*17*). This heparin-like nature of HDL was shown to be dependent upon both the lipid and apolipoprotein composition of HDL and has given rise to a novel mechanism by which HDL may impact lipolysis and TG clearance (*3*, *15*). This work has led to a clearer understanding of

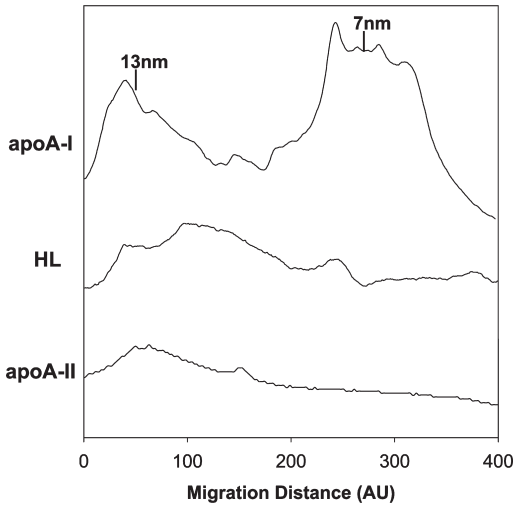

FIGURE 4: Association of HL with apoA-I and apoA-II on HDL. Nondenaturing gradient gel electrophoresis was performed on conditioned media from DLPC-treated HepG2 cells and immunoblotted for apoA-I, apoA-II, and HL. Densitometry profiles show an association of HL with 9−14 nm HDL complexes containing both apoA-I and apoA-II. Results are representative of at least three independent experiments.

why low HDL levels, high post-heparin HL activity, and elevated plasma TG levels are related (*17*, *18*). It appears that the heparin-like displacement ability of HDL may play a central role in regulating TG clearance.

Cell culture studies showed that HDL, when added to the cell media, will displace cell surface HL and liberate the enzyme into the media (*3*). As such, it would be expected that an accumulation of newly synthesized HDL in the media would have a similar effect on HSPG-bound lipases. This view has now been confirmed. Experiments show that LAPL are able to stimulate both HDL secretion and HL release from hepatocytes. Factors that stimulate HDL accumulation in the cell media also stimulate HL accumulation. HL accumulation therefore appears to be a product of HDL-dependent release from the cell membrane. The displacement process appears to be due to protein−protein interactions and related to the apolipoprotein composition of HDL (*15*, *16*). ApoA-I can directly displace HL by binding to the lipase (*15*), and apoA-II can enhance HL displacement by increasing the association of HL with HDL (*15*, *16*).

HL release and HDL secretion are sensitive to LAPL and incubation time (Figure 3). DLPC treatment over a 24 h period showed two different phases of protein secretion. During the first 12 h period, a low level of steady accumulation was observed for both apoA-I and HL in the media. After the first 12 h, however, there was a marked increase in both apoA-I and HL accumulation in the media. This high-output accumulation of HL could be attributed to an increased protein synthesis or decreased degradation of cellular HL. Since analyses showed no change in cellular HL mRNA levels and total HL protein expression after DLPC treatment (Figures 7 and 2B), HL synthetic pathways did not appear to have been upregulated. Increased media HL mass may instead be related to an increased displacement of cell surface HL and a decrease in the cellular recycling and degradation of the enzyme. Both cell surface biotinylation and displacement experiments conducted 24 h after DLPC treatment showed that cell surface HL levels are unaffected by DLPC treatment (Supporting Information Figure 1). Cell surface HL levels therefore also remain somewhat constant. This suggests that

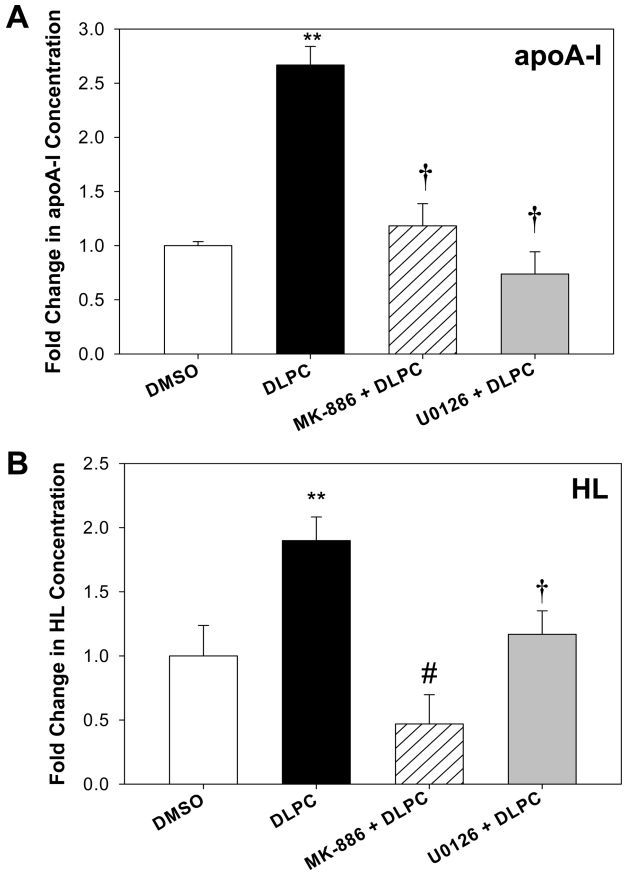

FIGURE 5: MEK 1/2 and PPARα inhibition block DLPC-induced apoA-I secretion and HL release. HepG2 cells were pretreated with 10 μM MEK 1/2 inhibitor, U0126, or the PPARα inhibitor, MK-886, for 30 min prior to addition of 12 μM DLPC for 24 h. The conditioned media were collected, and an ELISA was performed to determine the amount of apoA-I that was secreted (panel A). Immunoblotting was conducted to determine HL accumulation (panel B). Both apoA-I and HL accumulation results are normalized to total cell protein amounts and shown as fold change relative to DMSO controls. Values are expressed as mean ± SD. At least three independent experiments were performed. **, $P < 0.001$ vs DMSO, #, $P < 0.05$, and †, $P < 0.001$ vs DLPC.

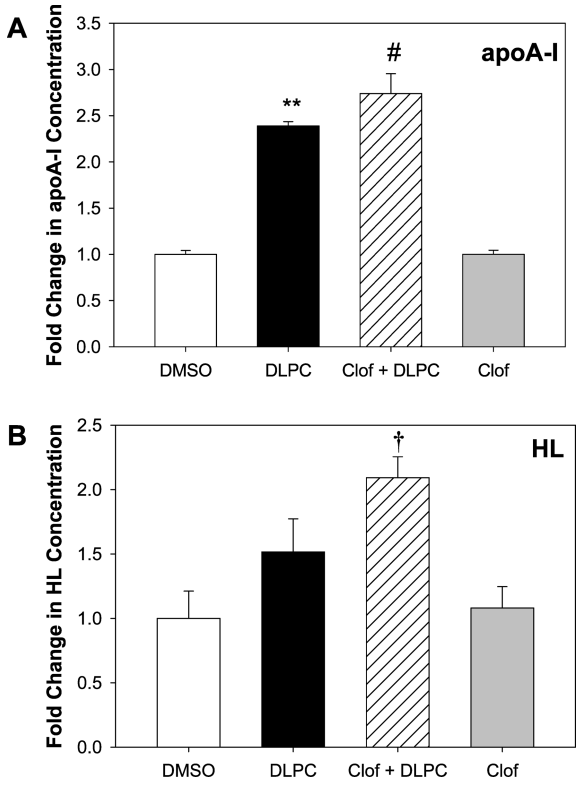

FIGURE 6: Clofibrate augments DLPC-induced apoA-I and HL release. HepG2 cells were pretreated with 10 μM clofibrate for 30 min prior to addition of 12 μM DLPC for 24 h. The conditioned media were collected, and an ELISA was performed to determine the amount of apoA-I that was secreted (panel A). Immunoblotting was conducted to quantify HL in the media. Both apoA-I and HL accumulation results are normalized to total cell protein amounts and shown as fold change relative to DMSO controls. Values are expressed as mean ± SD. At least three independent experiments were performed. **, $P < 0.001$ vs DMSO, #, $P < 0.05$, and †, $P < 0.001$ vs DLPC.

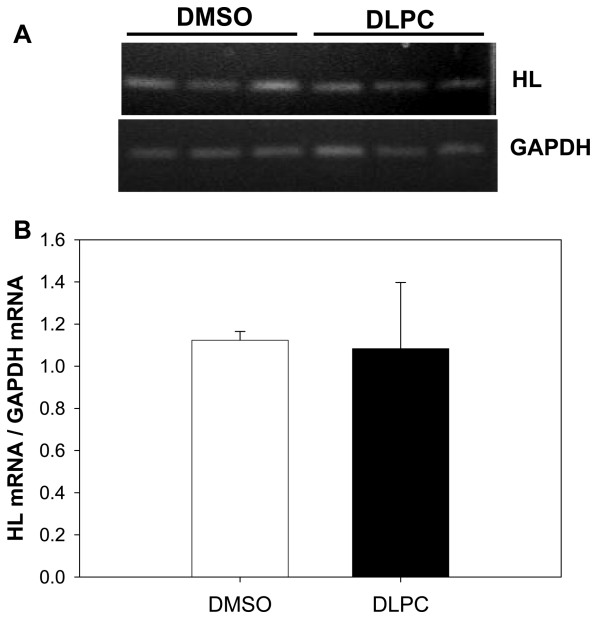

FIGURE 7: Steady-state HL mRNA levels are unaffected by LAPL. HepG2 cells were treated with 12 μM DLPC for 24 h. Total RNA was isolated, and RT-PCR was carried out with primers specific for human HL and GAPDH, respectively. The RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide (panel A). The results were then confirmed by real-time quantitative RT-PCR (panel B). Values are expressed as mean ± SD. At least three independent experiments were performed.

hepatocytes are able to maintain membrane HL levels by quickly replenishing any cell surface HL lost to the media.

HL release appears to be the result of the accumulation of extracellular HDL, which in turn acts similarly to heparin to displace HL from the HSPG of the hepatocyte cell surface (*2*). The direct effect of apoA-I on HL release into the media was confirmed by both overexpression of pre-pro-apoA-I and siRNA knockdown of apoA-I in HepG2 cells. As with LAPL studies, a stimulation in apoA-I secretion was shown to be directly related to the accumulation of HL in the cell media (Figure 8). Accumulation of the overexpressed apoA-I appears to have the same effect at liberating cell surface HL as adding exogenous apoA-I or HDL to the media (*2*, *5*). ApoA-I siRNA knockdown studies showed the opposite effect. A knockdown of apoA-I secretion resulted in a decrease in HL release into the media (Figure 9).

DLPC also increased secretion of another apolipoprotein, apoA-II (data not shown), and our previous work has shown that apoA-II directly stimulates HL displacement (*15*). ApoA-II has also been shown to inhibit HL activity (*16*), which may explain why the HL in HepG2 cell media was shown to be inactive (data not shown). HL is associated with large HDL complexes containing both apoA-I and apoA-II (Figure 4).

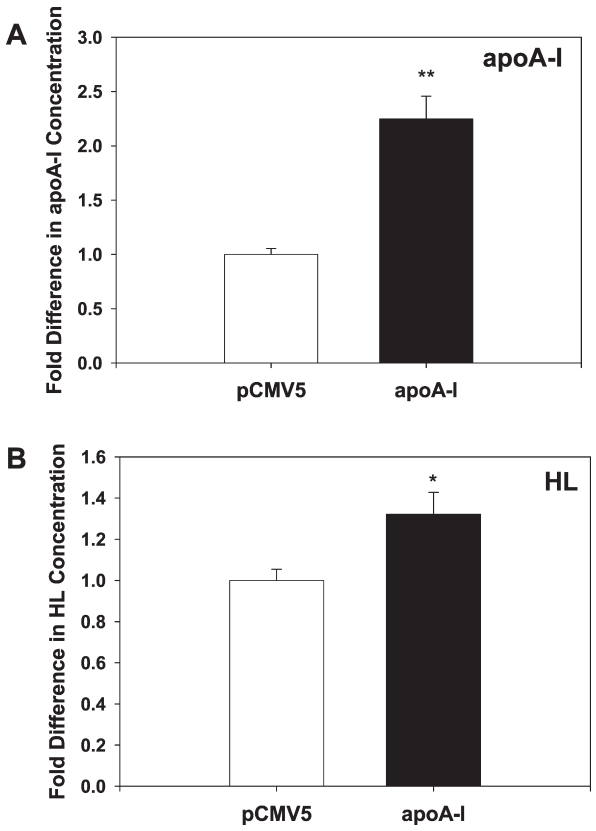

FIGURE 8: Effect of pre-pro-apoA-I overexpression on HL release. HepG2 cells were transiently transfected with pre-pro-apoA-I-pCMV5 or the control pCMV5 plasmid by reverse transfection. Conditioned media were collected 48 h posttransfection, and an ELISA was performed to determine the amount of apoA-I that was secreted (panel A). Nontransfected and pCMV5 transfected HepG2 cells secreted ~ 6−7 μg apoA-I/mg cell protein, whereas pre-pro-apoA-I transfected HepG2 cells secreted ~15−17 μg apoA-I/mg cell protein. Immunoblotting was conducted to quantify HL in the media (panel B). Both apoA-I and HL quantification results are normalized to total cell protein and shown as fold change relative to pCMV5. Values are expressed as mean ± SD. At least three independent experiments were performed. *, $P < 0.05$, and **, $P < 0.001$, vs pCMV5.

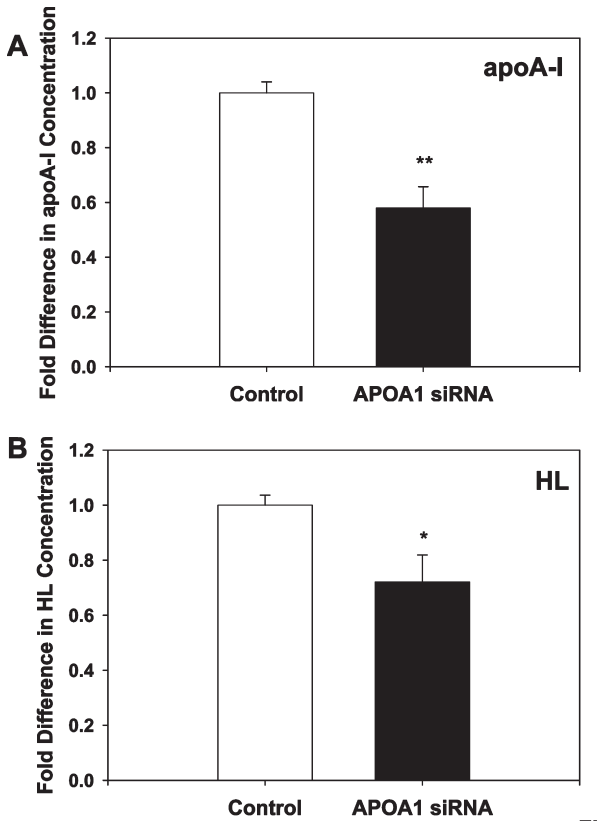

FIGURE 9: Effect of siRNA knockdown of apoA-I on HL release. HepG2 cells were transiently transfected with four different siRNA sequences for apoA-I or the control negative siRNA by reverse transfection. Conditioned media were collected 48 h posttransfection, and an ELISA was performed to determine the amount of apoA-I that was secreted (panel A). Immunoblotting was conducted to quantify HL in the media (panel B). Both apoA-I and HL quantification results are normalized to total cell protein amounts and shown as fold change relative to control negative siRNA and nontransfected cell media. Values are expressed as mean ± SD of the four different siRNA sequences. At least two independent experiments were performed, averaging the results of the four siRNA sequences. *, $P < 0.05$, and **, $P < 0.001$, vs control.

The average size of the complex, 11 nm, may indicate an HL−HDL stoichiometric composition of a dimer of HL and a 7−8 nm HDL particle. This is similar to that reported by Rouhani et al., where HL and apoA-I were also shown to be associated with a 11 nm complex (*15*). Since HL is associated with an apoA-II-enriched HDL, this interaction would be expected to result in a catalytically inactive enzyme. HL in preheparin plasma has also been shown to be catalytically inactive (*2*, *3*) and associated with HDL (Young et al., unpublished observation). HDL inactivation of circulating HL may be important to preventing adverse membrane lytic events from the enzyme phospholipase activity. HL may then be activated in the vascular compartment by factors that reduce the association of HL with HDL. We have shown that HL activity is regulated by HDL charge and activated by anionic lipids, such as free fatty acids, which promote the release of HL from HDL (*12*).

Numerous studies have shown linkage between the cellular metabolic pathways thought to regulate HDL and TG metabolism (*17*−*20*). Drugs that have been shown to affect plasma HDL levels are also known to affect TG levels (*21*, *22*). Both niacin and the fibric acid derivatives (fibrates) are examples of drugs that affect plasma HDL and TG. The fibrate drugs act through PPARα to impact HDL through a transcriptional regulation of the apoA-I gene and TG metabolism by inhibiting TG synthesis and stimulating TG clearance (*22*, *23*). PPARα agonists have been shown to enhance TG clearance by increasing plasma TG hydrolytic activity (*24*, *25*). They stimulate lipoprotein lipase (LPL) expression (*26*) and inhibit apoC-III secretion (*27*−*29*). LAPL appear to act similarly to the fibrate PPARα agonists, and stimulate HDL secretion and HL release, in a PPARα-dependent fashion. Clofibrate has a small effect on HL release but appears to act synergistically with DLPC to enhance HL release from HepG2 cells (Figure 6, panel B). Niacin treatment is believed to raise HDL levels without impacting apoA-I gene transcription (*30*). LAPL may act similarly, as DLPC does not appear to affect cellular apoA-I and HL mRNA levels. It therefore seems that therapeutic compounds that increase hepatic HDL secretion and plasma HDL levels would be expected to promote the simultaneous release of HL.

A stimulation of HDL secretion therefore appears to impact plasma TG metabolism by promoting the release of cell surface bound lipases. LAPL are more effective than fibrates at stimulating hepatic HDL secretion (*9*) and therefore would be expected to have a greater impact on plasma TG levels. Soy phosphatidylinositol is enriched in linoleic acid, much like PLPC, and oral administration of the lipid to healthy normal subjects for 2 weeks

caused an almost 40% reduction in plasma TG levels (*31*). LAPL therefore appear to have value as both HDL and TG regulatory therapeutics.

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SUPPORTING INFORMATION AVAILABLE

Plasma membrane HL levels after DLPC treatment (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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