Phospholipid transfer protein deficiency in mice impairs macrophage reverse cholesterol transport *in vivo*

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Abstract

Phospholipid transfer protein is expressed in various cell types and secreted into plasma, where it transfers phospholipids between lipoproteins and modulates the composition of high-density lipoprotein particles. Phospholipid transfer protein deficiency *in vivo* can lower high-density lipoprotein cholesterol level significantly and impact the biological quality of high-density lipoprotein. Considering high-density lipoprotein was a critical determinant for reverse cholesterol transport, we investigated the role of systemic phospholipid transfer protein deficiency in macrophage reverse cholesterol transport *in vivo*. After the littermate phospholipid transfer protein KO and WT mice were fed high-fat diet for one month, they were injected intraperitoneally with ³H-cholesterol-labeled and acLDL-loaded macrophages. Then the appearance of ³H-tracer in plasma, liver, bile, intestinal wall, and feces over 48 h was determined. Plasma lipid analysis indicated phospholipid transfer protein deficiency lowered total cholesterol, high-density lipoprotein-C and apolipoprotein A1 levels significantly but increased triglyceride level in mice. The isotope tracing experiment showed ³H-cholesterol of plasma was decreased by 68% for male and 62% for female, and ³H-tracer of bile was decreased by 37% for male and 21% for female in phospholipid transfer protein KO mice compared with WT mice. However, there was no difference in liver, and ³H-tracer of intestinal wall was increased by 43% for male and 27% for female. Finally, ³H-tracer of fecal excretion in phospholipid transfer protein KO mice was reduced significantly by 36% for male and 43% for female during 0–24 h period, but there was no significant difference during 24–48 h period. Meanwhile, Western Blot analysis showed the expressions of reverse cholesterol transport -related protein liver X receptor α (LXR α), ATP binding cassette transporter A1, and cholesterol 7 α -hydroxylase A1 were upregulated in liver of phospholipid transfer protein KO mice compared with WT mice. These data reveal that systemic phospholipid transfer protein deficiency in mice impairs macrophage-specific reverse cholesterol transport *in vivo*.

Keywords: Phospholipid transfer protein, reverse cholesterol transport, high-density lipoprotein

Experimental Biology and Medicine 2016; 241: 1466–1472. DOI: 10.1177/1535370216641218

Introduction

Reverse cholesterol transport (RCT) is believed to inhibit atherogenesis by transporting excessive cholesterol from peripheral tissues to liver and intestine for excretion. An inverse association between high-density lipoprotein (HDL) level and atherosclerosis (AS) is found in many studies, which is owing to the ability of HDL to promote RCT. One key protein in HDL metabolism and possibly also in RCT is phospholipid transfer protein (PLTP).

PLTP is produced in various types of cells and secreted into plasma, where it transfers phospholipids between lipoproteins and modulates the size and composition of HDL particles, generating lipid-poor pre β -HDL (an efficient

acceptor of cellular cholesterol *in vitro*).¹ Systemic PLTP deficiency results in an obvious reduction of HDL cholesterol level in circulation.² Moreover, PLTP stabilizes ATP binding cassette transporter A1 (ABCA1), an important transporter to transfer cholesterol from peripheral tissues to apoA1.³ It has been reported that the absence of exogenous⁴ or endogenous⁵ PLTP impaired ABCA1-dependent efflux of cholesterol from macrophage foam cells. PLTP KO/apoE KO mice fed high-fat diet for seven weeks had higher plasma free cholesterol level and lower biliary cholesterol secretion than apoE KO mice.³ The pro-atherosclerotic effect resulted from PLTP deficiency is probably related to RCT.

In the present study, we observed the role of systemic PLTP knockout in antiatherosclerotic process of RCT

in vivo by a validated assay in which labelled cholesterol originating from mouse peritoneal macrophages was traced in plasma, liver, intestine, bile, and feces.

Materials and methods

Materials

Acetylated low density lipoprotein (acLDL) was prepared following a previous study with partial modification.⁶ [1,2-³H]-cholesterol was obtained from PerkinElmer (Waltham, MA, USA). Raw264.7 macrophages were bought from Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). RPMI1640 medium and fetal bovine serum were products of GIBCO (Canada). Plasma total cholesterol (TC), triglyceride (TG), HDL cholesterol (HDL-C) assay kits were obtained from Applygen (Beijing, China). Primary antibodies of apolipoprotein A1 (apoA1), liver X receptor α (LXR α), ABCA1, cholesterol 7 α -hydroxylase A1 (CYP7A1), scavenger receptor class B type 1 (SR-B1), and low-density lipoprotein receptor (LDL-R) were purchased from Abcam (Cambridge, MA, USA). Primary antibody for β -actin was obtained from Santa Cruz (USA). Enzyme-linked immunosorbent assay (ELISA) kit of apoA1 was purchased from Shanghai Langton Biotechnology Co. Ltd. (Shanghai, China).

Animal and diet

PLTP KO mice were presented by Dr Xiancheng Jiang, and all the animals were on a homogenous C57BL/6 background (nine generation backcrosses). PLTP KO mice (eight for male and eight for female) and WT mice (eight for male and eight for female), 10–12 weeks old, were applied to test RCT, and they were the offspring littermates of PLTP heterozygous mice. From 6–8 weeks old, the 32 mice were fed high-fat diet (15.8% fat and 1.25% cholesterol) for one month. These animals were housed in a temperature- and humidity-controlled room with a 12/12h light-dark cycle. All experiments were approved by the laboratory animals' ethical committee of Taishan Medical University and followed national guidelines for the care and use of animals.

³H-acLDL-loaded macrophages preparation for RCT

³H-acLDL-loaded macrophages were prepared for RCT as described previously.⁷ The procedure was as follows: Raw264.7 macrophages were loaded in RPMI1640 medium supplemented with 5 mCi/L ³H-cholesterol and 100 mg/L Ac-LDL for 24 h, then equilibrated and harvested for intraperitoneal injection to mice. The ratio of intracellular ³H-cholesterol to total ³H-cholesterol (both intracellular and extracellular) were obtained by a liquid scintillation counter. In this study, the ratio was more than 95%, which indicated ³H-cholesterol was mostly in macrophages injected.

In vivo RCT study

In vivo RCT study was performed essentially as published previously.⁸ After the littermate, PLTP KO and WT mice were fed high-fat diet for one month, they were injected intraperitoneally with the prepared ³H-acLDL-loaded macrophages (typically 7.5×10^5 cells containing 5.5×10^5 counts per minute in 0.5 ml minimum essential media per mouse). At 48 h after injection, blood was collected by retro-orbital puncture, and plasma was isolated and stored at -80°C . The mice were anesthetized and sacrificed. Bile was collected by microinjector. The small intestine from stomach pylorus to caecum was cut away and flushed with saline using a syringe and a feeding 20 G needle. Liver was taken out, weighed and stored at -80°C . Feces were collected during 0–24 h and 24–48 h after injection. Liver (0.5 g), the entire intestinal wall and feces were homogenized in a hexane-isopropanol (3:2, v:v) solution by an ultra-turrax, and the samples were rotated on a shaker overnight at 4°C . Then the samples were centrifuged and the upper fluid was carefully transferred to new vials. The vials were dried by speed vac. (Thermo Electron Corporation). ³H-tracer levels in plasma, liver, bile, intestinal wall, and feces were measured by liquid scintillation counting and expressed as percentages of injected ³H dose.

Plasma lipid and apoA1 levels

The levels of plasma TC, TG, and HDL-C were tested by enzymatic method. Non-HDL-C was calculated as TC minus HDL-C. To measure apoA1 level in plasma, both ELISA and Western blot analysis were applied. In Western blot, 0.2 μl of plasma was denatured at 90°C for 10 min and then subjected to SDS-PAGE.

Western blot

Total proteins from mice's liver were extracted with RIPA lysis buffer. The equal amounts of protein were subjected to 8% to 15% SDS-PAGE and then transferred onto PVDF membranes by electroblotting. After blocked with Tris-buffered saline (TBS) complement with 10% non-fat dry milk and 0.1% Tween 20 for 3 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C . After washed with TBS complement with 0.1% Tween 20, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoblots were exposed by ECL reaction and visualized by a high-performance chemiluminescence film. The IOD value of immunoreactive bands was determined using Image-Pro Plus software and normalized by house-keeping protein (β -actin).

Statistical analysis

Results are presented as mean $\pm$ SD. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Student-Newmann-Keuls multiple comparison tests with SPSS 13.0 software for Windows. *P* values less than 0.05 were considered statistically significant.

Results

PLTP deficiency decreased plasma TC, HDL-C, and apoA1 levels significantly but increased plasma TG level in mice fed high-fat diet

After the mice were fed high-fat diet for one month, the weight and plasma lipid levels of PLTP KO mice were compared with those of WT mice (both male and female). As indicated in Table 1, the body weight of male PLTP KO mice was lowered by 6% compared with that of male WT mice, whereas there was no significant difference in female mice. The liver weight of PLTP KO mice was not significantly different from that of WT mice of same gender. Plasma lipid analysis (PLTP KO mice versus WT littermates) showed a marked reduction in TC and HDL-C (55% and 79%, for male, respectively; 55% and 80%, for female, respectively). Compared with that of WT mice, TG level of PLTP KO mice was promoted by 23% for male and by 31% for female. Non-HDL-C level was not significantly altered between PLTP KO and WT mice for both male and female (Table 1). ApoA1 level in plasma was also determined by Western blot and ELISA analysis, and the data showed that it was obviously downregulated in PLTP KO mice compared with WT mice (Figure 1).

PLTP deficiency impaired macrophage-specific RCT *in vivo*

To evaluate the role of PLTP deficiency in RCT *in vivo*, we assessed the mobilization of radiolabeled cholesterol from macrophages to plasma, liver, intestine, bile, and feces by

the method which had been widely applied to physiological and pharmacological studies. First, ^3H -cholesterol-labeled and acLDL-loaded macrophages were injected into the peritoneum of littermate PLTP KO and WT mice fed high-fat diet. At 48 h after injection, blood, liver, intestine, bile, and feces of the mice were collected for analysis. The radioactivity in the samples was quantified to determine ^3H -cholesterol distribution along RCT pathway. Compared with that of WT mice of the same gender, ^3H -cholesterol in plasma of PLTP KO mice exhibited a significant decrease by 68% for male and 62% for female; however, there was no difference in ^3H -tracer of liver for both male and female; ^3H -tracer in intestinal wall of PLTP KO mice was increased by 43% for male and 27% for female; ^3H -tracer in bile of PLTP KO mice was lowered by 37% for male and 21% for female; finally, there was a significant reduction in ^3H -tracer of fecal excretion by 36% for male and 43% for female during 0–24 h period, but there was no statistical difference during 24–48 h period (Figure 2).

PLTP deficiency upregulated protein expressions of LXR α , ABCA1, and CYP7A1 involved in RCT in liver of mice

In this study, we also observed the effect of PLTP deficiency on the transporters, enzyme, and transcription factor of mice liver involved in RCT (ABCA1, SR-B1, LDL-R, CYP7A1, and LXR α) by Western blot. As shown in Figure 3, compared with those of WT mice of the same gender, the expression of transcription factor LXR α in liver of PLTP KO mice was promoted; the expressions of

Table 1 Weight and plasma lipid analysis of the littermate PLTP KO and WT mice fed high-fat diet

Type (sex)	Body weight (g)	Liver weight (g)	TC (mg/dl)	HDL-C (mg/dl)	non-HDL-C (mg/dl)	TG (mg/dl)
WT (male)	30.0 $\pm$ 1.0	1.44 $\pm$ 0.1	147.5 $\pm$ 13.0	90.1 $\pm$ 3.5	57.4 $\pm$ 13.2	83.2 $\pm$ 14.1
PLTP KO (male)	28.2 $\pm$ 1.5*	1.4 $\pm$ 0.2	67.0 $\pm$ 15.4***	18.5 $\pm$ 6.1***	48.5 $\pm$ 13.4	102.4 $\pm$ 13.7*
WT (female)	22.7 $\pm$ 1.4	1.1 $\pm$ 0.2	136.0 $\pm$ 19.6	83.6 $\pm$ 11.9	52.4 $\pm$ 9.7	78.5 $\pm$ 12.6
PLTP KO (female)	22.8 $\pm$ 2.3	1.0 $\pm$ 0.2	61.7 $\pm$ 8.6***	16.7 $\pm$ 2.4***	45.0 $\pm$ 9.1	103.0 $\pm$ 7.7**

Concentrations of plasma total cholesterol, HDL cholesterol, and triglyceride were determined by enzymatic method. Non-HDL-C was calculated as TC minus HDL-C. Data were given as mean $\pm$ SD ($n = 8$). TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglyceride. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs WT mice of the same gender.

Figure 1 PLTP deficiency decreased apoA1 level in plasma of mice fed high-fat diet. (a) Shows the representative image of apoA1 protein level in equal amounts of plasma by Western blot. (b) Shows the IOD ratios of apoA1 expression respectively by Western Blot. (c) It shows apoA1 level in plasma by ELISA. Data were presented as mean $\pm$ SD ($n = 8$). ** $P < 0.01$, *** $P < 0.001$ versus WT mice of the same gender

Figure 2 PLTP deficiency impaired macrophage-specific RCT *in vivo*. After the littermate PLTP KO and WT mice were fed high-fat diet for one month, they were injected intraperitoneally with ^3H -cholesterol-labeled and acLDL-loaded Raw264.7 cell. At 48 h after injection, macrophage-derived ^3H -tracer was quantified in plasma (a), liver (b), intestine (c), bile (d), and feces (e). All obtained counts were expressed as percentages of injected ^3H dose. Data were presented as mean $\pm$ SD ($n=8$). * $P < 0.05$, *** $P < 0.001$ versus WT mice of the same gender

ABCA1 and CYP7A1 were also significantly increased for both male and female; but there was no significant difference in protein expressions of LDL-R and SR-B1.

Discussion

Atherogenesis is triggered by accumulation of cholesterol-rich lipid strikes in the arterial wall.⁹ One defensive mechanism for AS is to remove excessive cholesterol from the artery intima via RCT. RCT can be completed via two pathways: hepatobiliary secretion pathway and transintestinal cholesterol efflux (TICE) pathway. The former is also named classic pathway. The process is as follows:¹⁰⁻¹² first, cholesterol from peripheral cells, including macrophages in the arterial wall, is effluxed to HDL via ABCG1 or SR-B1 and apoA1 via ABCA1 in plasma. Then the cholesterol is

transported by lipoproteins to liver where it can be taken up by hepatocytes via specific lipoprotein receptors SR-B1 and LDL-R. After uptake, the cholesterol can be converted into bile acid and its rate-limiting enzyme is CYP7A1. Both bile acid and cholesterol in liver can be delivered into bile duct and intestinal lumen where a large amount of bile acid and cholesterol is re-absorbed. The remaining is discharged from the body via fecal. Recently, it has become evident that there is a non-biliary cholesterol secretion pathway, i.e. TICE pathway. This pathway also contributes significantly to the evacuation of total fecal neutral sterol.¹³

In RCT process, HDL mediates the efflux of cholesterol from peripheral cells.¹⁴ Both characteristics and level of HDL are critical determinants for RCT. It is well known that PLTP impacts both HDL cholesterol level and biological quality of HDL molecule. Given its effect on HDL

Figure 3 PLTP deficiency upregulated the expressions of RCT-related protein LXR α , ABCA1, and CYP7A1 in mice's liver. The protein expressions were tested by western blot and normalized to β -actin level. (a), (c), (e), (g), and (i) show representative images of LXR α , ABCA1, CYP7A1, SR-B1, and LDL-R protein expressions by Western blot, respectively. (b), (d), (f), (h), and (j) show IOD ratios of LXR α , ABCA1, CYP7A1, SR-B1, and LDL-R to β -actin, respectively. Data were presented as mean $\pm$ SD ($n=8$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus WT mice of the same gender

metabolism, PLTP's role in RCT deserves to be addressed. In the present study, we focused on the potential role of PLTP deficiency in RCT progress by isotope tracing technique. For the first time, we found PLTP deficiency in mice led to the decrease of discharged ^3H -cholesterol in feces, which elucidated PLTP deficiency impaired macrophage-specific RCT *in vivo*. The mechanism was related to the following:

1. PLTP deficiency weakened ^3H -cholesterol efflux from macrophage to plasma. Our data indicated after injecting intraperitoneally with ^3H -acLDL-loaded

macrophages, plasma ^3H -cholesterol level of PLTP KO mice exhibited a significant decrease compared with that of the littermate WT mice (Figure 2(a)). The main reason of the decrease should be the sharp dive of plasma HDL and apoA1 in PLTP KO mice which were illustrated in Table 1 and Figure 1. Plasma HDL and apoA1, as major acceptors, can mediate cholesterol efflux from peripheral tissues and carry back to liver for further conversion and evacuation of cholesterol. Their concentration is the key determinant of RCT efficiency *in vivo*. Moreover,

the decreased ^3H -cholesterol level in plasma was probably related to the damaged ABCA1 function. It has been reported that absence of endogenous PLTP impairs ABCA1-dependent efflux of cholesterol from macrophage foam cells.⁴⁻⁵

2. PLTP deficiency significantly lowered the level of ^3H -tracer in bile. The possible reason involved two aspects. The cholesterol in liver can either be converted to bile acid via CYP7A1 and enter into bile or directly be secreted into bile via ABCG5/G8.¹⁵ In this study, the decrease of ^3H -cholesterol delivered into liver by plasma HDL may have resulted in a decrease of ^3H -cholesterol and ^3H -bile acid in bile of PLTP KO mice. A previous study indicated hepatic output of biliary cholesterol was significantly reduced in PLTP KO mice compared with WT controls.³ Thus, it is reasonable to conclude that ^3H -cholesterol secretion into bile may have been reduced for the PLTP KO mice.
3. PLTP deficiency lessened the secretion of ^3H -cholesterol via intestine. Our experiment showed ^3H -cholesterol in intestinal wall was increased in PLTP KO mice, which was probably caused by the dysfunction of secreting cholesterol into intestinal lumen via enterocytes. Jiang et al.¹⁶ have previously shown that PLTP deficiency specifically decreased cholesterol secretion in isolated PLTP KO enterocytes. Two pathways get involved in this process: (1) PLTP deficiency decreases cholesterol secretion of enterocytes in the form of apoB-containing lipoproteins because PLTP is involved in addition or remodeling of phospholipids on nascent apoB-containing lipoproteins.¹⁷ (2) PLTP deficiency also decreases cholesterol secretion of enterocytes in the form of HDL. ApoA1 and ABCA1 play roles in this pathway.¹⁸⁻²⁰ In PLTP KO mice, ApoA1 level is decreased (Figure 1). Meanwhile, ABCA1-dependent cholesterol export involves an initial interaction of apoA1 with lipid raft membrane domains.²¹ It is conceivable that PLTP deficiency might influence lipid composition on plasma membrane of enterocytes, thus lower cholesterol efflux.

These factors worked together, especially significant lowering of plasma HDL and apoA1, resulting in a decreased movement of cholesterol from macrophages to feces in mice with PLTP deficiency. The total excretion of ^3H -cholesterol via feces includes fecal neutral sterol fraction and bile acid fraction. Whether the decrease in ^3H -tracer of feces from PLTP KO mice is due to the alteration of ^3H -neutral sterol or ^3H -bile acid need to be further clarified.

Liver X receptor (LXR) is one of nuclear receptor superfamily which can regulate important lipid metabolic pathways.²²⁻²⁴ It has two isoforms, LXR α and LXR β . LXR α is mainly expressed in liver and kidney, whereas LXR β expression is ubiquitous with low level in liver. PLTP is a direct target gene of LXR. The previous study has confirmed that LXR agonist transcriptionally upregulates PLTP expression, which contributes to the increase of

HDL-C.²⁵ In this study, we found that the ablation of PLTP in mice promoted the protein expression of LXR α in a compensative way. The corresponding mechanism needs to be explored further. Besides PLTP, many other proteins involving RCT (ABCA1, CYP7A1, ABCG5/G8) are also regulated by LXR.²⁶ In this study, our data indicated the protein levels of ABCA1 and CYP7A1 were increased, which were probably upregulated by LXR α . It has been reported that PLTP deficiency also upregulated ABCG5/G8 expression in liver of mice on a chow or HFHC diet.²⁷ SR-B1 and LDL-R are important transporters involved in RCT. SR-B1 is HDL receptor on the surface of hepatocytes, and it can transport CE from the center of HDL into liver. LDL-R can combine with LDL and VLDL to internalize them into hepatocytes.²⁸ In this study, we found PLTP deficiency had no significant effect on their expression.

In summary, we provide evidence to show that PLTP deficiency in mice impairs macrophage-specific RCT *in vivo*. Meanwhile, PLTP deficiency upregulates the expressions of RCT-related protein LXR α , ABCA1, and CYP7A1 in mice's liver.

Authors contributions: All authors participated in the design and laboratory experiments of the study. YS and YZ analyzed data and wrote the manuscript. All authors approved the final version of the manuscript. YS and YZ equally contributed to this article.

ACKNOWLEDGEMENTS

This study was financially supported by Natural Science Foundations of China (81070247 and 31300639), Shandong Provincial Natural Science Fund (ZR2013HL063), Shandong Provincial Key Research and Development Program (2015GSF119008), and Tai'an City Research and Development planning (201440774-03).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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(Received September 13, 2015, Accepted February 16, 2016)