Diet-induced lipid accumulation in phospholipid transfer protein-deficient mice: its atherogenicity and potential mechanism[S]

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Abstract A high saturated fat diet induces free cholesterol and phospholipid accumulation in the plasma of phospholipid transfer protein (*Pltp*)-deficient mice. In this study, we examined the atherogenic consequence of this phenomenon and investigated the possible mechanism(s). *Pltp* KO/*Apoe* KO mice that were fed a coconut oil-enriched high-fat diet (COD) for 7 weeks had higher plasma free cholesterol (149%), phospholipids (15%), and sphingomyelin (54%) than *Apoe* KO controls. In contrast to chow-fed animals, COD-fed *Pltp* KO/*Apoe* KO mice had the same atherosclerotic lesion size as that of *Apoe* KO mice. Similar to *Pltp* KO mice, plasma from COD-fed *Pltp* KO/*Apoe* KO mice contained VLDL/LDL-sized lamellar particles. Bile measurement indicated that COD-fed *Pltp* KO mice have 33% less hepatic cholesterol output than controls. In conclusion, COD-fed, *Pltp*-deficient mice are no longer protected from atherosclerosis and have impaired biliary lipid secretion, which is associated with free cholesterol and phospholipid accumulation.—Yeang, C., S. Qin, K. Chen, D. Q-H. Wang, and X-C. Jiang. **Diet-induced lipid accumulation in phospholipid transfer protein-deficient mice: its atherogenicity and potential mechanism. *J. Lipid Res.* 2010.** 51: **2993–3002.**

Supplementary key words saturated fat-based diet • free cholesterol • atherosclerosis • bile production

Phospholipid transfer protein (PLTP) belongs to a gene family of lipid transfer/lipopolysaccharide-binding proteins, which includes cholesteryl ester transfer protein (CETP), lipopolysaccharide binding protein (LBP), and bactericidal/permeability-increasing protein (BPI) (1). PLTP transfers phospholipids from lipid vesicles or VLDL to HDL in vitro and in vivo (2). PLTP can also mediate net transfer of free cholesterol (3). Moreover, PLTP has been implicated in hepatic apoB-containing particle secretion (4). Consistent with its role in lipoprotein metabolism, *Pltp* is regulated by liver X receptor (LXR) (5) and farnesoid X receptor (FXR) (6), which are regulators of bile metabolism. *Pltp* overexpression has been associated with increased cholesterol and phospholipids output via the bile (7).

PLTP deficiency in mice results in a large decrease in plasma lipid levels, including total cholesterol, cholesteryl ester, and phospholipids, when the mice are fed a chow diet (2, 8) or Western-type diet consisting of 20% milk fat plus 0.15% cholesterol (8). However, when fed a high-fat diet consisting of 20% saturated fat from coconut oil and 0.15% cholesterol (COD), *Pltp* knockout (KO) mice accumulate more free cholesterol and phospholipids than wild-type (WT) controls (8). On chow diet, *Pltp* KO/*Apoe* KO mice have significantly less atherosclerosis than *Apoe* KO controls (4).

The role of plasma total cholesterol in atherogenesis has been well established. However, plasma cholesterol exists in two forms: cholesteryl ester and free (unesterified) cholesterol. There is evidence suggesting that free cholesterol plays an important role in atherosclerosis. Free cholesterol has been observed in human atherosclerotic lesions, both intracellularly and extracellularly (9). Furthermore, free cholesterol accumulates more in severe lesions than in mild ones in the same aorta (10). In a cell culture model, free

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Abbreviations: Apo, apolipoprotein; COD, coconut oil-enriched high-fat diet; FPLC, fast-phase liquid chromatography; FXR, farnesoid X receptor; KO, knockout; LXR, liver X receptor; oxLDL, oxidized phospholipids on LDL; PLTP, phospholipid transfer protein; SR, scavenger receptor; WT, wild-type.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two figures and two tables.

cholesterol loading induces macrophage death (11), which is considered to be atherogenic in a late lesion due to ineffective clearance of necrotic cells (12). Moreover, free cholesterol loading of macrophages induces synthesis and secretion of proinflammatory and proatherogenic cytokines, such as TNF-α and IL-6 (13). In general, the in vivo consequences of plasma free cholesterol accumulation on atherosclerosis have not been well studied due to the scarcity of mouse models with elevated plasma free cholesterol. So far, only one mouse model, the scavenger receptor B-I (*Srb1*) KO mice, showed the proatherogenic consequence of free cholesterol accumulation in the blood (14).

Elevated plasma phospholipids, especially on apoB-containing lipoproteins, can be atherogenic as well. Phospholipids, particularly those containing poly-unsaturated fatty acids (PUFA), are susceptible to free-radical or enzymatic oxidation by enzymes such as myeloperoxidase and lypoxygenase in the arterial vessel wall. Oxidized phospholipids on LDL (oxLDL) activate endothelial cells, leading to recruitment of monocytes into the vessel intima (15). Once inside the vessel wall, oxLDL can be taken up by macrophages, transforming them into proinflammatory and proatherogenic foam cells. Beyond oxidized phospholipids, sphingomyelin, the second most abundant plasma phospholipid, is also atherogenic. Sphingomyelin on apoB-containing lipoproteins can be hydrolyzed by sphingomyelinase in the vessel intima, leading to aggregation and retention of these particles in the vessel wall (16).

In this study, we examined the atherogenic consequence in COD-fed *Pltp/Apoe* KO mice and explored possible mechanisms leading to COD-induced lipid accumulation. We found that COD-fed *Pltp*-deficient mice are no longer protected from atherosclerosis and have impaired biliary lipid secretion.

METHODS

Animals and diets

All mice used in this study were in the C57BL/6J genetic background. At 13 weeks of age, female *Pltp* KO/*Apoe* KO and *Apoe* KO mice were fed a high-fat diet enriched with coconut oil (see below) for 7 weeks and were assessed for atherosclerosis. At 14–16 weeks of age, female *Pltp* KO or wild-type (WT) mice were used in the mechanism study. These mice were fed the same high-fat diet for 2 weeks. All animal procedures were approved by the SUNY Downstate Medical Centre Animal Care and Use Committee.

Mice were fed chow diet (Purina Laboratory Rodent Chow 5001) or chow diet supplemented with 20% hydrogenated coconut oil (100% saturated fat) and 0.15% cholesterol (COD) (Research Diets Inc., New Brunswick, NJ).

Lipid measurements

Fasted blood was collected for lipoprotein isolation and lipid measurement. Plasma total cholesterol, free cholesterol, phospholipids, and lipoproteins were assayed by enzymatic methods (Wako). Sphingomyelin was measured by an established method (17). HDL lipid concentrations were determined after nonHDL lipoproteins were precipitated from plasma using a 10× solution of 1% dextran sulfate (50,000 MW) and 500 mM $MgCl_2$ (pH 7.0), as described in (18).

Fast phase liquid chromatography

Lipoprotein profiles were obtained by fast-phase liquid chromatography (FPLC) using two Sepharose 6B columns in tandem. A 250 μl aliquot of pooled plasma was loaded onto the columns and eluted with FPLC buffer (50 mM Tris) at a constant flow rate of 0.35 ml/min. An aliquot of 100 μl from each fraction was used for the determination of lipids.

Quantitative analysis of atherosclerosis

Atherosclerosis was determined as previously described (19). Briefly, *Pltp* KO/*Apoe* KO and *Apoe* KO mice were fed the high-fat diet for 7 weeks. The heart was fixed in 4% formaldehyde and then embedded in paraffin. The aortic root was sectioned into 7 μm thick slices and then stained with Harris's hematoxylin and eosin. Five sections from each aortic root, 30 μm apart from each other, were imaged and captured with a Nikon Labophot 2 microscope equipped with a SPOT RT3 color camera. The average area of these aortic root lesions from each animal was quantified using Image J software.

Electron microscopy

Negative-stain electron microscopy was performed as described in Refs. 20 and 2 with modifications. NonHDL lipoprotein particles isolated by FPLC and fractions were concentrated using centrifugal filter devices (Millipore). Following concentration, the solvent was changed to 125 mM ammonium acetate, 2.6 mM ammonium carbonate, and 0.26 mM EDTA (pH 7.4), and this solution was mixed with an equal volume of 2% phosphotungstic acid (pH 7.4). Then 10 μl of lipoprotein solution was applied to a carbon/Formvar-coated copper grid (Electron Microscopy Sciences). Excess fluid was removed with filter paper after 20 s, and the grid was viewed immediately.

Conjugated dienes formation

NonHDL lipoprotein particles were isolated by FPLC, and fractions were concentrated using centrifugal filter devices (Millipore). Lipoproteins were oxidized in the presence of 5 μM CuSO4, and the formation of conjugated dienes was monitored at 234 nm every 5 min over a 5 h period using an UV spectrophotometer (Beckman DU 530).

In vivo clearance of nonHDL free cholesterol

NonHDL lipoprotein was labeled with [^{3}H] free cholesterol in a two-step procedure. First, 2.5 ml of pooled plasma from *Pltp* KO mice was incubated with 100×10^6 cpm of [^{3}H] free cholesterol in the presence of the LCAT inhibitor DTNB (final concentration 5 mM) for 16 h at room temperature. Total cholesterol and free cholesterol were assayed from plasma before and after labeling to check for cholesterol esterification. Second, labeled nonHDL lipoproteins were separated from other plasma components by density centrifugation at 1.063 g/l. The supernatant, containing nonHDL lipoproteins, was dialyzed against PBS. Finally, 1 million cpm of [^{3}H] free cholesterol labeled nonHDL lipoprotein was injected into each of COD-fed *Pltp* KO and WT mice (n = 4 per group) via the retro-orbital plexus. Plasma was counted for radioactivity at 5, 15, 30 min and at 1, 2, 4, 8 and 24 h after injection. The label should be distributed evenly throughout the circulatory system at 5 min after injection. Therefore, cpm in plasma at 5 min was considered to be the starting cpm. All data was expressed as a percentage of the respective 5 min plasma cpm.

VLDL lipid secretion

Secretion of lipids from VLDL was measured as the accumulation of VLDL lipids in plasma after VLDL clearance was inhibited. *Pltp* KO and WT mice (n = 4 per group) were fed COD for

2 weeks and then fasted for 16 h. An inhibitor of lipoprotein lipase, P407, was utilized to inhibit VLDL clearance (21). Plasma lipids were measured before and at 1 and 2 h after P407 injection (1 g/kg) ip. Lipid secretion rates are expressed as fold change of plasma lipid levels after P407 treatment.

Bile flow and lipid analysis

Pltp KO and WT mice (n = 4 per group) were utilized for biliary lipid secretion studies according to an established method (22). Biliary cholesterol, phospholipids, and total and individual bile salts were determined as described previously (23).

Plasma lipoprotein gel electrophoresis

Fresh plasma was either prestained with Sudan Black B or unstained and then spotted onto an agarose gel (Titan Lipoprotein Gel, Helena Laboratories) and subjected to electrophoresis according to the manufacturer's instructions. Plasma was prestained as previously described (24). Briefly, 2 μl of a saturated solution of Sudan Black B in 1 part petroleum ether and 4 parts ethyl alcohol were mixed with 20 μl of plasma. The mixture was centrifuged at 10,000 rpm for 5 min to remove any precipitated Sudan Black. Unstained plasma resolved on agarose gel was stained for free cholesterol using filipin as previously described (25). The gel was first fixed for 1 h in TCA, then washed five times with PBS. Subsequently, the gel was incubated with 0.01% filipin in PBS with 1% dimethylformamide for 16 h. Filipin was visualized using a UV transilluminator.

Plasma total bile acid measurement

Plasma total bile acids were measured by the enzyme cycling method (Diazyme) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were tested by two-tailed *t*-test, assuming unequal variance. A *P* value of less than 0.05 was considered significant.

RESULTS

Pltp KO/*Apoe* KO mice have a proatherogenic plasma lipid profile when fed a coconut oil-enriched, high-fat diet

Plasma lipid analysis showed that on chow diet, *Pltp* deficiency in *Apoe* KO background significantly reduced total cholesterol, free cholesterol, total phospholipids, and sphingomyelin but not triglyceride levels (**Table 1**). These results confirmed what we had observed before (4). We also noticed that the ratio of total cholesterol to free cholesterol was not significantly different between *Pltp* KO/*Apoe* KO and *Apoe* KO mice (Table 1).

We next examined the effect of COD on plasma lipid levels in *Pltp* KO/*Apoe* KO and *Apoe KO* mice, as we observed that feeding COD significantly increased plasma free cholesterol levels in all *Pltp*-deficient mice (2, 8). In response to COD, there was a significant increase, instead of decrease, in plasma free cholesterol (149%), sphingomyelin (54%), and total phospholipids (15%) in *Pltp* KO/*Apoe* KO mice compared with *Apoe* KO mice (Table 1). More importantly, we noticed that the ratio of total cholesterol to free cholesterol was significantly ($P < 0.01$) reduced from 3.48 ± 0.56 to 1.73 ± 0.27 (Table 1), indicating that a significant amount of free cholesterol accumulated in the circulation.

Next, we determined the distribution of free cholesterol and sphingomyelin in lipoproteins from *Pltp* KO/*Apoe* KO mice and *Apoe* KO mice. Using FPLC, we resolved plasma lipoproteins into 50 fractions for free cholesterol and sphingomyelin measurements. This analysis revealed that COD (**Fig. 1B**), but not chow diet feeding (Fig. 1A), caused more free cholesterol to accumulate in nonHDL-sized lipoproteins in *Pltp* KO/*Apoe* KO mice compared with *Apoe* KO mice. This was also true for sphingomyelin levels (Fig. 1C, D).

Negative-stain electron microscopy of plasma lipoprotein particles

COD feeding causes accumulation of plasma free cholesterol and phospholipids on 40–50 nm-sized, lamellar-shaped particles in *Pltp* KO mice (26). Here we characterized nonHDL-sized plasma lipoproteins from COD-fed *Pltp* KO/*Apoe* and *Apoe* KO mice using electron microscopy. There were numerous lamellar-shaped particles in plasma from COD-fed *Pltp* KO/*Apoe* KO mice, whereas these particles were very scarce in plasma from COD-fed *Apoe* KO mice (**Fig. 2**).

TABLE 1. Plasma lipid levels in *Pltp* KO/*Apoe* KO and *Apoe* KO mice on chow diet and COD

Mice	TC	FC	CE	PL	SM	TG	TC/FC	SM/PL
				mg/dl				
Chow Diet								
Apoe KO	538 ± 85	182 ± 23	356 ± 74	560 ± 39	107 ± 23	80 ± 7	2.96 ± 0.32	0.19 ± 0.03
Pltp KO/*Apoe* KO	391 ± 51[b]	141 ± 17[a]	250 ± 11[a]	444 ± 49[b]	71 ± 11[a]	73 ± 18	2.77 ± 0.42	0.16 ± 0.02
% difference with *Apoe* KO on chow diet	−27.32	−22.53	−29.78	−20.71	−33.64	−8.75	−6.42	−16.31
COD								
Apoe KO	823 ± 83	236 ± 63	587 ± 50	661 ± 71	155 ± 19	80 ± 11	3.48 ± 0.56	0.23 ± 0.03
Pltp KO/*Apoe*	1019 ± 56[a]	588 ± 90[b]	431 ± 69[a]	760 ± 91[a]	239 ± 26[a]	89 ± 12	1.73 ± 0.27[b]	0.31 ± 0.04[a]
% difference with *Apoe* KO on COD	23.82	149.15	−26.58	14.98	54.19	11.25	−50.29	34.11

Values are mean ± SD; n = 12 per group.

Abbreviations: Apo, apolipoprotein; CE, cholesterol ester; COD, coconut oil-enriched high-fat, high-cholesterol diet; FC, free cholesterol; KO, knockout; PL, phospholipid; PLTP, phospholipid transfer protein; SM, sphingomyelin; TC, total cholesterol; TG, triglyceride.

[a] $P < 0.05$.

[b] $P < 0.01$.

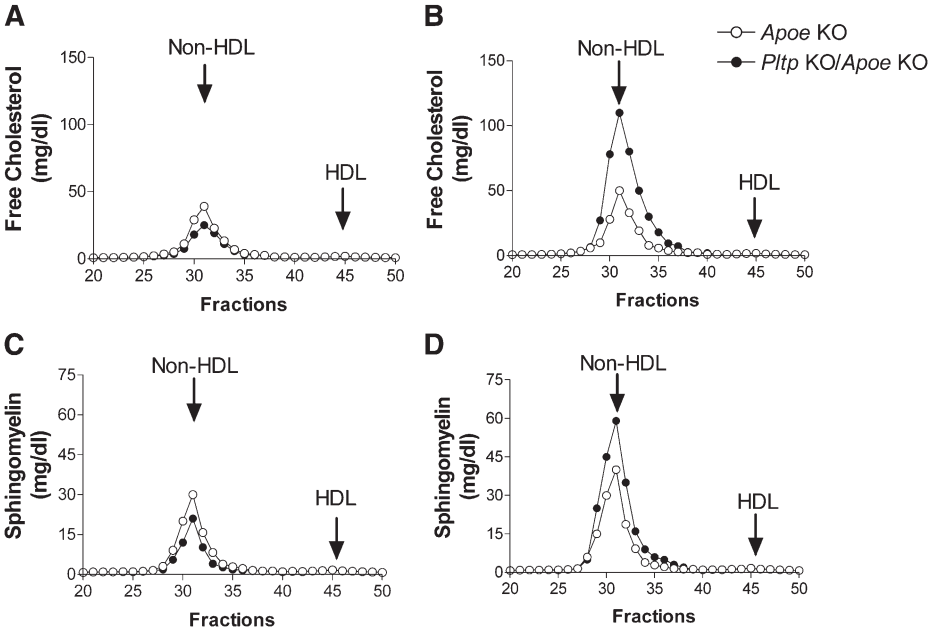

Fig. 1. *Pltp* KO/*Apoe* KO and *Apoe* KO mouse plasma lipid distributions measured by FPLC. A 250 μl aliquot of pooled plasma (from five animals) was loaded on two Sepharose 6B columns in tandem and eluted with 50 mM Tris, 0.15 M NaCl (pH 7.5). An aliquot of each fraction was used for the determination of cholesterol and sphingomyelin. A: Free cholesterol on chow. B: Free cholesterol on COD. C: Sphingomyelin on chow. D: Sphingomyelin on COD. Abbreviations: Apo, apolipoprotein; COD, coconut oil-enriched high-fat diet; FPLC, fast-phase liquid chromatography; KO, knockout; PLTP, phospholipid transfer protein.

Conjugated dienes formation

COD-fed *Pltp* KO/*Apoe* KO mice have elevated nonHDL plasma phospholipids (Table 1), which if oxidized, can become atherogenic. Therefore, we assayed for levels of oxidizable lipoproteins in these mice relative to controls. We isolated nonHDL particles from *Pltp* KO/*Apoe* and *Apoe* KO mice using FPLC and monitored conjugated diene formation during oxidation by copper sulfate (**Fig. 3**). On COD, nonHDL particles from *Pltp* KO/*Apoe* KO mice were more susceptible to oxidation than nonHDL particles from *Apoe* KO mice (Fig. 3).

Atherosclerosis in COD-fed *Pltp* KO/*Apoe* KO mice

It is known that on chow diet, *Pltp* KO/*Apoe* KO mice have less plasma cholesterol and less atherosclerotic lesion compared with *Apoe* KO mice (4). However, COD-fed *Pltp* KO/*Apoe* KO mice accumulated plasma nonHDL free cholesterol and sphingomyelin, which may promote atherogenesis. Moreover, nonHDL from COD-fed *Pltp* KO/*Apoe* KO mice were more susceptible to oxidation and, therefore, more atherogenic. We measured atherosclerotic lesions in COD-fed *Pltp* KO/*Apoe* and *Apoe* KO mice using two techniques: imaging the intact aortic arch (**Fig. 4A**, B) and examining tissue sections from the aortic root (Fig. 4C, D). We did not find significant differences in lesion size between two groups of animals (Fig. 4). These results showed that *Pltp* deficiency was no longer antiatherogenic in animals fed a coconut oil-enriched diet.

Potential mechanism(s) of free cholesterol accumulation in COD-fed *Pltp* KO mice

Feeding COD has been shown to cause nonHDL free cholesterol accumulation in multiple *Pltp*-deficient mouse models (8, 27), including *Pltp* KO/*Apoe* KO mice. More-

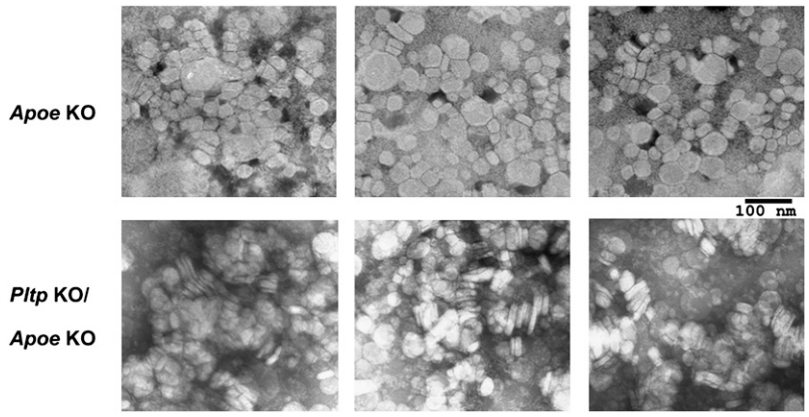

Fig. 2. Negative-stain electron microscopy. Pooled plasma from COD-fed *Apoe* KO and *Pltp* KO/*Apoe* KO mice (n = 3 per group) were fractionated by FPLC. Lipoproteins from nonHDL fractions were stained with phosphotungstic acid and viewed under electron microscope. Three representative fields of lipoproteins from *Apoe* KO (top panels) and *Pltp* KO/*Apoe* KO (bottom panels) are shown. Abbreviations: Apo, apolipoprotein; COD, coconut oil-enriched high-fat diet; FPLC, fast-phase liquid chromatography; KO, knockout; PLTP, phospholipid transfer protein.

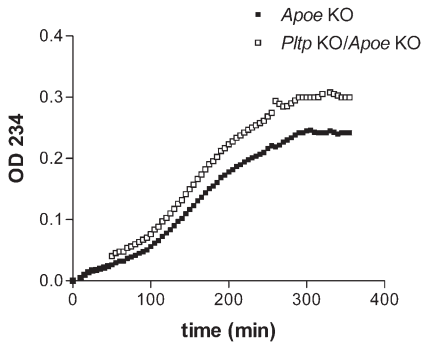

Fig. 3. Conjugated diene formation. Pooled plasma from COD-fed *Apoe* KO and *Pltp* KO/*Apoe* KO mice (n = 3 per group) were fractionated by FPLC. Lipoproteins from nonHDL fractions were subjected to 5 μM CuSO4-mediated oxidation. Conjugated diene formation was monitored in real time by absorbance at 234 nm. Abbreviations: Apo, apolipoprotein; COD, coconut oil-enriched high-fat diet; FPLC, fast-phase liquid chromatography; KO, knockout; PLTP, phospholipid transfer protein.

over, COD-fed *Pltp* KO (2, 26) mice and *Pltp* KO/*Apoe* KO mice accumulated nonHDL-sized, lamellar-shaped particles (Fig. 2). These findings suggest that mechanisms governing the COD-induced free cholesterol accumulation are common to *Pltp* KO and *Pltp* KO/*Apoe* KO mice. Therefore, we utilized the simpler and more generalizable *Pltp* KO mouse model to study how COD influences free cholesterol accumulation in *Pltp*-deficient mice. We confirmed that COD-fed *Pltp* KO mice have higher levels of free cholesterol than WT mice (supplementary Table I). In addition, we stained agarose gel-resolved plasma with filipin, a specific dye for free cholesterol, and found COD-fed nonHDL particles from *Pltp* KO mice stain more intensely compared with WT controls (**Fig. 5**). Therefore, we focused on the pathways relevant to the steady-state levels of nonHDL lipoprotein free cholesterol. First, we considered an increase in the clearance of nonHDL-particle free cholesterol in vivo as a potential mechanism for free cholesterol accumulation in the blood. We injected [^{3}H] cholesterol-nonHDL particles into *Pltp* KO and WT mice via the retro-orbital plexus, and [^{3}H] levels remaining in plasma were monitored over a course of 24 h. Interesting, this analysis demonstrated that COD-fed *Pltp* KO mice had slightly faster nonHDL-particle free cholesterol clearance rate than that of WT controls (**Fig. 6**).

Having ruled out nonHDL free cholesterol clearance as the potential mechanism, we next asked if the in vivo secretion of nonHDL lipoprotein free cholesterol from the liver was affected. The synthetic surfactant P-407 inhibits VLDL clearance from plasma (21) and is therefore a valuable tool for studying lipids of VLDL particles secreted from the liver. Although free cholesterol can also be secreted from the liver through ATP-binding cassette (ABC) transporters to HDL or ApoA1, HDL free cholesterol levels were not significantly changed in COD-fed *Pltp* KO mice compared with WT animals (8). Therefore, we be-

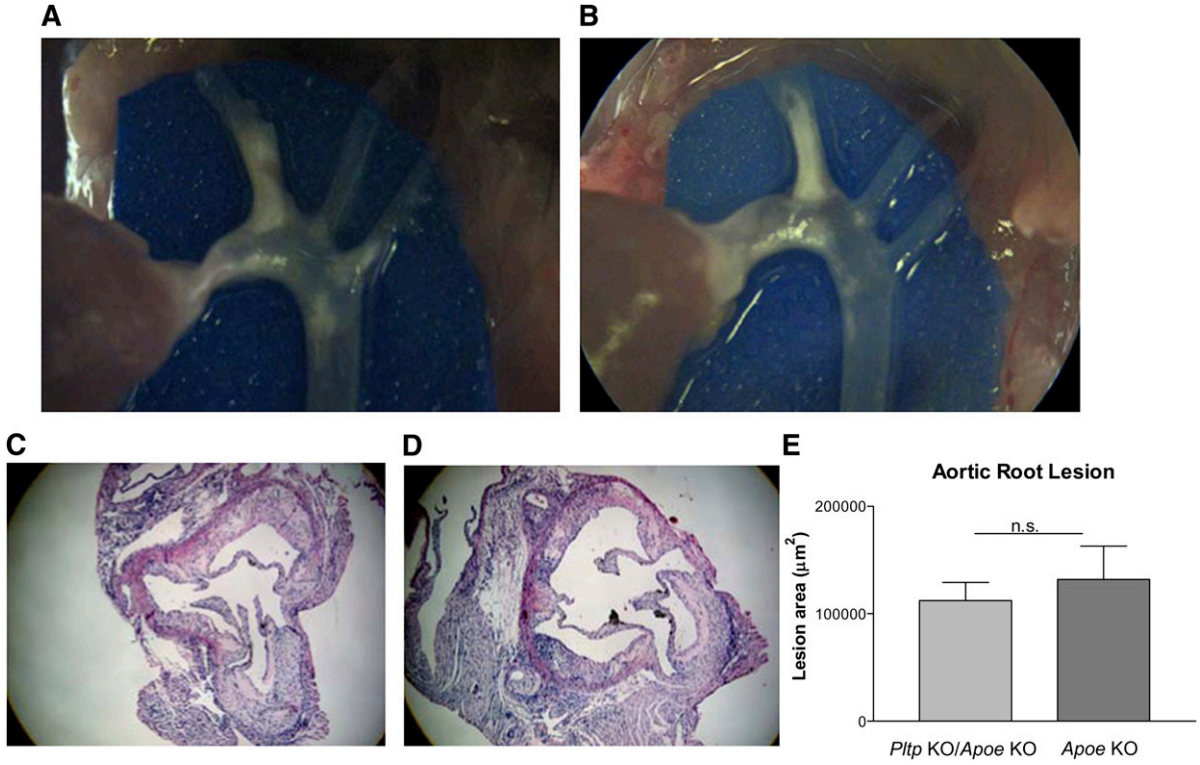

Fig. 4. Atherosclerosis in *Pltp* KO/*Apoe* KO and *Apoe* KO mice. Female mice were fed COD for 7 weeks starting at 13 weeks of age. Representative image shows lesion severity in the aortic arch of *Pltp* KO/*Apoe* KO (A) and *Apoe* KO (B) mice. Representative image of lesion size in aortic root sections by hematoxylin and eosin staining from *Pltp* KO/*Apoe* KO (C) and *Apoe* KO (D) mice. Summary of aortic root lesion size from these mice (n = 12 per group) fed COD (E). Values are mean ± SD. Abbreviations: Apo, apolipoprotein; COD, coconut oil-enriched high-fat diet; KO, knockout; PLTP, phospholipid transfer protein.

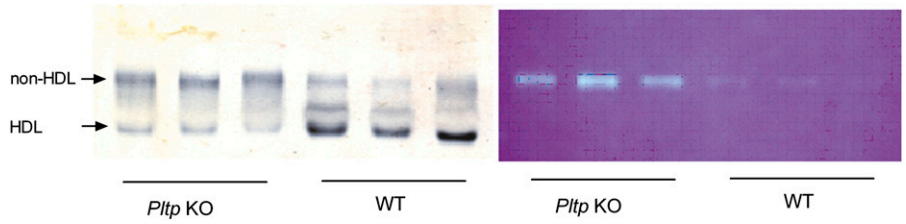

Fig. 5. COD-fed *Pltp* KO mice accumulate nonHDL free cholesterol. Plasma lipoproteins from COD-fed *Pltp* KO and WT mice (n = 3 per group) were resolved on agarose gel. Left panel: Plasma prestained with Sudan Black. Right panel: Unstained plasma probed with filipin (46). Abbreviations: COD, coconut oil-enriched high-fat diet; KO, knockout; PLTP, phospholipid transfer protein; WT, wild-type.

lieved that studying VLDL free cholesterol secretion could reflect the liver's contribution to plasma nonHDL lipoprotein free cholesterol concentrations. Mice were fasted for 16 h and then injected with P-407. Following the inhibitor administration, plasma lipids were measured at 1 and 2 h. We did not observe any significant increases in free cholesterol (**Fig. 7A**), total cholesterol (Fig. 7B), or phospholipid (Fig. 7C) secretion in *Pltp* KO versus WT mice. However, *Pltp* KO mice secreted less triglyceride than WT mice (Fig. 7D), consistent with a previous report on decreased ApoB secretion in *Pltp* KO mice (4).

After exhausting the obvious processes involved in free cholesterol metabolism, we further explored whether hepatic secretion of biliary free cholesterol was affected in COD-fed *Pltp* KO mice. Biliary secretion is a major pathway for removal of free cholesterol and phospholipids from the body. Patients with biliary cholestasis (28, 29) and mice having bile duct ligation (30, 31) experienced elevated levels of plasma free cholesterol and phospholipids. Therefore, we determined bile flow rates and hepatic outputs of biliary free cholesterol and phospholipids in COD-fed *Pltp* KO and WT mice.

Hepatic bile samples were collected at 1 and 2 h after a successful cannulation of the common bile duct, and bile flow rates were subsequently determined. Bile lipids from each time point were also measured. *Pltp* KO mice had a 42% lower bile flow rate compared with WT controls (**Fig. 8A**). Moreover, hepatic output of biliary cholesterol was significantly reduced by 33% in *Pltp* KO mice compared with WT controls (Fig. 8B). Biliary phospholipid output was also diminished by 25% in *Pltp* KO mice (Fig. 8C). These results indicate that in the COD-fed state, *Pltp* deficiency could reduce hepatic secretion of biliary cholesterol, which could contribute to the accumulation of plasma free cholesterol in these mice. However, there were no significant changes in lipid concentrations of hepatic biles between *Pltp* KO and WT mice (supplementary Table II). We also measured plasma total bile acids in these mice (**Fig. 9**). Feeding COD, compared with the chow diet, increased the levels of bile acids in plasma in both *Pltp* KO and WT mice. Although there was a trend for higher levels of plasma bile acids in COD-fed *Pltp* KO mice compared with WT controls, this difference was not statistically significant.

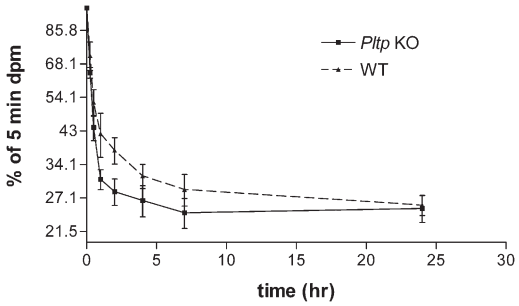

Fig. 6. Plasma free cholesterol clearance. One million dpm of [^{3}H] free cholesterol labeled nonHDL particles were injected (via retro-orbital plexus) into female KO or WT mice fed COD for 2 weeks, and dpm from plasma was measured at 5, 15, 30 min, and 1, 2, 4, 7, 24 h after injection. Data is represented as mean ± SD (n = 4 per group). Abbreviations: COD, coconut oil-enriched high-fat diet; KO, knockout; WT, wild-type.

DISCUSSION

In this study, we found that free cholesterol and phospholipid accumulation in plasma can overcome the antiatherogenic properties endowed by *Pltp* deficiency in *Apoe* KO mice. Moreover, we observed that under COD feeding conditions, hepatic secretion of biliary cholesterol is significantly reduced in *Pltp* KO mice, which could contribute to the accumulation of plasma free cholesterol in these mice.

Previously, we reported that on the chow diet, *Pltp* KO/*Apoe* KO mice have significantly less atherosclerotic lesions than *Apoe* KO mice, and we attributed this phenomenon to lower triglyceride-rich lipoprotein production, less lipoprotein oxidation, and anti-inflammation in *Pltp* KO/*Apoe* KO mice (4, 32). In this study, COD-fed *Pltp* KO/*Apoe* KO accumulate plasma free cholesterol and phospholipds. Under these conditions, *Pltp* deficiency offers no net protection against atherosclerosis.

NonHDL particles from COD-fed *Pltp* KO/*Apoe* KO mice were more susceptible to oxidation than the controls. Oxidized lipoproteins activate the vessel endothelium, induce foam cell formation, and promote atherosclerosis. When fed a chow diet, *Pltp* KO/*Apoe* KO mice have nonHDL particles that have more vitamin E and are less susceptible to oxidation than controls (32) However, when fed COD, it appears that *Pltp* KO/*Apoe* KO mice accumulate oxidizible phospholipids disproportionately to antioxidants on nonHDL lipoproteins, which may aggravate atherosclerosis.

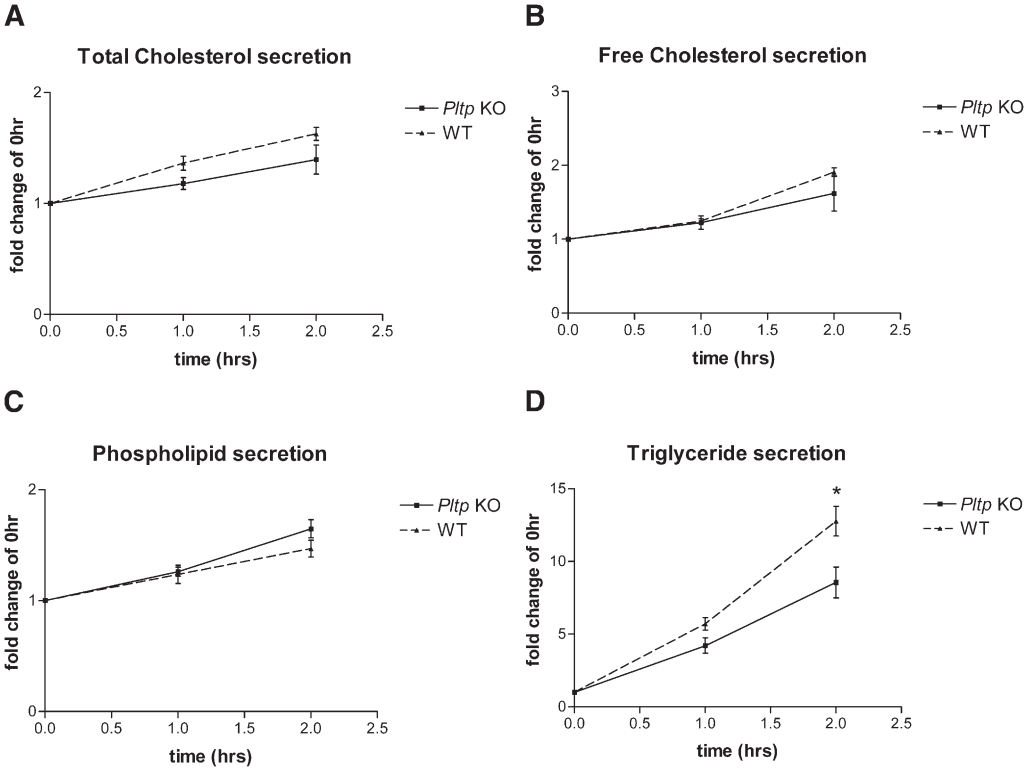

Fig. 7. Hepatic VLDL secretion. COD-fed female *Pltp* KO or WT mice were fasted for 16 h and then injected with P407 at 1g/kg ip. Free cholesterol (A), total cholesterol (B), phospholipid (C), and triglyceride (D) were measured before and after (1 and 2 h) P407 administration. Data is expressed as fold change in lipid concentration at the respective time point following P407 injection. Data is represented as mean ± SD (n = 4 per group). Abbreviations: COD, coconut oil-enriched high-fat diet; KO, knockout; PLTP, phospholipid transfer protein; WT, wild-type.

Free cholesterol is generally considered to be an atherogenic lipid. There is a body of evidence linking free cholesterol to cytotoxicity and inflammation, but there have been few animal models to test the in vivo consequences of elevated free cholesterol in plasma. Scavenger receptor B-I (SR-BI) deficiency accelerates plasma free cholesterol accumulation and leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in *Apoe* KO mice (14, 33). Lecithin:cholesterol acyltransferase (*Lcat*) deficiency decreases plasma total cholesterol levels, but it increases the free cholesterol/cholesteryl ester ratio with or without *Apoe*-deficient background (34). Reduced atherosclerosis was observed in the *Lcat* KO/*Apoe* KO mice (34). We have shown that COD-fed *Pltp* KO mice are an animal model with elevated plasma free cholesterol. Furthermore, free cholesterol accumulation in *Pltp* KO mice is associated with loss of protection against atherosclerosis.

In this study, we also observed that COD significantly increases nonHDL lipoprotein sphingomyelin, which may also promote atherogenesis. NonHDL lipoproteins are the major carriers of sphingomyelin (35). The ratio of sphingomyelin to phosphatidylcholine is increased by 5-fold in VLDL from hypercholesterolemic rabbits (36). We have found that plasma sphingomyelin levels in *Apoe* KO mice are 4-fold higher than those in WT mice (37), and this may contribute to increased atherosclerosis in the mice (38). We also found that human plasma sphingomyelin levels and sphingomyelin/phosphatidylcholine ratios are independent risk factors for coronary heart disease (39, 40). Furthermore, a sphingomyelin-enriched (1%) diet significantly increases plasma sphingomyelin levels, LDL aggregation, and atherosclerotic lesions in LDL-receptor KO mice (41). These data suggest that plasma sphingomyelin plays a critical role in the development of atherosclerosis.

We addressed the possible mechanisms involved in COD-induced free cholesterol, phospholipid, and sphingomyelin accumulation in *Pltp* KO mice. Given the role of SR-B1 in cholesterol metabolism and the increased plasma free cholesterol levels in *Srb1* KO mice, we assayed for liver SR-B1 levels and found no change between COD-fed *Pltp* KO/*Apoe* KO and control mice (supplementary Fig. I), Previously, we showed that COD-fed *Pltp* KO also have unaltered SR-B1 levels but might have dysfunctional SR-BI (8). We speculate that SR-B1 may also be dysfunctional in COD-fed *Pltp* KO/*Apoe* KO mice, which may at least partly explain the plasma free cholesterol accumulation. Separately, LCAT regulates plasma free cholesterol by converting free cholesterol into cholesteryl ester; therefore, LCAT activity should be relevant in COD-fed *Pltp*-deficient mice. However, in a previous study, we measured LCAT activity in COD-fed *Pltp* KO and WT mice and did

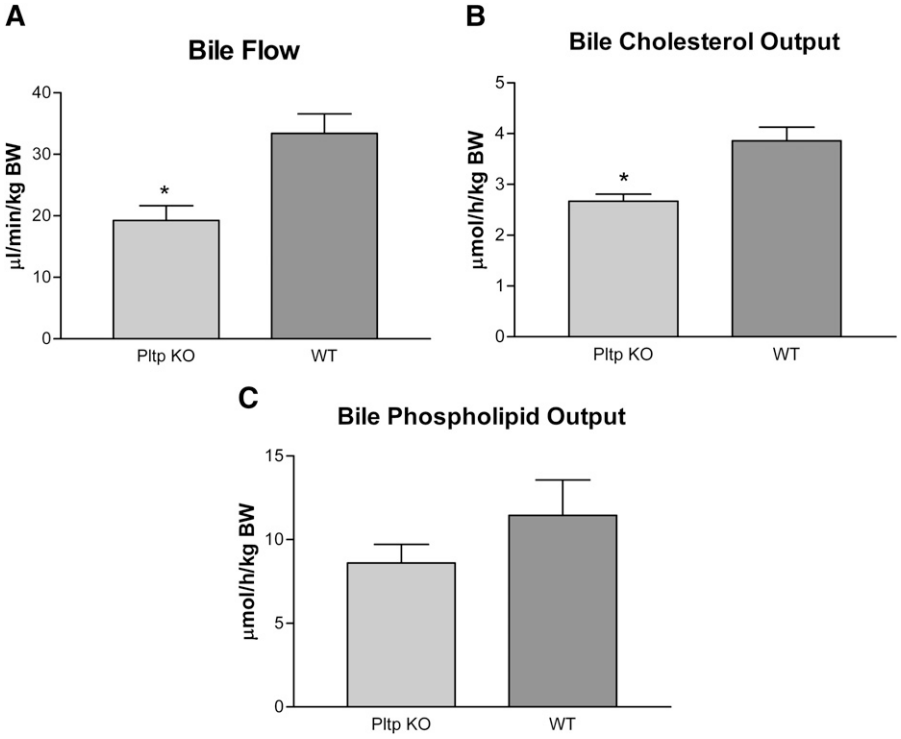

Fig. 8. COD-fed *Pltp* KO mice have decreased bile flow rates and biliary lipid outputs. The common bile ducts were cannulated in COD-fed *Pltp* KO and WT mice, and hepatic bile samples were collected at the first and second h of biliary washout. Compared with WT controls, COD-fed *Pltp* KO mice had a decrease in bile flow rate (A), biliary cholesterol output (B), and biliary phospholipid output (C). Data is represented as mean ± SD (n = 4 per group). $*P < 0.05$. Abbreviations: COD, coconut oil-enriched high-fat diet; KO, knockout; PLTP, phospholipid transfer protein; WT, wild-type.

not find significant differences (2). The liver and intestine are the major contributors of steady-state plasma lipid levels. Here we found that liver secretion of free cholesterol and phospholipids on VLDL was not different between COD-fed *Pltp*-deficient mice and WT controls. Furthermore, although changes in the levels of liver sphingomyelin synthase result in changes of plasma sphingomyelin (19), there was no change in liver sphingomyelin synthase (SMS) activity between COD-fed *Pltp* KO/*Apoe* KO mice and *Apoe* KO mice (supplementary Fig. II). Moreover, the mice used in these studies had been fasted, making intestinal absorption of free cholesterol from the diet a negligible factor.

In this study, we found that COD-fed *Pltp* KO mice have decreased hepatic outputs of biliary cholesterol and phospholipids. This is reminiscent of other scenarios in which biliary lipid outputs are impeded either through bile duct ligation (30, 31) or due to biliary cholestasis (28, 42), and plasma free cholesterol and phospholipids are consequently elevated. Under those circumstances, the excess free cholesterol and phospholipids circulate on an abnormal lipoprotein termed "lipoprotein X," which appear as lamellar structures under the electron microscope (42). Interesting, we have reported the existence of lamellar lipoproteins in *Pltp* KO mice fed COD (2), and we observed these particles in COD-fed *Pltp* KO/*Apoe* KO as well (Fig. 2).

The liver maintains free cholesterol homeostasis through esterification by ACAT, secretion into bile as free cholesterol or as bile acids after its conversion by liver enzymes, and secretion into the plasma via newly assembled VLDL lipoproteins or through plasma membrane bound ABC transporters. We observed that feeding COD results in accumulation of plasma free cholesterol and phospholipids on nonHDL particles in *Pltp* KO mice. KO mice do not secrete more free cholesterol via VLDL than WT controls. We propose that COD feeding impairs hepatic biliary secretion of free cholesterol in *Pltp* KO mice and

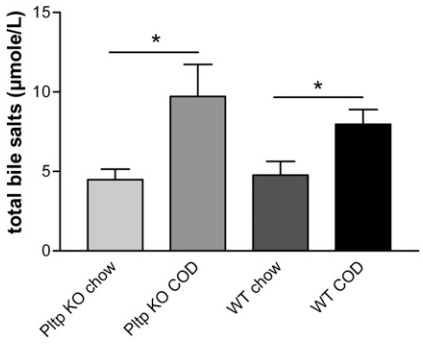

Fig. 9. Feeding COD increases plasma total bile acids. Total bile acids in fasting plasma were assayed in *Pltp* KO and WT mice (n = 5 per group), which were fed the chow diet or COD for 2 weeks. Data is represented as mean ± SD. $*P < 0.05$. Abbreviations: COD, coconut oil-enriched high-fat diet; KO, knockout; PLTP, phospholipid transfer protein; WT, wild-type.

results in secretion of free cholesterol on an abnormal lipoprotein into the plasma.

The role of *Pltp* in hepatic secretion of biliary lipids is an interesting topic which deserves more attention. *Pltp* is under the transcriptional control of FXR (6) and LXR (5), two important regulators of biliary lipid metabolism. *Pltp* overexpressing mice have higher biliary bile acid, cholesterol, and phospholipid outputs compared with WT controls (7). *Pltp* KO mice on the chow diet do not display altered biliary bile acid output (43). In this study, we show that *Pltp* KO mice on COD have a decrease in biliary cholesterol secretion and an increase in free cholesterol in plasma. Furthermore, phosphatidylcholine transfer protein (PC-TP), another protein which mediates transfer of phosphatidylcholine, has been implicated in biliary lipid secretion. *Pc-tp*-deficient mice have impaired biliary lipid secretion when fed a high-fat, high-cholesterol diet but not when fed a chow diet (44). Although PLTP is generally thought to function in the circulation, there is evidence for an intracellular role of PLTP (4, 45). Perhaps phospholipid transfer, by PLTP or PC-TP, becomes important for biliary lipid secretion when redundant factors are overwhelmed by certain high-fat diets.

In summary, COD-fed *Pltp* KO mice are no longer protected from atherosclerosis and have impaired biliary lipid secretion. These findings emphasize the complexity and importance of the interplay between diet and genetics on atherogenesis.

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