Phosphatidylinositol promotes cholesterol transport and excretion

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Abstract Administration of phosphatidylinositol (PI) to New Zealand white rabbits increases HDL negative charge and stimulates reverse cholesterol transport. Intravenously administered PI (10 mg/kg) associated almost exclusively with the HDL fraction in rabbits. PI promoted an increase in the hepatic uptake of plasma free cholesterol (FC) and a 21-fold increase in the biliary secretion of plasma-derived cholesterol. PI also increased cholesterol excretion into the feces by 2.5-fold. PI directly affects cellular cholesterol metabolism. In cholesterol-loaded macrophages, PI stimulated cholesterol mass efflux to lipid-poor reconstituted HDL. PI was about half as effective as cAMP at stimulating efflux, and the effects of cAMP and PI were additive. In cultured HepG2 cells, PI-enriched HDL also enhanced FC uptake from HDL by 3-fold and decreased cellular cholesterol synthesis and esterification. PI enrichment had no effect on the selective uptake of cholesterol esters or on the internalization of HDL particles. PI-dependent metabolic events were efficiently blocked by inhibitors of protein kinase C and the inositol signaling cascade. The data suggest that HDL-PI acts via cell surface ATP binding cassette transporters and signaling pathways to regulate both cellular and intravascular cholesterol homeostasis.—Burgess, J. W., J. Boucher, T. A-M. Neville, P. Rouillard, C. Stamler, S. Zachariah, and D. L. Sparks. **Phosphatidylinositol promotes cholesterol transport and excretion.** ***J. Lipid Res.*** **2003.** 44: **1355–1363.**

Supplementary key words atherosclerosis • bile • high density lipoprotein • hypercholesterolemia • lipid clearance • reverse cholesterol transport

It has been known for over 30 years that the altered lipoprotein metabolism in many dyslipidemic states is associated with abnormally charged lipoprotein particles (1). There is now accumulating evidence that this abnormal charge may directly contribute to aberrant lipoprotein metabolism (2–5). Lipoproteins all exhibit a net negative charge, and this charge is determined by both the apolipoprotein and lipid constituents of the lipoprotein particle (6–8). The primary anionic lipid in lipoprotein particles is phosphatidylinositol (PI). While PI is a minor constituent (3–7%) of lipoprotein phospholipids (7, 9), studies suggest that it may be a critical component of chyle and an important regulator of lipoprotein secretion (10, 11). Our work has shown that HDL charge directly affects lipid metabolism by controlling interactions with interfacial enzymes (12–15) and cell surface molecules (16, 17). It is now clear that PI affects lipoprotein metabolism both by controlling interfacial interactions and uniquely regulating intracellular signaling pathways.

We have previously reported that a single intravenous injection of PI liposomes into fasted rabbits increases the net negative surface charge of HDL and almost completely inhibits lecithin:cholesterol acyltransferase (LCAT) (18). PI therefore directly acts to block the synthesis and storage of cholesteryl ester in the blood stream. In addition, PI appeared to stimulate reverse cholesterol transport (RCT) by promoting a 30-fold increase in the rate of clearance of free cholesterol (FC) from the circulation (18). Previous work has shown that infusion of lecithin liposomes can also promote cholesterol transport; however, the doses utilized to obtain the effect were about 30-fold greater than that required with PI (19–22). Large unilamellar lecithin vesicles (~120 nm) appear to promote a rapid mobilization of tissue cholesterol with consequent increases in plasma cholesterol levels (20). The liver then acts to clear the cholesterol-enriched vesicles, and it is thought that the antiatherogenic propensity of this infusate results from their ability to act as mediators of RCT from the peripheral tissues to the liver (20, 21). A reversal of experimentally induced atherosclerosis has been re-

Manuscript received 5 February 2003 and in revised form 20 March 2003.

Published, JLR Papers in Press, April 16, 2003.
DOI 10.1194/jlr.M300062-JLR200

Abbreviations: EMEM, minimum essential medium with Earle's salts; FC, free cholesterol; KBr, potassium bromide; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; PI, phosphatidylinositol; RCT, reverse cholesterol transport.

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ported in several animal systems using repeated intravenous injections of lecithin liposomes (19–22). The present study shows that PI also stimulates RCT and results in a net elimination of cholesterol from the body.

In this study, we have investigated the ability of PI to directly affect RCT in rabbits, and have evaluated the mechanism of action of PI in macrophages and HepG2 cells. We report that a single intravenous injection of PI rapidly associates, almost exclusively, with HDL particles and promotes a marked increase in the movement of cholesterol from the plasma to the feces. The mechanism of action of PI appears to involve both membrane transporters and cellular signaling pathways.

MATERIALS AND METHODS

Materials

[1α, 2α-^{3}H(N)]cholesterol was purchased from DuPont NEN (Boston, MA). 1-Palmitoyl 2-oleoyl phosphatidylcholine (POPC) and bovine liver PI were obtained from Avanti Polar Lipids (Birmingham, AL). [^{14}C]acetic acid was obtained from Perkin-Elmer. [1α,2α(n)-^{3}H]cholesteryl oleate, 1,2-dipalmitoyl L-3-phosphatidyl-[*N*-methyl-^{3}H]choline, and L-3-phosphatidyl[2-^{3}H]inositol were obtained from Amersham. Propanolol was obtained from BioMol. Cyclic adenosine monophosphate, quercetin, chelerythrine chloride, wortmannin, verapamil, thapsigargin, nitrendipine, calcium ionophore A23187, *O*-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate (D609), U73122, U73343, and most other chemicals were obtained from Sigma. Polyclonal antibodies against scavenger receptor class B, type I (SR-BI) were obtained from Novus Biologicals (Littleton, Colorado). Tissue culture reagents were obtained from either Sigma or Life Technologies, Inc. (Burlington, Ontario, Canada), and culture plasticware was obtained from Life Technologies, Inc. or Falcon (St. Laurent, Quebec, Canada). HepG2 cells were from the ATCC. Lipoproteins were prepared from the plasma of normolipidemic subjects or from rabbit plasma by ultracentrifugation as described by Rall et al. (23).

Methods

Preparation of phospholipid vesicles and phospholipid-enriched HDL. POPC and PI vesicles in PBS (1 mg/ml) were prepared by sonication as previously described (18). To prepare phospholipid-enriched HDL, the lipoprotein was incubated for 24 h at 4°C with phosphatidylcholine (PC) or PI vesicles (0.1 mg phospholipid/mg HDL protein). To label the lipoprotein, [^{3}H]cholesterol was spotted on a filter paper disk (10 μCi/mg HDL protein), dried under nitrogen, and included in the phospholipid-HDL incubation. An alteration in HDL surface charge following these incubations was determined by electrophoresis on precast 0.5% agarose gels (Beckman, Paragon Lipo Kit).

Preparation of reconstituted spherical HDL particles by sonication. Purified apolipoprotein A-I (apoA-I) was isolated by size-exclusion chromatography and, prior to use, was resolubilized in 6 M guanidine hydrochloride and 10 mM Tris, pH 7.2, and dialyzed extensively against PBS. Reconstituted HDL particles were prepared by sonication of a fixed ratio of lipid-apoA-I. Specific amounts of lipids in chloroform were dried under nitrogen, 800 μl PBS was added, and the lipid-buffer mixture was successively sonicated with a Branson Sonifier 450 under nitrogen for 1 min at constant output, incubated at 37°C for 30 min, and sonicated again for 5 min at 95% duty cycle under nitrogen. ApoA-I (1.4 mg/ml) was added and the mixture was sonicated for 4 × 1 min at 90% duty cycle under nitrogen, with 1 min cooling periods between sonications. The particles were then filter sterilized.

Animal studies. To examine the plasma clearance of phospholipids, 54 μmol of POPC vesicles containing 100 μCi of [^{3}H]PC or 54 μmol of PI vesicles containing 50 μCi of [^{3}H]PI was injected via the marginal ear vein into fasted male New Zealand white (NZW) rabbits (weighing 3.5–4.0 kg). The total volumes injected were 3 ml per animal. Blood samples were drawn, and 0.4 ml of plasma was used for lipoprotein fractionation at each time point. Briefly, 0.155 ml of 1.21 g/ml potassium bromide (KBr) was added to adjust the density to 1.063 g/ml. Sufficient 1.063 g/ml density solution was added to give a final 5 ml volume. The mix was subjected to ultracentrifugation at 100,000 rpm for 5 h to yield a combined LDL and VLDL fraction and an HDL fraction. The distribution of radiolabeled phospholipids in the lipoprotein fractions was then determined by scintillation counting.

The effects of phospholipid vesicle injections on cholesterol accumulation in liver and bile were determined as follows. NZW rabbits (n = 3) were injected via the marginal ear vein with 40 μmol of PI or PC vesicles containing 250 μCi [^{3}H]cholesterol. At 20 min postinjection, the animals were anaesthetized, and at 35 min postinjection they received a lethal injection to arrest blood flow. The body cavity was opened and the cystic duct and common bile duct were ligated. The liver was then removed, washed in PBS, weighed, and bile was aspirated from the gall bladder. Radioactivity associated with bile was measured by direct β counter analysis in Eco-Lite. Liver values were determined based on the Bligh and Dyer extraction of homogenized tissues (24). The effects of injected phospholipids on fecal cholesterol and bile acid levels were determined in similar studies. Rabbits were injected with 40 μmol of PI or PC vesicles, and feces were collected every 24 h for 7 days. These samples were lyophilized and the dry weights determined. Fecal samples were extracted in triplicate by the method of De Wael et al. (25), and fecal cholesterol (Boehringer Mannheim Enzymatic kit) and bile acid (Sigma Enzymatic kit) assays were measured in duplicate.

Cholesterol efflux. J774 macrophages were maintained and seeded into 12-well plates (26), and cholesterol efflux from the macrophages was determined as previously described (27).

Cholesterol uptake in HepG2 cells. HepG2 cells were cultured in minimum essential medium with Earle's salts (EMEM) growth medium containing 10 mM HEPES, 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Confluent cultures were split 1:10 into 24-well plates and grown to 90–95% confluency for individual experiments. HepG2 cells were washed twice with warm EMEM-BSA and then incubated in the same medium containing 50 μg of PI- or PC-enriched and [^{3}H]cholesterol-labeled HDL prepared as described above. When included, inhibitors of phospholipase activity and protein kinase C or calcium channels were included at this step and at concentrations described in **Table 1**. The cells were incubated at 37°C, and at the appropriate time, were allowed to chill on ice. The cells were then washed twice with cold PBS-BSA and then twice with ice cold PBS. The PBS was replaced with 1 ml 0.2 N NaOH, and the cells were solubilized overnight on a tilting shaker. The cell lysates were counted for tritium and analyzed for protein content by the BCA assay (Pierce).

Cholesterol synthesis and esterification in HepG2 cells. HepG2 cells were grown to 60–70% confluency in 6-well plates. The cells were washed twice with warm EMEM-BSA and then incubated at 37°C with EMEM-BSA (2 ml/well) containing ^{14}C-labeled acetate (0.3 μCi/ml) and HDL (25 μg HDL/ml) that was enriched in either PC or PI as described above. After 4 h, the cells were chilled on ice, the medium discarded, and the cell layers washed twice with

TABLE 1. The effects of chemical inhibitors on PI-mediated cholesterol uptake

Inhibitor	Concentration	PI-Dependent Cholesterol Uptake (% Control)
EMEM-BSA		100
Glyburide	100 μM	6.0 ± 1.7
Vanadate	2.7 mM	0 ± 19.0
U73122	50 μM	29.2 ± 11.8
D609	50 μM	121 ± 29
Propanolol	200 μM	36.1 ± 9.6
Quercetin	10 μM	31 ± 16
Wortmannin	100 nM	40.8 ± 29
Chelerythrine chloride	10 μM	3.1 ± 5.3
Verapamil	100 μM	17.4 ± 8.7
Thapsigargin	50 μM	26.9 ± 0.1
Nitrendipine	100 μM	15.3 ± 4.7

EMEM, minimum essential medium with Earle's salts; PI, phosphatidylinositol. HepG2 cell monolayers were incubated at 37°C with EMEM-BSA containing phosphatidylcholine (PC)- or PI-enriched [^{3}H]cholesterol-labeled HDL. Inhibitors were included in the incubations at the indicated concentrations. After 4 h, the cells were chilled on ice, washed, and then solubilized overnight in 0.2 N NaOH. The data are expressed as a percentage of the difference between PI-derived uptake and PC-derived uptake, with 100% representing incubations without inhibitors. All data represent the mean and SD of at least three determinations.

cold PBS-BSA and twice with cold PBS. The cells were then solubilized with 0.2 N NaOH (1 ml/well) for 48 h at 4°C. Seven hundred fifty microliters of the cell suspension was subjected to the Bligh and Dyer extraction (24) and analyzed by thin layer chromatography on ITLC™ SG plates using a solvent system composed of hexane-diethylether-acetic acid, 90:10:1 (v/v/v). The areas of the plates containing cholesterol and cholesterol ester were scraped into vials, and radioactivity was determined by scintillation counting.

RESULTS

We have previously reported that intravenous injections of the anionic phospholipid, PI, into NZW rabbits promoted marked changes in plasma cholesterol metabolism and an accelerated flux of cholesterol from the plasma compartment (18). To examine the fate of intravenously administered PI in rabbits, radiolabeled PI was administered and blood samples were collected at various times for the preparation of HDL and combined VLDL+LDL fractions by KBr density gradient ultracentrifugation. Analysis of plasma samples showed that greater than 90% of the radioactive label remained with the organic phase of a Bligh and Dyer extraction, indicating that the radioactive PI remained as a lipid and was not degraded intravascularly over the time course. **Figure 1A** demonstrates the clearance of labeled PI and PC from the circulation as a function of time. PI is cleared more rapidly than PC and is essentially gone by 7 h postinjection. In contrast, about 12% of the injected dose of PC remains at 8 h, and 6% at 12 h. Analysis of the plasma lipoproteins shows that [^{3}H]PI associated almost exclusively with the HDL fraction (93% at 5 min and 82% after 12 h; Fig. 1B). This is in agreement with our previous studies that demonstrated

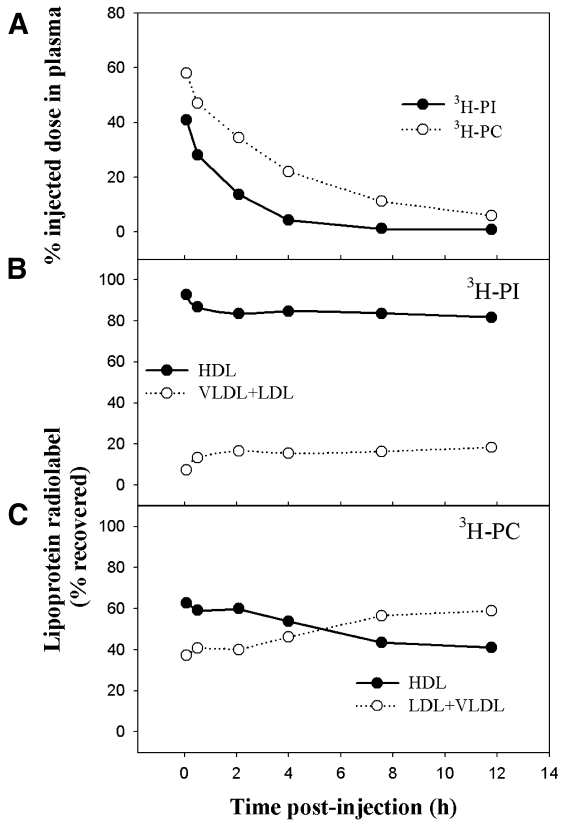

Fig. 1. Intravenous phosphatidylinositol (PI) localizes predominantly with the HDL fraction. Male New Zealand white (NZW) rabbits (n = 2) weighing ~4 kg were injected via the marginal ear vein with 54 μmol of PI vesicles containing 50 μCi of [^{3}H]PI or 54 μmol of 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) vesicles containing 100 μCi of [^{3}H]phosphatidylcholine (PC). Blood was sampled at the indicated times, red blood cells were separated, and plasma radioactivity was determined. Clearance curves (A) illustrate the percent of initial dose at the times indicated. VLDL+LDL were separated from HDL fractions by density centrifugation, and the relative association of [^{3}H]PI and [^{3}H]PC with the different lipoprotein pools is shown in B and C, respectively. Values are the average of duplicate determinations.

increased PI mass in HDL fractions by TLC (18). HDL PI was cleared rapidly ($t_{1/2}$ ~1 h) and reached baseline levels by 8 h postinjection. Intravenous PC distributed less with HDL at early time points (63% at 5 min) and more with the other lipoprotein classes (Fig. 1C). HDL-associated PC was cleared from the circulation slowly, relative to PI, with a $t_{1/2}$ ~4 h. Substantial amounts of labeled PC were found associated with the combined VLDL and LDL fractions throughout the time course and were cleared very slowly from the circulation with a $t_{1/2}$ ~10 h.

To determine whether the enhanced cholesterol clearance effects of PI could be the result of increased hepatic uptake and biliary output of cholesterol, male NZW rabbits were injected with 1 mg POPC vesicles containing 300 μCi [^{3}H]cholesterol, and then 5 min later with a second injection containing 40 μmol of PI or PC. After 30 min, the animals were euthanized and liver and bile samples obtained and analyzed for the content of radiolabel. After 30 min, 52.1% and 31% of the injected dose had accumu-

lated in the livers of PI- and PC-injected rabbits, respectively. The accumulation of radiolabeled cholesterol in liver and bile is shown in **Fig. 2**. In liver tissues, there was a 1.5-fold increase in labeled cholesterol in PI-injected animals compared with PC-injected controls. Analysis of bile samples demonstrated a 21-fold increase in radiolabeled cholesterol content in PI-injected animals versus controls. In a second study, HDL was PI-enriched and cholesterol-labeled in vitro and then administered intravenously to NZW rabbits. In this study, we observed an 8.7-fold increase in biliary [^{3}H]cholesterol when compared with PC control animals (Burgess and Sparks, unpublished observations).

To determine if enhanced biliary sterol output may affect fecal excretion, fecal cholesterol and bile acid levels were determined at 24 h intervals for 5 days after a single bolus injection of PI- or PC-vesicles. PI injection increased fecal cholesterol output significantly ($P \leqslant 0.001$) and maximally at 48 h postinjection, representing a 2.5-fold increase over control injections with PC (**Fig. 3**, upper panel). Fecal cholesterol levels remained elevated on Day 3 (1.6-fold over PC-injected animals), and returned to control levels by Day 4. In contrast, control animals receiving either injections of PC (Fig. 3) or PBS (data not shown) did not demonstrate any alterations in fecal cholesterol output over the 5 day study. Injections of PI- or PC-vesicles had no significant effect on the output of fecal bile acids (Fig. 3, lower panel).

Our previous study showed that PI caused a markedly enhanced cholesterol clearance from the blood, but did not significantly reduce the amount of cholesterol in the plasma over a 24 h period (18). This suggests that PI may stimulate a flux, comprising an enhanced mobilization or efflux of cholesterol into the blood stream and a parallel clearance of cholesterol from this pool. To determine if HDL-PI content directly affects cholesterol efflux pathways, we examined the effect of PI on cholesterol efflux from cholesterol-loaded macrophages. Reconstituted HDL particles deficient in cholesterol were utilized to prevent cholesterol exchange and were prepared to contain 100

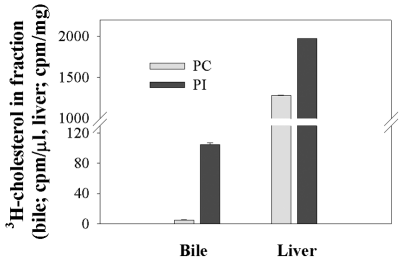

Fig. 2. PI stimulates hepatic uptake and output of free cholesterol into the bile. Male NZW rabbits weighing ~4 kg were injected with 1 mg POPC vesicles containing 300 μCi [^{3}H]cholesterol. This was followed 5 min later by a second injection containing 40 μmol of PI (black bars, n = 2 animals) or PC (gray bars, n = 2 animals). After 30 min, liver and bile were harvested and radioactivity determined as described in Materials and Methods. The data are the mean ± SE of triplicate determinations for each animal.

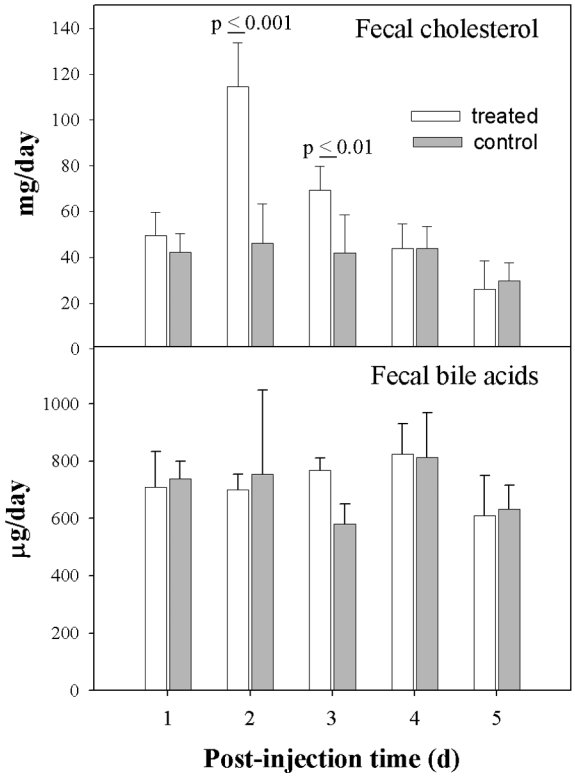

Fig. 3. PI enhances the fecal output of cholesterol. Male NZW rabbits weighing ~4 kg were injected with 40 μmol of PI (gray bars; n = 6 animals) or PC (white bars, n = 6 animals). Fecal cholesterol and bile acid levels were determined at 24 h intervals for 5 days as described in Materials and Methods.

mol of PC per two molecules of apoA-I and an additional 20 mol of PI or PC. These complexes were then incubated with cholesterol-loaded macrophages, and the efflux of radioactive cholesterol from the cells was measured, with or without pretreatment with cAMP. **Figure 4** shows that PI stimulated cholesterol efflux in a similar manner to that shown for cAMP, to a lesser extent, however. The lipid was only about half as effective as the nucleotide. However, the PI-stimulatory effect on cholesterol efflux was also evident after cAMP pretreatment and appeared greater in magnitude (>2-fold) than in the absence of cAMP. PI-dependent cellular effects were shown to be completely blocked by ABC transporter inhibitors glyburide and vanadate (Table 1).

To determine how PI may affect sterol uptake and transport pathways in the liver, we also examined the effect of PI on the uptake of HDL cholesterol and cholesterol ester by hepatic cell line HepG2. In these studies, we tracked the effect of PI on the transfer of [^{3}H]cholesterol or [^{3}H]cholesteryl ester into HepG2 cells. **Figure 5A** demonstrates that PI-enriched HDL transferred ~3-fold more cholesterol to HepG2 cells than did PC-enriched HDL. PI-enrichment had no effect on cholesterol uptake from LDL or VLDL (data not shown). The enrichment of HDL with PI also had no effect on the uptake of cholesteryl ester by HepG2 cells when compared with PC-enriched HDL (Fig. 5B). This may suggest that CLA-1, the human

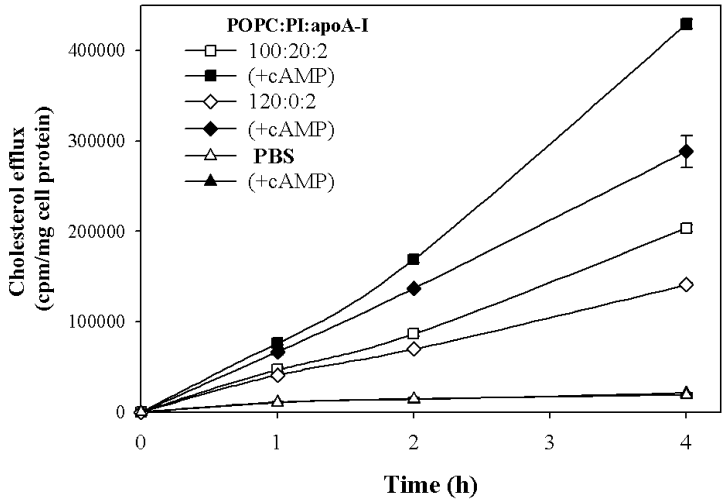

Fig. 4. PI enrichment of reconstituted HDL increases cholesterol efflux from cholesterol-loaded J774 macrophages. J774 macrophages were loaded with cholesterol by incubation with [^{3}H]cholesterol-labeled acetylated LDL for 48 h. After the loading period, the cells were washed and then pretreated with 0.15 mM cAMP for 10 h (solid symbols) or with media alone (open symbols). The cells were then incubated with different reconstituted HDL particles [POPC-PI-apoA-I; 100:20:2, v/v/v (closed square) and 120:0:2, v/v/v (closed diamond)] for the indicated times. The media was then removed and analyzed for radioactivity. All data are the mean and SE of at least three separate determinations.

homolog of SR-BI, is not involved in PI-mediated cholesterol uptake. In agreement with this view, PI-dependent cholesterol uptake in HepG2 cells was unaffected by inclusion of an anti-SR-BI antibody that inhibits both SR-BI and CLA1 uptake activities (data not shown). In addition, PI was also able to stimulate cholesterol uptake from HDL to ldlA7 CHO cells deficient in SR-BI (data not shown). We also examined the effect of PI enrichment on the cell association of ^{125}I-labeled HDL (Fig. 5C). PI enrichment of HDL resulted in a small decrease of about 20% in the cell association of HDL at all tested time points. This suggests that the increased cholesterol uptake in response to PI enrichment was not the result of particle internalization and metabolism.

PI-mediated cholesterol uptake from HDL was dramatically reduced by the inclusion of U73122, an inhibitor of PI-dependent phospholipase C, and propanolol, an inhibitor of phosphatidic acid phosphohydrolase, into the assay mix (Table 1). In contrast, D609, a reported PC-phospholipase C (PLC) inhibitor, did not inhibit the reaction. We also tested inhibitors of the inositol kinases and protein kinase C, quercetin, and wortmannin, which inhibit PI-3 kinase activity and reduced cholesterol uptake in response to PI by 70% and 65%, respectively. The protein kinase C inhibitor chelerythrine chloride fully blocked PI-mediated cholesterol uptake. Conversely, cholesterol uptake was shown to be stimulated, to much the same extent as PI, by the PKC agonist, dioctanoyl glyceride, a short-chain diglyceride (data not shown). The inositol signaling path is reported to alter intracellular calcium metabolism through the release of calcium from the endoplasmic reticulum. Table 1 also demonstrates that thapsigargin, which promotes IP3-independent release of calcium, also inhibited PI-mediated cholesterol uptake, as did the calcium channel blockers verapamil and nitrendipine. The results suggest that the cholesterol metabolic effects of PI are probably mediated through the actions of specific phospholipases, PKC activation, and inositol phosphate-induced fluxes in intracellular calcium levels.

To determine if the ability of PI to promote the excretion of cholesterol in rabbits may also be linked to an inhibition of cholesterol synthesis or storage, we examined the effects of PI on cholesterol synthetic pathways in HepG2 cells. In these studies the incorporation of ^{14}C-labeled acetate into cholesterol or cholesterol ester was determined in the absence or presence of PI-enriched HDL. Inclusion of 50 μg of HDL in the medium prompted a 2-fold increase in the incorporation of acetate into cholesterol when compared with incubations performed with EMEM-BSA alone (**Fig. 6**, upper panel). In contrast, incubations performed with PI-enriched HDL demonstrated only background levels of cholesterol synthesis. Similarly, comparison of the FC-to-cholesterol ester ratios demonstrated a 30% increase in cholesterol esterification with the addition of HDL, which again was blocked with enrichment of HDL with PI (Fig. 6, lower panel).

DISCUSSION

This study confirms the view that lipoprotein metabolism and RCT can be selectively manipulated by the administration of the anionic phospholipid, PI. We previously reported that intravenous injections of PI into rabbits produced a significant but transient increase in the net negative surface charge of the plasma lipoproteins (18). We now show that intravenously administered PI associates almost exclusively with the HDL fraction and is cleared more rapidly from the circulation than HDL-PC. This is apparently in contrast to previous studies that suggested that PI may be metabolized more slowly than PC (10). However, we also found no evidence of [^{3}H]PI degradative products in the plasma compartment, which agrees with this earlier study and suggests that PI is probably not degraded in the blood stream, but is predominantly metabolized intracellularly. Indeed, our cell culture studies show that exogenous PI rapidly turns on intracellular signaling pathways. Therefore, PI performs two potentially related functions in HDL particles: that of a surface charge modulator and that of a cell-signaling stimulant.

Intravenous injections of the vesicles composed of the anionic phospholipid PI into rabbits also caused a profound increase in the clearance of FC from plasma and

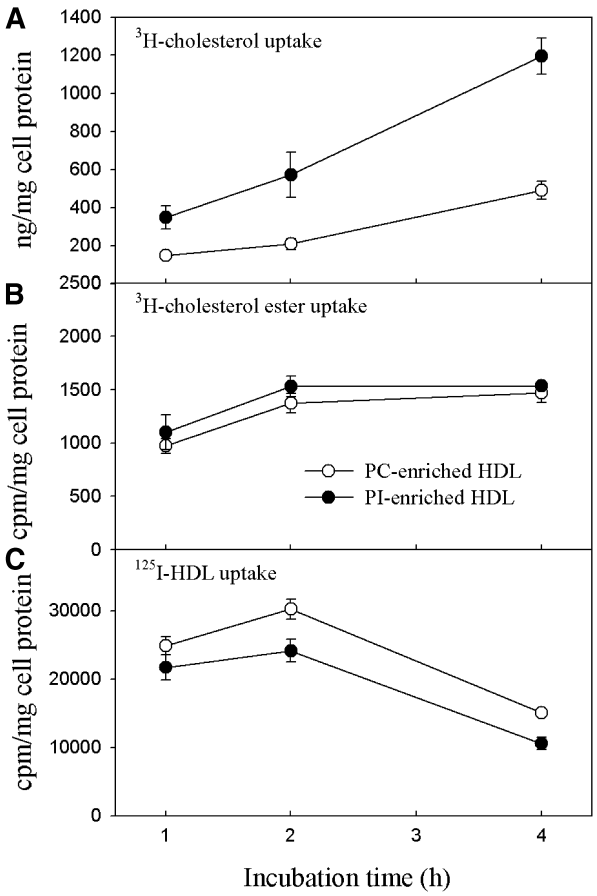

Fig. 5. PI increases cholesterol uptake from HDL in HepG2 cells but has no effect on cholesterol ester or HDL uptake. HepG2 cells were grown to 90–95 confluence in 24-well plates. The cells were washed twice with warm minimum essential medium with Earle's salts (EMEM)-BSA and then incubated with EMEM-BSA containing [^{3}H]cholesterol-labeled HDL (A), [^{3}H]cholesterol oleate-labeled HDL (B), or ^{125}I-labeled HDL (C). The cells were incubated for the indicated times at 37°C and then chilled on ice. The cells were washed sequentially with cold PBS-BSA and cold PBS, and then solubilized overnight with 0.2 N NaOH before determining the cell-associated radioactivity. All data are the mean and SE of at least three separate determinations.

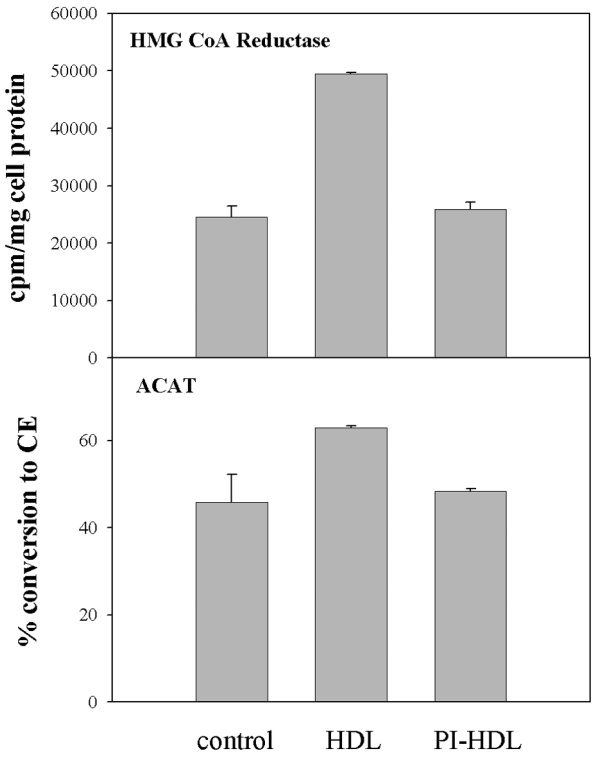

Fig. 6. Enrichment with PI eliminates stimulation of cholesterol synthesis and esterification by HDL. HepG2 cells, grown to 60–65% confluence, were washed twice with EMEM-BSA and then incubated at 37°C with EMEM-BSA containing ^{14}C-labeled acetate (0.3 μCi/ml) and HDL (25 μg HDL/ml) that was enriched in either PC or PI as described above. After 4 h, the cells were chilled on ice, washed sequentially with cold PBS-BSA and cold PBS, and then solubilized with 0.2 N NaOH for 48 h at 4°C. The cell suspensions were extracted by the Bligh and Dyer method (24) and then analyzed by thin layer chromatography on ITLC™ SG plates using a solvent system composed of hexane-diethylether-acetic acid, 90:10:1 (v/v/v). The areas of the plates containing cholesterol and cholesterol ester were scraped into scintillation vials, and counted in a β counter. All data are the mean and SE of at least three separate determinations.

suggested that PI may be stimulating RCT (18). This has been confirmed in the present study. Bolus injections of PI promoted an increase in the hepatic uptake of plasma cholesterol and a major increase in the biliary secretion of the plasma-derived cholesterol. Evidence suggests that this RCT excretion pathway is stimulated by a PI-enriched HDL fraction. As stated, we have shown that the majority of the injected PI associates with the HDL pool and directly affects the enzymes (LCAT) and cell surface events that regulate the metabolism of HDL cholesterol. Intravenous injection of PI-enriched HDL was shown to promote a significant increase in hepatic uptake and biliary secretion of [^{3}H]cholesterol similar to that observed with injection of PI vesicles. Cell culture studies showed that PI is only able to stimulate cholesterol uptake from HDL, and not from LDL or VLDL. This work, therefore, appears consistent with previous reports that have shown that HDL cholesterol is the primary source of biliary cholesterol (28). PI administration appears to stimulate RCT to such a magnitude that significant amounts of cholesterol are excreted from the body in the feces. This is similar to that observed after infusions of apoA-I (29). Infusions of pro-apoA-I liposomes into humans also promoted a net cholesterol excretion from the body (29); however, the magnitude of the increase stimulated by the apoA-I infusate was much smaller (~40% increase) than that observed in this study (>250% increase). Since PI modifies the whole HDL pool in vivo, this may suggest that PI harnesses the cholesterol mobilizing and transport capacity of the entire HDL pool. This may prove of therapeutic value as previous work has shown that stimulating RCT with infusions of HDL into atherosclerotic rabbits can directly induce regression of preestablished aortic fatty streaks and lipid deposits (30).

RCT is thought to comprise both an initial efflux step, in which cholesterol is liberated from peripheral cell membranes by HDL, and a final clearance step, in which

the sterol is taken up and metabolized in the liver. The role of HDL in promoting cholesterol efflux is now established and thought to be linked to the actions of the scavenger receptor, SR-BI, and the membrane transporter, ABCA-1 (31). PI enrichment of reconstituted HDL significantly stimulates the efflux of cholesterol from cholesterol-loaded macrophages. Pretreatment of macrophages with cAMP has been previously shown to upregulate expression of ABCA-1 and stimulate cholesterol efflux (32). In the present study, we show that PI also increases cholesterol efflux and that this effect is additive with cAMP stimulation. In other experiments we have found that inclusion of glyburide, an inhibitor of ABCA-1 activity, promoted a marked inhibition of PI-mediated cholesterol uptake into HepG2 cells, suggesting that PI may act by altering either the expression or activity of the transporter. PI also increased cholesterol efflux when poorly lipidated apoA-I particles (5 PC or PI/apoA-I) were used as cholesterol acceptors; however, efflux rates were almost 50% that shown for the Lp2A-I (120:2) reconstituted HDL (data not shown). This is also consistent with previous reports and shows that efflux is sensitive to the amount of phospholipid in acceptor particles (33–35). These results suggest that PI regulates the actions and/or expression of ABCA-1, and the observations with glyburide appear to support this view.

Previous reports by others have identified changes in the intracellular metabolism of inositol in response to the exogenous addition of PI (50, 51). This appears consistent with our results in hepatocytes, which suggest that HDL-PI is metabolized by a PI-specific PLC to produce the PKC activators, diacylglyceride, and inositol phosphate (Table 1). Activation of the inositol cascade may result from an increased concentration of intracellular PI and increased substrate for the actions of PI-specific phospholipase C. In this regard, PI-mediated cholesterol uptake was almost fully inhibited by U73122, an inhibitor of PI-specific phospholipase C (36–38), but was unaffected by D609, a PC-specific phospholipase C inhibitor (39, 40). Cholesterol uptake was also shown to be stimulated by PKC agonists, and PI-dependent cholesterol uptake was fully inhibited by PKC inhibitors, such a chelerythrine chloride, quercetin, and wortmannin, inhibitors of inositol kinase activity (41–45), and verapamil, thapsigargin, and nitrendipine, which affect calcium channel function (46–49); all efficiently inhibited cholesterol uptake in response to PI. This indicates that PI promotes its actions through inositol signaling and by alterations in intracellular calcium levels. It has also been reported previously that the addition of PI liposomes to the extracellular medium of fetal lung fibroblasts increases calcium uptake into these cells (52).

In addition to its effect on cholesterol uptake by cells, PI-mediated signal transduction also directly inhibited the synthesis of cholesterol in HepG2 cells (Fig. 6). In agreement with the findings of others, the addition of HDL to cultured cells promoted an increase in cholesterol synthesis (53, 54); however, this stimulatory effect of HDL was fully inhibited by the enrichment of HDL with PI. Calcium mobilization from the ER has been reported to promote the catabolism of HMG-CoA reductase (55–58), and therefore PI may decrease cholesterol synthesis in hepatocytes by modulating intracellular calcium levels. Alternatively, increased cholesterol influx into the cells could directly decrease the activity of HMG-CoA reductase at a gene-regulatory level (59–61). PI-enriched HDL also decreased the intracellular CE-to-cholesterol ratio, but whether this effect is the result of a direct inhibition of ACAT activity in cells or simply the result of decreased cholesterol synthesis is not known.

In summary, our studies indicate that the administration of the anionic lipid PI promotes effects on both intravascular cholesterol storage and RCT. PI effectively inhibits cholesteryl ester production by LCAT and its storage in the blood stream. PI simultaneously stimulates an RCT pathway that results in the net excretion of cholesterol from the body. Therefore, it appears that the actions of LCAT can determine whether cholesterol is stored in the blood stream or excreted from the body. The mechanism of PI action, derived from studies with cultured cells, appears to involve both the efflux and the clearance of cholesterol from HDL particles through processes that involve membrane transporters, PI, and PKC signaling pathways, and alterations in cellular calcium levels. PI, therefore, acts through a regulation of lipoprotein charge and cell signaling pathways to alter both vascular and cellular cholesterol homeostasis.

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