Linoleic Acid-Enriched Phospholipids Act through Peroxisome Proliferator-Activated Receptors α To Stimulate Hepatic Apolipoprotein A-I Secretion[†]

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ABSTRACT: A uniquely formulated soy phospholipid, phosphatidylinositol (PI), is under development as a therapeutic agent for increasing plasma high-density lipoprotein (HDL) levels. Soy PI has been shown to increase plasma HDL and apolipoprotein A-I (apoA-I) levels in phase I human trials. Low micromolar concentrations of PI increase the secretion of apoA-I in model human hepatoma cell lines, through activation of G-protein and mitogen-activated protein (MAP) kinase pathways. Experiments were undertaken to determine the importance of the PI head group and acyl chain composition on hepatic apoA-I secretion. Phospholipids with choline and inositol head groups and one or more linoleic acid (LA) acyl chains were shown to stimulate apoA-I secretion by HepG2 cells and primary human hepatocytes. Phospholipids containing two LA groups (dilinoleoylphosphatidylcholine, DLPC) were twice as active as those with only one LA group and promoted a 4-fold stimulation in apoA-I secretion. Inhibition of cytosolic phospholipase A2 with pyrrolidine 1 (10 μM) resulted in complete attenuation of PI- and DLPC-induced apoA-I secretion. Pretreatment with the peroxisome proliferator-activated receptor α (PPARα) inhibitor MK886 (10 μM) also completely blocked PI- and DLPC-induced apoA-I secretion. Hepatic PPARα expression was significantly increased by both PI and DLPC. However, in contrast to that seen with the fibrate drugs, PI caused minimal inhibition of catalytic activities of cytochrome P450 and UGT1A1 enzymes. These data suggest that LA-enriched phospholipids stimulate hepatic apoA-I secretion through a MAP kinase stimulation of PPARα. LA-enriched phospholipids have a greater apoA-I secretory activity than the fibrate drugs and a reduced likelihood to interfere with concomitant drug therapies.

Low plasma high-density lipoprotein (HDL)[1] levels and elevated low-density lipoprotein (LDL), cholesterol, and triglycerides (TG) are associated with increased risk of cardiovascular disease (*1*). Even with aggressive therapy to reduce plasma levels of LDL-C (i.e., statin therapy), significant residual cardiovascular risk remains (*2*, *3*). Recent

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[1] Abbreviations: apoA-I, apolipoprotein A-I; cPLA2, cytosolic Ca^{2+}-dependent phospholipase A2; CYP, cytochrome P450; DLPC, dilinoleoylphosphatidylcholine; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular-regulated protein kinases 1 and 2; HDL, high-density lipoprotein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; LA, linoleic acid; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B (Akt); PKC, protein kinase C; PLC, phospholipase C. PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PPARs, peroxisome proliferator-activated receptors; PPRE, peroxisome proliferator response element; TG, triglyceride; UDPGA, uridine 5′-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase.

focus has shifted to targeting HDL elevation as an adjunctive therapy and have focused on the peroxisome proliferator-activated receptor (PPAR) activation profile of drugs including fibrates, statins, and niacin drugs (*4*) by increasing or modifying levels of HDL components, cholesterol, and apoA-I (*5*). Niacin (nicotinic acid) and fibrates (PPARα agonists) increase HDL cholesterol levels by 20−30% and 10−15%, respectively (reviewed in ref *5*), as well as reducing plasma triglyceride levels by ∼30%. The statin drugs effectively reduce plasma triglyceride (TG) and LDL cholesterol levels (*2*, *6*), while niacin and fibrates have been used to reduce plasma TG levels and raise HDL levels (*7*, *8*). We previously have shown that soy PI increases apoA-I and HDL-cholesterol levels and decreases plasma triglycerides in healthy human subjects (*9*). The therapeutic effects of PI appeared similar to that of niacin and the fibrate drugs. PI uniquely affects hepatic lipid metabolism in rabbits (*10*) and in human hepatoma cell systems (*11*).

PPARs comprise a three-member subgroup (α, γ, and β/δ) within the nuclear hormone receptor family of ligand-activated transcription factors. Fibrates are considered to be PPAR agonists due to their ligand-specific activation of PPARα and heterodimerization of the 9-*cis*-retinoic acid receptor RXR and are able to uniquely regulate apolipoprotein C-III and lipoprotein lipase gene expression, key players

in triglyceride metabolism (*12−14*). PPARα binds to specific response elements, peroxisome proliferator response elements (PPREs), in the regulatory regions of target genes (*15*). PPREs consist of a direct repeat of the degenerated hexamer AGGTCA sequence separated by one nucleotide (DR-1). Fibrates exert their effects on plasma lipids by altering the transcription of genes involved in lipoprotein metabolism (*16*). Fibrate action on lipoprotein metabolism is mediated by PPARα, the principal PPAR form in liver as demonstrated in PPARα-deficient mice (*17*). Functional PPREs have been identified in the promoters of the genes involved in plasma triglyceride (*18*), in lipoprotein lipase and apoC-III genes (*19*), and in HDL metabolism, in both apoA-I (*20*) and apoA-II genes (*21*). Fibrate-induced HDL regulation differs in various animal species and is associated with opposite changes in apoA-I expression due to differences in cis-element sequence (*15*). In human plasma, HDL cholesterol and apoA-I levels increase upon fibrate treatment (*22*), while decreases have been observed in rats (*23*). PPAR regulation is known to differ in small animal models, relative to humans, and as such the human hepatoma cell line HepG2 has been a useful model for studying hepatic lipoprotein metabolism (*24, 25*) and apoA-I secretion (*26*). The fibrate drugs appear to interfere in the metabolism of the statin drugs, partly by inhibiting statin hydroxyl acid glucuronidation (*27, 28*). The statin drugs are metabolized by the cytochrome P450 enzymes. Several fibrate drugs have been shown to inhibit some of the CYP P450 enzymes and as such may exacerbate some of the known toxicities of the statin drugs (*28−31*). It is for this reason that statin−fibrate combination therapies are undertaken cautiously.

Tracking the effect of the PPARα agonists on HDL levels has been difficult in animal models, as the regulation of PPARα and its effect on gene transcription are unique in rodent models and generally species-specific (*32*). For this reason, the model human hepatocyte cell lines (i.e., HepG2) and primary human cells have been the preferred choice for evaluating the effect of PPARα agonists on HDL synthetic activity. The human apoA-I gene promoter has been shown to have a PPARα-responsive element (*33*), and agonists have been shown to increase apoA-I gene transcription (*16, 20*). Unsaturated long-chain fatty acids, notably arachidonic acid and its eicoisanoid metabolites, are thought to be potent natural ligands for PPARα (*34*). Intracellular production of these ligands is controlled through the action of cytosolic phospholipase A_2 (cPLA$_2$), and as such, the enzyme plays an important role in regulating PPAR-mediated gene transcription (*35−38*).

Soy phosphatidylinositol (PI) is being developed as a therapeutic agent for the treatment and prevention of heart disease associated with dyslipidemia. PI has been shown to stimulate reverse cholesterol transport in animals (*10*) and to increase HDL and decrease triglyceride levels and in human subjects (*9*). We have shown that a G-protein-dependent activation of mitogen-activated protein kinase (MAPK) by PI is required for apoA-I secretion by HepG2 cells (*11*). Statins and PPAR ligands have also been shown to induce phosphorylation of MAPK family members (*39−44*). We therefore sought to evaluate whether PI-induced apoA-I secretion is also mediated by MAPK-dependent activation of PPARα or PPARγ and whether other phospholipids with related acyl chain compositions would act

similarly to impact apoA-I secretion. We show that linoleic acid- (LA-) enriched phospholipids stimulate hepatic apoA-I secretion through a MAPK stimulation of PPARα. LA-enriched phospholipids have a greater apoA-I secretory activity than clofibrate and do not inhibit the cytochrome P450 enzymes.

MATERIALS AND METHODS

Chemicals. All phospholipids, namely, soy PI, POPC, DLPC, PLPC, and LA, were procured from Avanti Polar Lipids Inc., Alabaster, AL. MK886 (a noncompetitive PPARα inhibitor), clofibrate (a PPARα agonist), and GW9662 (a PPARγ inhibitor) were from Cayman Chemicals, Ann Arbor, MI. cPLA2 inhibitor pyrrolidine 1 was from Calbiochem. Antibodies for PPARα and β-actin were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, and phospho- and non-phospho-specific PKB/Akt were from Cell Signaling, Beverly, MA. Antibodies for apoA-I were obtained from Biodesign, Saco, ME. Unless otherwise stated, drugs and inhibitors were of analytical grade and were solubilized in dimethyl sulfoxide (DMSO).

Cell Culture. HepG2 cells were cultured in 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. High glucose experiments were performed with DMEM containing 25 mM D-glucose, 10% FBS, and 1% penicillin/streptomycin and with corresponding serum-free medium.

Human Primary Hepatocytes. Collagen-coated, and HIV-I, hepatitis B and C, mycoplasma, bacteria, yeast, and fungi test-negative human primary hepatocytes (HPH) were obtained from freshly donated livers supplied by Lonza Walkersville (Walkersville, MD). HPH 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, and then conditioned medium and cell protein were collected for analysis.

Preparation of Phospholipid Vesicles. Phospholipid vesicles in phosphate-buffered saline (PBS; 1 mg/mL) were prepared by sonication as previously described (*45*). Briefly, phospholipids in chloroform were dried down under N_2 and 1 mL of PBS was added by vortexing. The mix was then sonicated (Branson sonicator set at 100% duty cycle and 10% power) for 1 min. The sonicated preparation was incubated for 30 min at 37 °C in a water bath, and samples were resonicated for 5 min at 95% duty cycle and 10% power and filtered before use. Purity of all phospholipids was >99% (Avanti Polar Lipids) and was verified by HPLC.

ApoA-I ELISA. Protein in conditioned medium from each stimulation was analyzed by ELISA on a 96 well plate according to manufacturer's instructions, with minor modifications. 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 was added to each well and the reaction was stopped with a 1 M HCl solution; and the

Table 1: Effect of Soy PI on Cytochrome P450 and UGT Inhibition

enzyme	substrate (concn, μM)	positive control (inhibitor concn, μM)	IC_{50}^{a} for PI inhibition, μM
CYP1A2	phenacetin (50.0)	7,8-benzoflavone (0.3)	*b*
CYP1A2	phenacetin (50.0)	7,8-benzoflavone (3.0)	>10*c*
CYP2C9	diclofenac (6.0)	sulfaphenazole (100.0)	>10
CYP2C19	(S)-mephenytoin (50.0)	tranylcypromine (1.0)	>10
CYP2D6	bufuralol (10.0)	quinidine (1.0)	>10
CYP3A4	testosterone (120.0)	ketoconazole (1.0)	>10
CYP3A4	midazolam (3.0)	ketoconazole (1.0)	>10
UGT1A1	7β-estradiol (150.0)	bilirubin (50.0)	>100

a IC_{50} values were determined with human liver microsomes as the enzyme source. *b* IC_{50} value could not be determined because the test article interfered in the quantitation of the metabolite. *c* Data shown were analyzed by LC/MS. Positive controls are compounds that inhibit the corresponding enzyme−substrate reaction by 50% at the indicated inhibition concentration.

absorbance was recorded at 450 nm. The assay conditions were optimized to minimize any apoA-I conformation interference with the apoA-I ELISA.

Western Blot Analysis. After incubation with drugs for the indicated times and doses, cells were washed twice with ice-cold PBS-T on ice. Cells were lysed by adding buffer [NaF 1 mmol/L, NaCl 5 mmol/L, EDTA 1 mmol/L, NP40 1 mmol/L (Roche Diagnostics, Indianapolis, IN), HEPES 10 mmol/L, pepstatin A 1 mg/mL, leupeptin 1 mg/mL, aprotinin 1 mg/mL, Na_3VO_4 1 mmol/L, and PMSF 1 mmol/L] and total protein was extracted. An equal amount of cell proteins were separated by SDS−12% PAGE and were analyzed by Western blot with specific antibody PPARα (SantaCruz Biotechnology, Santa Cruz, CA), Akt antibody phosphorylated at residue Ser473, and total Akt antibody. Blots for the similar experiments were also subjected to β-actin for a loading control. Band intensity was analyzed with spot densitometer by AlphaImager software, and obtained PPARα values were normalized to the value of corresponding β-actin values.

Cytochrome P450 Study. Inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and UGT1A1 enzymes by PI were examined by using model substrates and pooled human liver microsomes (CYPs) (BD Gentest catalogue no. 452161) and cDNA-derived UGT1A1 (BD Gentest catalogue no. 456411) in microsomes prepared from baculovirus-infected insect cells, as a source of enzyme. The assay consisted of determination of a 50% inhibitory concentration (IC_{50}) for PI and enzyme/substrate pair as indicated in Table 1. Positive controls are compounds that are known to inhibit the corresponding P450 enzymes. We determined the degree of enzyme inhibition by various concentrations of PI using a single concentration of each model substrate (near the apparent K_M). Final PI concentrations were 0.0, 0.001, 0.003, 0.01, 0.3, 1.0, 3.0, and 10.0 μM for CYP inhibition and 0.0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, and 100.0 μM for UGT inhibition. A 0.25 mL reaction mixture containing various dosages of PI, 0.025−0.8 mg/mL microsomal protein, 1.3 mM NADP$^+$, 3.3 mM glucose 6-phosphate, 0.4 unit/mL glucose-6-phosphate dehydrogenase, 3.3 mM MgCl$_2$ and substrates (as indicated in Table 1) were incubated at 37 °C for 15 min. For UGT1A1, 50 mM Tris-HCl (pH 7.5), 2 mM UDPGA, 10 mM MgCl$_2$, 25 μg/mL alamethicin, 150

μM 17β-estradiol, and 0.4 mg/mL enzyme proteins were taken in a final volume of 0.20 mL and were incubated at 37 °C for 20 min. The reaction was stopped and centrifuged to pellet the protein, and the supernatant was injected into a 5 μM, C18 HPLC column and separated at 45 °C with a mobile phase of methanol/water at a flow rate of 1 mL/min for each samples. The catalytic activities for CYP1A2, CYP2C9, CYP2D6, CYP3A4, and UGT1A1 were calculated by measurement of metabolite formation in the presence and absence of the inhibitor, quantified by comparison to standard curves of known concentrations of analytes using the coefficient of variation of the metabolite standard peak areas. HPLC absorbance/fluorescence peak areas was converted into picomoles of metabolite formed on the basis of the peak area of the standards. LC/MS peak ratio (metabolite/internal standard) in the samples was converted into picomoles of metabolite formed on the basis of the peak area ratios of the standards. Catalytic activity for CYP2C19 was determined radiometrically on the basis of the specific activity of the substrate and the detector's response to radioactivity. For each isoform, the IC_{50} was calculated by linear interpolation. The linear interpolation used the mean percent inhibition for each concentration of test substance.

Statistical Analysis. Values are shown as mean ± SEM, 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 post-hoc test (SigmaStat; Systat Software, Inc., San Jose, CA) Results in Figure 3 were evaluated by two-way analysis of variance (ANOVA) on ranks by a pairwise multiple comparison using Bonferroni *t*-test.

RESULTS

Effect of LA-Enriched Phospholipids on ApoA-I Secretion. Low micromolar doses of soy PI (12 μM) promote a significant increase of apoA-I secretion in HepG2 cells, after a 24-h incubation (Figure 1). Experiments were undertaken to determine the importance of the phospholipid head group and acyl chain composition on apoA-I secretion. Phosphatidic acid, phosphatidylethanolamine, and phosphatidylserine all had no effect on apoA-I secretion (data not shown). Phospholipids with choline and inositol head groups and one or more linoleic acid (LA) acyl chains were shown to stimulate apoA-I secretion in HepG2 cells (Figure 1). The major phospholipid species in soy PI is 1-palmitoyl-2-linoleoylphosphatidylinositol. PI deficient in LA (bovine PI and dioleoyl-PI) had no effect on apoA-I secretion (data not shown). Phosphatidylcholine with a similar acyl chain content (PLPC) to soy PI showed a similar ability to promote HepG2 apoA-I secretion (Figure 1). Phospholipids containing two LA groups, such as dilinoleoylphosphatidylcholine (DLPC), were about twice as active as those with only one. In contrast, twice the molar concentration of pure LA (equivalent mass) had no effect on apoA-I secretion and no toxic effect on the cells. PI and DLPC also significantly stimulated apoA-I secretion into the medium of human primary hepatocytes (Figure 1 inset).

Effect of Phospholipid Concentration on ApoA-I Secretion. Various doses of PI, DLPC, and clofibrate were incubated with HepG2 cells and apoA-I secretion was determined
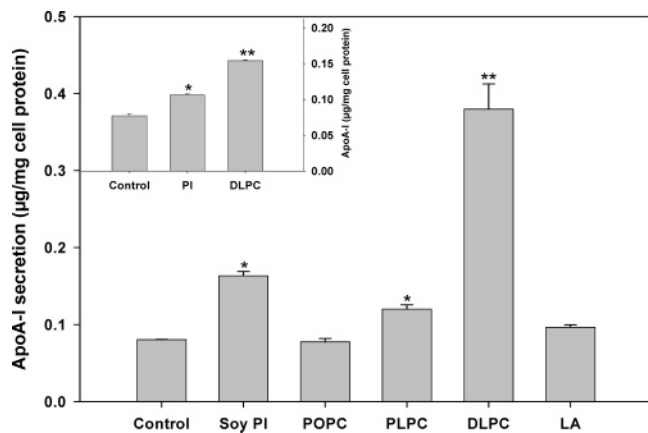
FIGURE 1: LA-enriched phospholipids increase hepatic apoA-I secretion. Confluent and quiescent HepG2 cells were incubated with various pure lipids and apoA-I secretion was measured. Aqueous vesicular mixtures of 12 μM soy phosphatidylinositol (PI), 13 μM 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 13 μM 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC), 13 μM dilinoleoylphosphatidylcholine (DLPC), or 24 μM linoleic acid (LA) were added to the cells, and the samples were incubated for 24 h; an equal volume of medium was taken as control. Primary human hepatocytes (inset) were stimulated with or without 12 μM PI or 13 μM DLPC for 24 h. Conditioned medium was collected and apoA-I concentration was determined by ELISA. ApoA-I secretion is presented relative to total cell protein values. Values are expressed as mean $\pm$ SEM of at least 4 independent experiments; *$P < 0.05$; **$P < 0.001$ vs control.
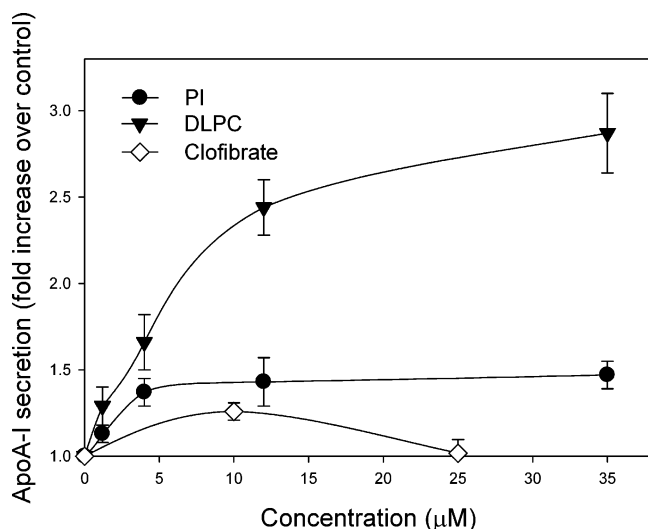

FIGURE 2: Effect of phospholipid dose on hepatic apoA-I secretion. HepG2 cells were incubated with various amounts of soy phosphatidylinositol (PI), dilinoleoylphosphatidylcholine (DLPC), or clofibrate and apoA-I secretion was measured. Medium was collected after a 24 h incubation and apoA-I concentration was determined by ELISA. ApoA-I secretion is presented relative to total cell protein values as an *x*-fold increase relative to control incubations. Values are expressed as mean $\pm$ SEM of at least 4 independent experiments.

(Figure 2). PI and DLPC showed a saturable dose−response. PI dose−response began to plateau at a dose of 4 μM, while DLPC response began to plateau at 12 μM but was still increasing by 35 μM. DLPC was almost twice as effective as PI and promoted an almost 3-fold stimulation in apoA-I secretion. Clofibrate was less effective at stimulating apoA-I secretion, and only about a 1.25-fold stimulation was observed at a dose of 10 μM. Increasing the dose beyond
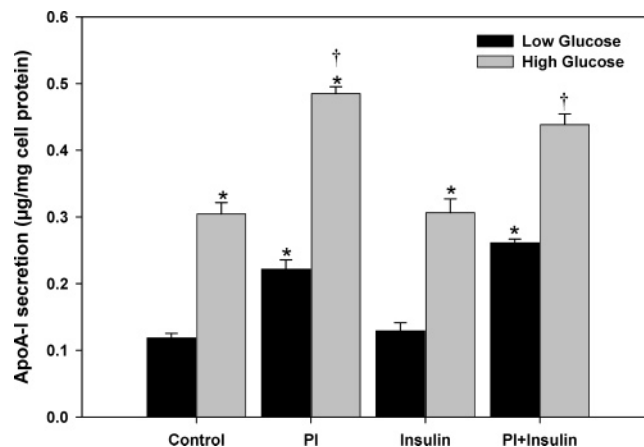
FIGURE 3: Effect of PI, insulin, and glucose concentration on apoA-I secretion in HepG2 cells. HepG2 cells were cultured under low-glucose (5.6 mM) or high-glucose (25 mM) conditions and incubated with PI (12 μM) and/or insulin (100 nM) for 24 h; an equal volume of medium was taken as control. Conditioned medium was collected and subjected to apoA-I quantification by ELISA. ApoA-I secretion is presented relative to total cell protein values. Values are expressed as mean $\pm$ SEM of at least 4 independent experiments; *$P < 0.001$ vs low-glucose control, †$P < 0.001$ vs high-glucose control (two-way ANOVA).
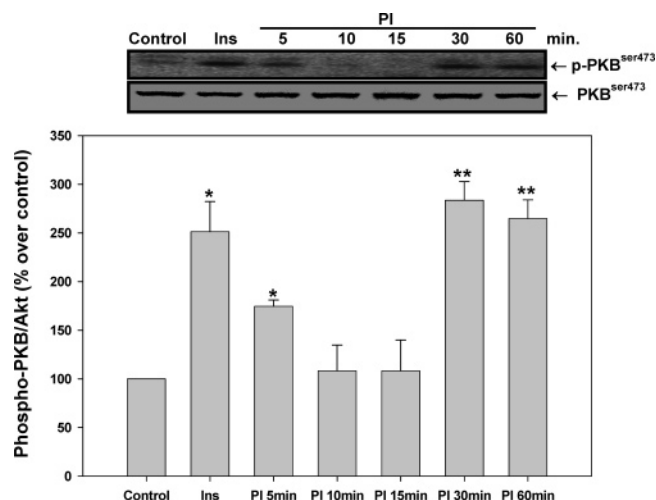

FIGURE 4: Effect of PI on PKB phosphorylation in HepG2 cells. HepG2 cells were incubated with insulin (100 nM) for 5 min or with PI (12 μM) for the indicated times, and phosphorylated (upper panel) and total (middle panel) PKB were analyzed by Western blot with specific antibodies. The lower panel is a histogram of analyzed values. Values are expressed as mean $\pm$ SEM of at least 4 independent experiments; *$P < 0.05$, **$P < 0.001$ vs control.

10 μM blocked the stimulation of apoA-I secretion in HepG2 cells.

Effect of Glucose and Insulin on PI-Induced ApoA-I Secretion. The effect of low (5 mM) and high (25 mM) glucose levels in the medium, and of insulin, on PI-induced apoA-I secretion were evaluated. Elevated glucose levels in the medium showed a significant effect on apoA-I secretion, with a high glucose level resulting in an almost 3-fold stimulation (Figure 3). PI stimulated apoA-I secretion under both low- and high-glucose conditions; however, a lesser stimulation was observed in high-glucose medium. PI doubled apoA-I secretion under low-glucose conditions but stimulated secretion by only 1.5-fold under high-glucose conditions.
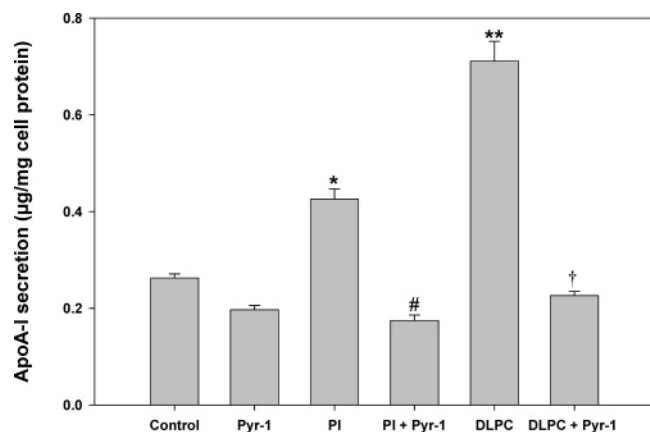
FIGURE 5: cPLA2 inhibition attenuates PI- and DLPC-induced apoA-I secretion. HepG2 cells were incubated with or without PI (12 μM) and DLPC (13 μM) and cPLA2 inhibitor, pyrrolidine 1 (10 μM) for 24 h; and an equal volume of DMSO was taken as control. Conditioned medium was collected and apoA-I concentration was determined by ELISA. ApoA-I secretion is presented relative to total cell protein values. Values are expressed as mean $\pm$ SEM of at least 4 independent experiments; *$P < 0.05$, **$P < 0.001$ vs control, #$P < 0.001$ vs PI, and †$P < 0.001$ vs DLPC.

Effect of PI on PKB Phosphorylation. Insulin is known to promote the phosphorylation of protein kinase B (PKB/Akt) to induce glucose transport (*46*). Fibrate drugs are believed to inhibit hepatic PKB phosphorylation (*47, 48*). Experiments were undertaken to determine how PI may impact PKB. Figure 4 shows that both insulin (100 nM) and PI (12 μM) induce a significant increase in PKB phosphorylation by 5 min. With PI, PKB phosphorylation returns to basal levels at 10 and 15 min and then peaks again at 30 and 60 min.

Effect of cPLA2 Inhibition on PI-Induced ApoA-I Secretion. Cytosolic phospholipase A2 (cPLA2) has been shown to play a central role in PPARα-mediated gene transcription in HepG2 cells (*21−24*). We therefore tested whether inhibition of cPLA2 with pyrrolidine 1 (10 μM) would impact the ability of LA-enriched phospholipids to promote apoA-I secretion. As shown in Figure 5, inhibition of cPLA2 with pyrrolidine 1 resulted in complete attenuation of PI- and DLPC-induced apoA-I secretion in HepG2 cells.

Effect of LA-Enriched Phospholipids on PPARα and ApoA-I Secretion. We also tested whether inhibition of PPARα or PPARγ would impact LA-enriched phospholipid stimulation of apoA-I synthesis and secretion. As shown in Figure 6, inhibition of PPARα with the inhibitor MK866 (*49*) attenuated both PI- and DLPC-induced apoA-I secretion. In contrast, inhibition of PPARγ with the inhibitor GW9662 had no effect on apoA-I secretion. The data show that PPARα is involved in the LA-rich phospholipid induction of apoA-I secretion. PI concentrations of 6 and 12 μM significantly increased PPARα protein expression (Figure 7) over a 24-h incubation. PI (12 μM) promotes a 1.7-fold increase in PPARα protein level, similar to that observed with 10 μM clofibrate.

Effect of PI on Cytochrome P450 and UGT Inhibition in Human Liver Microsomes. The PPARα agonist gemfibrozil has been shown to be a potent inhibitor of human cytochrome P450 enzymes (*28, 29, 50*). Other fibrate drugs are also metabolized by hepatic cytochrome P450 (*13, 14*). Therefore, we investigated the effect of soy PI on the inhibition of
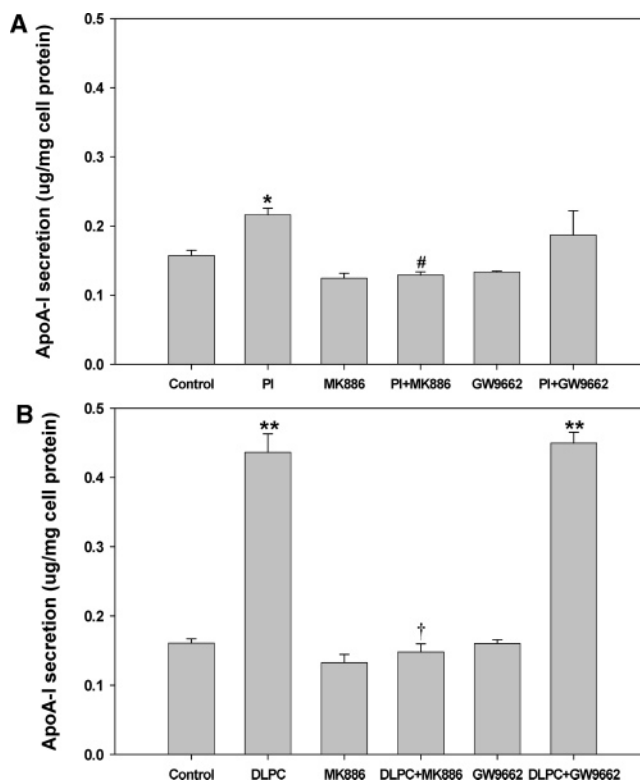

FIGURE 6: LA-enriched phospholipid stimulation of apoA-I secretion is attenuated by PPARα inhibition. HepG2 cells were incubated with PI (12 μM, panel A) or DLPC (13 μM, panel B) and the PPAR inhibitors MK886 (PPARα) and GW9662 (PPARγ) (10 μM) for 24 h; an equal volume of DMSO was taken as control. Conditioned medium was collected and apoA-I concentration was determined by ELISA. ApoA-I secretion is presented relative to total cell protein values. Values are expressed as mean $\pm$ SEM of at least 4 independent experiments; *$P < 0.05$, **$P < 0.001$ vs control, #$P < 0.001$ vs PI, and †$P < 0.001$ vs DLPC.

catalytic activity for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and UGT1A1. Inhibition was calculated by measuring metabolite formation for the indicated substrate (Table 1) in the presence and absence of the inhibitor. Positive controls listed in the table are known to induce the indicated CYP P450 enzymes; the micromolar concentration is that needed for the positive response. Human liver microsomes were utilized as the enzyme source. Table 1 shows that the IC$_{50}$ values for PI inhibition of the CYP P450 enzymes were >10 μM, while the IC$_{50}$ for UGT1A1 was >100 μM. The data shows that PI has minimal inhibitory effects on the cytochrome P450 and glucuronidation enzymes. In contrast, the fibrate drugs are thought to be metabolized by CYP3A4 (*31*) and gemfibrozil is known to inhibit CYP1A2, CYP2C9, and CYP2C19 (*28−30*).

DISCUSSION

LA-enriched phospholipids are able to increase hepatic apoA-I secretion (Figure 1A). PI and DLPC significantly increased apoA-I secretion in HepG2 cells and in human primary hepatocytes. The data show that phospholipids with choline and inositol head groups and one or more LA acyl chains are able to effectively stimulate apoA-I secretion in cultured hepatic cells. PC containing two LA groups were twice as active as those with only one. Other phospholipid classes had no effect on apoA-I secretion. PI devoid of 18:2 fatty acyl chains also had no activity. Bovine PI (mostly 20:3
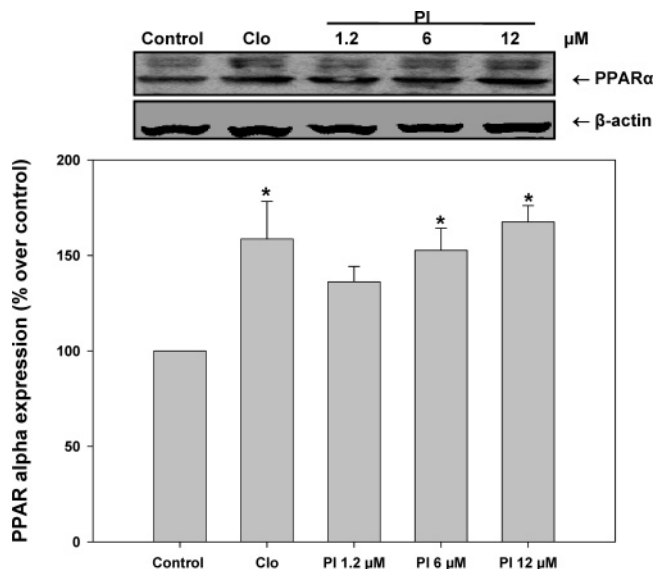
FIGURE 7: LA-enriched phospholipids increase PPARα expression. HepG2 cells were incubated with clofibrate (10 μM) or various amounts of PI (1.2, 6, and 12 μM) for 24 h; an equal volume of DMSO was taken as control. Cells were harvested and cell lysates were electrophoresed on SDS−12% polyacrylamide gels and then analyzed by Western blot with an antibody specific for PPARα (upper panel). Membranes were also analyzed for β-actin (middle panel) as a loading control, and band intensity was quantified (AlphaImager). Values obtained for PPARα expression were corrected with corresponding β-actin values and are presented as a histogram (lower panel). Values are expressed as mean ± SEM of at least 4 independent experiments; *P < 0.05 vs control.

and 20:4 fatty acyl chains) and DOPI ($2 \times 18:1$ fatty acyl chains) had no effect on apoA-I secretion. LA-enriched phospholipids were, however, more effective than the PPARα agonist clofibrate at promoting hepatic apoA-I secretion (Figure 2).

We do not yet know what cell surface receptors/targets are involved in this LA-rich phospholipid stimulation. Experiments have demonstrated that the process involves phospholipase C (PLC): both PI- and PC-PLC inhibitors block apoA-I secretion (11). We have also shown PI-induced apoA-I secretion is regulated by G-protein stimulation of mitogen-activated protein kinase (MAPK) (Figure 8) (11). Activation of MAPK (28) has been shown to catalyze PPARs through the activation of Ca^{2+}-dependent cytosolic phospholipase A2 (cPLA2) in HepG2 cells (37, 38). PI- and DLPC-induced apoA-I secretion was attenuated by the inhibition of cPLA2 (Figure 5). PI- and DLPC-induced hepatic apoA-I secretion was also blocked by PPARα inhibition with the specific inhibitor MK886, while PPARγ inhibition with GW9662 had no effect (Figure 6). This suggests that LA-enriched phospholipid induction in apoA-I secretion is mediated by PPARα but that PPARγ is not involved. Like clofibrate, PI also increased PPARα expression in the HepG2 cells (Figure 7) in a dose-dependent manner.

Decreased expression of PPARα is implicated in increased risk of cardiovascular diseases and metabolic defects in animals and humans, due to a downregulation of fatty acid oxidation (51). LA is a ligand for PPARα (52) and may directly interact with the receptor or may activate the receptor through a stimulation of MAPK and cPLA2. LA has also been shown to increase PPARα protein expression in HepG2 cells (53) but the lipid has no effect on apoA-I secretion (Figure 1). This suggests that a stimulation of apoA-I secretion appears to require more than just increased PPARα expression. A stimulation of PPARα has been shown to increase apoA-I gene transcription (54). LA-enriched phospholipids, however, do not increase hepatic apoA-I transcription. We have shown that LA-enriched phospholipids do not directly affect HepG2 cell apoA-I mRNA levels (11). ApoA-I
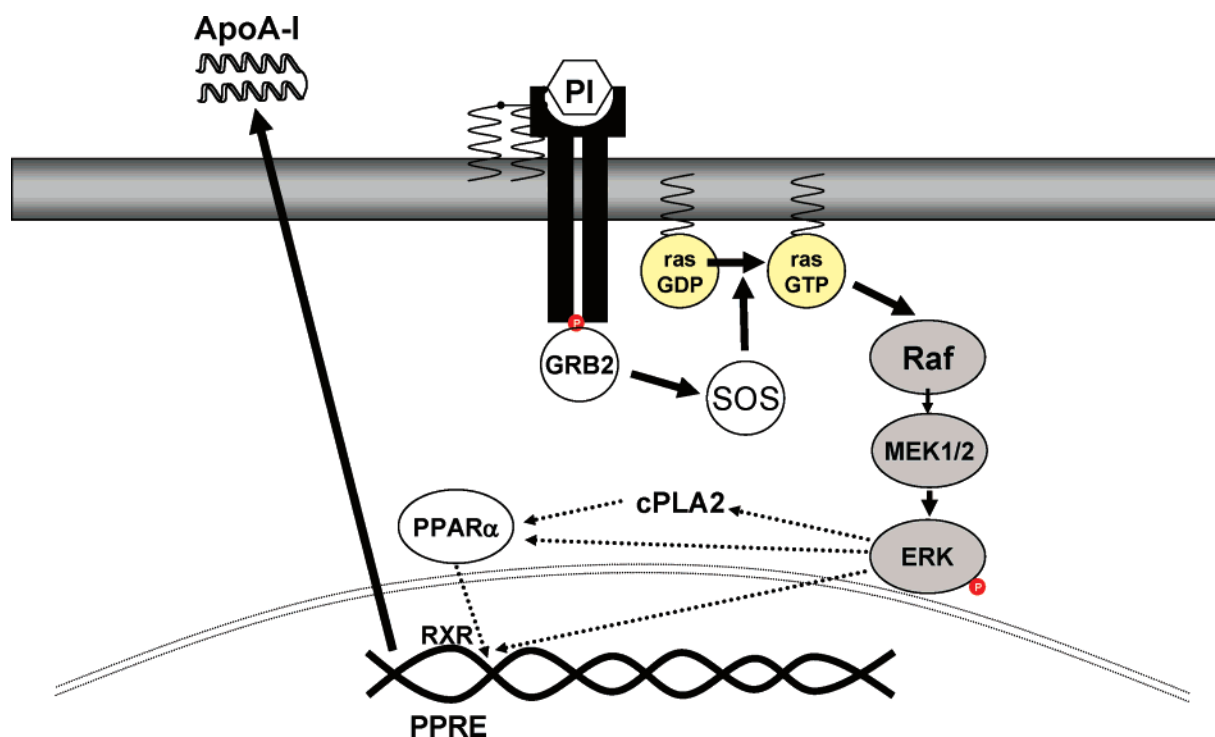

FIGURE 8: LA-enriched phospholipids induce apoA-I secretion through PPARα. LA-enriched phospholipids have been shown to act through both G-protein and MAP kinase pathways. The MAP kinase pathway activates cPLA2 and PPARα to promote apoA-I synthesis and secretion.

mRNA levels were similar to those of control cells at time points up to and including a 24-h incubation (*11*). In contrast, apoA-I degradation is significantly attenuated by PI treatment (*11*). Similar observations have been made for niacin, which also has little effect on apoA-I transcription (*55*).

PPARα activation by the fibrates has been shown to increase cellular apoptosis by the inhibition of protein kinase B (PKB) phosphorylation (*47*, *48*). These authors suggested that this may partly cause the hepatotoxic side effects of the fibrate drugs. Soy PI does not have the same effects on HepG2 cells. In contrast, PI increased PKB phosphorylation in a time-dependent manner (Figure 4). The stimulation was biphasic, showing a peak at 5 min and then again at 30−60 min. Induction of the insulin signaling cascade involving PKB and ERK1/2 phosphorylation has been reported to have biphasic responses in various cell lines, including HepG2 cells (*56−58*). Early-phase activation of phosphorylation is believed to impact rapid signaling events, while later phases are associated with transactivation of other protein targets to induce signaling.

Insulin has been shown to positively impact apoA-I synthesis and secretion (*59*, *60*). In addition, high glucose increases mRNA levels for several genes that are functionally important in HDL metabolism, including human ATP-binding cassette transporter A1, apoA-I, scavenger receptor BI, and hepatic lipase (*61*). We therefore tested whether insulin and high-glucose conditions (25 mM) may impact the PI induction of apoA-I secretion. We show that increased glucose in the medium was able to significantly increase apoA-I secretion by about 3-fold, while insulin had minimal effects (Figure 3). The effect of PI on apoA-I secretion was additive to the effect of glucose. PI induction was also evident in cells treated with insulin. It is noteworthy that PI significantly increased apoA-I secretion in the hyperglycemic HepG2 cells. This may suggest that the HDL-raising efficacy of soy PI may be unaffected by the hyperglycemia of diabetic patients. These patients would significantly benefit from increased plasma HDL levels (*62*).

PI also does not impact the cytochrome P450 enzymes. The statin drugs are metabolized by the cytochrome P450 enzymes, notably CYP3A4. Several fibrate drugs have been shown to inhibit some of the CYP P450 enzymes and as such may exacerbate some of the known toxicities of the statin drugs. It is for this reason that statin−fibrate combination therapies are undertaken cautiously (*63*, *64*). While PI appeared to activate PPARα like a fibrate, PI does not inhibit any of the other major CYP P450 (1A2, 2C9, 2C19, 2D6, 3A4) or glucuronidation enzymes like the fibrate drug, gemfibrozil. PI also does not have induction effects on these enzymes (data not shown). Soy PI would therefore be considered less problematic in combination therapies with other CYP P450 metabolizing drugs.

Research shows that the LA content of PI and PC is critical for apoA-I secretory activity. The importance of LA in human nutrition is well established, but its potential utility as a phospholipid therapeutic agent is novel. LA-enriched phospholipids act through MAPK and PPARα pathways to stimulate the hepatic secretion of apoA-I, in similar fashion to other PPAR agonists (Figure 8) but with greater potency. The compounds have been shown to be safe and efficacious in human trials. LA-enriched phospholipids do not interact with cytochome P450 and other drug metabolic enzymes, and as such, these lipids would be considered less problematic for combination therapies. LA-enriched phospholipids are therefore novel PPARα therapeutic agents, which have comparable or better efficacy than the fibrate drugs but less potential for hepatic side effects.

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