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Hepatic uptake and metabolism of phosphatidylcholine associated with high density lipoproteins

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ABSTRACT

Background: Phosphatidylcholine (PC) is the predominant phospholipid associated with high density lipoproteins (HDL). Although the hepatic uptake of cholesteryl esters from HDL is well characterized, much less is known about the fate of PC associated with HDL. Thus, we investigated the uptake and subsequent metabolism of HDL-PC in primary mouse hepatocytes.
Methods and results: The absence of scavenger receptor-BI resulted in a 30% decrease in cellular incorporation of [^{3}H]PC whereas [^{3}H]cholesteryl ether uptake was almost completely abolished. Although endocytosis is not involved in the uptake of cholesteryl esters from HDL, we demonstrate that HDL internalization accounts for 40% of HDL-PC uptake. Extracellular remodeling of HDL by secretory phospholipase A_2 significantly enhances HDL lipid uptake. HDL-PC taken up by hepatocytes is partially converted to triacylglycerols via PC-phospholipase C-mediated hydrolysis of PC and incorporation of diacylglycerol into triacylglcyerol. The formation of triacylglcerol is independent of scavenger receptor-BI and occurs in extralysosomal compartments.
Conclusions and general significance: These findings indicate that HDL-associated PC is incorporated into primary hepatocytes via a pathway that differs significantly from that of HDL-cholesteryl ester, and shows that HDL-PC is more than a framework molecule, as evidenced by its partial conversion to hepatic triacylglycerol.

1. Introduction

Plasma high density lipoprotein (HDL) levels are inversely correlated to the risk of atherosclerosis and cardiovascular disease [1]. The anti-atherogenic properties of HDLs include a key role in reverse cholesterol transport [2], a process by which excess cholesterol is transported from the periphery back to the liver for disposal. HDL also has anti-inflammatory and anticoagulant effects, as well as an established anti-oxidant action [3]. The lipid moiety of HDL is predominantly composed of cholesteryl esters (CE) (24–45%) and phospholipids (42–51%), the majority of which is PC [4]. When HDL reaches the liver, its interaction with the plasma membrane is essential for the delivery of lipid molecules to the cells. Lipid transfer from lipoproteins to intracellular sites involves a number of possible pathways. First, lipid molecules might be transferred to the plasma membrane. This event is unlikely to occur for the hydrophobic CE [5]. However cholesterol from HDL can enter the plasma membrane pool very rapidly, possibly after desorption from HDL [6]. Endocytosis of HDL particles by hepatic cells has been reported [7,8]. Although endocytosis does not contribute to HDL cholesterol uptake [9], the significance of receptor mediated endocytosis for the cellular incorporation of other lipids associated with HDL has yet to be described.

Another well characterized HDL lipid uptake mechanism is the selective CE uptake pathway [10]. The scavenger receptor class B, type I (SR-BI), known as the HDL receptor, is largely expressed in hepatocytes where it mediates the selective uptake of HDL lipids [i.e. the transfer of lipids without significant degradation of the whole particle [11–13]]. SR-BI-mediated selective lipid uptake is believed to facilitate the excretion of excess cholesterol into bile [14]. Indeed, SR-BI expression in mice is highly correlated with the magnitude of HDL-derived cholesterol secretion into bile [14,15]. Furthermore, even if SR-BI$^{-/-}$ mice have increased plasma HDL levels, they are highly susceptible to developing atherosclerosis [15–18]. CE uptake has been suggested to occur at the cell surface, with SR-BI facilitating the creation of a hydrophobic channel favorable to the trans-bilayer movement of CE.

Abbreviations: ATK, arachidonyl trifluoromethyl ketone; CE, cholesteryl ester; CEt, cholesteryl ether; CETP, cholesterol ester transfer protein; DGAT, diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoprotein; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; LDLr, low density lipoprotein receptor; LPL, lipoprotein lipase; PBS, phosphate buffered saline; PC, phosphatidylcholine; PLA_2, phospholipase A_2; PLC, phospholipase C; SR-BI, scavenger receptor class B type I; TG, triacylglycerol

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This model was proposed to accommodate the low activation energy required for the selective cellular transfer of HDL lipids [19–21]. In addition to CE, SR-BI participates in the translocation of other lipophilic substrates [22], including phospholipids [21] and α-tocopherol [23]. SR-BI can directly interact with phospholipids of the donor particles, independently of the presence of apolipoproteins and SR-BI appears to have different binding sites for apolipoproteins and phospholipids [21]. A specific role for SR-BI in the uptake of lipoprotein-associated PC has been described in various cell types [21,24,25]. However, much less is known about its involvement in hepatic metabolism of HDL-PC.

In addition to facilitating lipid transfer, there is evidence that entire HDL particles can be internalized via SR-BI. Indeed, Silver et al. [26] have provided confirmation that SR-BI participates in intracellular cholesterol trafficking. After HDL binds at the plasma membrane, SR-BI and the lipoprotein can be both internalized with redistribution of CE to the endosomal compartment and to the canalicular membrane of polarized hepatocytes [27]. In contrast to low density lipoprotein (LDL) uptake via classical receptor-mediated endocytosis [28], the internalization of HDL by SR-BI does not result in the delivery of HDL content to lysosomes [29,30]. Lipid-depleted apoA1 appears to be recycled back to the plasma membrane, together with SR-BI [26,31].

Another pathway has been described for HDL internalization by hepatic cells, involving the β subunit of the F_1-ATPase complex, ectopically expressed at the plasma membrane of hepatocyte [8,32]. Cell surface ATP hydrolase activity of the β-chain can be stimulated by the binding of apoA1, generating ADP which then activates the G-protein coupled receptor $P2Y_{13}$ [33]. This pathway is specific for HDL and is thought to contribute to approximately 50% of total hepatic HDL endocytosis in livers of rodents [33,34]. Interestingly, inhibition of SR-BI does not affect the basal or the ADP-enhanced endocytosis of HDL [35]. The significance of the ATP synthase β-chain pathway for uptake of HDL-PC by hepatocytes remains to be established.

Other factors that are potentially involved in hepatic HDL metabolism include the extracellular lipolysis of HDL lipids [36,37]. Lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), phospholipid transfer protein, and several lipases such as lipoprotein lipase (LPL), endothelial lipase, hepatic lipase (HL) and secretory phospholipase A_2 (PLA_2) have been implicated in the regulation of plasma HDL levels. LCAT, synthesized and secreted by the liver, mediates the transfer of a fatty acid from PC to cholesterol to form CE [38]. CETP, expressed in humans but not in mice, catalyzes the transfer and exchange of neutral lipid between lipoproteins whereas phospholipid transfer protein mediates HDL remodeling by facilitating the exchange of phospholipids [39]. LPL hydrolyzes lipoprotein-associated TG to supply surrounding tissues with fatty acids [40]. Because of its high phospholipase activity, endothelial lipase has been suggested to play a crucial role in HDL metabolism by hydrolyzing HDL-associated phospholipids [37]. HL, synthesized and secreted primarily by hepatocytes, also has significant phospholipase A_1 activity. HL has been proposed to regulate the selective uptake of HDL lipids *in vivo* [41–43] as evidenced by increased plasma HDL levels in HL-deficient mice [44]. The catalytic activity of HL contributes to the remodeling of HDL [45], potentially accelerating the clearance of HDL by making the particle a better substrate for hepatic receptors. In addition, the non-lipolytic properties of HL also appear to be important, as it "bridges" lipoproteins and components of the plasma membrane [46]. Finally, secreted PLA_2, an acute-phase protein, can significantly accelerate HDL metabolism: *in vitro* studies with Chinese hamster ovary cells expressing SR-BI have shown that HDL remodeling by $sPLA_2$ generates a particle that is twice as efficient for HDL lipid uptake [47].

Although the importance of HDL for cellular cholesterol homeostasis is well documented, much less is known about its relevance for hepatic phospholipid metabolism [21]. Phosphatidylcholine (PC) is the major phospholipid component of all plasma lipoproteins and, in addition to its role in maintaining the structure of the particle, PC participates in diverse cellular pathways. For example, lipoprotein-derived PC represents a significant source of polyunsaturated fatty acids for eicosanoid production in the brain [24]. More recently, the hepatic fate of LDL-associated PC was examined in our laboratory and it was demonstrated that LDL-PC can serve as a precursor for TG synthesis via a metabolic pathway that involves hydrolysis of PC by PC-PLC and further processing by DGAT2 [48]. The intracellular fate of HDL-associated PC is largely unknown. Therefore, we have investigated the mechanism(s) by which PC is taken up by the liver and the subsequent metabolic fate of HDL-associated PC in primary hepatocytes.

2. Experimental procedures

2.1. Materials

HDL ($d = 1.07–1.21$ g/ml) was obtained by ultracentrifugation of pooled plasma of healthy male and female volunteers [49]. Lab rodent chow diet #5001 was from PMI Nutrition International (St-Louis, MO). Collagen-coated dishes for hepatocyte culture were from BD Biosciences (Mississauga, ON). [^{3}H]choline chloride, [^{14}C]choline chloride, [^{3}H]oleate, [^{3}H]palmitate and the enhanced chemiluminescence detection system for immunoblotting were from Amersham Biosciences (Buckinghamshire, UK). [^{3}H]Cholesteryl oleoyl ether was purchased from Perkin Elmer (Massachusetts, USA). Thiomerosal, chloroquine, monensin, E600 (diethyl p-nitrophenyl phosphate), Western Diamondback rattlesnake venom extract, DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), Orlistat (tetrahydrolipstatin), deoxyglucose, colchicine and collagenase (*C. histolyticum* type IV) for liver perfusion were purchased from Sigma (Oakville, ON). Silica gel G60 plates for thin-layer chromatography (TLC Silica Gel 60) were from Merck (Darmstadt, Germany). The Bio-Rad protein assay reagent was from Bio-Rad Laboratories (Hercules, CA). The cell surface protein labeling kit (biotinylation kit) and secondary antibodies conjugated to horseradish peroxidase (goat anti-rabbit and rabbit anti-goat IgG) were obtained from Pierce (Rockford, IL). Goat polyclonal anti-SR-BI (NB 400-131) and rabbit polyclonal SR-BI blocking antibody (NB 400-113) were purchased from Novus Biologicals (Littleton, CO). Rabbit polyclonal anti-calnexin antibody was from StressGen (Victoria, BC). Protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). Arachidonyl trifluoromethyl ketone (ATK) and D609 (O-tricyclodec-9-yl dithiocarbonate potassium salt) were obtained from BioMol Research Laboratory (Plymouth Meeting, PA). Poloxamer 407 (Pluronic F-127) was from BASF (Mississauga, ONT). Me-Indoxam was acquired from Dr. M.H. Gelb (University of Washington, Seattle, WA) and the polyclonal rabbit anti-LDL receptor antibody was a gift from Dr. G. Ness (University of South Florida, Tampa, FL). The anti-ATP synthase β subunit antibody (anti-OxPhos Complex V subunit β, Mouse IgG_1, monoclonal 3D5), Prolong Antifade Reagent, Alexa-Fluor 488 transferrin conjugate and the secondary antibodies for immunofluorescence (Alexa-Fluor 488 goat anti-rabbit and Texas Red-X goat anti-mouse IgG) were from Molecular Probes (Invitrogen) (Burlington, ONT). C57BL/6 mice lacking the LDL receptor were purchased from Jackson Laboratories (Bar Harbor, Maine). SR-BI$^{-/-}$ mice with a C57BL/6 background were originally provided by Dr. M. Krieger (Massachusetts Institute of Technology) and were bred in the central animal facility of McMaster University. C57BL/6 HL-deficient mice were generated by targeted disruption of the mouse hepatic lipase gene [44].

2.2. Care and feeding of mice

C57BL/6 wildtype, SR-BI-, LDL receptor- and hepatic lipase-deficient mice used in this study were males, 12 to 24 weeks old and were fed *ad libitum* a chow diet. All procedures were approved by the Institutional Animal Care Committee of the University of Alberta and followed guidelines from the Canadian Council on Animal Care.

2.3. Hepatocyte isolation and culture

Primary hepatocytes were isolated from mice after liver perfusion with collagenase [50]. Cells (1.5×10^6 cells/collagen-coated 60 mm dish) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum for at least 3–4 h.

2.4. Immunoblot analyses

Equal amounts of proteins from cellular extracts were separated by electrophoresis on 12% polyacrylamide gels (containing 0.1% SDS) and the resolved proteins were transferred onto a nitrocellulose membrane. SR-BI and LDLr were detected using specific primary antibodies (dilutions used for SR-BI and LDLr antibodies were respectively 1:1000 and 1:500). For each immunoblot, calnexin was used as a loading control. Membranes were also stained with Ponceau Red to confirm equal loading.

2.5. Radiolabeling of phosphatidylcholine

McArdle 7777 rat hepatoma cells (90% confluent) were incubated for 8 h at 37 °C with 50 μCi [^{3}H]choline chloride, 10 μCi [^{14}C]choline chloride and/or 100 μCi [^{3}H]oleate, or 100 μCi [^{3}H]palmitate. Cells were scraped into phosphate-buffered saline and lipids were extracted in $CHCl_3$:CH_3OH (2:1). Phospholipids were separated by thin-layer chromatography in the solvent system $CHCl_3$:CH_3OH:CH_3COOH:H_2O (25:15:4:2 v/v) and stained with iodine vapor. [^{3}H]PC was scraped from the plate and $CHCl_3$:CH_3OH was added to extract PC from the silica. After centrifugation at 600 ×*g* for 5 min the supernatant containing [^{3}H]PC was collected.

2.6. Isolation and radiolabeling of HDL

Radiolabeled cholesteryl oleoyl ether (CEt) (50 μCi) and PC (50 μCi) ([^{3}H]PC-choline, [^{3}H]PC-oleate, [^{3}H]PC-palmitate or double labeled [^{3}H]PC-oleate/[^{14}C]PC-choline) were dried under nitrogen and liposomes prepared by sonication in a warm water bath in 1 ml phosphate buffered saline for 10 min. The donor liposomes were incubated with 25 mg of HDL and freshly isolated lipoprotein-deficient human plasma ($d>1.21$ g/ml) was heated at 60 °C to inactivate LCAT and was used as a source of CETP [19]. Glutathione (0.01%), thiomerosal (0.03 mM) and protease inhibitors were added to the lipoprotein preparation and mixed at 37 °C for 48 h on an orbital shaker at 200 rpm. [^{3}H]^{H}HDLs were re-isolated by sequential centrifugation [49]. The [^{3}H]HDLs were allowed to percolate through a heparin-Sepharose column to remove apoE/apoB-containing lipoproteins. After extensive dialysis, the protein concentration was determined [51] and appropriate controls (lipid extraction and isolation of HDL-associated PC/CEt by thin-layer chromatography, SDS-PAGE analysis of apolipoprotein content) were performed to ensure the purity of the radiolabeled HDL preparation. [^{3}H]HDL was kept at 4 °C under nitrogen.

2.7. HDL lipid uptake by hepatocytes

Primary cultured hepatocytes were incubated for 1 h at 37 °C in DMEM. Media were removed and cells were incubated for another hour in DMEM in the absence or presence of the following: deoxyglucose (50 mM), monensin (20 μM), Poloxamer 407 (6 mg/ml), Orlistat (100 μM), DTNB (250 μM), Me-Indoxam (50 μM), E600 (100 μM), chloroquine (50 μM), ATK (50 μM), butanol (0.03%), D609 (50 μg/ml), secreted PLA_2-inactivated rattlesnake venom (4 μg/ml), oleate supplementation (0.5 mM with 0.5% bovine serum albumin), anti-SR-BI blocking antibody (4 μl/ml) or anti-ATP synthase β-chain blocking antibody (10 μg/ml). After this pre-incubation period, [^{3}H]HDL (100 μg/ml) was added to each dish and cells were incubated for an additional 2 h to allow lipid uptake. The radioactive media were removed, cells were washed, then incubated 20 min with unlabeled HDL (250 μg protein/dish) to eliminate non-specific binding of HDL to cellular membranes. Cells were scraped into phosphate-buffered saline, and lipids were extracted with $CHCl_3$:CH_3OH (2:1) then washed with CH_3OH:H_2O:$CHCl_3$:CH_3COOH (480:470:30:9.6 v/v) [52]. The solvents were evaporated under nitrogen and lipids were dissolved in $CHCl_3$. Phospholipids were separated by thin-layer chromatography using a developing solvent of $CHCl_3$:CH_3OH:CH_3COOH:H_2O (25:15:4:2 v/v) until the solvent reached 7 cm up the plate. The solvent was evaporated from the plate and the neutral lipids were separated using heptane:diisopropyl ether:acetic acid (60:40:4 v/v) which developed to the top of the plate. Lipids were visualized with iodine vapor and the bands corresponding to PC, TG and CEt were scraped into scintillation vials for measurement of radioactivity.

2.8. Immunofluorescence assays

Primary hepatocytes from C57Bl/6 wildtype mice were plated directly onto a sterile cover slip (100,000 cells/cover slip) and incubated in DMEM supplemented with 10% fetal bovine serum for 3–4 h. Cells were incubated for 1 h in DMEM, then washed three times with 0.2% gelatin-containing phosphate buffered saline (washing buffer). Cells were fixed at room temperature with 4% paraformaldehyde for 10 min before the primary "blocking" antibody (anti-SR-BI or anti-ATP synthase β-chain), diluted in the washing buffer, was added. The cells were incubated for 1 h at 37 °C with the primary antibodies, washed quickly 3× and incubated for another hour with the secondary antibody (Texas-Red anti-mouse IgG antibody and Alexa 488 anti-rabbit IgG antibody). Cells were examined by confocal fluorescence microscopy to confirm the binding of the blocking antibodies to hepatic plasma membranes.

2.9. Inhibition of the endocytosis of Alexa-488 transferrin

Primary hepatocytes from C57Bl/6 wildtype mice were plated directly onto a sterile cover slip (100,000 cells/cover slip) and incubated in DMEM supplemented with 10% fetal bovine serum for 3–4 h. Cells were incubated at 37 °C for 1 h in glucose-free media (5 mM NaN_3 and 50 mM deoxyglucose), or at 4 °C for 1 h in DMEM. Alexa-Fluor 488-labeled transferrin conjugate (5 μg/ml) was added to each well and endocytosis of the fluorophore was measured after 1, 5 and 10 min. Hepatocytes were carefully washed with phosphate buffered saline, fixed with 4% paraformaldehyde and kept at 4 °C, protected from the light, before analysis by confocal fluorescence microscopy.

2.10. Biotinylation of cell surface receptors

Primary cultured hepatocytes were incubated for 1 h in DMEM in the absence or presence of monensin (20 μM). The cell surface protein labeling kit (Pierce) was used for this procedure. The cells were washed with PBS, then biotin solution (2 ml/dish) was added to each dish and cells were incubated at 4 °C for 30 min. A quenching solution was added to each dish and cells were lysed and sonicated for 20 s. Protein concentration was determined and 300 μg aliquots were incubated for 1 h with neutravidin beads at room temperature. Samples were washed 4× with 0.5% Triton-containing phosphate buffered saline after which 300 μl of buffer (with DTT) was added. After 1 h at room temperature, samples were centrifuged at 10,000 rpm for 5 min and biotinylated proteins were collected in the supernatant and loaded directly onto a 10% polyacrylamide gel. Immunoblotting for SR-BI (goat anti-SR-BI) and the LDLr (rabbit anti-LDLr) was performed as described above, using calnexin (from whole cellular lysates) as a loading control.

3. Results

The aim of the present study was to elucidate the mechanism(s) responsible for the cellular incorporation and metabolism of HDL-associated PC by hepatic cells. We used primary mouse hepatocytes as an experimental model because the liver is the predominant organ involved in HDL uptake [53]. Furthermore, we could isolate hepatocytes from livers of different genetically modified mice. The uptake of HDL-PC and further hepatic metabolism was determined by means of radioactive tracers [54]. Human HDL particles were labeled with a non-hydrolyzable analogue of CE, [^{3}H]cholesteryl oleoyl ether ([^{3}H]CEt) and [^{3}H]PC. For phosphatidylcholine, either [^{3}H]palmitate (*sn*-1), [^{3}H]oleate (*sn*-2), [^{3}H]choline or [^{14}C]choline were incorporated into PC molecules as radioactive labels.

3.1. SR-BI is partially involved in HDL-associated PC uptake by the liver

SR-BI mediates the selective uptake of CE from HDL but its role in the cellular incorporation of HDL-PC by hepatocytes is less clear. Therefore, we examined the role of SR-BI in the hepatic uptake of HDL-PC using either a blocking antibody against SR-BI or primary hepatocytes isolated from SR-BI-deficient mice (Fig. 1). First, wildtype hepatocytes were incubated in the absence (control) or in the presence of a SR-BI blocking antibody (4 μl/ml) to inhibit SR-BI activity [30]. After 1 h of pre-incubation with the antibody, [^{3}H]HDL particles (100 μg/ml) containing [^{3}H]PC-choline and [^{3}H]CEt were added, and the cells were incubated for an additional 2 h at 37 °C to allow cellular uptake of [^{3}H]HDL lipids (Fig. 1A). The blocking antibody significantly reduced [^{3}H]CEt uptake by 52% whereas the incorporation of HDL-derived [^{3}H]PC was decreased by 35%. The binding of the SR-BI blocking antibody to the hepatic plasma membrane was confirmed by immunofluorescence (Fig. 1B). Cells were incubated with (upper panel) or without (lower panel) the rabbit-derived blocking antibody and subsequently with a fluorescent secondary antibody (Alexa-Fluor 488 goat anti-rabbit IgG). Fluorescence was detected by confocal microscopy.

To investigate further the participation of SR-BI in HDL-associated PC uptake and metabolism, we performed [^{3}H]HDL lipid uptake assays in wildtype or SR-BI$^{-/-}$ hepatocytes (Fig. 1C). The HDL preparation used for these experiments was labeled with [^{3}H]CEt and with either [^{3}H]PC-choline or [^{3}H]PC-oleate. In the absence of SR-BI, the reduction in [^{3}H]CEt uptake was striking (85%) (Fig. 1C), validating the importance of the HDL receptor in cholesterol metabolism [55]. When PC molecules were labeled with [^{3}H]oleate, the uptake of HDL-associated PC was significantly (49%) lower in SR-BI-deficient hepatocytes than in wildtype hepatocytes and [^{3}H]TG production from [^{3}H]PC-oleate in these cells was decreased by 50% (Fig. 1C). The absence of SR-BI in hepatocytes from SR-BI$^{-/-}$ mice was confirmed by immunoblot analysis (Fig. 1D).

3.2. Energy-depletion significantly reduces the uptake of HDL-associated PC

The importance of energy supply for the hepatic uptake of HDL-PC was examined by incubating C57Bl/6 wildtype primary hepatocytes in energy-depleting culture conditions (Fig. 2). [^{3}H]HDL lipid uptake assays were performed in 50 mM deoxyglucose and 5 mM NaN_3 (Fig. 2A) to determine the relative amount of HDL-associated PC entering hepatocytes via an energy-dependent pathway. The hepatic uptake of [^{3}H]PC-choline from HDL was reduced by 40% whereas [^{3}H]

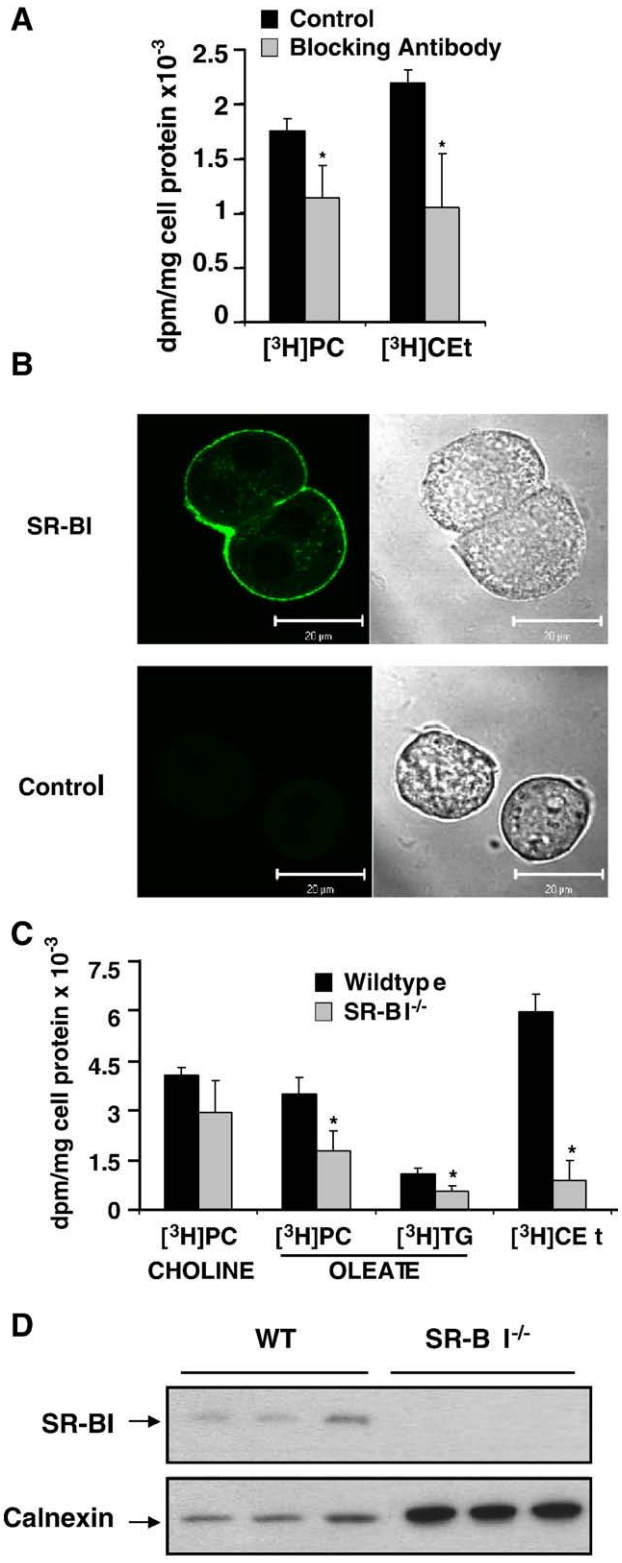

Fig. 1. The cellular incorporation of HDL-associated PC partially involves the HDL receptor SR-BI. (A) Hepatocytes were incubated for 1 h in the absence or presence of rabbit-derived SR-BI blocking antibody (4 μl/ml) to inhibit lipid uptake from HDL. Cells were then incubated with [^{3}H]HDL (containing [^{3}H]PC-choline and [^{3}H]cholesteryl oleoyl ether) and lipid uptake assays were performed as described under "Experimental procedures". Values are expressed as means ± S.E. for 8 experiments. *p<0.001. (B) The binding of the SR-BI blocking antibody to the hepatic plasma membrane is demonstrated by immunofluorescence using an Alexa 488-labeled anti-rabbit SR-BI antibody and visualizing the cells by confocal fluorescence microscopy (upper panel). Bottom panel is showing control cells incubated with the secondary antibody alone. The right panels show phase-contrast images. (C) [^{3}H]HDL lipid uptake assay was performed in wildtype and SR-BI$^{-/-}$ hepatocytes. CHOLINE indicates the cells were labeled with [^{3}H]choline and OLEATE indicates that cells were labeled with [^{3}H]oleate. Values are expressed as means ± S.E. for 3 mice of each genotype. *p<0.001. (D) Immunoblot analysis of SR-BI protein in wildtype and SR-BI$^{-/-}$ hepatocytes. Calnexin is shown as a loading control.

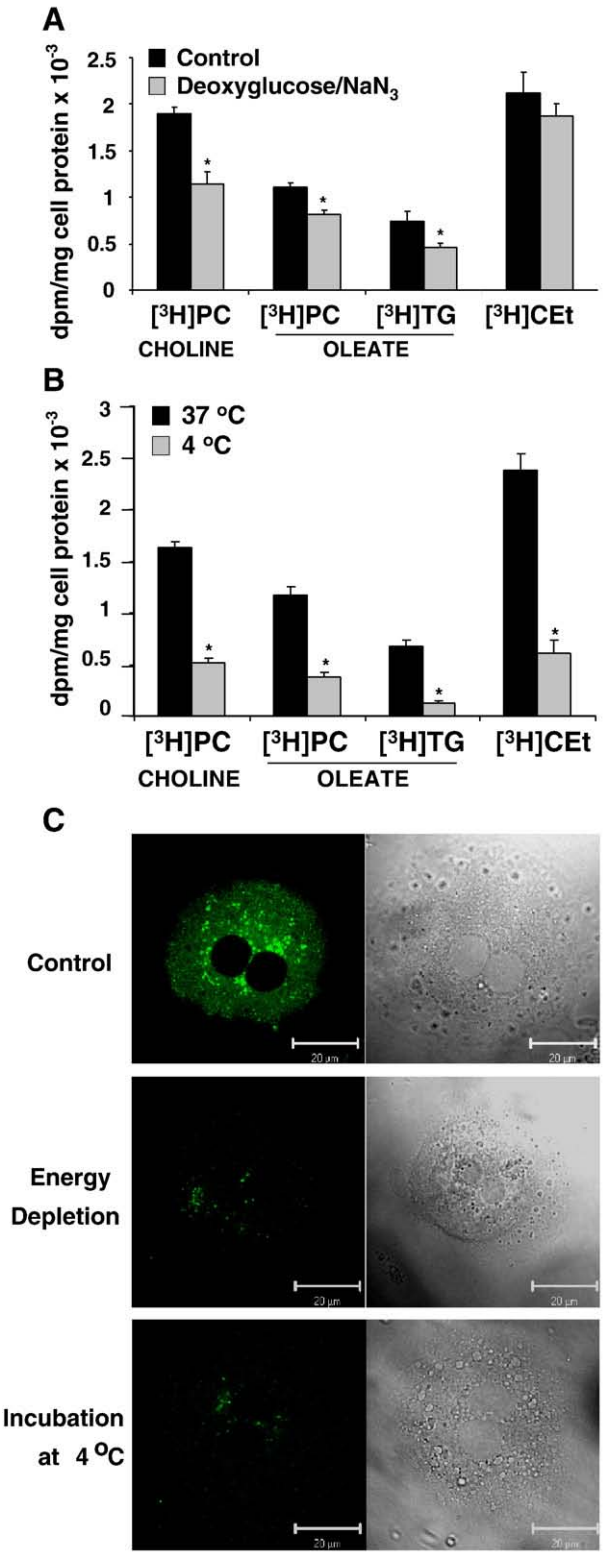

Fig. 2. Incubation of hepatocytes during energy-depletion or at 4 °C reduces the cellular incorporation of HDL-associated PC. (A) Hepatocytes were incubated in glucose-free media containing 50 mM deoxyglucose and 5 mM NaN_3 prior to the [^{3}H]HDL lipid uptake assay. (B) Hepatocytes were incubated at 4 °C or 37 °C for 1 h prior to the uptake assay and during incubation with [^{3}H]HDL. For A and B, independent experiments were performed to acquire the [^{3}H]PC-choline and [^{3}H]PC-oleate data. For both assays, values are expressed as means ± S.E. for 4–5 experiments. *$p<0.001$. (C) The uptake of Alexa-488-labeled transferrin in control cells (upper panel), in energy-depleted hepatocytes (middle panel), or in cells incubated at 4 °C (bottom panel). Fluorescence (left panels) was analyzed by confocal microscopy, using the same laser settings for all slides. The right panel shows phase-contrast images. The results are representative of 2 independent experiments.

PC-oleate uptake was decreased by 27% in energy-depleted cells. [^{3}H]TG production from HDL-derived [^{3}H]PC-oleate was also reduced by 40% upon energy-depletion. It is noteworthy that [^{3}H]CEt uptake was not significantly affected by the energy-depletion treatment.

The cellular incorporation of HDL-associated lipids was also studied in primary hepatocytes incubated at 4 °C. As shown in Fig. 2B, the uptake of [^{3}H]PC and [^{3}H]CEt is dramatically decreased by reduction of the growth temperature to 4 °C. We detected approximately 70% less [^{3}H] radioactivity associated with both [^{3}H]PC-choline and [^{3}H]PC-oleate and the conversion of [^{3}H]PC-oleate to hepatic [^{3}H]TG was reduced by 80%. There was also an 80% decrease in [^{3}H]CEt uptake from HDL, when cells were incubated with the [^{3}H]HDL preparations at 4 °C compared to 37 °C. The efficiency of these treatments to inhibit endocytic processes was evaluated by confocal fluorescence microscopy (Fig. 2C). Primary hepatocytes were cultured for 1 h at 37 °C in DMEM (control, upper panel) or in energy-depleted media supplemented with 50 mM deoxyglucose and 5 mM NaN_3 (middle panel) or at 4 °C (bottom panel). Alexa-Fluor 488-labeled transferrin (10 μg/ml) was added to the media for 5 min before cells were fixed with 4% paraformaldehyde. The uptake of the fluorescent transferrin conjugate was detected using confocal microscopy. It is evident that either energy-depletion or incubation at 4 °C effectively inhibited the uptake of fluorescent transferrin.

3.3. Alteration of the endosomal/recycling compartment by monensin reduces the uptake of HDL-associated PC by hepatocytes

Energy-depletion treatment was used to block classical endocytic processes, resulting in a significant decrease in PC uptake (Fig. 2A). However, when endocytosis was blocked by incubating cells with deoxyglucose-NaN_3 and/or at 4 °C, many metabolic pathways are altered. Monensin is known to cause inhibition of receptor recycling to the cell surface and to decrease ligand–receptor dissociation in endocytic vesicles. Thus, to further investigate the importance of endocytic/recycling receptors and to determine whether HDL-associated PC is taken up during endocytosis of the whole particle, primary hepatocytes were cultured in the presence of 20 μM monensin (Fig. 3A). [^{3}H]HDL uptake assays revealed a significant 24% reduction in [^{3}H]CEt uptake by hepatocytes treated with monensin compared to control cells (Fig. 3A). Monensin also decreased [^{3}H]PC uptake from HDL by 54% (Fig. 3A). To control for the efficiency of monensin treatment, plasma membrane proteins were biotinylated and the reduction in cell surface expression of SR-BI and of the LDL receptor was verified by immunoblot analysis (Fig. 3B). Our data indicate that monensin treatment efficiently decreases receptor expression at the plasma membrane, likely as a result of impaired endosomal vesicle recycling, further supporting a role for particle internalization in hepatic uptake of HDL-associated PC.

We also considered the possibility of exchange between HDL and the hepatocytes. This was assessed by incubating wildtype hepatocytes with [^{3}H]choline for 15 h and then chasing with medium ± 100 μg HDL/ml for 1 to 3 h. The amount of [^{3}H]PC in cells and in HDL in the medium was measured. After 3 h, 1.75% of the labeled PC in the hepatocytes was recovered in added HDL. In the absence of exogenous HDL, approximately 0.5% of PC was recovered in the HDL fraction in the medium (presumably HDL secreted by the hepatocytes). Thus, approximately 1.25% of the labeled PC in the cells had exchanged on to the exogenous HDL within 3 h. It seems that exchange between exogenous HDL and hepatocytes can account for a small incorporation of labeled PC from HDL into the hepatocytes.

3.4. The ATP synthase β-chain expressed at the cell surface of hepatocytes does not mediate the uptake of HDL-associated lipids

The ATP synthase β-chain is ectopically expressed at the hepatic plasma membrane. The β-subunit has been shown to bind to apoA1

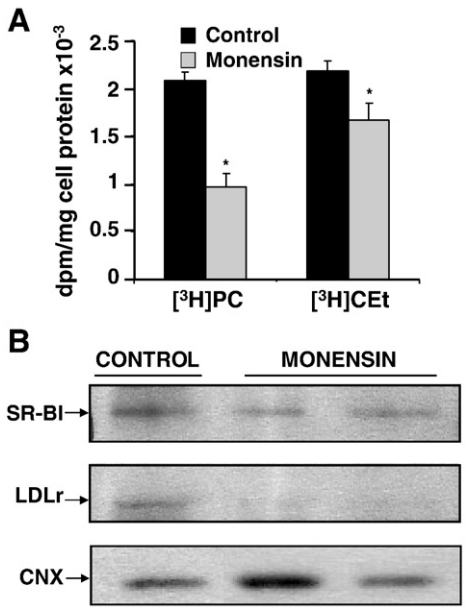

Fig. 3. Monensin significantly reduces the uptake of HDL-associated PC. (A) Hepatocytes were incubated in the absence or presence of 20 μM monensin for 1 h then [^{3}H]HDL (containing [^{3}H]PC-choline and [^{3}H]cholesteryl oleoyl ether) were added for an additional 2 h. Lipids were extracted and isolated. Values are expressed as means ± S.E. for 4 experiments. *$p<0.001$. (B) The cell surface expression of lipoprotein receptors SR-BI and LDLr was determined by biotinylation of cell surface proteins and SDS-PAGE analysis. Also shown in the bottom panel is an immunoblot of calnexin (CNX) present in 25 μg of total cellular lysates.

with high specificity and is thought to be involved in HDL internalization [8,35]. We analyzed the participation of this potential pathway in the cellular incorporation and metabolism of HDL-associated PC (Fig. 4). Radioactive HDL (containing [^{3}H]PC-choline and [^{3}H]CEt) were used for the uptake experiments, which were conducted in the presence of 10 μg/ml of a blocking antibody that recognizes the β subunit of the ATP synthase complex. This primary murine monoclonal antibody (Clone 3D5 from Molecular Probes), when incubated with HepG2 cells at a concentration of 10 μg/ml, inhibited [^{125}I]HDL uptake by 35% [32]. In contrast, our results with primary mouse hepatocytes show that the blocking antibody treatment did not decrease HDL-associated lipid uptake (Fig. 4A), suggesting that the cellular incorporation of HDL lipids is not mediated by the ectopic β chain of the ATP synthase. The binding of the blocking anti-β-chain antibody to hepatic plasma membranes was confirmed by immunofluorescence. Hepatocytes were incubated with (upper panel) or without (lower panel) the mouse-derived blocking antibody. A secondary antibody (Texas Red-X goat anti-mouse IgG) was then added and fluorescence was detected by confocal microscopy (Fig. 4B).

3.5. Extracellular remodeling of HDL significantly enhances HDL-associated PC uptake by hepatocytes

We next evaluated the importance of PC hydrolysis in the extracellular compartment for efficient lipid uptake from HDL (Fig. 5). In order to determine whether or not extracellular lipolysis influenced HDL lipid uptake, primary C57Bl/6 wildtype hepatocytes were incubated with 6 μg/ml Poloxamer 407 (P407), a non-toxic hydrophilic and non-ionic surface-active agent [56] that inhibits the activity of many plasma enzymes, including HL, endothelial lipase, and LPL [56]. Results from this approach should establish the possible role of extracellular remodeling of HDL in facilitating the cellular incorporation of HDL-associated lipids. Indeed, uptake of [^{3}H]PC-choline and [^{3}H]PC-oleate was decreased by 37% and 57% respectively by incubation of cells with Poloxamer 407 and [^{3}H]CEt uptake was reduced by approximately 60% (Fig. 5A). [^{3}H]TG production from HDL-derived [^{3}H]PC-oleate was similarly reduced by 55%, reflecting the extent of the 57% reduction observed for the incorporation of [^{3}H]PC-oleate. Thus, P407 inhibits extracellular metabolism of PC without affecting its intracellular fate following uptake by hepatocytes.

In addition to catalyzing the hydrolysis of TG associated with lipoproteins, HL possesses a phospholipase A_1 activity. To explore the role of HL in HDL-associated PC uptake by primary hepatocytes, [^{3}H] HDL uptake assays were performed in wildtype and HL$^{-/-}$ hepatocytes (Fig. 5B). It is noteworthy that when rat hepatoma cells are incubated with [^{3}H]palmitate, the label is preferentially incorporated at the *sn*-1 position of PC whereas [^{3}H]oleate is incorporated mainly at the *sn*-2 position [57]. Thus, because saturated fatty acids on PC are preferentially hydrolyzed by HL [43], and because palmitate predominantly is incorporated into the *sn*-1 position, we chose [^{3}H] palmitate instead of [^{3}H]oleate as a label for PC for this experiment. As shown in Fig. 5B, the absence of HL in primary hepatocytes did not affect the uptake of [^{3}H]PC-choline. Similarly, the uptake of [^{3}H]CEt was not significantly decreased in HL$^{-/-}$ hepatocytes compared to wildtype cells. However we did observe a small 17% reduction in [^{3}H]

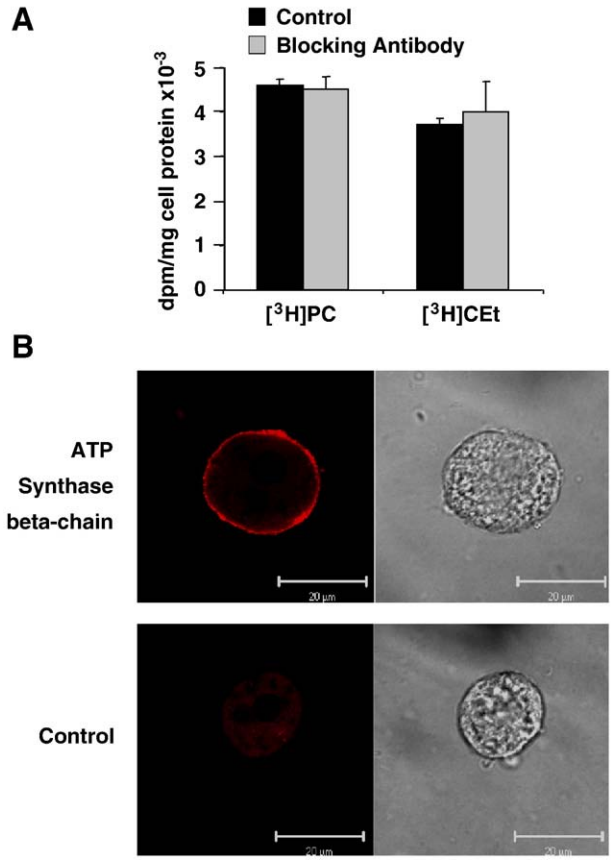

Fig. 4. The cell surface ATP synthase β-chain is not involved in the cellular incorporation of HDL-associated PC. (A) Hepatocytes from C57Bl/6 mice were incubated for 1 h in the absence or presence of 10 μg/ml of a mouse monoclonal anti-ATP synthase β-chain blocking antibody (Clone 3D5) to inhibit uptake of HDL via this pathway. Hepatocytes were then incubated with [^{3}H]HDL (containing [^{3}H]PC-choline and [^{3}H]cholesteryl oleoyl ether) for 2 h and uptake of radiolabeled lipids was determined. Values are expressed as means ± S.E. for 4 separate experiments. (B) The binding of the ATP synthase β-chain blocking antibody to the hepatic plasma membrane is confirmed by immunofluorescence using a Texas Red-labeled anti-mouse IgG secondary antibody and examining the cells by confocal fluorescence microscopy (upper left panel). Bottom left panel is showing control cells incubated exclusively with the fluorescent secondary antibody. The right panels show phase-contrast microscopy images of the same hepatocytes.

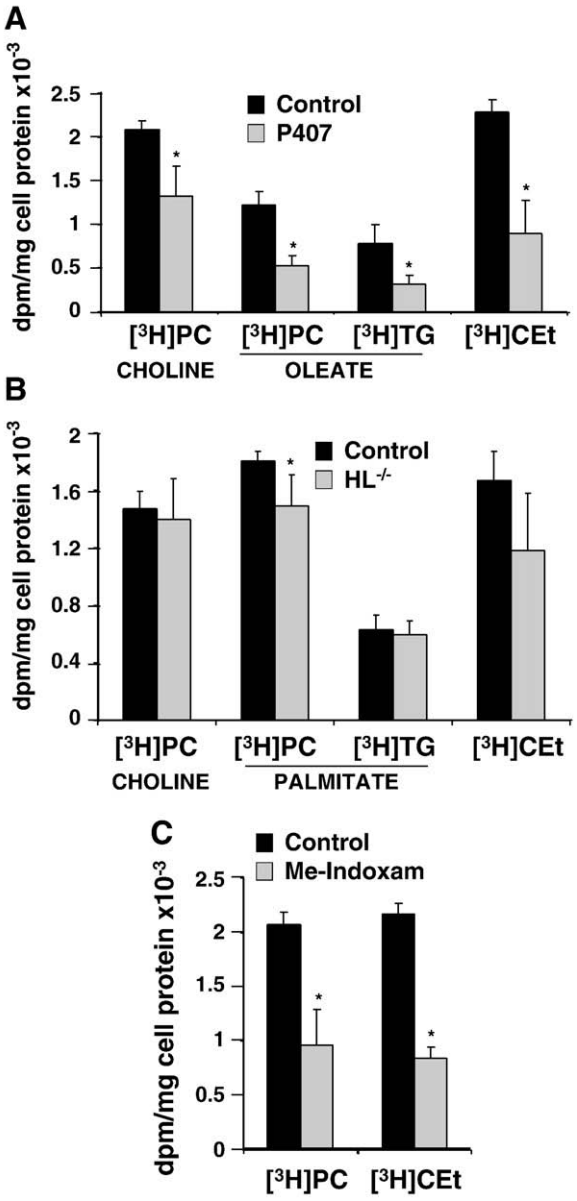

Fig. 5. Extracellular lipolysis significantly enhances HDL lipid uptake. (A) Hepatocytes were incubated for 1 h with or without P407 (6 mg/ml) then 100 μg/ml of [^{3}H]HDL was added to each dish for 2 h. Lipids were extracted and separated by thin-layer chromatography. Values are expressed as means ± S.E. for 6–10 experiments. *$p<0.001$. (B) [^{3}H]HDL lipid uptake assays were performed in wildtype and HL$^{-/-}$ hepatocytes. Values are expressed as means ± S.E. for 5 mice of each genotype. *$p<0.01$. For both A and B, the results for [^{3}H]PC-choline and [^{3}H]PC-palmitate were obtained from independent experiments. (C) Primary hepatocytes were incubated in the absence or presence of Me-Indoxam (50 μM) for 1 h prior to and during the [^{3}H]HDL lipid uptake assay; HDL was labeled with [^{3}H]PC-choline and [^{3}H]CEt. *$p<0.001$.

associated with PC when labeled with [^{3}H]palmitate (Fig. 5B). Moreover, the data from these uptake experiments indicate that the intracellular metabolism of HDL-associated PC was not affected by the absence of HL, as evidenced by the similar rates of [^{3}H]TG production in hepatocytes of both genotypes (Fig. 5B). Thus, HL appears to play a very minor role in HDL-PC metabolism by primary hepatocytes.

To further characterize the extracellular remodeling of HDL, and therefore to attempt to identify which lipase plays an important role in HDL-associated PC uptake by the liver, we used Me-Indoxam (50 μM) to selectively inhibit secreted PLA_2 activity. This treatment resulted in a 61% decrease in [^{3}H]CEt uptake and a 53% reduction in [^{3}H]PC-choline uptake (Fig. 5C).

3.6. HDL-associated PC taken up by hepatocytes can serve as a precursor for TG synthesis

When we used [^{3}H]choline as tracer, over 95% of cellular radioactivity was associated with PC. The remaining radioactivity was distributed between lyso-PC and water-soluble choline-containing compounds (data not shown). However, when we used [^{3}H]oleate to label PC, we found a significant percentage (25–40%) of cellular [^{3}H] associated with TG (Figs. 1C, 2A, 2B, 5A, 5B). We next determined if there was incorporation of [^{3}H]oleate into other neutral lipids. The uptake of HDL-associated [^{3}H]PC-oleate resulted in significant production of [^{3}H]TG (23% of total counts were associated with TG) with minimal transfer of PC-derived [^{3}H]oleate to cholesterol (2–3% of radioactivity) (Fig. 6A, B). [^{3}H]PC represented ~60% of total radioactivity incorporated into hepatocytes.

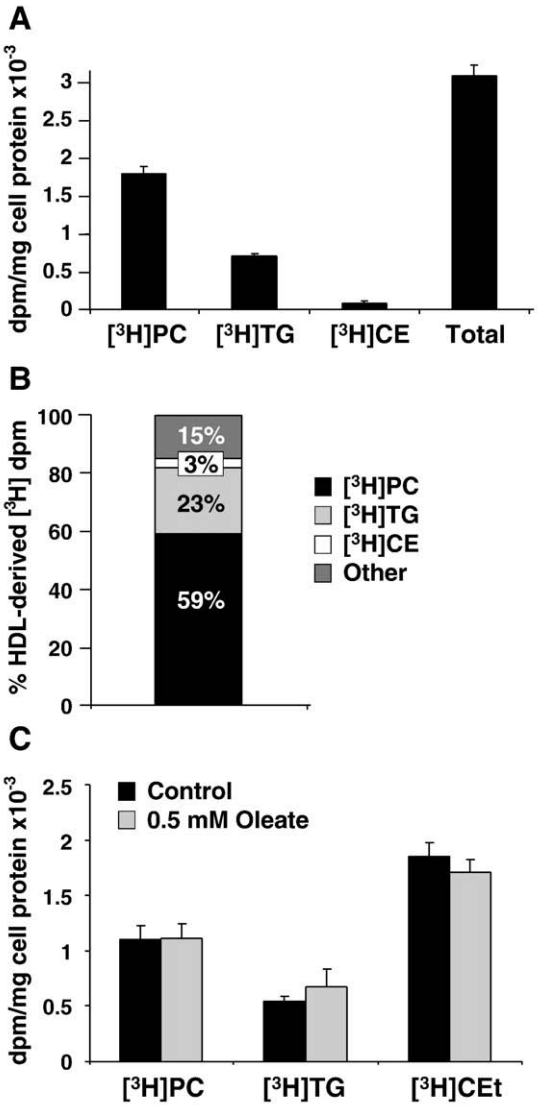

Fig. 6. HDL-associated PC taken up by hepatocytes can serve as a precursor for TG synthesis. (A) Primary hepatocytes from C57Bl/6 mice were cultured in the presence of HDL containing [^{3}H]PC-oleate. Following the lipid uptake assay, [^{3}H]lipids were extracted and isolated by thin-layer chromatography. Values are means ± S.E. for 3 experiments. (B) The results in A were normalized to total cellular radioactivity and expressed as % of HDL-derived [^{3}H] dpm. "Other" include [^{3}H] associated with diacylglycerol, other phospholipids, fatty acids and lyso-PC. (C) Cells were cultured in DMEM (0.5% albumin) or supplemented with 0.5 mM oleate (0.5% albumin) prior to and during the [^{3}H]HDL uptake assay. Values are expressed as means ± S.E. for 3 experiments.

To further investigate the mechanism responsible for the hepatic conversion of HDL-derived PC to TG, we evaluated the contribution of a phospholipase A-mediated pathway by measurement of the liberation of a fatty acid. Hepatocytes were incubated with unlabeled oleate (from 0.25 to 1 mM) in 0.5% BSA. The addition of 0.5 mM oleate during the [^{3}H]HDL lipid uptake assay had no effect on [^{3}H]PC metabolism; unlabeled oleate was unable to dilute radioactivity in TG and did not alter [^{3}H]PC and [^{3}H]CEt hepatic uptake (Fig. 6C).

3.7. Hepatic intracellular metabolism of HDL-derived PC is mostly extralysosomal

It is believed that, in contrast to LDL CEs which are degraded in lysosomes, CEs from HDL are hydrolyzed extralysosomally [58,59]. To address the potential role of lysosomal degradation of HDL-derived PC in the conversion of [^{3}H]PC to [^{3}H]TG, primary hepatocytes from C57Bl/6 mice were incubated in the absence or presence of chloroquine (50 μM). [^{3}H]HDL lipid uptake was measured using either [^{3}H]PC-choline or [^{3}H]PC-oleate to trace PC molecules (Fig. 7). Chloroquine treatment did not significantly decreased the uptake of [^{3}H]PC (Fig. 7). However, the transfer of [^{3}H]oleate from PC to TG was slightly decreased (25%) by chloroquine (Fig. 7).

3.8. Inhibition of intracellular lipolysis by E600 alters HDL-derived PC metabolism by hepatocytes

We next analyzed the consequences of inhibiting intracellular lipolysis on HDL lipid uptake and the subsequent metabolism of HDL-associated PC. Hepatocytes were incubated with 100 μM E600 (diethyl p-nitrophenyl phosphate) to block serine-containing esterase activity [60] and the uptake and metabolism of HDL lipids was analyzed. The cellular incorporation of HDL-associated [^{3}H]PC-choline and [^{3}H]CEt was unaffected by E600 (Fig. 8). However, intracellular metabolism of [^{3}H]PC-oleate was evidently perturbed, as demonstrated by the 2.2-fold increase in [^{3}H]PC-oleate and the 40% reduction in [^{3}H]TG production (Fig. 8). Thus, an intracellular lipase is implicated in the conversion of [^{3}H]PC-oleate to [^{3}H]TG.

3.9. The production of [^{3}H]TG from HDL-derived [^{3}H]PC does not require cytosolic PLA$_2$ or phospholipase D

To gain further insights into the mechanism underlying the conversion of HDL-associated PC into hepatic TG, we used ATK, a potent and selective inhibitor of the 85 kDa cytosolic PLA$_2$ (Fig. 9A). Cells were also incubated with 1-butanol to study the role of phospholipase D in the conversion of PC-derived [^{3}H]DG to [^{3}H]TG (Fig. 9B). In the presence of

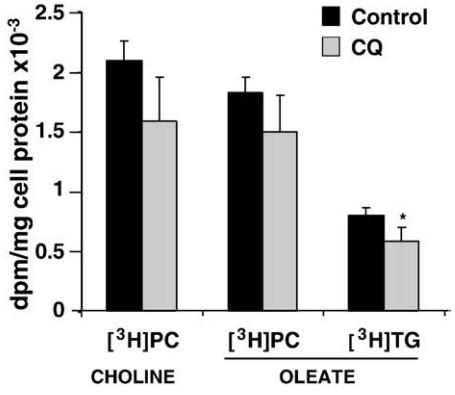

Fig. 7. Metabolism of HDL-derived PC in hepatocytes occurs mostly in extralysosomal compartments. Cells were incubated in the absence or in the presence of 50 μM chloroquine (CQ) for 1 h before 100 μg/ml of [^{3}H]HDL was added to each dish for an additional 2 h. Data for [^{3}H]PC-choline and [^{3}H]PC-oleate were acquired from separate experiments. Values are expressed as means ± S.E. for 6 experiments. *p<0.05.

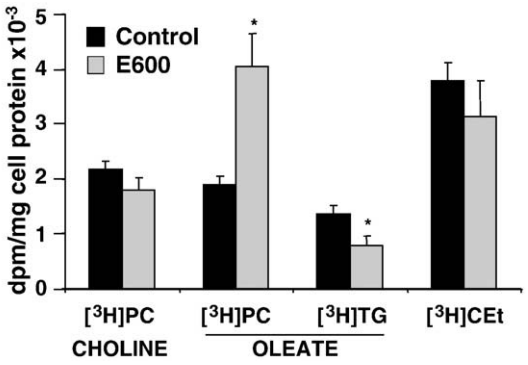

Fig. 8. The inhibition of lipolysis by E600 reduces the production of [^{3}H]TG from HDL-associated [^{3}H]PC. Hepatocytes were cultured in the absence or presence of 100 μM E600 for 1 h prior to and during [^{3}H]HDL lipid uptake assays. Independent experiments were performed to obtain the [^{3}H]PC-choline and [^{3}H]PC-oleate data. Values are expressed as means ± S.E. for 8 experiments. *p<0.05.

butanol, phospholipase D catalyzes the hydrolysis of PC to form choline and phosphatidylbutanol [61]. Phosphatidylbutanol is not a substrate for phosphatidate phosphohydrolase and should thus reduce the availability of PC-derived [^{3}H]DG intermediate [62], resulting in reduced [^{3}H]TG counts. There was no significant difference in radioactivity associated with [^{3}H]PC-choline, [^{3}H]PC-oleate and [^{3}H]TG between ATK- or butanol-treated cells and their equivalent controls, indicating that

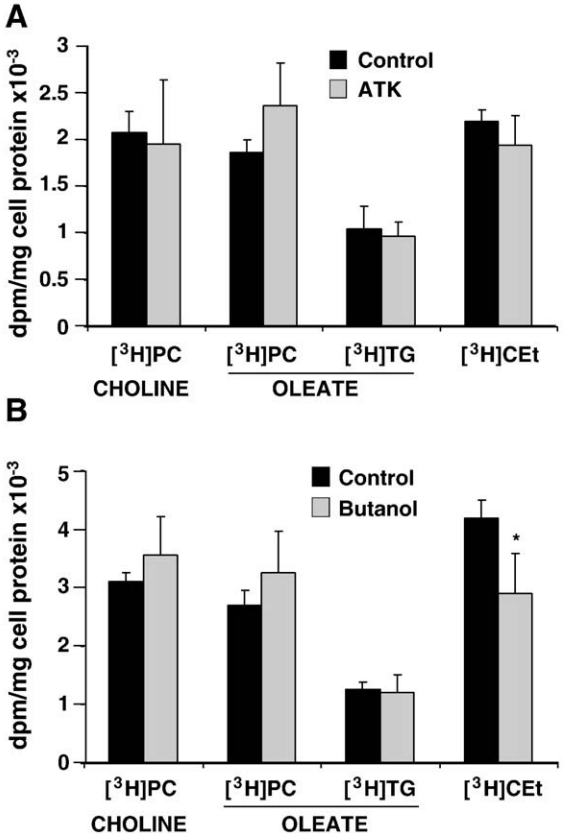

Fig. 9. The fate of HDL-associated PC following lipid uptake by hepatocytes is unaffected by inhibition of PLA$_2$ or phospholipase D. (A) Cells were incubated with or without 50 μM arachidonyl trifluoromethyl ketone (ATK) to inhibit the activity of the 85 kDa cytosolic PLA$_2$. Lipid uptake assays were performed. Values are expressed as means ± S.E. for 3–6 experiments. (B) Primary hepatocytes were cultured in the absence or presence of 0.03% butanol to block the conversion of PC-derived [^{3}H]DG to [^{3}H]TG via phosphatidic acid. Lipid uptake assays were performed. Values are expressed as means ± S.E. for 4 experiments. *p<0.05. For A and B, independent experiments were performed to obtain [^{3}H]PC-choline and [^{3}H]PC-oleate data.

neither cPLA$_2$ nor PLD are implicated in conversion of HDL-derived PC to TG. Unexpectedly, [^{3}H]CEt uptake by hepatocytes was 31% lower in the presence of butanol (Fig. 9B).

3.10. Remodeling of PC via PLC hydrolysis contributes to hepatic TG production from HDL-associated PC

The importance of PLC-mediated hydrolysis of PC was examined using D609, an inhibitor of PC-specific PLC [63,64]. Cells were incubated with 50 μg/ml D609 and the fate of HDL-derived [^{3}H]PC-oleate was determined (Fig. 10A). Radioactivity associated with [^{3}H]PC-oleate did not change upon D609 treatment. In contrast, [^{3}H]TG production was reduced by 55% in the presence of D609 (Fig. 10A), suggesting that PLC hydrolysis plays a significant role in mediating [^{3}H]TG synthesis from PC-derived [^{3}H]diacylglycerol.

To further analyze the importance of this pathway, primary hepatocytes were treated with rattlesnake venom (2 μg/ml), an activator of PC-PLC [65]. Because rattlesnake venom also contains significant PLA$_2$ activity, Me-Indoxam was added to the venom preparation in order to neutralize this PLA$_2$ activity. Hepatocytes were also treated with a combination of rattlesnake venom and D609 (Fig. 10B). The addition of rattlesnake venom to the culture media did not affect [^{3}H]CEt uptake compared to control cells and the cellular [^{3}H] associated with PC was similar in venom-treated and untreated cells. [^{3}H]TG production was dramatically (6.7-fold) increased by the rattlesnake venom. Inhibition of PC-PLC with D609 effectively abolished this effect, bringing [^{3}H]TG synthesis to levels comparable to that of untreated hepatocytes (Fig. 10B). The sum of the radioactivity associated with PC and TG clearly shows that more PC was taken up by the hepatocytes treated with venom (6379 ± 879 dpm/mg protein) compared to controls (2245 ± 127 dpm/mg protein). An unexplained observation of this experiment is the 35% (Fig. 10A) and the 27% (Fig. 10B) reduction in [^{3}H]CEt upon treatment with D609. To determine whether or not the conversion of HDL-derived [^{3}H]PC to [^{3}H]TG in hepatocytes involves the SR-BI-mediated uptake pathway, we performed HDL lipid uptake assays in SR-BI$^{-/-}$ hepatocytes in the absence or presence of the PC-PLC inhibitor D609 (Fig. 11). D609 reduced the amount of [^{3}H]TG to a similar extent (64% and by 68%) in wildtype and SR-BI$^{-/-}$ hepatocytes, respectively.

Finally, to confirm that a PLC-mediated mechanism is involved in producing [^{3}H]TG from [^{3}H]PC, we used radiolabeled HDL particles containing [^{3}H]oleate/[^{14}C]choline as tracers of PC. The ratio of [^{3}H]/[^{14}C] was 4.1 for the HDL preparation. The [^{3}H]/[^{14}C] ratio decreased to 2.3 in cellular PC recovered during the uptake assay (Fig. 12). This lower ratio suggests that HDL-derived PC is indeed metabolized intracellularly and that the hepatic remodeling of PC results in the segregation of the fatty acid and head group moieties.

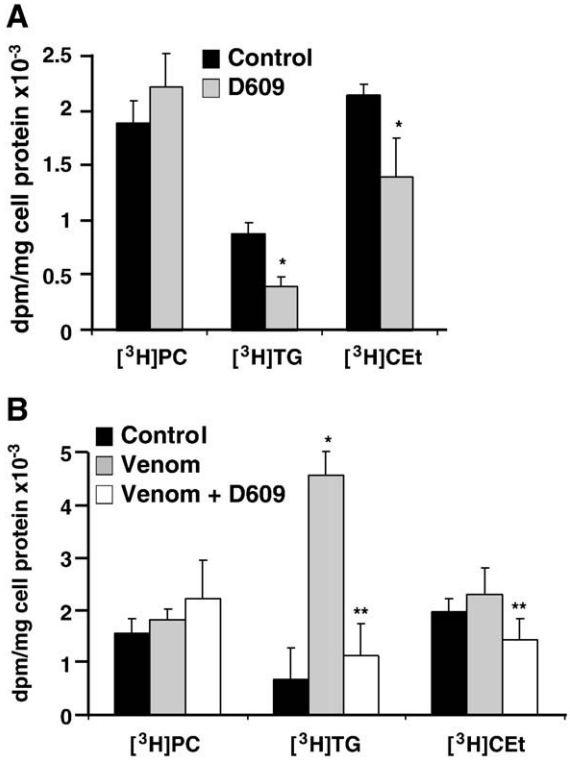

Fig. 10. Remodeling of PC via PLC hydrolysis contributes to the production of [^{3}H]TG from HDL-associated [^{3}H]PC. (A) Primary hepatocytes were either cultured in DMEM or exposed to 50 μg/ml D609 to inhibit PLC activity for 1 h before 100 μg/ml of [^{3}H]HDL (labeled with [^{3}H]PC-oleate and [^{3}H]CEt) was added. Lipid uptake assays were performed. (B) PLC-mediated hydrolysis of PC was enhanced by incubating cells with rattlesnake venom (2 μg/ml) that had been previously treated with Me-Indoxam to inactivate secreted PLA$_2$. Hepatocytes were also incubated with a venom preparation containing the inhibitor D609 as a control. HDL was labeled with [^{3}H]PC-oleate and [^{3}H]cholesteryl oleoyl ether. For A and B, values are expressed as means ± S.E. for 4 experiments. *p<0.001 compared with untreated (control) cells. **p<0.001 compared with rattlesnake venom-treated cells.

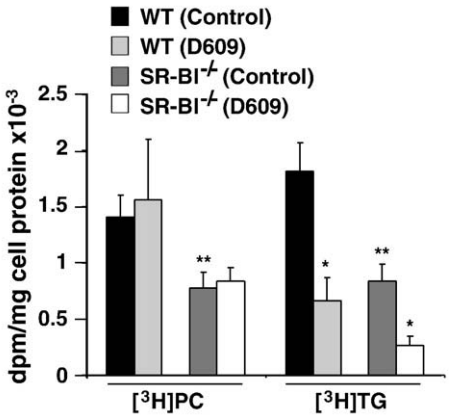

Fig. 11. The conversion of HDL-associated PC to TG is independent of SR-BI. [^{3}H]HDL lipid uptake assays were performed in wildtype and SR-BI$^{-/-}$ hepatocytes, in the absence (control) or presence of the PC-PLC inhibitor D609 (50 μg/ml). 100 μg/ml of [^{3}H]HDL radiolabeled with [^{3}H]PC-oleate was added to the culture media and cells were incubated for 2 h at 37 °C. Values are expressed as means ± S.E. for 2–3 mice of each genotype. *p<0.001 compared to untreated (control) cells of the same genotype. **p<0.05 compared to wildtype untreated cells.

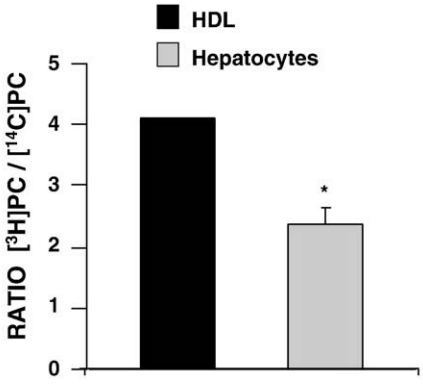

Fig. 12. Double-labeled PC ([^{3}H]PC-oleate/[^{14}C]PC-choline) confirms hepatic remodeling of HDL-derived PC by PLC. Primary hepatocytes from wildtype C57Bl/6 mice were incubated with radiolabeled HDL (containing [^{3}H]PC-oleate/[^{14}C]PC-choline and [^{3}H]CEt) for 2 h. [^{3}H] and [^{14}C] radioactivity associated with PC was measured and the ratio compared to the initial ratio [^{3}H]/[^{14}C] of the radiolabeled HDL preparation. For the final ratios determined at the end of the lipid uptake assay, values are expressed as means ± S.E. for 7 experiments. *p<0.001.

4. Discussion

Although the metabolism of plasma HDL cholesterol is well characterized, from particle formation [66] to hepatic selective lipid uptake [10,11] and biliary secretion [6,67], much less was known about the fate of phospholipids associated with HDL. In the present study, we investigated the uptake and subsequent metabolism of HDL-associated PC in primary mouse hepatocytes. Our data support a model by which HDL-associated PC is taken up by hepatocytes via pathways that mostly differ from that of HDL-CE (Fig. 13). Indeed, SR-BI only mediates approximately one third of PC uptake while being the major mediator of clearance of HDL cholesterol by the liver [55]. Whereas HDL internalization does not contribute to cholesterol uptake in mouse hepatocytes [9,68], our data suggest that endocytosis participates significantly in the cellular incorporation of HDL-PC. Moreover, extracellular remodeling of HDL-associated PC by secreted PLA_2 efficiently enhances HDL-PC uptake. Lastly, following uptake by hepatic cells, PC can supply diacyglycerol for TG synthesis via a PLC-mediated pathway.

4.1. SR-BI partially mediates the hepatic uptake of PC associated with HDL

SR-BI is important in facilitating the selective uptake of HDL-CE by the liver and steroidogenic tissues [25,55]. Even if SR-BI has been shown to participate in HDL retroendocytosis [69], selective CE uptake is usually considered to occur at the cell surface with the involvement of a hydrophobic channel favorable to the movement of cholesterol [20,21]. A role for SR-BI in the uptake of lipoprotein-associated PC has been described in endothelial cells [24], adrenal glands [25] and monocytes [21]. Much less was known about the role of SR-BI in metabolism of HDL-PC by the liver, the predominant tissue responsible for clearance of HDL.

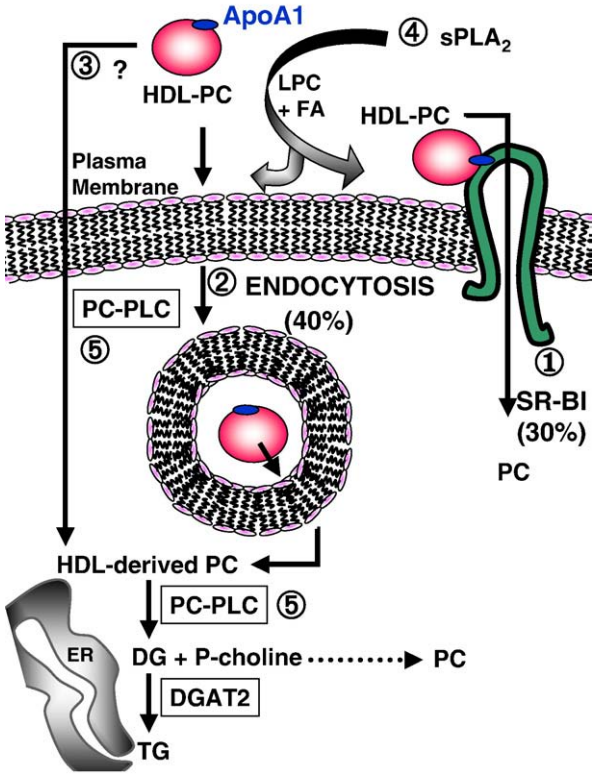

Fig. 13. Proposed model for HDL-associated PC uptake and metabolism in primary hepatocytes. PC can be delivered to the liver via SR-BI (~30%) ① or via an SR-BI-independent pathway (40%) ②. Passive diffusion of PC from HDL to the plasma membrane, or even an unknown pathway for lipid uptake, might facilitate some incorporation of HDL-PC into hepatocytes ③. Extracellular hydrolysis of HDL-associated PC by secreted PLA_2 ④ contributes to the generation of products that can readily diffuse through the membrane, lyso-PC (LPC) and fatty acid (FA). Moreover, secreted PLA_2-mediated HDL remodeling might produce HDLs that are better substrates than intact HDL for hepatic lipoprotein receptors. Following uptake from HDL, PC can be hydrolyzed by PC-PLC (suggested to be localized either at the plasma membrane or in the cytosol) ⑤ and further metabolized to TG via mechanisms that are largely unknown. Dotted arrow indicates re-utilization of phosphocholine for biosynthesis of PC. DGAT, diacylglycerol: acyl-CoA acyltransferase.

We hypothesized that the vast majority of PC is transferred to hepatocytes via a similar pathway as HDL-CE, via SR-BI. However, when using a blocking antibody or SR-$BI^{-/-}$ hepatocytes, cellular incorporation of HDL-associated [^{3}H]PC was only reduced by a third (27–35%) whereas there was an 85% decrease in hepatic [^{3}H]CEt incorporation. Our [^{3}H]CEt data agree with many reports suggesting that hepatic SR-BI represents the major uptake pathway for HDL-associated CE [55,70]. Therefore, approximately one third of HDL-PC hepatic transfer occurs via SR-BI. The remaining [^{3}H]PC uptake from HDL involves different mechanism(s) from CE uptake from HDL.

4.2. Endocytosis represents another important pathway for uptake of HDL-associated PC by primary hepatocytes

SR-BI mediates HDL endocytosis/retroendocytosis [26,27], but most HDL particle uptake in hepatic cells appears to be SR-BI-independent [8]. To address the role of endocytosis in the incorporation of HDL-associated PC, primary hepatocytes were incubated with deoxyglucose/NaN_3 to inhibit energy-dependent holoparticle uptake [30]. Inhibition of endocytosis decreases [^{125}I]HDL uptake but does not affect SR-BI-mediated selective CE uptake from HDL [9,71–73]. We observed similar [^{3}H]CEt uptake in hepatocytes cultured in energy-depleted conditions compared to control cells. However, energy-depletion significantly decreased the cellular incorporation of [^{3}H]PC from HDL by 40%.

The results discussed so far suggest that HDL-associated [^{3}H]PC is taken up partially via a SR-BI-mediated pathway and partially via the internalization of HDL, independent of the uptake of [^{3}H]CEt. Because SR-BI can be endocytosed along with HDL particles, we examined the effects of energy-depletion on [^{3}H]PC uptake in hepatocytes isolated from SR-$BI^{-/-}$ mice. The extent of inhibition was comparable to that seen in wildtype cells (data not shown), suggesting that this endocytosis-dependent component of HDL-associated [^{3}H]PC uptake is not mediated by SR-BI. Thus, [^{3}H]CEt and ~2/3 of [^{3}H]PC are incorporated into hepatic cells via different mechanisms.

In additional studies hepatocytes were exposed to monensin, an inhibitor of clathrin-coated pit endocytosis and receptor recycling to the cell surface [74]. Monensin abolished the recycling of LDLr to the plasma membrane and led to decreased SR-BI expression at the cell surface. Interestingly, monensin slightly reduced HDL-[^{3}H]CEt uptake by hepatocytes. This 24% decrease is in contrast with the current concept of selective HDL-CE uptake in rodents, occurring independently of HDL endocytosis [68,75]. We believe that this result might simply reflect the reduced expression of SR-BI at the cell surface caused by monensin. As for the effect of monensin on [^{3}H]PC uptake, [^{3}H]PC-choline uptake from HDL is reduced by 54%. Energy-depletion caused a 40% decrease in the cellular incorporation of HDL-PC. The greater effect seen with monensin (54% reduction versus 40% for deoxyglucose/NaN_3 treatment) might be due to the reduced levels of SR-BI at the plasma membrane since SR-BI does participate, to some extent, in the uptake of hepatic HDL-PC.

We also determined that our observations were not due to HDL endocytosis mediated by the LDL receptor (LDLr) or LDLr-related proteins. Internalization of HDL particles can indeed occur via those pathways when lipoproteins are associated with apoE [76]. Our [^{3}H]HDL preparations were devoid of apoE. However, HDL may acquire newly secreted apoE from the cell surface and thus be internalized into an intracellular compartment via an apoE-dependent pathway [59,77]. We performed [^{3}H]HDL lipid uptake experiments in LDLr$^{-/-}$

hepatocytes and those cells were as efficient as wildtype cells in mediating lipid uptake from HDL (data not shown). Therefore, there must be another endocytic pathway, sensitive to monensin, that is able to trigger HDL internalization and facilitate the uptake of PC associated with HDL. Possibly there is a yet to be identified receptor for HDL affected by monensin treatment.

We examined a possible role of the ADP-dependent endocytosis of HDL via the newly described ATP synthase β-chain pathway [8]. Incubations of hepatocytes with a blocking antibody had no effect on [^{3}H]HDL lipid uptake suggesting that this pathway is not likely to be involved in HDL-associated PC uptake.

4.3. Remodeling of HDL phospholipids by extracellular lipases represents a significant mechanism for the hepatic uptake of HDL lipids

Another potential uptake pathway for HDL-PC involves extracellular hydrolysis, thereby allowing lyso-PC to diffuse into the plasma membrane. The fate of PC associated with HDL has been studied in rats; PC was hydrolyzed by a phospholipase A_1, most likely HL, and by a phospholipase A_2 mechanism (either secreted PLA_2 or LCAT) [40]. A specific role for secreted PLA_2 in SR-BI-mediated HDL lipid uptake has also been described [47,78].

We hypothesized that the remodeling of HDL particles by extracellular lipases, could enhance HDL-associated PC uptake. Poloxamer 407 (P407) has been shown to inhibit the activity of many lipases [79]. When added to culture media, P407 decreased the uptake of [^{3}H]PC-choline by 37% and [^{3}H]CEt by 61%. P407 had a greater effect in interfering with cellular incorporation of HDL-associated PC (57% lower in treated cells) when PC molecules were labeled with [^{3}H]oleate. It is likely that extracellular remodeling of HDL lipids, with liberation of a fatty acid and generation of lyso-PC molecules, creates an alternative route of entry for the fatty acid. It is also possible that the transacylation of oleate from PC to cholesterol by LCAT [80], explains the reduced cellular incorporation of [^{3}H]PC-oleate.

In attempt to identify which lipase(s) are involved in PC uptake from HDL, we analyzed the role of HL in HDL-associated PC metabolism. HL is a secreted acylglycerol-hydrolase/phospholipase A_1 that regulates the selective uptake of HDL lipids [41–43] via a mechanism that may [81] or may not [82] involve SR-BI. Both lipolytic and non-lipolytic functions of HL have been described as being important to HDL metabolism [83]. Therefore, our experiments were designed to distinguish between the effects of HL-mediated lipolysis versus the non-enzymatic role of HL in HDL-associated PC uptake. First, hepatocytes from wildtype mice were incubated with Orlistat, an active site-directed inhibitor of lipases that blocks the activity of HL and other lipases [82,84]. Orlistat had no effect on HDL lipid uptake by hepatocytes (data not shown). By interactions with both lipoproteins and cell surface proteins, HL might be important in bridging HDL to the plasma membrane and facilitating lipid transfer [83]. To address this possible non-enzymatic role, we used HL-deficient mice. The absence of hepatic lipase did not alter the cellular incorporation of [^{3}H]CEt or [^{3}H]PC-choline, although a slight decrease for [^{3}H]PC-palmitate was observed. Thus, HL does not play a major role in uptake of PC from HDL.

We inhibited LCAT activity by incubating the cells with the classical inhibitor DTNB [80]. No effect of DTNB was detected (data not shown). Secreted PLA_2 has been proposed to play a significant role in enhancing SR-BI-mediated CE uptake from HDL [78]. Therefore, Me-Indoxam was utilized as a selective inhibitor of secreted PLA_2 [85]. The effect of Me-Indoxam on [^{3}H]CEt and [^{3}H]PC-choline uptake was striking, showing a respective 61% and 53% reduction in cellular incorporation. The significance of extracellular hydrolysis of HDL-associated PC is thus highlighted by these results and reveals a much more important role for secreted PLA_2 than initially anticipated.

4.4. HDL-derived PC can be metabolized to triacylglycerols in primary hepatocytes

Phospholipids associated with LDL and HDL are clearly more than just components of a lipoprotein framework. For example, HDL-derived PC can supply cells with polyunsaturated fatty acids required for eicosanoid synthesis [86]. Also, PC from LDL can be metabolized to hepatic TG [48]. Similarly, studies with [^{3}H]PC-oleate or [^{3}H]PC-palmitate demonstrated that 25–40% of HDL-derived PC can be converted to TG by hepatocytes. Less than 3% of [^{3}H]oleate was detected as [^{3}H]CE, indicating that neither LCAT nor acyl-CoA: cholesterol acyltransferase are significantly involved in HDL-PC hepatic metabolism.

CEs delivered to hepatocytes via HDL are hydrolyzed mainly in an extralysosomal compartment [58,59] although lysosomes might also be involved [87]. Chloroquine raises the intracellular pH, thus altering lysosomal function and perturbing endosomal/recycling pathways [88]. Moreover, chloroquine can affect the activity of lysosomal phospholipases [88,89] that have an optimal pH in the acidic range. In our experiments, chloroquine did not significantly alter [^{3}H] associated with PC. However, chloroquine treatment resulted in a small, but significant 25% decrease in [^{3}H]TG synthesis from HDL-PC. We believe that the lower [^{3}H] associated with TG is not caused by the disturbance in lysosomal function. Alterations in cell surface expression of HDL receptor(s) are probably responsible for the slightly lower levels of overall [^{3}H] radioactivity taken up by hepatocytes.

We propose that the vast majority of hepatic metabolism of HDL-associated PC occurs in extralysosomal compartments. When the cellular incorporation of HDL-associated [^{3}H]PC was examined in SR-BI$^{-/-}$ hepatocytes, the extent of inhibition seen in [^{3}H]PC-oleate uptake is very similar to that of [^{3}H]TG production. Thus, the conversion of HDL-associated [^{3}H]PC to [^{3}H]TG is independent of SR-BI. We speculate that SR-BI partially mediates the uptake of HDL-associated PC but subsequent metabolism of PC does not require SR-BI.

4.5. Hepatic metabolism of HDL-derived PC to TG involves a PC-PLC-mediated pathway

Our results indicate that hydrolysis of fatty acids from PC is not the major pathway involved in converting HDL-associated PC to hepatic TG. First, [^{3}H] radioactivity associated with free fatty acids and lyso-PC is almost negligible. Second, oleate supplementation did not affect the amount of [^{3}H] radioactivity recovered in TG. Unlabeled oleate should dilute the [^{3}H]oleate hydrolyzed by PLA_2 and thus result in reduced [^{3}H]TG, which we did not observe. Moreover, ATK, an inhibitor of cytosolic PLA_2, did not alter the intracellular metabolism of HDL-derived PC [90].

To study a possible role of phospholipase D in production of TG from HDL-derived PC, we measured the hepatic distribution of [^{3}H] oleate following incubation with 1-butanol. Butanol did not affect the metabolism of HDL-associated PC indicating that phospholipase D activity is not required for TG production from HDL-PC. The metabolic fate of LDL-PC in primary hepatocytes was shown to be phospholipase D-independent as well [48]. In addition, CHO cells can metabolize lipoprotein-associated PC and generate DG intermediates that are directly incorporated into TG [91].

Synthesis of TG from diacylglycerol generated by PLC is likely mediated by DGAT2 as a similar metabolic pathway has been confirmed for LDL-associated PC using siRNA-mediated gene silencing [48]. The involvement of PC-PLC was indicated by using the inhibitor D609 [64] that reduced significantly the incorporation of [^{3}H]oleate in hepatic TG. We tested the importance of PC-PLC by treating primary hepatocytes with rattlesnake venom extract, an activator of the enzyme [65]. The venom extract caused a 7-fold induction in TG synthesis, agreeing with our previous study with LDL-PC [48].

Finally, we can conclude that the conversion of HDL-derived PC to TG is mostly an energy-independent mechanism, as evidenced by the 40% decrease in [^{3}H]TG in deoxyglucose-treated cells, reflecting the 40% reduction seen in HDL-associated [^{3}H]PC-choline uptake. Incubating the cells at 4 °C caused a similar inhibition of TG synthesis as that observed for [^{3}H]PC cellular incorporation.

Approximately 60% of biliary PC is supplied by an extrahepatic source [92]. Since both biliary PC and HDL-PC contain mainly palmitate (16:0) at the sn-1 position and oleate (18:1) or linoleate (18:2) at the sn-2 position [93], it was proposed that HDL provides phospholipids for bile secretion [94]. In our study, we have discovered that PC delivered to primary murine hepatocytes via HDL can be utilized for TG synthesis. Interestingly, our laboratory has recently shown that lipid uptake from HDL is greater in the livers of mice lacking phosphatidylethanolamine *N*-methyltransferase, an enzyme involved in hepatic PC biosynthesis [95].

In summary, we have examined the mechanisms by which HDL-associated PC is taken up by the liver. We have demonstrated that SR-BI-mediated uptake pathway and HDL internalization both contribute to the hepatic incorporation of PC, along with extracellular remodeling of HDL phospholipids by secreted PLA_2 (Fig. 13). Our findings bring further insight into the metabolism of HDL-associated PC, an important molecule often overlooked in HDL metabolism. PC influences the interactions of HDL with plasma enzymes [38] and cell surface receptors [96], is necessary for adequate biliary sterol secretion [97] and participates in HDL formation along with ABCA1 [98,99].

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