The Role of Phospholipid Transfer Protein (PLTP)

in Lipoprotein Metabolism and Atherosclerosis

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Table of content

Abbreviations

PLTP	**phspholipid transfer protein**
LDL	**low density lipoprotein**
HDL	**high density lipoprotein**
TG	**triglyceride**
NPC1l1	**Niemann-Pick C1 like 1**
ABCG5/G8	**ATP-binding cassette transporter G5/G8**
ABCA1	**ATP-binding cassette transporter A1**
MTP	**microsomal lipid transfer protein**
Apo	**apolipoprotein**
WT	**wild type**
KO	**gene knockout**
LXR	**liver X receptor**

Abstract

Plasma phospholipids transfer protein (PLTP) is a member of the lipid transfer/lipopolysaccharide binding protein gene family. PLTP is an independent risk factor for coronary artery disease. PLTP deficiency decreases and PLTP overexpression increases atherosclerosis in mouse models. Therefore, PLTP is considered as a potential target for pharmacological or gene therapy. However, this is hampered by the fact that the mechanism by which PLTP is atherogenic is not completely understood. PLTP is a multi-functional protein that is expressed in a variety of tissues. Some effects of PLTP are considered atherogenic, while others are thought to be anti-atherogenic. In order to unravel the mechanisms, we have utilized two approaches to study the impact of PLTP deficiency on lipid metabolism and atherosclerosis.

First, we explored the role of PLTP deficiency in macrophages, in terms of lipids metabolism and atherosclerosis. It is known that formation of macrophage-derived foam cells (which highly express PLTP as well as apoE, a well-known anti-atherogneic apolipoprotein) is the critical step in the process of atherosclerosis. To study the relationship between PLTP and apoE in macrophages, we transplanted PLTP-deficient mouse bone marrow into apoE-deficient mice, creating a mouse model with PLTP deficiency, and apoE expression exclusively in the macrophages. We found that macrophage PLTP deficiency significantly decreased PLTP activity, compared with controls. Moreover, macrophage-produced plasma apoE was significantly decreased in apoE-deficient recipients that received PLTP-deficient bone marrow relative to apoE-deficient recipients that received

PLTP expressing bone marrow. On a western type diet, macrophage PLTP deficiency increased plasma cholesterol and phospholipid, mainly on non-HDL particles, thus increasing atherosclerotic lesions in the aortic arch and root, as well as the entire aorta. These results provided novel information concerning the effect of PLTP on apoE secretion in macrophages and its atherogenic consequences.

Second, we investigated the role of PLTP deficiency in cholesterol absorption in small intestine, a process closely related to plasma cholesterol levels and atherosclerosis. We show that the absorption of a bolus of radiolabeled cholesterol (short-term) but not triglyceride in PLTP KO mice was significantly lower than that of wild type (WT) mice. Also, cholesterol transport to the plasma, small intestine, and liver was significantly lower in PLTP KO mice than that of WT ones. Long-term studies involving multiple feedings of radiolabeled lipids also showed significant reduction of cholesterol but not triglyceride absorption and transport to the plasma, small intestine, and liver in PLTP KO mice, compared to WT ones. Moreover, we found that, compared to WT mice, PLTP KO primary enterocytes secrete and absorb significant less cholesterol, Thus, PLTP KO mouse small intestine secretes less and accumulates more cholesterol, which, in turn, inhibit cholesterol absorption and this may provide a new mechanism for the reduction of atherosclerotic lesions in these mice.

Chapter 1

Background and Significance

PLTP Characteristics Initially, it has been suggested that there might be transfer of surface components (phospholipids, soluble apolipoproteins) from apoB-containing lipoprotein particle (BLp) into high density lipoprotein (HDL) (1, 2). This was subsequently demonstrated by injecting or perfusing of chylomicrons or VLDL in intact rats or their organs (3-6). In humans, Schaefer et al (7) showed transfer of radiolabeled apoAI from BLp into HDL (7). It was thought that phospholipids (PL) transfer into HDL might involve the dissociation of discrete surface remnants, followed by the action of LCAT on these particles, or spontaneous incorporation of PL into HDL (8). However, Tall et al (9) characterized a novel plasma PL transfer activity and showed that it stimulated the transfer and exchange of PL between VLDL and HDL, but did not facilitate cholesteryl ester (CE) and triglyceride (TG) transfer. Although cholesteryl ester transfer protein (CETP) also has the ability to stimulate PL exchange between different lipoprotein classes, studies of the plasma of subjects with complete CETP deficiency showed that at least 50% of this activity was due to a distinct gene product (10). Subsequently, Albers and co-workers purified and cloned the PLTP (11). PLTP is expressed ubiquitously (11-12). The highest expression levels in human tissues were observed in ovary, thymus, placenta and lung (11). Taking into account the organ size, liver, adipose tissue (12) and small intestine (Jiang, X.C. unpublished results) also appear to be important sites of PLTP expression. Recently, it is shown that PLTP is highly expressed in

macrophages in atherosclerotic lesions (13-15).

The relationship between PLTP structure and function is far from resolved. However, some progress has been made. PLTP belongs to a family of lipid transfer/lipopolysaccharide binding proteins including lipopolysaccharide binding protein (LBP), bactericidal/permeability increasing protein (BPI) and CETP (16). These proteins show sequence homology, related gene structures and probably share a similar protein fold (16-17). Despite the fairly low level of amino acid sequence identity between the family members (20%-26%) (16) certain common structural characteristics, as well as similar gene organization (18-21) indicate functional relatedness and suggest a common evolutionary origin of these proteins.

In terms of lipid transfer activity, PLTP has its own characteristics. PLTP has no neutral lipid transfer activity (22-25). PLTP circulates bound to HDL and mediates the net transfer of phospholipids between unilamellar vesicles into HDL and also the exchange of phospholipids between lipoproteins. The net transfer of phospholipids into HDL results in the formation of larger, less dense species (25). In human plasma, PLTP is responsible for about half of the activity mediating phospholipids exchanges between lipoproteins (22, 25), and CETP accounts for the other half (22, 25). Plasma PLTP is also a non-specific lipid transfer protein. Several studies have indicated that PLTP is capable of transferring all common phospholipids (23-25). Besides phospholipids, diacylglycerol, α-tocopherol, cerebrodide and lipopolysaccharides are also transferred efficiently (26). Although CETP also can transfer phospholipids, there is no redundancy in functions of PLTP and CETP in mouse model (27). Two forms of PLTP exist in human plasma, one

catalytically active and the other inactive (28-29). The relationship between the two forms is unknown.

PLTP regulation PLTP activity and mRNA can be regulated by many factors. A high fat, high cholesterol diet caused a significant increase in PLTP activity and its mRNA levels (12). After lipopolysaccharide injection, plasma PLTP activity was significantly decreased. This decrease in activity was associated with a similar decrease in PLTP mRNA levels in the liver and adipose tissue (12). PLTP expression and activity can be upregulated by glucose (30) and downregulated by insulin (31-32). It is reported that diacylglyceride can also regulate PLTP activity (33).

PLTP promoter shows no strong homology to known steroid response elements (SREs), but contains farnesoid X-activated receptor (FXR) and peroxisome proliferators-activated receptor (PPAR) binding motifs. The promoters of human and mouse PLTP genes show 5 consensus sequences for the transcription factors Sp1 and AP2 that are necessary for PLTP transcription (34-35). The transcriptional activity of PLTP gene was significantly increased by chenodeoxycholic acid and fenofibrate, suggesting that FXR and PPAR are likely involved in the process (36). We (15) and another group (37) independently showed that PLTP expression can also be upregulated by liver X receptor (LXR). The PLTP promoter contains a high-affinity LXR response element that is bound by LXR/RXR heterodimers in vitro and is activated by LXR/RXR in transient-transfection studies (37).

The effect of PLTP overexpression on HDL metabolism. The transgenic mice expressing moderate levels (~30% increase) of human PLTP do not exhibit marked changes in lipoprotein metabolism, whereas the PLTP transgenic mice expressing human apo A-I showed a significant increase in the plasma levels of ß-HDL and preß-HDL (38-39). The little changes in lipoprotein distribution in the PLTP transgenic mice may be explained by the substantial levels of PLTP that are already present in control animals, but these data suggest that preß -HDL particles are generated by PLTP reaction. Overexpression of PLTP in mice was achieved by adenovirus and adenovirus associated virus mediated infection. The former resulted in a 10- to 40-fold increase in plasma PLTP activity (40-41). These mice were characterized by the increased preß-HDL levels, but decreased ß-HDL levels due to an increased fractional catabolic rate of HDL, and enhanced hepatic uptake of HDL-CE compared with the wild type (WT) mice, suggesting the role of PLTP in stimulating reverse cholesterol transport *in vivo.* PLTP expression mediated by adenovirus-associated virus showed a prolong overexpression pattern and resulted in a significant decrease of total cholesterol and HDL cholesterol in C57BL/6 mice (42). Transgenic mice that overexpress human PLTP at high levels were also generated. Compared with WT mice, these mice show a 2.5- to 4.5- fold increase in PLTP activity in plasma. This results in a 30% to 40% reduction of plasma HDL cholesterol levels. Incubation of plasma from transgenic animals at 37^0C reveals a 2- to 3-fold increase in the formation of preß-HDL compared with plasma from WT

mice (43). Overall, PLTP overexpression causes significant reduction of HDL levels in the circulation.

The effect of PLTP deficiency on HDL metabolism So far there is no PLTP deficiency found in human. The allelic frequencies of six intragenic polymorphisms and two polymorphisms located in the immediate vicinity of the PLTP gene were determined and there were no significant associations between these polymorphisms and plasma PLTP activity (44). The most informative knowledge about PLTP deficiency was obtained from PLTP gene knock out (KO) mice (15). PLTP KO mice showed complete loss of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM), and partial loss of free cholesterol (FC) transfer activities. Moreover, the *in vivo* transfer of [^{3}H] phosphatidylcholine ether from VLDL to HDL was abolished in PLTP KO mice. On a chow diet, the mice showed a marked decrease in HDL PL, FC and apoA-I, demonstrating the important role of PLTP-mediated transfer of surface components of triglyceride-rich lipoprotein in the maintenance of HDL levels (45). Furthermore, the HDL of the PLTP KO was enriched with protein and poor in PC, and turnover studies showed a 4-fold increase in the catabolism of HDL protein and CE compared to that of WT (46). Thus, the impairment of PL transfer from triglyceride rich lipoprotein into HDL may lead to hypoalphalipoproteinemia characterized by hypercatabolism of HDL particles.

PLTP and atherosclerosis The physiological and pathological roles of PLTP in atherosclerosis development are still lacking. In patients with coronary heart

disease, a positive correlation was found between PLTP activity and HDL particles containing apo A-I but not apo A-II (47). In our own study, we found that coronary artery disease (CAD) patients with the highest quintile of PLTP activity had a 1.9-fold increase in risk compared to the lowest quintile (48). On the other hand, PLTP mass has been shown as a possible protective factor for CAD (49). The limitation of this study is the small number of CAD cases due to the short follow up and the PLTP mass measurement might not reflect the real situation, since the major form of PLTP in the circulation is an inactive one (28-29). In terms of plasma HDL cholesterol levels, the existing reports are contradictory. In one set of studies, plasma PLTP activity was positively related to HDL cholesterol (47). However, in another study, it is reported that the activity of PLTP in human plasma was negatively correlated with HDL cholesterol and apoA-I (50).

Recently, immunoreactive PLTP was found in histological sections of human carotid artery (13). Immunoreactive PLTP colocalized with CD-68 positive macrophages, suggesting its production *in situ.* Synthesis of PLTP was further demonstrated in cultured macrophages, as was its upregulation by acetylated LDL (37). In sections of human coronary arteries, PLTP immunostaining was seen in plaques colocalizing with macrophages and smooth muscle foam cells (14). Moreover, in the atherosclerotic segments, PLTP accumulated in extracellular matrix, colocalizing with apo A-I, apo E and biglycan. These findings suggested that PLTP might promote the binding of HDL_3 to biglycan (14). PLTP enhances cell-surface binding and remodeling of HDL to improve its ability to promote cholesterol and phospholipid efflux (51).

Genetic mouse models have played a crucial role in elucidating the role of PLTP in atherosclerosis. Lie et al reported that overexpression of PLTP in mice with LDL receptor deficient background causes a dose-dependent increase of atherosclerotic lesion (52). The mechanism might be due to 1) significant reduction of HDL levels; and 2) a moderate stimulation of VLDL secretion (</=1.5-fold) (52-53). PLTP-deficient mice provided *in vivo* evidence for PLTP-mediated lipid transfer in the maintenance of lipoprotein levels (45) and in modulating the development of atherosclerosis. PLTP deficiency resulted in markedly decreased atherosclerosis (54) attributable in part to 1) decreased production and levels of BLp (54), 2) increased bioavailability of vitamin E in atherogenic lipoproteins (55), and 3) improved the anti-inflammatory properties of HDL in mice and reduced the ability of LDL to induce monocyte chemotactic activity (46).

Cholesterol accumulation in macrophage and atherosclerosis*:* A critical event in the developing atherosclerotic lesion is the entry of monocytes into the subendothelium of focal areas of the arterial wall (56). After entry, the monocytes are exposed to endothelium-derived growth and differentiation factors, such as macrophage-colony stimulating factor (M-CSF), leading to their differentiation into macrophages. Macrophages express several scavenger receptors that are capable of taking up oxidized LDL (57-58), an atherogenic form of LDL, in an unrestricted fashion. The oxidized LDL taken up by macrophage scavenger receptors is delivered to lysosomes, where its cholesterol ester content is hydrolyzed to free cholesterol (FC) and fatty acids. FC loading in macrophages is cytotoxic and thus

FC-induced toxicity may be an important cause of lesional macrophage death (59-60). Excess FC is subjected to esterification to form cholesterol ester (CE), which is important because the CE resulting from this reaction is the major lipid that accumulates in lesional foam cells. (61-62). The development of macrophage "foam cells" that contain massive amounts of cholesterol esters is a hallmark of atherosclerotic lesions and foam cell formation is the critical step in atherogenesis. Since macrophages highly express PLTP (15) and PLTP activity is involved in phospholipids and cholesterol metabolism (63, 64, 40-43, 52), it is conceivable that PLTP should play a role in macrophage lipid accumulation, thus influencing the process of foam cell formation.

ApoE and atherosclerosis: ApoE is a glycoprotein with a molecular size of approximately 34KD. It is mainly synthesized in the liver and brain. Macrophages produced about 10% of the apoE. ApoE is a structural component of all lipoprotein particles except low density lipoprotein. It functions as a high affinity ligand for apoB and apoE receptors and chylomicron-remnant receptor in the liver, thereby allowing the specific uptake of apoE-containing particles by the liver. ApoE knockout mice are severely hypercholesterolaemic compared to the wild-type counterparts (65, 66) On chow diet, apoE knockout animals have plasma cholesterol levels of about 500 mg/dl compared with about 100 mg/dl in control animals, and when challenged with a high fat Western-type diet, these animals have plasma cholesterol levels of about 1800 mg/dl compared with about 200 mg/dl in controls. (66). The accumulation of cholesterol-rich lipoproteins leads to the development of complex atherosclerotic

lesions, even when the mice are fed a chow diet. (66) ApoE knockout mice have become the most commonly used atherosclerotic model. Several lines of studies demonstrated that atherosclerosis in apoE knockout mice are prevented by increasing circulating apoE levels through either recombinant adenovirus-mediated gene transfer to the liver (67, 68), or injection of synthetic apoE peptide mimics (69). Monocytes/macrophages are the only haematopoietic cells that express apoE, therefore bone marrow transplantation has been used to investigate the role of macrophage-derived apoE on atherosclerosis in vivo. Researches have revealed that macrophage-derived apoE makes a contribution to the plasma apoE levels, which in turn influence plasma cholesterol levels and atherogenesis (70-72**).**

PLTP and apoE: Although PLTP and apoE are deeply involved in lipid and lipoprotein metabolism the relationship between PLTP and apoE was not well established. It has been reported that PLTP is associated with apoE in human cerebrospinal fluid, and that exogenous addition of recombinant PLTP to primary human astrocytes significantly increases apoE secretion to the conditioned medium (73**)** It has been also reported that PLTP secreted from HepG2 cells is associated with apoE but not apoA-I (74). These phenomena may also exist in macrophages, thus playing a role in cholesterol metabolism and the process of atherosclerosis. Two forms of PLTP are present in plasma: one with high activity (HA-PLTP) and the other with low activity (LA-PLTP). (75). HA-PLTP co-purifies with apolipoprotein E (apoE), whereas LA-PLTP is associated with apoA-I (76). It was reported that apoE activates the low-activity form of human PLTP (77). However more recently,

another group reported that PLTP activity in plasma was not changed after apoE was removed from plasma by immunoadsorbance of apoE. (78)

Cholesterol absorption in small intestine: Epidemiological studies have clearly established a direct relationship between plasma cholesterol level and risk of coronary artery disease. Plasma cholesterol is derived from two sources, endogenous (hepatic and extra-hepatic synthesis of cholesterol) and exogenous (intestinal absorption of dietary and biliary cholesterol). Pharmacological approaches to inhibit cholesterol absorption are intriguing. There are three main steps in intestinal cholesterol absorption. The first step occurs in the lumen of small intestine. Cholesterol ester is hydrolyzed to free cholesterol by pancreatic enzymes. Free cholesterol, which is only minimally soluble in aqueous environment (79-80), needs to be partitioned into bile salt micelles prior to its transport to the brush border membranes where it can be absorbed into the mucosa. The second step is the uptake of free cholesterol by enterocytes. The mechanism by which micellar cholesterol are taken up by enterocytes is still enigmatic and being actively investigated. A recent study (81) revealed that mice with targeted inactivation of the Niemann-Pick C-1 like-1 (NPC1L1) gene reduced cholesterol absorption and that the NPC1L1 mRNA expression in the intestine parallels the efficiency of cholesterol absorption along the gastrocolic axis, with the highest levels of NPC1L1 expression and cholesterol absorption observed in the proximal intestine and little or no NPC1L1 expression and cholesterol absorption observed in the ileum, suggesting that NPC1L1 plays a key role in this process. The third step is the assembly of cholesterol and cholesterol ester with lipoproteins in enterocytes. Free cholesterol

is esterified to cholesteryl ester in endoplasmic reticulum. The newly synthesized cholesteryl esters are transferred to nascent apolipoprotein B48 via the action of the microsomal triglyceride transfer protein (MTP) (82). Finally cholesterol esters are secreted to lymph then to the blood stream in the form of chylomicrons.

Cholesterol transport across the intestinal epithelial cells has been shown to involve apoB-dependent and apoAI-dependent pathways (83). It has been shown that enterocytes isolated from mice and monkeys also secrete cholesterol by two pathways (84). Cholesterol secretion by apoAI deficient enterocytes via apoB-dependent pathways was unaffected but apoAI-dependent pathway was significantly reduced. Supplementation of purified apoAI increased cholesterol secretion by these enterocytes indicating that the efflux pathway was functional. Acute studies revealed that cholesterol absorption was significantly reduced and was occurring over longer lengths of the intestine in the apoAI KO mice compared to controls (84). Since PLTP activity influences both apoB and apoAI production (40-43), it is conceivable that PLTP should play roles in apoB-dependent and apoAI-dependent pathways in cholesterol absorption in small intestine.

Significance: One highly important implication of our work is the discovery that plasma PLTP is an independent risk factor for CAD (85), and that inhibiting PLTP activity may have therapeutic value for the treatment of atherosclerosis. Although presently known risk factors have some predictive value for CAD, a major part of the variability in this process remains unexplained (86). In addition, therapy aimed at lowering LDL cholesterol only reduces a fraction (roughly 30%) of the burden of

atherosclerotic disease (87).

In the current studies, I have utilized PLTP knockout mice to investigate the role of macrophage PLTP in the development of atherosclerosis and the effect of PLTP in cholesterol absorption in small intestine. It has been anticipated that the proposed experiments in this thesis should provide a test of the hypothesis that PLTP has atherosclerosis-related properties, and that these studies should provide insights into the underlying mechanisms. This in turn could suggest novel antiatherogenic therapies for clinical use.

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Chapter 2

Macrophage Phospholipid Transfer Protein Deficiency and ApoE Secretion: Impact on Mouse Plasma Cholesterol Levels and Atherosclerosis

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Running title: macrophage PLTP deficiency, apoE secretion and atherosclerosis

Key Words: Phospholipid transfer protein; apoE; bone marrow transplantation; macrophage; lipoprotein; atherosclerosis

Objective--Phospholipid transfer protein (PLTP) and apolipoprotein E (apoE) play important roles in lipoprotein metabolism and atherosclerosis. It is known that formation of macrophage-derived foam cells (which highly express PLTP and apoE) is the critical step in the process of atherosclerosis. We investigated the relationship between PLTP and apoE in macrophages and the atherogenic relevance in a mouse model.

Methods and Results--We transplanted PLTP-deficient mouse bone marrow into apoE-deficient mice, creating a mouse model with PLTP deficiency and apoE expression exclusively in the macrophages. We found that macrophage PLTP deficiency significantly decreased PLTP activity, compared with controls (20%, $P<0.01$). Moreover, plasma apoE derived from macrophages was significantly decreased in apoE-deficient recipients that received PLTP-deficient bone marrow relative to apoE-deficient recipients that received PLTP expressing bone marrow (73%, $P<0.001$). On a western type diet, macrophage PLTP deficiency increased plasma cholesterol (124%, $P<0.001$) and phospholipid (152%, $P<0.001$), mainly on non-HDL particles, thus increasing atherosclerotic lesions in the aortic arch and root (403%, $P<0.001$), as well as the entire aorta (298%, $P<0.001$).

Conclusions--Macrophage PLTP deficiency causes a significant reduction of apoE secretion from the cells, and this in turn promotes the accumulation of cholesterol in the circulation and accelerates the development of atherosclerosis.

Condensed Abstract

Macrophage PLTP deficiency causes a significant reduction of apoE secretion from the cells, and this in turn promotes the accumulation of cholesterol in the circulation and accelerates the development of atherosclerosis.

Plasma phospholipid transfer protein (PLTP) is a member of the lipid transfer/lipopolysaccharide binding protein gene family (1). PLTP is known to be an independent risk factor for coronary artery disease (2), and is significantly increased in obesity, as well as in type I and II diabetes (3,4). Moreover, PLTP deficiency decreases (5), and PLTP overexpression increases (6,7), atherosclerosis in mouse models, so that it is considered a potential target for pharmacologic or gene therapy. However, research toward this goal is hampered by the fact that the mechanism of PLTP's atherogenicity is not completely understood. This is a multi-functional protein that is expressed in a variety of tissues, with some of its effects considered proatherogenic (8,9), and others antiatherogenic (10-12).

Apolipoprotein (Apo) E is a multifunctional protein that is synthesized by the liver and several peripheral tissues and cell types (13,14). ApoE is to serve as a ligand for receptor-mediated uptake of lipoproteins through the LDL receptor, the LDL receptor-related protein, and heparan sulfate proteoglycans (15,16). ApoE also plays a key role in intracellular lipid metabolism, influencing processes such as the assembly and secretion of lipoproteins (17,18), and cholesterol efflux to HDL (19).

The relationship between PLTP and apoE is mostly unknown. There are two forms of PLTP that exist in human plasma, one catalytically active and the other inactive (20). Of the circulating PLTP mass only a minor portion is in the active form in normolipidemic subjects (20). It has been reported that active PLTP in plasma is associated with apoE but not with apoA-I (21) and apoE proteoliposomes can convert inactive PLTP into active one (22). There is a hypothesis that transferring of

active PLTP from apoE-containing lipoproteins to apoA-I-containing ones results in the conversion of active PLTP to inactive PLTP (21). However, this is not confirmed by a recent report, indicating that active plasma PLTP is associated primarily with apoA-I but not apoE-containing lipoproteins (23).

The formation of foam cells from lipid-accumulated macrophages is a critical step in atherogenesis. Both macrophages and macrophage-derived foam cells express PLTP (24). It has recently been shown that PLTP is highly expressed in macrophages from atherosclerotic lesions (25,26). It has also been speculated that PLTP in human atherosclerotic plaque might participate in the cellular cholesterol efflux from atherosclerotic tissue, thus preventing the process of atherosclerosis (26). However, there is as yet no direct evidence to support this hypothesis. Macrophages synthesize and secrete apoE, which makes a contribution to the apoE pool in the blood circulation and associates with plasma lipoproteins and accelerates their clearance *in vivo* (27,28). Macrophage-derived apoE can also act as a cholesterol acceptor to remove it from cholesterol-loaded cells (29-32). The effect of macrophage-derived apoE on cholesterol metabolism may be critical in protecting the artery wall from atherosclerotic lesion formation (27,28). The relationship between PLTP and apoE in macrophages is unknown. However, it has been reported that PLTP is associated with apoE in human cerebrospinal fluid, and that exogenous addition of recombinant PLTP to primary human astrocytes significantly increases apoE secretion to the conditioned medium (33). It has been also reported that PLTP secreted from HepG2 cells is associated with apoE but not apoA-I (34). These phenomena may also exist in macrophages, thus playing a role

in cholesterol metabolism and the process of atherosclerosis.

To evaluate the specific relationship between PLTP and apoE in macrophages, we transplanted PLTP-deficient mouse bone marrow into apoE-deficient mice, creating a model with PLTP deficiency and apoE expression exclusively in the macrophages. As a control, we also transplanted wild-type mouse bone marrow into apoE-deficient mice, creating a model with apoE expression exclusively in the macrophages. We investigated plasma apoE, cholesterol, and phospholipid levels, as well as atherosclerosis development in these animals.

Methods

Mice and Diets

ApoE-deficient (apoE$^{-/-}$) mice (8-week-old females) of the C57BL/6 background and wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). PLTP-deficient (PLTP$^{-/-}$) animals were available in our laboratory. All were initially fed a chow diet (Purina Rodent Chow, No. 5001, Research Diets Inc). Three months after bone marrow transplantation (see below), all mice were switched to a western type diet (0.15% cholesterol, 20% saturated fat) for 7 months. Experiments involving animals were conducted with the approval of SUNY Downstate Medical Center IACUC.

Bone marrow transplantation to replace peripheral macrophages

Bone marrow cells were harvested from the tibias of donor mice (PLTP$^{-/-}$ and WT), as previously described.[27] Twenty apoE$^{-/-}$ mice were lethally irradiated with 1,000 rads (10Gy). Ten of these animals were transplanted with PLTP$^{-/-}$ mouse bone marrow cells (5 X 10^6 cells), and the other 10 with WT mouse ones, via the femoral vein within 3 hours of irradiation. We monitored the process of cell replacement by PCR, using genomic DNA from mouse white blood cells as a template (35). The genotype of PLTP and apoE were determined with PCR. PLTP primer sequences: 1) TGGTCATGCATCTAGAACGGAGT; 2) AAAGGCTGCTGGACCCGCG; 3) GCAGCGCATCGCCTTCTATC. ApoE primer sequences: 1) GCCTAGCCGAGGGAGGACCG 2) TGTGACTTGGGAGCTCTGCAGC 3). GCCGCCCCGACTGCATCT.

PLTP activity assay

PLTP activity was measured with an assay kit (Cardiovascular Target, Inc). The kit includes both donor and acceptor particles. Incubation of both with 3 μL of mouse plasma results in the PLTP-mediated transfer of fluorescent phospholipid, which is present in a self-quenched state when associated with the donor. The transfer is determined by the increase in fluorescence intensity as the fluorescent lipid is removed from the donor and transferred to the acceptor.

Lipid and lipoprotein assays

The total cholesterol and phospholipid in plasma were assayed by enzymatic methods (Wako Pure Chemical Industries Ltd., Osaka, Japan). Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC), using a Sepharose 6B columns.[38]

Western blot for mouse apoE

SDS PAGE was performed on a 4% to 20% SDS-polyacrylamide gradient gel, using 3 μl of mouse plasma or isolated lipoprotein solutions ($1.006 < d < 1.063$ g/ml and $1.063 < d < 1.21$ g/ml, 200 μg protein), and the separated proteins were transferred to nitrocellulose membrane. Western blot analysis for mouse apoE was performed, using a polyclonal antimouse apoE antibody (Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-conjugated rabbit polyclonal antibody to mouse IgG (Novus Biologicals) was used as a secondary antibody. The SuperSignal West detection kit (Pierce) was used for the detection step. The maximum intensity of

each band was measured by Image–Pro Plus version 4.5 software (Media Cybernetics Inc.), and used for analysis.

Mouse atherosclerotic lesion measurement

The aorta was dissected and the arch photographed, as previously reported (36). Aortic root assay: sequential sections 10 μm thick were taken, using a cryostat. These were stained in Oil Red O, and the mean area of lipid staining per section per animal from five sections was determined for each animal. *En face* assay: the whole aorta was isolated, and the fatty streaks in the lumen were stained in Oil Red O. The mean lesion area of root assay and the percentage of lesion area on *en face* assay were determined, using Image-Pro-Plus software.

Statistical analysis. Each experiment was conducted at least three times. Data are typically expressed as mean ± SD. Data between two groups were analyzed by the unpaired, two-tailed Student's *t* test, and among multiple groups by ANOVA followed by the Post-Hoc test.

Results

Macrophage PLTP makes a contribution to PLTP activity in the circulation

Twenty apoE$^{-/-}$ mice were lethally irradiated. After 3 hours, half the animals (group 1) were transplanted with PLTP$^{-/-}$ mouse bone marrow cells, and the other half (group 2) with WT ones. We monitored the process of cell replacement by PCR, using genomic DNA from the mouse white blood cells as a template. In group 1, by 8 weeks after transplantation, the peripheral cells had been replaced by donor cells with a PLTP deficiency and an apoE expression (PLTP$^{-/-}$→apoE$^{+/+}$) genotype (Fig. 1). In group 2, the replaced peripheral cells had both a PLTP and an apoE expression (WT→apoE$^{-/-}$) genotype (Fig. 1).

After 10 weeks of bone marrow transplantation, we measured plasma PLTP activity in both group of animals using NBD-PL vesicles as donors and HDL as acceptors. We found that PLTP$^{-/-}$→apoE$^{+/+}$ mice had 20% less plasma PLTP activity than did WT→apoE$^{-/-}$ mice ($P<0.01$), suggesting that macrophages make about a 20% contribution to PLTP activity in the circulation.

Plasma lipoprotein analysis

It is known that through replacement of apoE$^{-/-}$ peripheral cells with WT ones, the hyperlipidemia of these animals can be completely corrected, owing to apoE secretion into the circulation from macrophages (27). On chow diet, we confirmed this observation. WT→apoE$^{-/-}$ mice have normal plasma lipid levels, compared with WT mice (Table 1). However, we did not find differences in the lipid profile of

PLTP$^{-/-}$→apoE$^{-/-}$ mice compared to WT→apoE$^{-/-}$ animals (Table 1), indicating that, on chow diet, macrophage PLTP deficiency has no detectable contribution to the plasma lipid metabolism.

We next sought to determine whether high fat high cholesterol diet could alter the lipid metabolism in those mice. After 12 weeks of bone marrow transplantation, we fed the mice with a western type diet (0.15% cholesterol, 20% saturated fat) for 4 weeks. We then determined the plasma PLTP activity and lipid levels. PLTP$^{-/-}$→apoE$^{-/-}$ mice have 22% lower PLTP activity than that of WT→apoE$^{-/-}$ mice ($P<0.01$). We also confirmed that WT→apoE$^{-/-}$ mice have normal lipid levels (WT→apoE$^{-/-}$ vs. WT, $P>0.05$; WT→apoE$^{-/-}$ vs. apoE$^{-/-}$, $P<0.0001$)(Table 2). Moreover, PLTP$^{-/-}$→apoE$^{-/-}$ mice have significantly higher plasma cholesterol and phospholipid levels, compared with WT→apoE$^{-/-}$ mice (124% and 152%, $P<0.001$, respectively) (Table 2). The distribution of lipids was determined by FPLC of pooled plasma samples (Fig. 2). This revealed that both non-HDL and HDL cholesterol and phospholipid were higher in PLTP$^{-/-}$→apoE$^{-/-}$ mice than in WT→apoE$^{-/-}$ animals. Since non-HDL lipoproteins are the major ones in the mice, the accumulated cholesterol is mainly located on those particles (Fig.2). Assessment of apolipoprotein composition of centrifugally isolated lipoproteins by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) revealed a increase of apoA-I and an increase of apoB levels (Fig. 3). All these results indicate that, on the high fat high cholesterol diet, macrophage PLTP deficiency has a significant contribution to the plasma lipid and lipoprotein metabolisms.

We then sought to determine whether macrophage PLTP deficiency has an

impact on apoE levels in the circulation, thus influences the lipoprotein metabolism. We utilized Western blot to measure apoE in the plasma, finding that PLTP$^{-/-}$→apoE$^{-/-}$ mice have significantly less apoE in the circulation (PLTP$^{-/-}$→apoE$^{-/-}$ vs WT→apoE$^{-/-}$, 73%, $P<0.01$) (Fig. 4A). We also isolated non-HDL ($1.006 < d < 1.063$ g/ml) and HDL particles ($1.063 < d < 1.21$ g/ml) and performed the Western blot on them. We found that apoE is located on non-HDL portion and PLTP$^{-/-}$→apoE$^{-/-}$ mouse non-HDL particles carry significant less apoE than that of WT→apoE$^{-/-}$ mouse (67%, $P<0.01$)(Fig. 4B). There is no detectable apoE on HDL portion in both PLTP$^{-/-}$→apoE$^{-/-}$ and WT→apoE$^{-/-}$ mice (Fig. 4B). All these results revealed that PLTP deficiency in the macrophages significantly influences apoE secretion from the cells.

Evaluation of atherosclerosis

To evaluate the impact of macrophage PLTP deficiency on atherogenesis, we dissected mouse aortas and photographed them. We also measured proximal and whole aortic lesion areas. After 7 months on the western type diet, we found that all 10 PLTP$^{-/-}$→apoE$^{-/-}$ mice (10/10) had very obvious lesions in the aortic arch, while only two of the 10 WT→apoE$^{-/-}$ animals (2/10) had observable lesions there (Fig. 5A). We also found that PLTP$^{-/-}$→apoE$^{-/-}$ mice had a 4-fold ($P<0.0001$) larger lesion area in the proximal aorta, and a 3-fold ($P<0.0001$) larger lesion area in the whole aorta, compared with WT→apoE$^{-/-}$ mice (Fig.5B and 5C). These results indicate that macrophage PLTP deficiency causes a significant reduction of apoE secretion from the cells, and this in turn promotes the accumulation of cholesterol in the circulation

and accelerates the development of atherosclerosis.

Discussion

In this study, we utilized bone marrow transplantation techniques to create a mouse model with PLTP deficiency and apoE expression exclusively in the macrophages. We investigated plasma apoE, cholesterol, and phospholipid levels, as well as atherosclerosis development in these animals. We found that macrophage PLTP deficiency significantly: 1) decreased PLTP activity in the circulation; 2) increased plasma cholesterol and phospholipid levels mainly on non-HDL lipoproteins on a western type diet; 3) decreased plasma apoE contents which are located on non-HDL lipoproteins; and 4) increased atherosclerotic lesions in the aortic arch, root, and the entire aorta.

The expression of PLTP in macrophages contributes to PLTP levels in the circulation. It has been reported that macrophages express PLTP (25), but the contribution of that PLTP to the plasma PLTP has been uncertain, for other tissues including the liver, adipose tissue, lung, and small intestine all express PLTP mRNA (37,38). Since our results show that macrophage PLTP deficiency decreases plasma PLTP activity, we know that mouse macrophage PLTP can be secreted into the circulation, making about a 20% contribution to plasma PLTP activity.

There is a relationship between PLTP and apoE secretion in macrophages. The mice used in this study were PLTP expressed, and apoE-deficient in all the tissues and cells except the macrophages. Because of this, we could specifically study the impact of macrophage PLTP on apoE secretion and on plasma cholesterol levels. Our results indicate that, on chow diet, there was no obvious difference between $PLTP^{-/-}$→$apoE^{-/-}$ and WT→$apoE^{-/-}$ mice, in terms of lipid levels

(Table 1), indicating that the apoE in the circulation is sufficient enough for the lipid clearance. It has been reported that apoE levels, that are only 12.5% of those in normal mice, are sufficient to achieve normalization of plasma lipoproteins in $apoE^{-/-}$ mice after WT bone marrow transplantation (27). However, when the animals were challenged with a high fat and high cholesterol diet, the $PLTP^{-/-} \rightarrow apoE^{-/-}$ mice cannot properly catabolize the dietary lipids owing to the defect of apoE secretion from the macrophage, thus cholesterol (mainly non-HDL cholesterol) accumulated in the circulation (Table 2, Fig.2).

The mechanism by which PLTP deficiency decreases apoE secretion from the macrophages is not yet clear. ApoE secretion from macrophage needs assistance from other proteins. It was reported that alpha-helix-containing apolipoproteins (apoA-I, apoA-II, apoA-IV, apoE2, apoE3, apoE4) stimulate apoE secretion, implying a positive feedback autocrine loop for apoE secretion (39). PLTP also is an alpha-helix-containing protein (40) and is involved in lipoprotein metabolism. It is conceivable that PLTP may also be needed for proper apoE secretion from cells. Indeed, PLTP secreted from HepG2 cells is associated with apoE but not apoA-I (34). PLTP in the circulation (21,22) or in human cerebrospinal fluid is associated with apoE (33). Exogenous addition of PLTP to primary human astrocytes significantly increases apoE secretion (33). Our results indicate that macrophage PLTP deficiency decreases apoE secretion from the cells (Fig.4). Since PLTP deficiency also decreases apoB secretion from hepatocytes (5), there may be a PLTP-medicate mechanism for both apolipoproteins in the secreting pathway. This phenomenon deserves further investigation.

It has been reported that the increase in mouse atherosclerotic lesion area is correlated with decreased cholesterol efflux from apoE-deficient macrophage (28). Previous reports also indicated replacement of apoE$^{-/-}$ peripheral cells with WT ones, the hyperlipidemia of these animals can be completely corrected and atherosclerosis can be dramatically diminished, owing to apoE secretion into the circulation from macrophages (27,28). Macrophage-derived apoE *per se* has an antiatherogenic property. Our results revealed that macrophage PLTP deficiency blocks cellular apoE secretion and reduces apoE-mediated cholesterol clearance from the circulation, thus promoting atherosclerosis (Fig.5). In terms of apoE secretion and function, macrophage-derived PLTP *per se* has a proatherogenic property.

It seems contradictory that macrophage-specific PLTP deficiency is proatherogenic, while a general PLTP deficiency is antiatherogenic (5). PLTP is a multifunctional protein that is expressed in a variety of tissues. Some of its effects are considered proatherogenic, and others antiatherogenic. The final atherosclerotic lesion formation is the consequence of this combination. Recent data indicate that PLTP deficiency in mice is associated with a decrease in atherosclerotic susceptibility, despite concomitant decreases in plasma HDL levels (5). Complementary metabolic studies have revealed that at least three distinct molecular mechanisms could account for the reduction of atherosclerosis in PLTP-deficient animals. They include: 1) the reduction in liver production and plasma levels of potentially atherogenic apoB-containing lipoproteins (5), 2) the rise in the antioxidative potential of apoB-containing lipoproteins attributable to the

accumulation of vitamin E (41), and 3) improvements in the anti-inflammatory properties of HDL in mice, which reduce the ability of LDL to induce monocyte chemotactic activity (42). Published reports have also indicated that PLTP overexpression in mice increases atherosclerotic lesions (6,7), despite the increase of preβ-HDL (11), a known factor involved in reverse cholesterol transport (30,43). The major contributor in this scenario is plasma HDL levels, which are significantly decreased (6).

As shown in Figure 6, macrophage $PLTP^{-/-}$→$apoE^{-/-}$ mice had significant less apoE and more cholesterol (mainly non-HDL-cholesterol) in the circulation than that of WT→$apoE^{-/-}$ mice, and this in turn accelerates the development of atherosclerosis in the mice.

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FOOTNOTES

[1]Abbreviations used in this paper: PLTP, phospholipid transfer protein; apo, apolipoprotein; PL, phospholipids; Chol, cholesterol; TG, triglyceride; FPLC, fast protein liquid chromatography.

[2]This work was supported by NIH HL-69817.

Table 1. Plasma and lipoprotein lipid analysis in mice on a chow diet.

Mice	Cholesterol	Phospholipid	Triglyceride
WT	135±22[a]	177±29[a]	82±19[a]
WT →apoE$^{-/-}$*	131±32[a]	170±44[a]	80±24[a]
PLTP$^{-/-}$→apoE$^{-/-}$**	139±36[a]	184±33[a]	76±36[a]
ApoE$^{-/-}$***	532±81[b]	671±101[b]	97±29[b]

*WT mouse bone marrow was transplantation into apoE$^{-/-}$ mouse; **PLTP$^{-/-}$ mouse bone marrow was transplanted into apoE$^{-/-}$ mouse; ***Conventional apoE knockout mice. In each column, $p<0.001$ by ANOVA. Value, mean ± SD, mg/dl, n=5-10. Columns labeled with different lower-case letters are statistically different by the Post-Hoc test ($P<0.01$).

Table 2. Plasma and lipoprotein lipid analysis in mice on a western type diet.

Mice	Cholesterol	Phospholipid	Triglyceride
WT	195±39[a]	237±57[a]	144±18[a]
WT →apoE$^{-/-}$*	189±51[a]	220±44[a]	138±21[a]
PLTP$^{-/-}$→apoE$^{-/-}$**	424±79[b]	555±107[b]	169±16[b]
ApoE$^{-/-}$***	1038±251[c]	1267±361[c]	198±19[c]

*WT mouse bone marrow was transplantation into apoE$^{-/-}$ mouse; **PLTP$^{-/-}$ mouse bone marrow was transplanted into apoE$^{-/-}$ mouse; ***Conventional apoE knockout mice. In each column, $p<0.001$ by ANOVA. Value, mean ± SD, mg/dl, n=5-10. Columns labeled with different lower-case letters are statistically different by the Post-Hoc test ($P<0.02$).

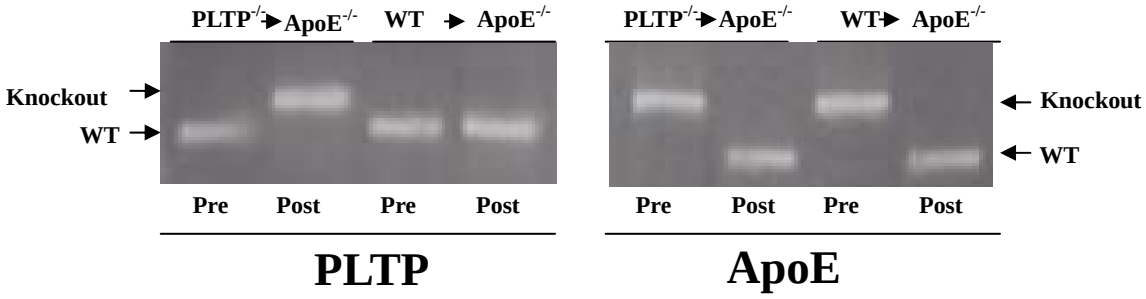

Figure 1. Genotype determination of mouse peripheral cells. Genomic DNA was extracted from white blood cells of the same mouse, before and post irradiation. The genotype of PLTP and apoE were determined with PCR, as described in "Experimental Procedure." Pre: pre-transplantation; Post: 8 week post-transplantation. $PLTP^{-/-}$→$apoE^{-/-}$, $PLTP^{-/-}$ mouse bone marrow was transplanted into $apoE^{-/-}$ mouse; WT→$apoE^{-/-}$, WT mouse bone marrow was transplanted into $apoE^{-/-}$ mouse.

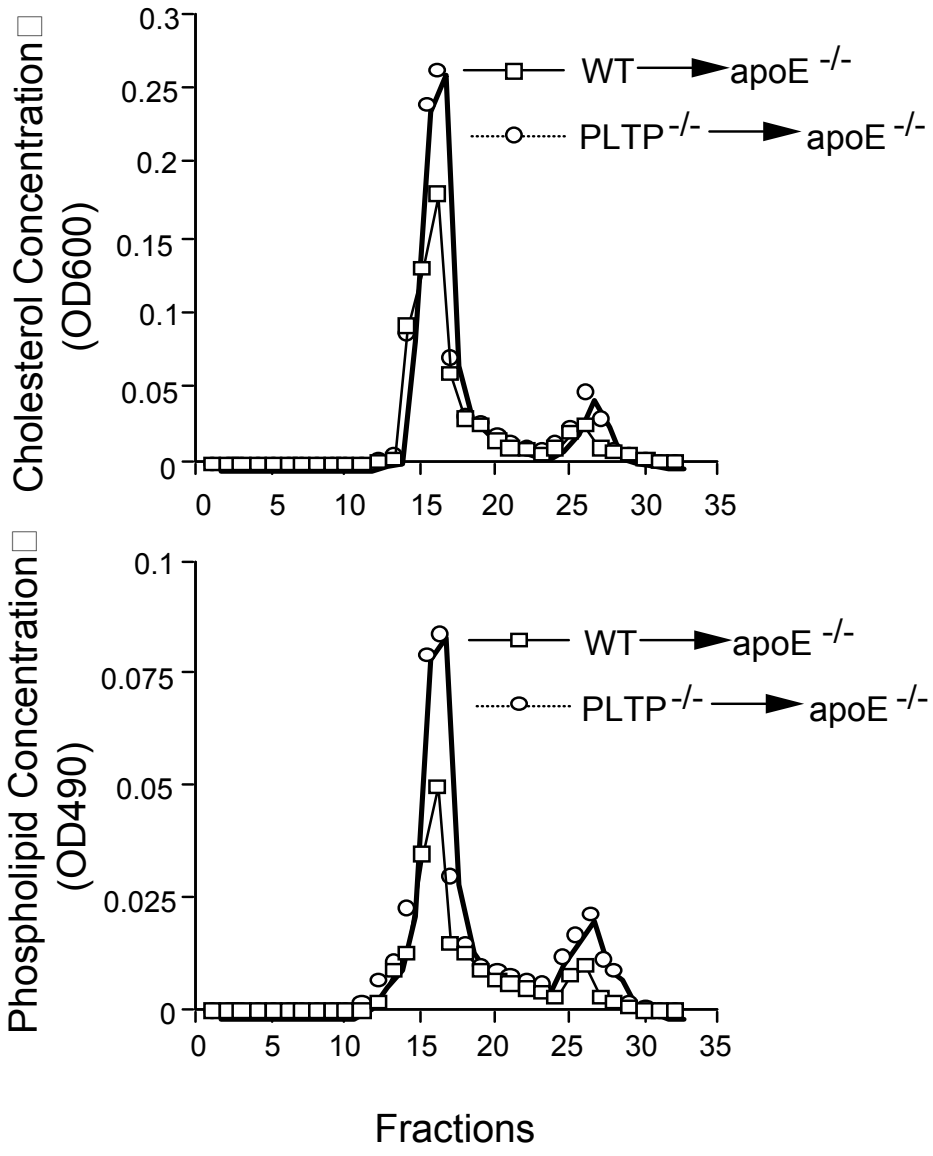

Figure 2. Mouse plasma lipoprotein analysis by fast protein liquid chromatography (FPLC). A 200-µl aliquot of pooled plasma (from 10 animals) was loaded onto a Sepharose 6B column and eluted with 50 mM Tris, 0.15 M NaCl (pH 7.5). An aliquot of each fraction was used for the determination of cholesterol and phospholipid.

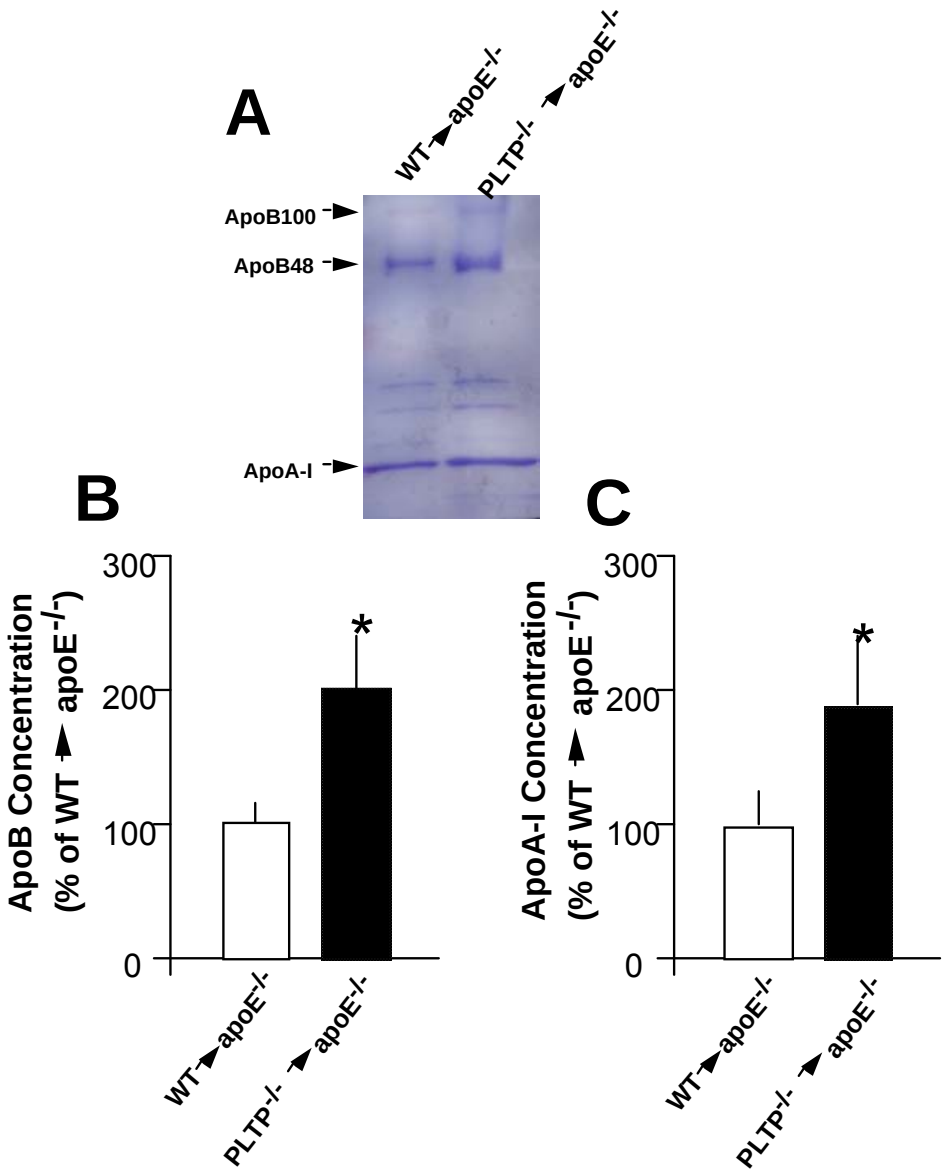

Figure 3. SDS PAGE analysis of apolipoproteins. Plasma lipoproteins (density = 1.006–1.21 g/ml) were separated by preparative ultracentrifugation as described (38). SDS PAGE was performed on 4%–20% SDS–polyacrylamide gradient gel, and the apolipoproteins were stained by Coomassie brilliant blue as described (38). Values are mean ± SD, n=4. Data were analyzed with Student's *t*-test, P<0.01.

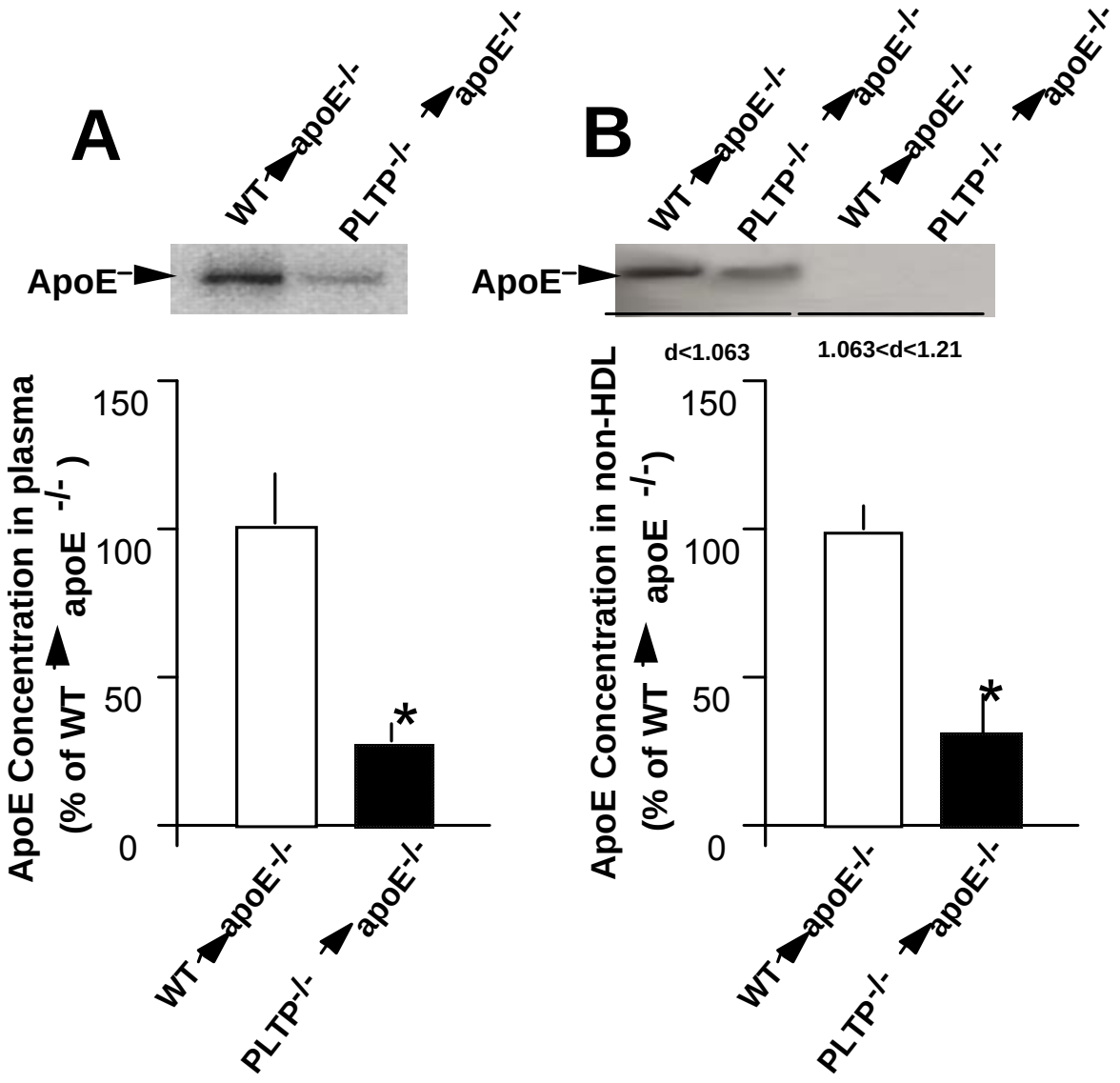

Figure 4. Western blot analysis for apoE. SDS PAGE was performed on a 4% to 20% SDS-polyacrylamide gradient gel, using 3 µl of mouse plasma, or isolated lipoprotein fractions (1.006 < d < 1.063 g/ml, and 1.063 < d < 1.21 g/ml, 200 µg protein). The separated proteins were transferred to nitrocellulose membrane. Western blot analysis for mouse apoE was performed as described in "Experimental Procedure**."** *A*, plasma. *B*, lipoproteins. Values are mean ± SD, n=4. Data were analyzed with Student's *t*-test, P<0.001.

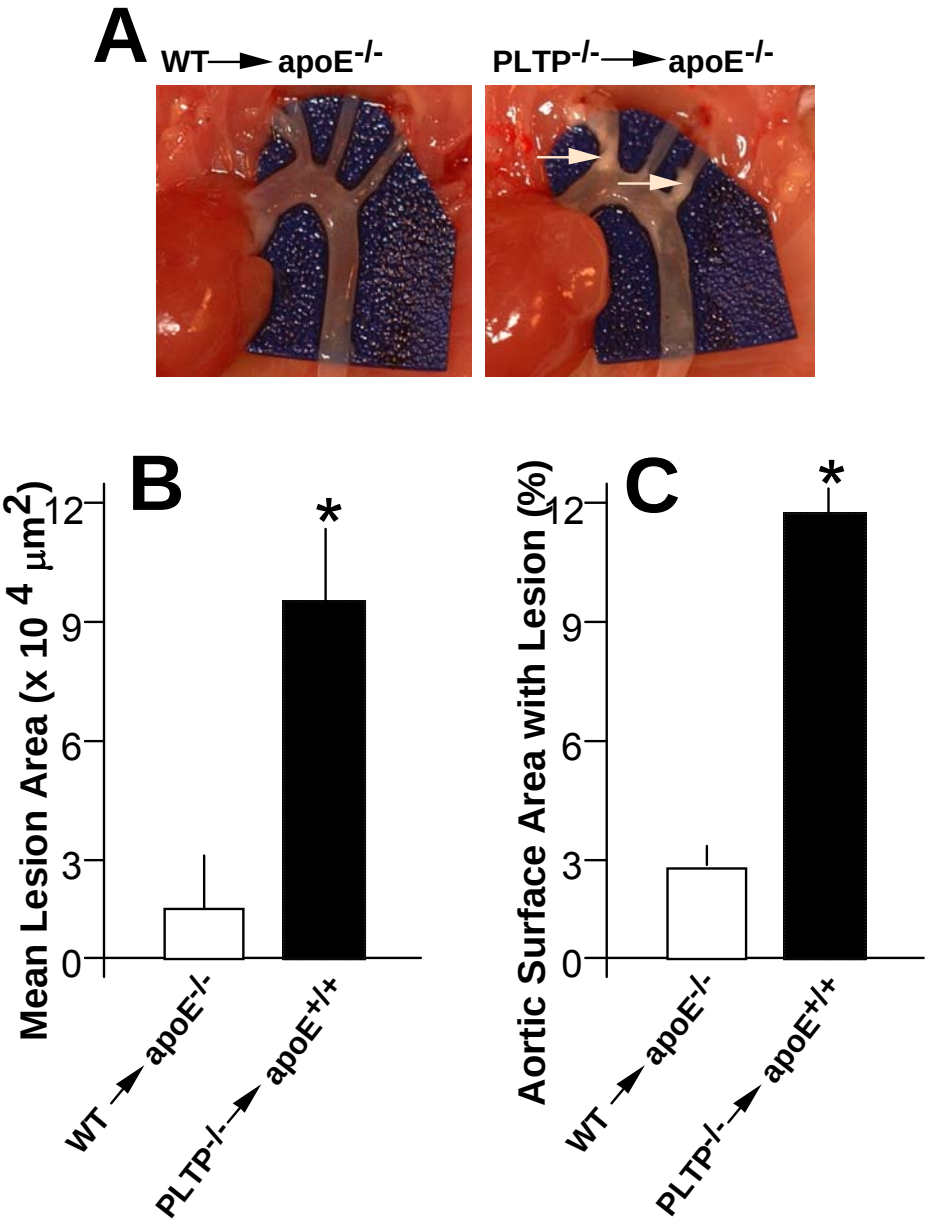

Figure 5. Mouse atherosclerotic lesion determination. *A*, mice were euthanized and the aortas dissected and photographed. This set of pictures is representative of 10 sets. *B and C*, quantification of atherosclerotic lesions in the proximal aorta by root assay and whole aorta by *en face* assay in mice fed a Western type diet for 7 months. The procedures for root assay and *en face* assay were performed. Values are mean ± S.D. *P<0.001; n=10. Arrows indicate the lesions.

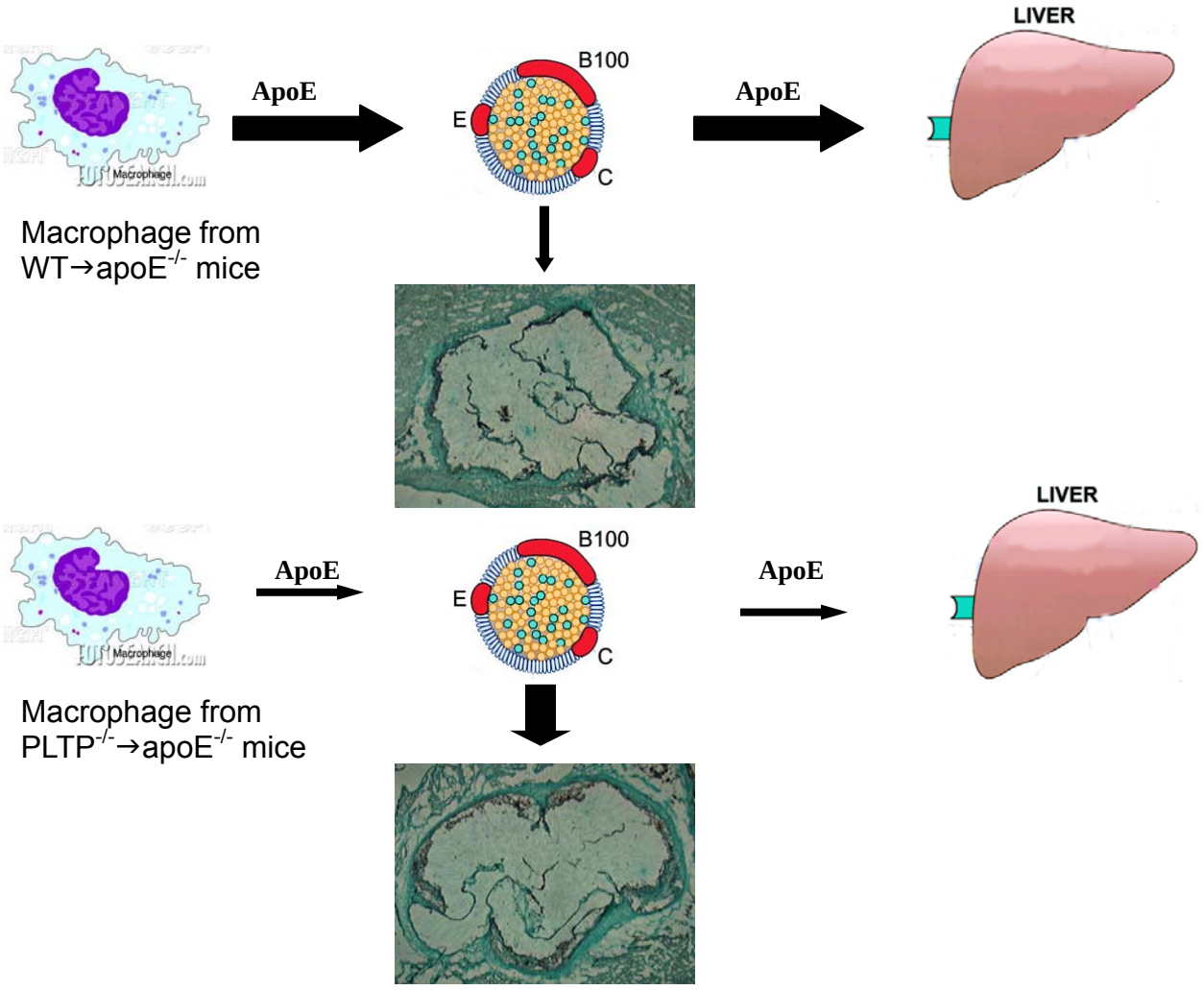

Figure 6. Proposed model. Macrophage is the only source for apoE secretion in both WT→apoE$^{-/-}$ and PLTP$^{-/-}$→apoE$^{-/-}$ mice. Macrophages from WT→apoE$^{-/-}$ mice (upper panel) secret apoE which can be added on lipoproteins in the circulation. The apoE-containing lipoproteins can be degraded in the liver through apoE receptors. Macrophages from PLTP$^{-/-}$→apoE$^{-/-}$ mice (lower panel) secret significant less apoE and more cholesterol (mainly non-HDL-cholesterol) was accumulated in the circulation than that of WT→apoE$^{-/-}$ mice, and this in turn accelerates the development of atherosclerosis in the mice.

Chapter 3

Phospholipid transfer protein (PLTP) deficient mice absorb less cholesterol

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Running title: Reduced cholesterol absorption in PLTP deficient mice

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ABSTRACT

Phospholipid transfer protein (PLTP) knockout (KO) mice have significantly lower levels of plasma cholesterol, apoB, and apoA-I. Here, we show that PLTP KO mice absorbed significantly less radiolabeled cholesterol but not triglyceride than wild type (WT) mice after multiple feedings of lipids during 5 days (35%-45%, $P < 0.01$). During this period of time PLTP KO mouse plasma, small intestine, and liver contain significant less radiolabeled cholesterol than those of WT (57%, 23%, and 35%, $P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Absorption of a bolus of radiolabeled cholesterol (short-term) but not triglyceride in PLTP KO mice was also significantly lower than that of WT mice (38%, $P < 0.01$). Also, PLTP KO mouse plasma, small intestine, and liver contain significant less radiolabeled cholesterol than those of WT (51%, 24%, and 30%, $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively). Moreover, we found that, compared to WT mice, PLTP KO primary enterocytes secrete significant less cholesterol through apoB-dependent and -independent pathways and PLTP KO enterocytes assimilate significantly less cholesterol but not triglyceride. To elucidate the mechanism involved in decreased cholesterol absorption, we quantified the mRNA levels of Niemann-Pick C1 like 1 (NPC1L1), ATP-binding cassette transporter G5/G8 (ABCG5/G8), and ABCA1. We also measured the activity of microsomal triglyceride transfer protein (MTP) in small intestine. We found that NPC1L1 and ABCA1 mRNA levels as well as MTP activity were significantly decreased, while ABCG5 and G8 mRNA levels were significantly increased in PLTP KO enterocytes, compared with WT enterocytes. Thus, decreased cholesterol absorption observed in PLTP KO mice is due to the

INTRODUCTION

It is estimated that a 60% reduction in plasma cholesterol level could be achieved by 100% inhibition of cholesterol absorption (1). Cholesterol absorption is a three-step process in which cholesterol is emulsified in intestinal lumen, taken up by the enterocytes and assembled into lipoproteins. Accumulating evidence demonstrates that Niemann-Pick C1-like 1 (NPC1L1) plays a key role in the uptaking of cholesterol by enterocytes. NPC1L1 deficiency reduces cholesterol absorption by about 70% (2), and has been shown to be the target of ezetimibe, a well-known cholesterol absorption inhibitor (3). Some of the cholesterol in the enterocytes is effluxed into the intestinal lumen through ATP-binding cassette transporters G5/G8 (ABCG5/G8) (4), Most of cholesterol is transported out of the cells through an apoB-dependent pathway which requires microsomal lipid transfer protein (MTP) activity (5, 6) and an apoB-independent pathway which requires ABCA1 but not MTP activity (7).

Phospholipid transfer protein (PLTP) circulates bound to HDL, and mediates the transfer of phospholipids from apoB-containing lipoprotein onto HDL (8). PLTP is expressed in the liver (9) and small intestine, and its PLTP proatherogenic potency has been demonstrated in humans (10). However, the underlying mechanisms of PLTP involvement in atherogenesis are not fully understood. PLTP deficiency in mice results in markedly decreased atherosclerotic lesions (11), probably due to: 1) decreased total plasma cholesterol (8, 11); 2) decreased apoB-containing particle secretion from the liver (11,12), and 3) increased plasma HDL clearance rate, which in turn increase reverse cholesterol transport (13). Since PLTP is expressed in

intestine which is a major organ for cholesterol absorption (14), it is conceivable to hypothesize that PLTP may play a role in cholesterol absorption and make a contribution to the plasma cholesterol levels.

In this study, we provided evidence that exogenous cholesterol absorption in PLTP KO small intestine is impaired, which can explain partially the reduction of plasma cholesterol level in these mice.

MATERIALS AND METHODS

Materials. [1,2-^{3}H] cholesterol was from NEN Life Science Products. [4-^{14}C] cholesterol was from Amersham and [5,6-^{3}H]sitostanol was from American Radiolabeled Chemicals. Oleic acid (OA) and HDL reagent was from Sigma. DMEM was from Invitrogen.

Animals and diets. Age and sex matched wild type (WT) and PLTP KO mice, with C57BL/6 background, were used in these studies. WT mice were from the Jackson Laboratory (Maine). PLTP KO mice were created and bred in our laboratory. Mice had free access to water and rodent chow.

Cholesterol absorption studies. The cholesterol absorption rate was determined by the fecal dual-isotope ratio method, using [^{3}H] sitostanol as a nonabsorbable reference sterol (15,16). PLTP KO and WT mice (10–12-week-old) were placed in individual cages that had wire mesh floors. For a single bolus cholesterol absorption study: A mixture of [^{14}C]-labeled (0.1 μCi) and unlabeled cholesterol (1 mg) and [^{3}H]-labeled sitostanol (0.2 μCi) in 15 μl of olive oil was fed to WT and PLTP KO mice. Feces were collected after 24 hours and isotope ratio was determined. For long-term cholesterol absorption studies: a mixture of [^{14}C]-labeled (0.1 μCi) and unlabeled cholesterol (0.2 mg) and [^{3}H]-labeled sitostanol (0.2 μCi) in 15 μl of olive oil was fed to each mouse three times a day, at 11 AM, 2 PM, and 5 PM for 5 days. Feces were collected every 24 h, and isotope ratio was determined.

Mouse plasma was collected at designed time point. HDL and non-HDL were

separated by precipitation as reported before (8) and the radioactivity was counted in each lipoprotein fraction. At the end of both short-term and long-term studies, mice were sacrificed. Small intestine (from duodenum to ileum) was cut into 15 segments. Each segment of small intestine and a part of liver were digested and radioactivity was counted, as described before (7). Cholesterol absorption ratio was calculated as: % absorption = {1-[fecal($^{14}C/^{3}H$)] /administrated($^{14}C/^{3}H$)}x100

Isolation of primary enterocytes. Primary enterocytes were isolated from control and knockout mice by the method of Weiser (17), as described before (7). Briefly, overnight-fasted mice were anesthetized, and small intestines were used for enterocyte isolation. Contents from the intestinal lumen were removed, washed with 117 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, and 5.5 mM glucose, and then filled with 67.5 mM NaCl, 1.5 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, 27 mM sodium citrate, and 5.5 mM glucose (buffer A). Intestines were then bathed in oxygenated saline at 37°C for 10 min. The buffer was discarded, and intestinal lumen were refilled with buffer A containing 1.5 mM EDTA and 0.5 mM dithiothreitol and incubated in 0.9% sodium chloride solution at 37°C for 10 min. Lumenal contents were collected and centrifuged at 1,500 rpm for 5 min. All buffers were adjusted to pH 7.4, gassed with 95% O_2/5% CO_2 for 20 min, and maintained at 37°C prior to use.

Cholesterol transport across the primary enterocytes. Enterocytes were resuspended in 4 ml of DMEM containing 1 µCi/ml of [^{3}H] cholesterol and incubated at 37°C in a cell culture incubator with 5% CO_2. Cells were gassed for 1 min with 95% O_2/5% CO_2 at regular intervals of 15 min. After 1 h, enterocytes were centrifuged (1,500 rpm for 5 min), washed twice with DMEM, and incubated with micelles containing 0.14 mM sodium cholate, 0.15 mM sodium deoxycholate, 0.17 mM phosphatidylcholine, 1.2 mM oleic acid, and 0.19 mM monopalmitoylglycerol at 37°C with regular gassing. After 2 h, enterocytes were centrifuged (3,000 rpm, 5 min), supernatants were subjected to density gradient ultracentrifugation, fractions were collected, and radioactivity was measured in a Beckman liquid scintillation counter. Cell pellets were incubated overnight at 4°C with 1 ml of isopropanol to isolate total lipids. After lipid extraction, 1 ml of 0.1 N NaOH was added to dissolve proteins. Protein was measured by the Bradford method (18), using Coomassie reagent (Pierce Chemical Company; Rockford, IL).

Density gradient ultracentrifugation. Ultracentrifugation involves the separation of lipoproteins between d<1.006 and 1.063 g/ml. This procedure separates large, triglyceride-rich apoB-containing lipoproteins (19,20) and has been used for cell culture media (7).

Absorption of cholesterol by primary enterocytes. Enterocytes were resuspended in 4 ml of DMEM containing 0.05 μCi/ml of [^{14}C] cholesterol, [^{3}H] triglyceride and unlabeled cholesterol (0.5 mg/ml). Enterocytes were collected at 5 min, 10 min, and 20 min, and washed with DMEM twice. The radioactivity was counted.

Secretion of cholesterol by primary enterocytes. Enterocytes were isolated from small intestine of PLTP KO and WT mice and resuspended in 4 ml of DMEM containing 0.2 μCi/ml of [^{3}H] cholesterol and unlabeled cholesterol (0.5 mg/ml). Enterocytes were collected at 0 hr, 0.5 hr, 1 hr and 2 hr after incubation, and the cells were pelleted and the medium radioactivity was counted. The radioactivity in the cell at time 0 was considered as total loaded [^{3}H] cholesterol (100%).

Real time PCR examined genes' expression. Mice were sacrificed with cervical dislocation. Jejunum was dissected and total RNA was extracted using Trizol (Invitrogen). CDNA was synthesized with kit of Invitrogen. Polymerase chain reaction (PCR) was performed in total volume of 20 μl by the sybergreen kit of Applied Biosystems. 18S was used as internal control. The amplification program consisted of an activation of 95^{o}C for 10 min; followed by 40 amplification cycles: 95^{o}C for 15 sec, 60^{o}C for 1 min. Each sample was triplicated. The genes' relative expression was expressed as mean ± SD.

Mouse NPC1L1 primers: forward, ATCCTCATCCTGGGCTTTGC; reverse, GCAAGGTGATCAGGAGGTTGA. Mouse ABCG5 primers: forward,

GCAGGGACCAGTTCCAAGACT; reverse, ACGTCTCGCGCACAGTGA. Mouse ABCG8 primers: forward, AAAGTGAGGAGTGGACAGATGCT; reverse, TGCCTGTGATCACGTCGAGTAG. Mouse ABCA1 primers: forward, TTGGCGCTCAACTTTTACGAA; reverse, GAGCGAATGTCCTTCCCCA. 18S rRNA, forward, AGTCCCTGCCCTTTGTACACA; reverse, GATCCGAGGGCCTCA CTAAAC.

Northern Blot Analysis. Thirty μg of total RNA was used for Northern blot analysis as described previously (9).

Microsomal triglyceride transfer protein (MTP) activity assay. The MTP activity assay was described elsewhere (21). In brief, WT and PLTP knockout small intestines (n=5) were homogenized and the supernatants were used for MTP transfer assay. The homogenates are incubated with small unilamellar donor vesicles containing quenched fluorescent lipids (triacylglycerols) and acceptor vesicles made up of phosphatidylcholine or phosphatidylcholine and triacylglycerols. Increases in fluorescence attributable to MTP-mediated lipid transfer are measured after 30 min.

Statistical Analysis. Each experiment was conducted at least three times. Data are typically expressed as mean ± SD. Data between two groups were analyzed by the unpaired, two-tailed Student's *t* test, and among multiple groups by ANOVA followed by the Post-Hoc test. P values less than 0.05 were considered

significant.

RESULTS

PLTP expression in small intestine: Liver is known to be one of the major organs expressing PLTP (9). To determine whether the intestine also expresses PLTP, we performed Northern blot analysis using total RNA extracted from the liver and intestine of mice (Fig. 1). Both the intestine as well as the liver contained a single transcript of approximately 1.8 kilobases, similar in length to that of human PLTP (9) (Fig.1). The amount expressed in the small intestine is comparable to that in the liver (Fig. 1). To understand the role of PLTP in the small intestine, we used PLTP KO mice that have no PLTP expression in the small intestine and the liver (Fig.1), as well as the rest of the tissues.

Long-term cholesterol absorption study: Since the major function of the intestine is absorption, we hypothesized that the intestinal expression of PLTP might be related to its role in lipid absorption. To explore the relationship between PLTP deficiency and cholesterol absorption, we utilized a long-term protocol designed to quantify smaller changes in cholesterol absorption (7). In this protocol, animals are gavaged 3 times a day with radiolabeled cholesterol and sitostanol along with unlabeled cholesterol in olive oil (7). The amounts of cholesterol absorbed were calculated by isotopic ratio method (7, 15,16). During the first 24 h, WT mice absorbed ~ 60% of the cholesterol (Fig. 2B). This absorption was slightly decreased to ~50% on day 5. To our surprise, PLTP KO mice absorbed significantly lower amounts on day 1 and continued to absorb significantly lower amounts throughout the feeding schedule (Fig. 2B). Overall, we found that PLTP KO mice

absorbed 35%-45% lesser amounts of [^{14}C]-cholesterol than the WT mice ($P < 0.01$, respectively) (Fig. 2B). These studies indicated that PLTP KO mice are less proficient in cholesterol absorption compared to their WT counterparts.

To understand the mechanism involved in decreased cholesterol absorption, we measured the uptake and secretion of cholesterol by the intestinal cells. Mice were fed 0.1 µCi of [^{14}C] cholesterol together with 0.2 mg of unlabeled cholesterol in 15 µl of olive oil three times a day for 2 days. Plasma, small intestine and liver were collected 12 hours after the 6th feeding. We measured the amounts of cholesterol present in the intestinal segments and found that almost every part of the small intestine from PLTP KO mice contained significantly lesser amounts of [^{14}C]-cholesterol than the WT mice (Fig. 3). Overall, the small intestines from PLTP KO mice contained 23% less [^{14}C]-cholesterol than WT animals ($P < 0.05$) (Table 1). In addition, livers from PLTP KO mice contained significantly less [^{14}C]-cholesterol than WT mice (35%, $P < 0.05$) (Table 1). We also found that PLTP KO plasma contained significantly less total [^{14}C]-cholesterol, [^{14}C] HDL-cholesterol, and [^{14}C] non-HDL-cholesterol (Table 1). These data indicate that PLTP KO mice take up less cholesterol and also secrete less cholesterol into the plasma.

To investigate whether the effect of PLTP deficiency is specific for cholesterol absorption, we fed PLTP KO and WT mice with 0.1 µCi [^{3}H] triglyceride instead of [^{14}C]-cholesterol three times a day for 2 days. Mouse plasma, small intestine, and liver were collected and we did not find any significant changes of [^{3}H] glycerolipids in the circulation and the tissues between the two groups of animals (Table 1), indicating that PLTP deficiency specifically inhibits the cholesterol absorption.

Short-term cholesterol absorption study: We had performed long-term multiple feeding studies anticipating that PLTP deficiency would have significant effect on lipid absorption. The studies described above indicated that a significant difference between the WT and PLTP KO mice could be observed as early as within one day. Thus, we performed short-term cholesterol absorption studies in these mice using a more conventional fecal dual-isotope ratio method (7,15,16) after the gavage of a single bolus of 0.1 μCi [^{14}C] cholesterol and 0.2 μCi [^{3}H] sitostanol together with 1 mg cold cholesterol in 15 μl olive oil. We found that PLTP KO mice absorbed 38% less of [^{14}C] cholesterol than the WT mice (Fig. 4). Furthermore, small intestines from PLTP KO mice contained 24% less [^{14}C]-cholesterol than WT mice ($P<0.05$) (Table 2). We also observed that PLTP KO plasma contained 51% less [^{14}C]-cholesterol. This was due to 41% and 55% reduction in cholesterol present in non-HDL and HDL, respectively ($P < 0.01$) (Table 2). Moreover, PLTP KO livers contained significantly lower [^{14}C]-cholesterol than WT livers (30%, $P < 0.05$).

To study the specificity, we also fed PLTP KO and WT mice with 0.1 μCi [^{3}H] triglyceride instead of [^{14}C]-cholesterol. Twenty-four hours later, mouse plasma, small intestine, and liver were collected and we did not find any significant changes of [^{3}H] glycerolipids in the circulation and the tissues between the two groups of animals (Table 2). These studies again indicate that PLTP deficiency specifically reduces cholesterol absorption.

Role of PLTP deficiency in cholesterol transport across the enterocytes:

To determine the role of PLTP deficiency in cholesterol secretion, enterocytes were isolated from PLTP KO and WT mice, incubated with radiolabeled cholesterol for 1 hour, and then chased in the presence of oleic acid for 2 hours (Fig. 5). PLTP KO enterocytes secreted significantly less (44%, $p<0.01$) cholesterol than WT enterocytes (Fig. 5A). It is known that cholesterol is secreted by enterocytes as part of chylomicrons and HDL (5,7). To determine whether cholesterol secretion with these lipoproteins was affected by PLTP deficiency, we subjected the conditioned media to density gradient ultracentrifugation (Fig. 5B). Cholesterol secreted by control enterocytes was distributed in two separate fractions, corresponding to apoB lipoproteins (fractions 1 and 2) and HDL (fractions 8-10) (Fig. 5B). Similar analysis with PLTP KO enterocytes revealed that cholesterol secretion in both apoB lipoproteins and HDL were significantly reduced (51% and 33%, $P < 0.01$, respectively). It is possible that PLTP KO enterocytes secrete less cholesterol due to decreased cellular uptake. To test this, enterocytes were incubated with [^{3}H] cholesterol for various times and its assimilation with time was studied (Fig. 6A). PLTP KO enterocytes took up significantly reduced amounts of cholesterol at all time points. Similar experiment was also performed for [^{3}H] triglyceride assimilation, there was no significant difference between PLTP KO and WT mice (Fig.6B).

To determine whether there is indeed defect of cholesterol secretion in PLTP KO mice, enterocytes were isolated and incubated with [^{3}H] cholesterol for one hour. The cells were washed and incubated with fresh medium. Media was collected at

different time points (0 hr, 0.5 hr, 1 hr, and 2 hr) to measure the radioactivity. The radioactivity in the cell at 0 hr was considered as total loaded [^{3}H] cholesterol (100%). We found that PLTP KO enterocytes secreted significantly less [^{3}H] cholesterol than WT cells at all tested time points (Fig.7).

To elucidate the possible mechanisms of reduction of cholesterol absorption in PLTP KO small intestines, we measured NPC1L1, ABCG5/G8, and ABCA1 mRNA levels, as well as MTP activity in both PLTP KO and WT small intestine, since NPC1L1 and ABCG5/G8 are directly involved in cholesterol assimilation (2-4), while MTP and ABCA1 are directly involved in cholesterol secretion (5-7). We found that small intestine from PLTP KO mice had significantly lower NPC1L1 and ABCA1 (40% and 70%, $P < 0.01$, respectively), and significantly higher ABCG5/ABCG8 mRNA levels (80% and 75%, $P < 0.01$, respectively) (Fig.8), than WT mice. Moreover, MTP activity in PLTP KO small intestine was significantly decreased, compared with WT animals (27%, $P < 0.01$) (Fig.9). Thus, decreased cholesterol absorption observed in PLTP KO mice is due to both reduction of assimilation and secretion.

Discussion

In this study, we demonstrated for the first time that, compared with WT mice, PLTP deficiency in small intestine significantly caused: 1) less cholesterol absorption and less cholesterol in small intestine, plasma, and the liver, after a bolus or multiple administration of the radiolabeled lipid; 2) less cholesterol secretion from primary enterocytes through apoB-dependent and -independent pathways; and 3) less cholesterol uptake in primary enterocytes. Thus, decreased cholesterol absorption observed in PLTP KO mice is due to the reduction of assimilation and secretion in enterocytes.

Previously, we provided evidence for multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells in culture and *in vivo* (5,7). Here, we show that ablation of PLTP specifically decreases cholesterol absorption (Fig.2 and Fig.4). There are three possibilities for this phenomenon. First, enterocytes absorb less cholesterol; second, enterocytes secrete less cholesterol; and third both assimilation and secretion are diminished.

Another possibility for the decreased cholesterol absorption in PLTP KO mice could be related to the reduction of bile acids in the intestinal lumen. Post et al. have shown that elevation of PLTP activity in transgenic mice results in rapid disposal of cholesterol from the body via increased conversion into bile acids and subsequent excretion. (22). However, we did not find significant differences (Table 1 and Table 2) in triglyceride absorption, which is also dependent on bile acids. Thus, decreased cholesterol absorption in knockout mice is probably not due to bile acid deficiency.

How does PLTP deficiency play a role in cholesterol secretion? We have shown that abundant PLTP activity in the Golgi apparatus of hepatocytes may play a role in the addition of lipid to developing apoB-containing lipoproteins (11). The biosynthesis of apoB-containing lipoproteins is a two-step process: 1) initial lipidation of apoB occurs in the endoplasmic reticulum (ER) and requires the activity of MTP (23), and 2) a poorly understood second step involves the addition of further lipid to the nascent VLDL particle (24). In this study, we found that PLTP deficiency significantly decrease intestinal MTP activity (Fig.9), indicating that PLTP may play a role in the first step of lipidation. The Golgi apparatus is a major site of phospholipid synthesis (25), and our previous observations indicate that addition or remodeling of phospholipid on nascent apoB-containing lipoprotein could involve PLTP (11). We also found that PLTP deficiency decreases liver vitamin E content, increases hepatic oxidant tone, and substantially enhances reactive oxygen species-dependent destruction of newly synthesized apoB via a post-ER process (12). Since chylomicron assembly and secretion from enterocytes shares common features with VLDL from hepatocytes (26), PLTP deficiency may well play same role in the maturation and degradation of apoB-containing lipoprotein in enterocytes as in hepatocytes. Our findings in this study are likely to be broadly relevant to apoB secretory control *in vivo.*

It is known that apoA-I is secreted independent of lipids and that the lipid-poor apoA-I can accept cholesterol from ABCA1 (27). Others and we have shown that ABCA1 plays a role in apoB-independent cholesterol transport across the intestinal epithelial cells (7,28,29). It has been reported that PLTP interacts with and stabilizes

ABCA1 and enhances cholesterol efflux from the cells (30). PLTP and apoA-I bind to ABCA1 at the same or closely related sites and PLTP acts more as an intermediary in the transfer of cellular lipids to lipoprotein particles (30). It is conceivable that PLTP in enterocytes may also play the same role as in other cells, in terms of ABCA1-mediated and apoA-I-dependent cholesterol efflux. Indeed, in this study, we found that PLTP deficiency results in significant reduction of ABCA1 mRNA levels in small intestine (Fig. 8). The detailed mechanism deserves further investigation.

PLTP deficiency regulates some genes, which are directly involved in cholesterol absorption in small intestines. NPC1L1 and ABCG5/G8, located on the apical membrane of enterocytes (31), are the major players in this process. Dietary cholesterol is taken up by the enterocytes through NPC1L1 (2) and some of it is effluxed back into the lumen through ABCG5/G8 (31). The rest of the cholesterol is either assembled or secreted with apoB-containing lipoproteins or effluxed through ABCA1 to apoA-I (5-7). NPC1L1 (32) and ABCG5/G8 (33) can be respectively down- and up-regulated by liver X receptor (LXR), a nuclear hormone receptor which is the major regulator of cholesterol homeostasis. It is still unknown why PLTP deficiency decreases NPC1L1 and increases ABCG5/G8 expression levels. The cholesterol transport across enterocytes may have a feedback loop: the reduction of cholesterol secretion from enterocytes could cause cholesterol accumulation inside the cells, then reduce NPC1L1 and induce ABCG5/G8 expression, rendering the enterocytes either assimilate less exogenous cholesterol or pump cholesterol back to the lumen. PLTP deficiency decreases apoB-containing

particle secretion from hepatocytes (11,12) and contributes cholesterol but not triglyceride accumulation in the liver (34). Same mechanism might also exist in the small intestine.

PLTP is a multifunctional protein, its impact may be far beyond the lipoprotein metabolism in the circulation. Accumulated results indicated that PLTP may play a role in lung surfactant metabolism (35), in brain lipid metabolism (36), and in the formation of the tear film (37). In this study, we provide evidence that PLTP is involved in cholesterol transport across the enterocytes. The question deserves to be answered is: how PLTP works in different cells, including enterocytes, hepatocyte, brain cells, and so on, which express PLTP.

As shown in Figure 10, we provided evidence to show that PLTP KO mouse enterocytes secrete and assimilate less cholesterol, which, in turn, inhibit small intestine cholesterol absorption. These results may provide another mechanism for the reduction of atherosclerotic lesions in mouse models.

Table 1. Absorption of cholesterol and triglycerides after multiple feedings: Counted cholesterol or triglyceride in plasma, small intestine and liver after six feedings of [^{14}C] cholesterol or [^{3}H] triglyceride administration.

	WT	PLTP KO	%Change	*p*
[^{14}C]cholesterol				
Total (dpm/ml)	23852±4312	10247±2222**	- 57	<0.01
HDL (dpm/ml)	14136±2810	3524±1302**	- 75	<0.01
Non-HDL (dpm/ml)	9716±1059	6723±1915*	- 31	<0.05
Intestine (dpm/g tissue)	39987±3231	30702±2173*	- 23	<0.05
Liver (dpm/g tissue)	35188±4978	22710±2766*	-35	<0.01
[^{3}H]glycerolipids				
Total (dpm/ml)	16339±3015	19773±4090	+ 21	NS
HDL (dpm/ml)	13731±3667	16206±3876	+ 18	NS
Non-HDL (dpm/ml)	2608±436	3567±539	+ 24	NS
Intestine (dpm/g tissue)	28698±3004	25330±5255	- 12	NS
Liver (dpm/g tissue)	393517±22005	372747±22289	- 5	NS

Mice were fed 0.1 μCi of [^{14}C] cholesterol or 1 μCi of [^{3}H] triglyceride together with 0.2 mg of unlabeled cholesterol in 15 μl of olive oil at 11am, 2pm and 5pm for 2 days. Plasma, small intestine and liver were collected 12 hours after the 6th feeding. Values are Mean ± SD, n=3.

Table 2. Counted cholesterol or triglyceride in plasma, small intestine and liver after a single bolus of [^{14}C] cholesterol or [^{3}H] triglyceride administration.

	WT	PLTP KO	%Change	*p*
[^{14}C]-cholesterol				
Total(dpm/ml)	4896±257	2380±267	-51	<0.01
HDL(dpm/ml)	2581±333	1169±230	-55	<0.01
Non-HDL(dpm/ml)	2315±77	1211±323	-41	<0.01
Intestine(dpm/g tissue)	10299±613	7811±912	-24	<0.05
Liver(dpm/g tissue)	9714±1184	6771±783	-30	<0.05
[^{3}H]-glycerolipids				
Total(dpm/ml plasma)	8928±2095	10514±2610	+18	NS
HDL(dpm/ml)	7331±1789	8925±1739	+22	NS
Non-HDL(dpm/ml)	1597±406	1589±520	-0.5	NS
Intestine(dpm/g tissue)	24275±3875	21581±1190	-11	NS
Liver(dpm/g tissue)	63400±4469	55790±3067	-12	NS

Mice were fed 0.1 μCi of [^{14}C] cholesterol and 1 μCi of [^{3}H] triglyceride together with 1 mg of unlabeled cholesterol in 15 μl of olive oil. After 24 h, plasma, small intestine and liver were collected. Values are Mean ± SD n=3.

Figure 1. Liu et al.

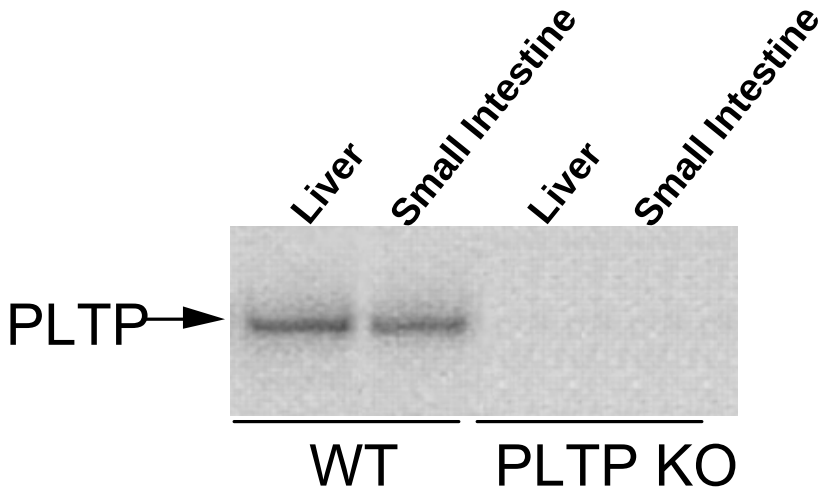

Figure 1. Expression of PLTP in the mouse small intestine: Thirty μg/lane of total RNA from small intestine and liver were subjected to Northern blot analysis with a 750 bp mouse PLTP cDNA (nucleotides 50-798) that was random primer-labeled. The size of mouse PLTP transcripts is 1.8 kb.

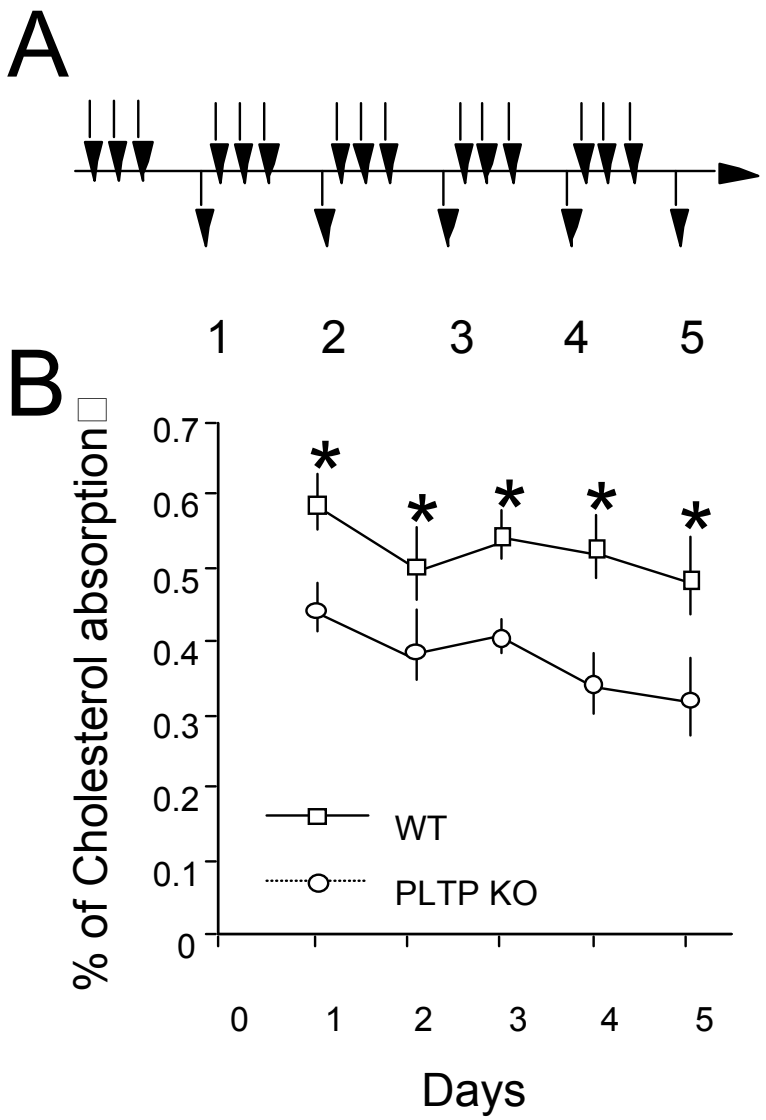

Figure 2. PLTP knockout mice absorbed less cholesterol in a long-term study. PLTP KO and WT mice (n=5, 12-14 weeks old) were fed with 0.1 μCi [^{14}C] cholesterol and 0.2 μCi [^{3}H] sitostanol together with 0.2 mg cold cholesterol dissolved in 15 μl of olive oil three times a day at 11 AM, 2 PM, and 5 PM everyday for 5 days. Feces were collected every 24 h, and isotope ratio was determined. A, the feeding schedule. B, cholesterol absorption in both groups of animals. Data was analyzed by ANOVA ($P < 0.001$), followed by the Post-Hoc test. Values are mean $\pm$ SD. *$P < 0.01$.

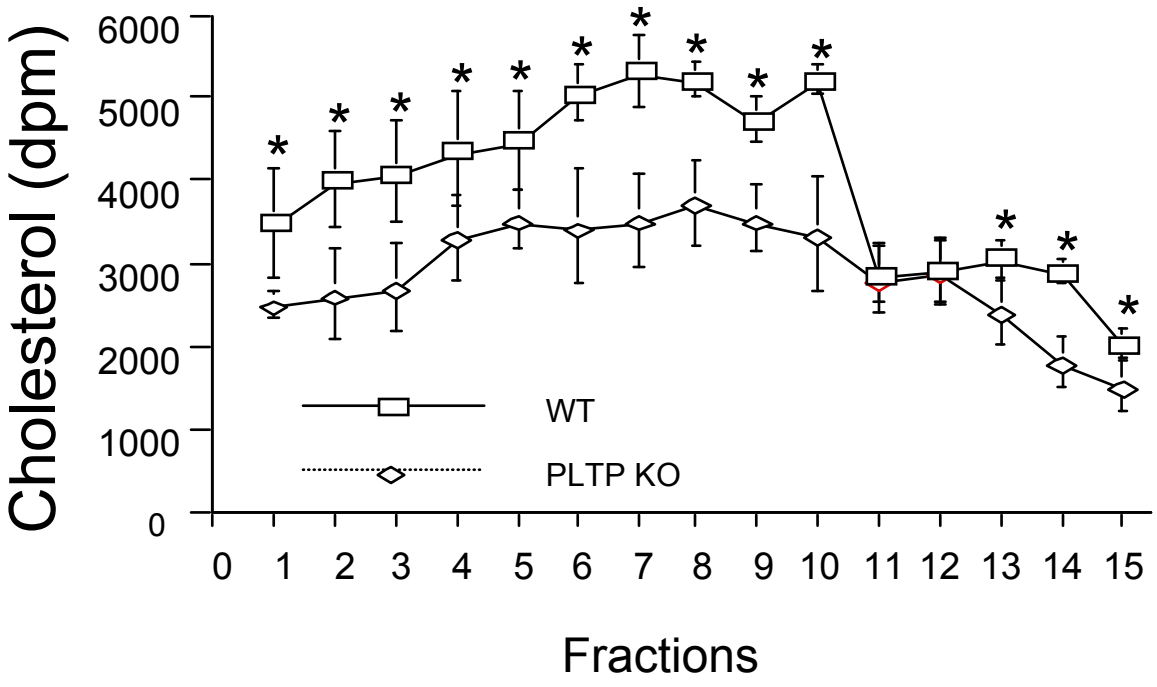

Figure 3. Distribution of cholesterol along the length of the small intestine during long-term absorption studies. PLTP KO and WT mice (n=3, 12-14 weeks old) were fed with 0.1 μCi [^{14}C] cholesterol and 0.2 μCi [^{3}H] sitostanol and 0.2 mg cold cholesterol dissolved in 15 μl of olive oil at 11 AM, 2 PM, and 5 PM everyday for 2 days. Twelve hours after the 6th feeding, small intestines were collected from the base of the stomach and cut into 2 cm segments. Each segment was digested with 1 ml of OptiSolv and mixed with 5 ml of liquid scintillation cocktail and counted. Data was analyzed by ANOVA ($P < 0.001$), followed by the Post-Hoc test. Values are mean $\pm$ SD. *$P < 0.01$.

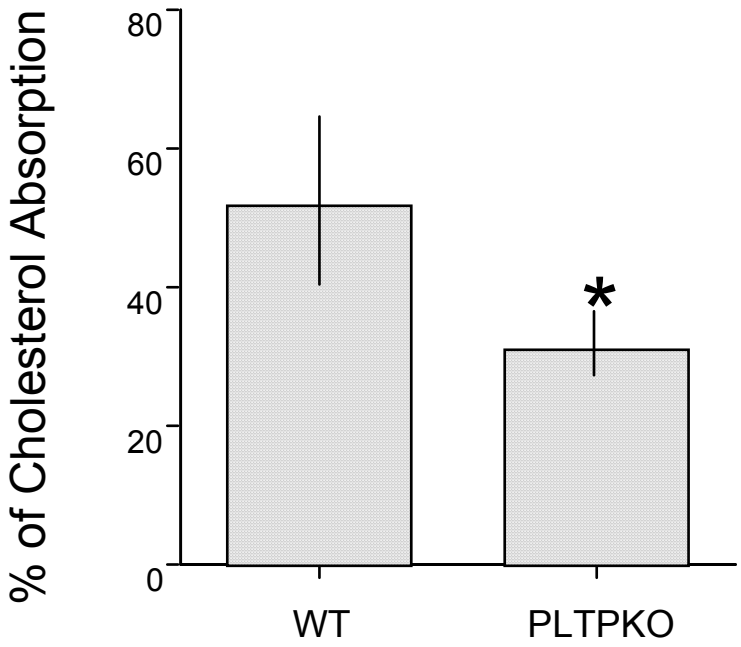

Figure 4. PLTP knockout mice absorbed less cholesterol with a single bolus of cholesterol feeding. PLTP KO and WT mice (n=5, 10-12 weeks old) were fed with 0.1 μCi [^{14}C] cholesterol and 0.2 μCi [^{3}H] sitostanol and 1 mg cold cholesterol dissolved in 15 μl of olive oil. 24 hours later feces were collected and lipids were extracted and counted. The cholesterol absorption was determined as described in "Materials and Methods". Value is mean ± SD. *P < 0.01.

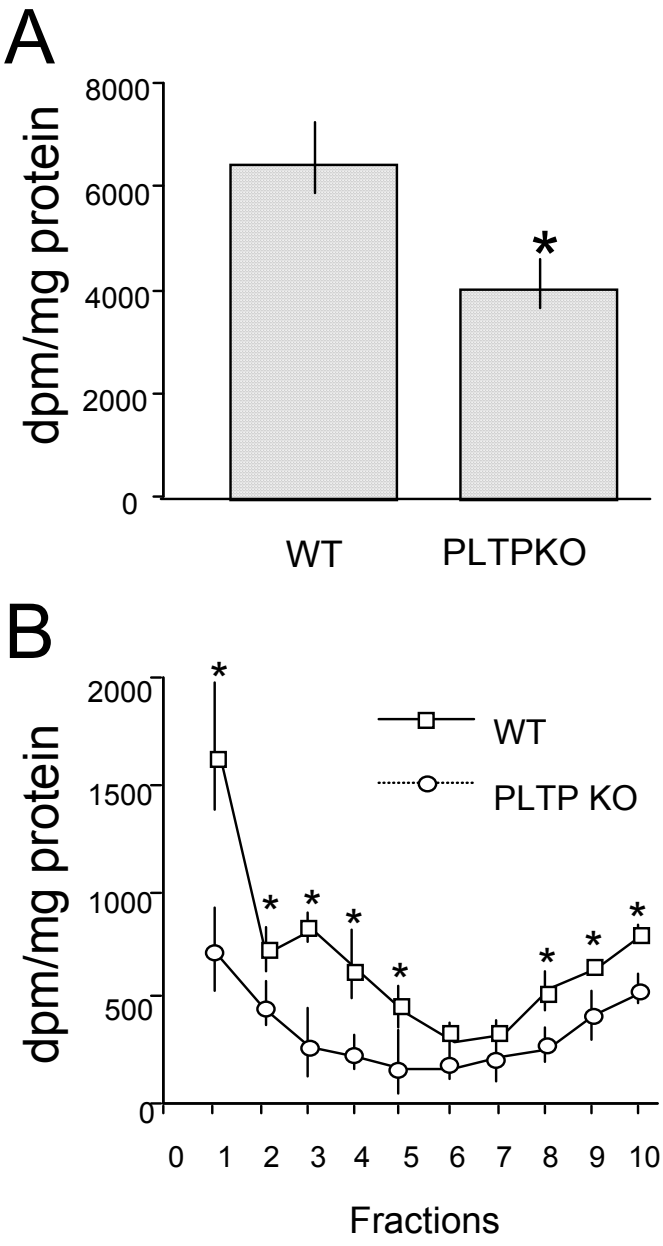

Figure 5. Cholesterol transport across enterocytes isolated from the PLTP KO and WT. small intestine: Enterocytes were isolated from small intestine of PLTP KO and WT mice (n=3, 12-14 weeks old) and used for cholesterol transport studies as described in "Material and Methods". A, total counts in both conditional medium. B, the conditioned medium was subjected to ultracentrifuge to separate various lipoproteins. Fractions were collected from the top and used to measure cholesterol in triplicate. Data was analyzed by ANOVA ($P < 0.001$), followed by the Post-Hoc test. Values are mean $\pm$ SD. *$P < 0.01$

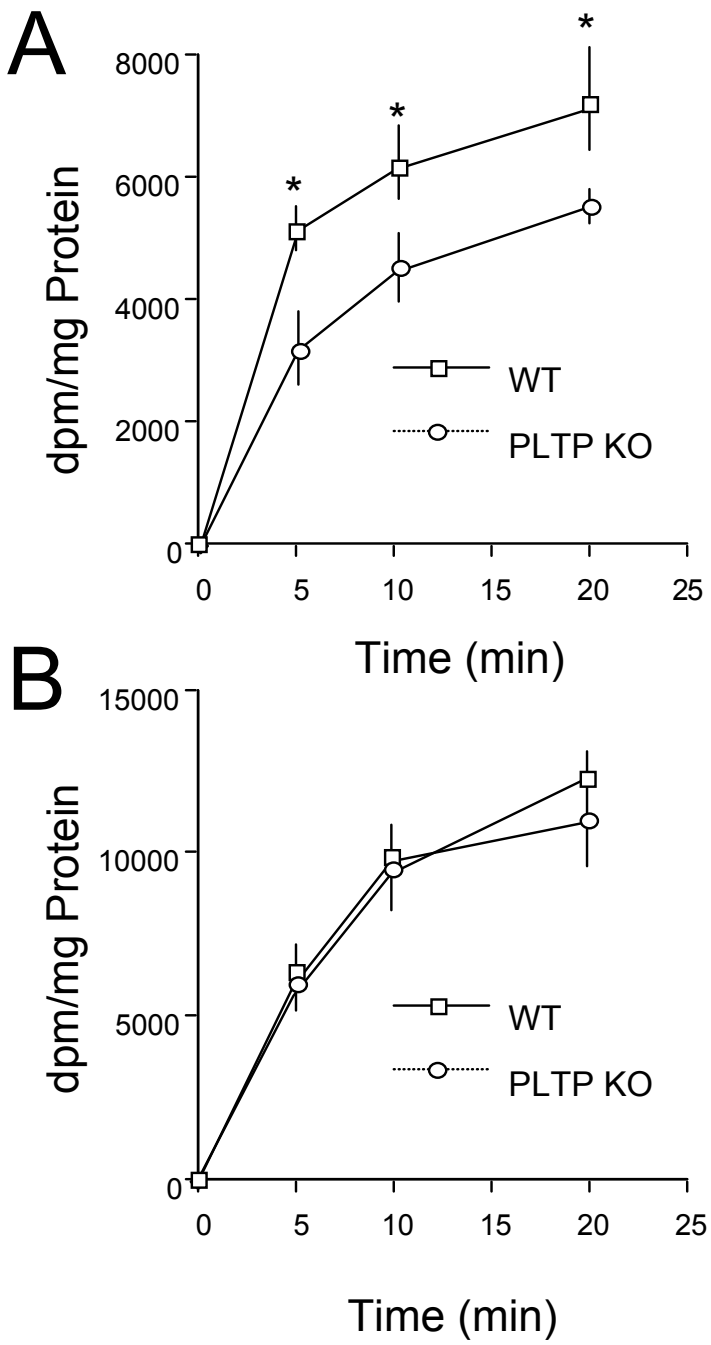

Figure 6. Cholesterol uptake by enterocytes isolated from the PLTP KO and WT. small intestine: Enterocytes were isolated from small intestine of PLTP KO and WT mice (n=3, 12-14 weeks old) and resuspended in 4 ml of DMEM containing 0.05 μCi/ml of [^{14}C] cholesterol and [^{3}H] triglyceride together with unlabeled cholesterol (0.5 mg/ml). Enterocytes were collected at 5 min, 10 min, and 20 min after incubation, and washed with DMEM twice. The cellular radioactivity was counted. A, [^{14}C] cholesterol uptake, Data was analyzed by ANOVA ($P< 0.001$), followed by the Post-Hoc test; B, [^{3}H] triglyceride uptake. Values are mean $\pm$ SD. *$P < 0.01$.

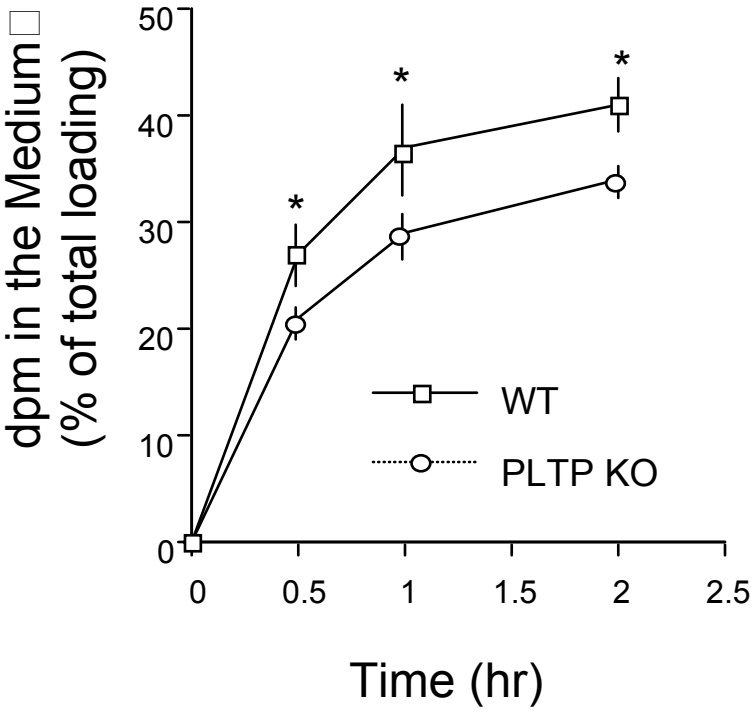

Figure 7. Choleterol secretion by enterocytes isolated from the PLTP KO and WT small intestine: Enterocytes were isolated from small intestine of PLTP KO and WT mice (n=3, 12-14 weeks old) and resuspended in 4 ml of DMEM containing 0.05 µCi/ml of [^{14}C] cholesterol and unlabeled cholesterol (0.5 mg/ml). Enterocytes were collected at 0 hr, 0.5 hr, 1 hr and 2 hr after incubation, and the cells were pelleted and the medium radioactivity was counted. The radioactivity in the cell at time 0 was considered as total loaded [^{3}H] cholesterol (100%). Data was analyzed by ANOVA ($P < 0.001$), followed by the Post-Hoc test. Values are mean $\pm$ SD. *$P < 0.01$.

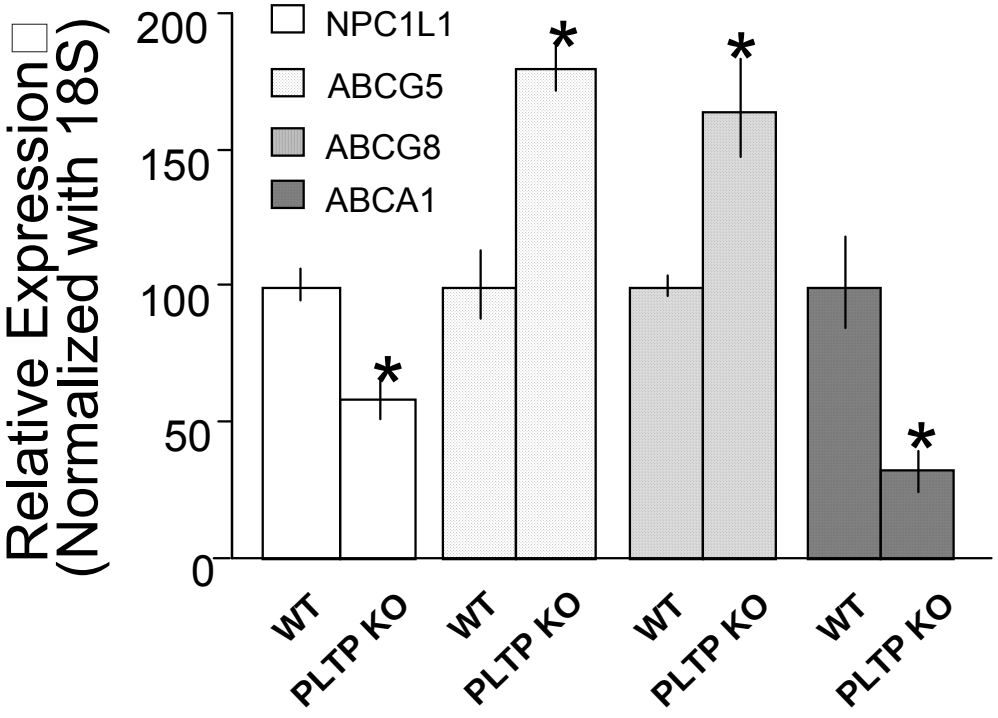

Figure 8. Real time PCR examined genes' expression. Total RNA was isolated from PLTP KO and WT mice (n=3, 12-14 weeks old) with TriZol (Invitrogen). NPC1L1, ABCG5, ABCG8, and ABCA1mRNA levels were measured by real-time PCR. Each mRNA level was expressed as a ratio to 18S rRNA. Values are mean $\pm$ SD. *P < 0.01.

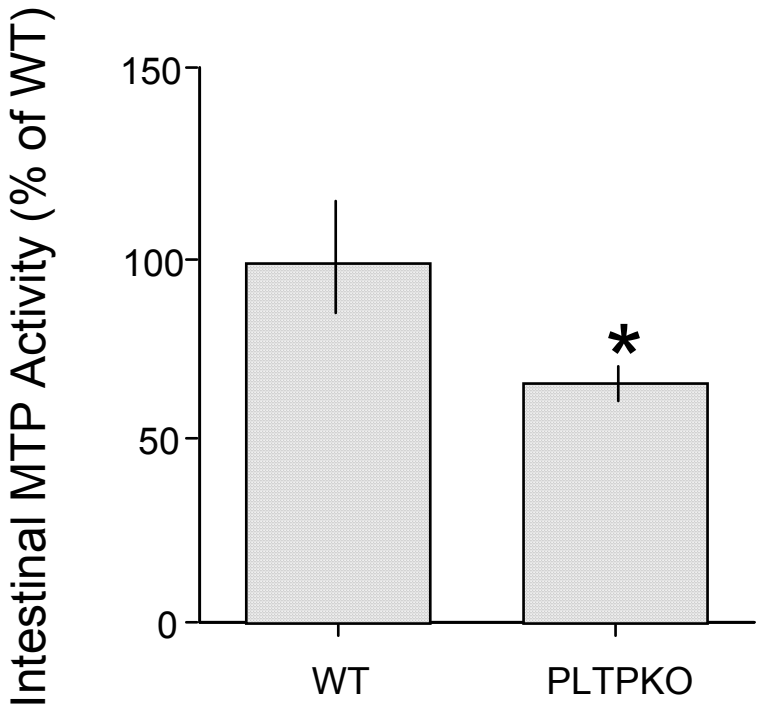

Figure 9. MTP activity in mouse small intestine. The intestinal MTP activity was measured as described before (21). Values are mean ± SD. *P < 0.01.

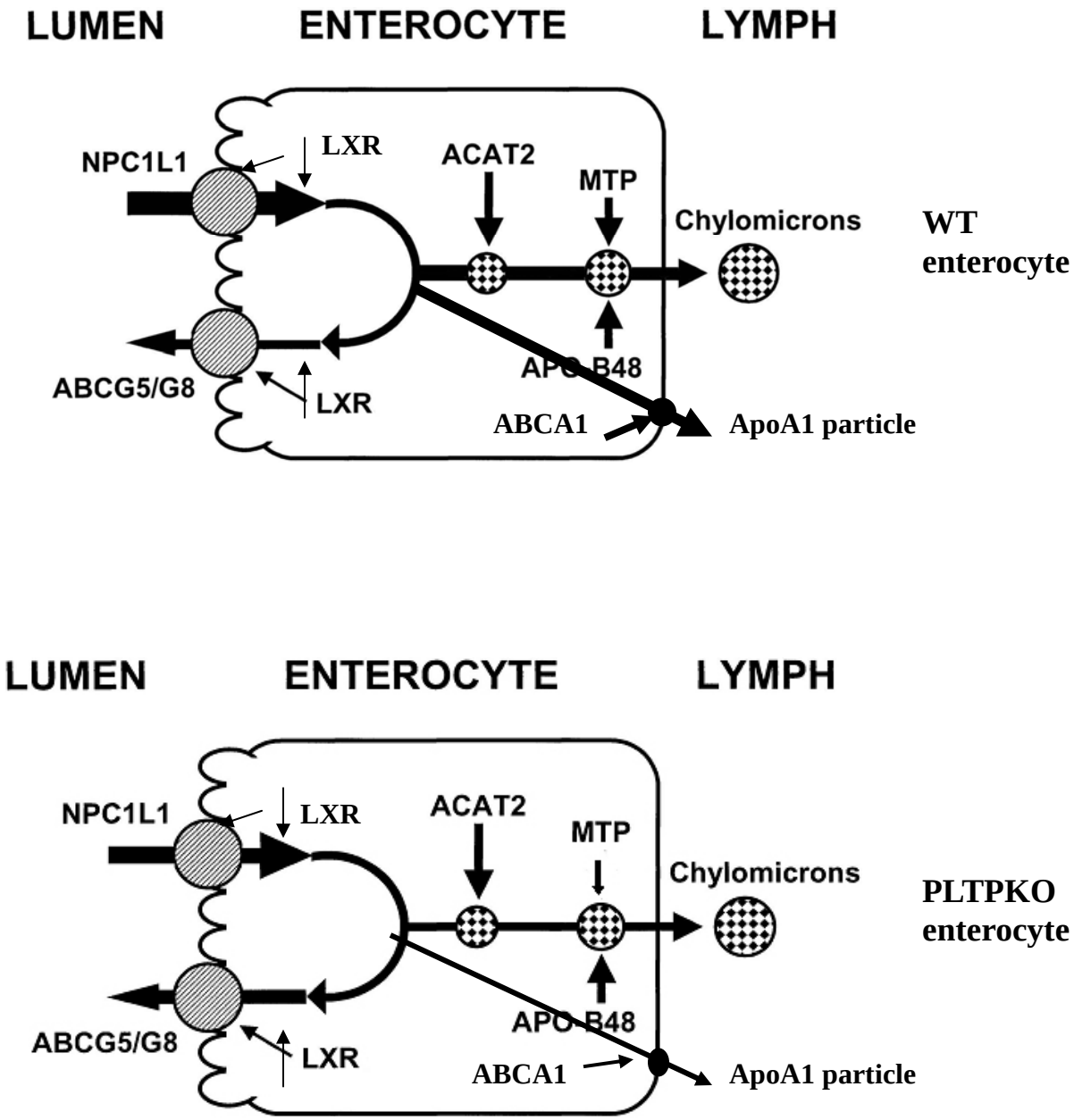

Figure 10. Proposed model. Wild type enterocytes (upper panel) absorbed cholesterol from intestinal lumen via NPC1L1 (downregulated by LXR). Some cholesterol returned to lumen via ABCG5/8 (upregulated by LXR) and some was assemlied into lipoproteins and secreted by apoB dependent pathway, which required MTP, and apoA1 dependent pathway, which required ABCA1. PLTP KO enterocytes (lower panel) uptake less cholesterol and pumped more cholesterol back to the lumen. Moreover, PLTP KO enterocytes had reduced MTP activity andABCA1 mRNA expression, which caused impaired cholesterol secretion. As a result, PLTP KO small intestine absorbs less cholesterol than WT.

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Chapter 4

Summary and Perspective

PLTP is an independent risk factor for coronary artery disease, and is significantly increased in those suffering from obesity, and well as from types I and II diabetes. Moreover, PLTP deficiency decreases and PLTP overexpression increases atherosclerosis in mouse models. PLTP is therefore considered a potential target for pharmacological or gene therapy. However, this possibility is hampered by the fact that the substance's atherogenic mechanism is not completely understood. PLTP is a multifunctional protein that is expressed in a variety of tissues. Some of its effects are considered atherogenic, while others are thought to be antiatherogenic. Furthermore, PLTP activity can be altered by multiple factors (including a high-fat high-cholesterol diet, oxidized steroids, lipopolysaccharide, chenodeoxycholic acid, fenofibrate, glucose, and insulin) and by multiple pathological conditions (including coronary artery disease, obesity, and diabetes). In order to unravel the mechanisms involved, I investigated the impact of macrophage PLTP deficiency on lipoprotein metabolism and atherosclerosis and the impact of small intestine PLTP deficiency on cholesterol absorption, a process directly related to lipoprotein metabolism and atherosclerosis.

In chapter 2, I demonstrated that macrophage PLTP deficiency has pro-atherogenic property. I transplanted PLTP-deficient mouse bone marrow into apoE-deficient mice, creating a mouse model with PLTP deficiency, and apoE expression exclusively in the macrophages. I found that PLTP deficient

macrophages secreted less apoE, leading to reduction of atherogenic lipoprotein clearance, subsequently, increased atherosclerosis in the mouse model.

In chapter 3, I revealed that small intestine PLTP deficiency has anti-atherogenic property. I showed that 1) PLTP KO mice absorb significantly less cholesterol than WT mice; 2) compared with WT mice, PLTP KO primary enterocytes secrete significant less cholesterol through apoB-dependent and -independent pathways and PLTP KO enterocytes assimilate significantly less cholesterol but not triglyceride; and 3) NPC1L1 and ABCA1 mRNA levels as well as MTP activity were significantly decreased, while ABCG5 and G8 mRNA levels were significantly increased in PLTP KO small intestine, compared with WT. These results may provide another mechanism for the reduction of atherosclerotic lesions in mouse models.

It seems contradictory that macrophage-specific PLTP deficiency is proatherogenic, while a general or small intestine PLTP deficiency is antiatherogenic. Since PLTP has many functions, in terms of lipoprotein metabolism and atherosclerosis, the final atherosclerotic lesion formation is the consequence of the combination of those effects. Recent data indicate that PLTP deficiency in mice is associated with a decrease in atherosclerotic susceptibility, despite concomitant decreases in plasma HDL levels (1). Published reports have also indicated that PLTP overexpression in mice increases atherosclerotic lesions (2,3) despite the increase of preβ-HDL (4) a known factor involved in reverse cholesterol transport (5). The major contributor in this scenario is plasma HDL level, which is significantly decreased.

To further study the impact of PLTP deficiency in macrophage and small intestine, we are going to perform following experiments:

1). We will explore if there is/are mechanism (s) other than decreased apoE secretion from PLTP knockout macrophages, which cause(s) induction of atherosclerosis in mice. We plan to transplant $PLTP^{-/-}/apoE^{-/-}$ bone marrow cells and $apoE^{-/-}$ bone marrow into $apoE^{-/-}$ mice. There is no apoE in both animal models. If no difference in atherosclerosis is found between these 2 groups of animals, we then can draw the conclusion that reduction of apoE secretion from PLTP knockout macrophages is the only mechanism causing the induction of atherosclerosis. Otherwise, different mechanism (s) exist(s).

2). We have scheduled to create small intestine specific PLTP transgenic or knockout mice. In these animal models, only small intestine does not express PLTP or overexpresses PLTP. Those mice will provide a unique opportunity for us to explore how PLTP plays a role in cholesterol absorption in small intestine.

In conclusion, I have explored the effects of macrophage PLTP deficiency on atherosclerosis and enterocyte PLTP deficiency on cholesterol absorption. Hopefully, these studies will broaden our view of the role of PLTP in lipoprotein metabolism and atherosclerosis.

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