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THE EFFECT OF PHOSPHOLIPID TRANSFER PROTEIN ON LIPOPROTEIN METABOLISM AND ATHEROSCLEROSIS

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1. ABSTRACT

Phospholipid transfer protein (PLTP) is a member of the lipid transfer/lipopolysaccharide binding protein gene family. Recently, the crystal structure of one of the members of the gene family, bactericidal permeability increasing protein, was solved, providing potential insights into the mechanisms of action of PLTP. These molecules contain intrinsic lipid binding sites and appear to act as carrier proteins that shuttle between lipoproteins to redistribute lipids. The phenotype of PLTP transgenic and gene knock out mice indicates that PLTP plays a major role in the metabolism of high-density lipoprotein (HDL) and apoB-containing lipoproteins and thereby influences the concentration, apolipoprotein content, and size of lipoprotein particles in plasma. Recent data indicate that PLTP deficiency in mice is associated with a decrease of atherosclerosis, despite decreased HDL levels. At lease two underlined mechanisms are involved in the reduction of atherosclerosis in PLTP deficient status, 1) reduction of apoB-containing lipoprotein production and levels; and 2) increase of anti-oxidation potential. Human studies indicated that PLTP activity positively correlated with aging, obesity, diabetes and coronary artery disease. A challenge for the future will be the inhibition of PLTP for therapeutic benefit.

2. PLTP CHARACTERISTICS

Havel and colleagues (1,2) initially suggested that there might be transfer of surface components (phospholipids, soluble apolipoproteins) from apoB-containing lipoprotein (BLp) into high density lipoprotein (HDL). This was subsequently demonstrated by injection or perfusion of chylomicrons or VLDL in intact rats or their organs (3-6). It was thought that phospholipid (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 (7). However, Tall et al (8,9) 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 (11,12).

The PLTP is expressed ubiquitously (11,13). 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 (13) and small intestine (Jiang, X.C. unpublished results) also appear to be the most important sites of PLTP expression.

The relationship between PLTP structure and function is far from resolved. However, some progresses have been made. Human PLTP contains 476 amino acid residues and has an 83% homology with mouse PLTP (13)

and a 93% homology with pig PLTP (14). PLTP belongs to a family of lipid transfer/lipopolysaccharide binding proteins including lipopolysaccharide-binding protein (LBP), bactericidal/permeability increasing protein (BPI) and CETP (11). These proteins show sequence homology, related gene structures and probably share a similar protein fold (15,16). Despite the fairly low levels of amino acid sequence identity between the family members (20%-26%) (11) certain common structural characteristics, as well as similar gene organization (17-20) indicate functional relatedness and suggest a common evolutionary origin of these proteins. The N-terminal regions of PLTP, CETP, LBP and BPI are markedly homologous, while the C-terminal regions are less conserved. It is suggested that C-terminal region may possess the specific properties of PLTP and CETP, namely the binding and transfer of surface lipids in the case of PLTP and the binding and transfer of polar lipids in the case of CETP, respectively (21). Human PLTP contains four cysteine residues and six potential N-glycosylation sites (11). Using cell-culture studies and site-directed mutagenesis, it was demonstrated that the cysteine residues 146 and 185 form a disulphide bridge in PLTP which is essential for the correct folding and secretion of the protein (15,22,23). The major secreted forms of PLTP carry complex N-glycans and N-glycosylation is crucial for efficient PLTP secretion (22). However, when PLTP was expressed in baculovirus/insect-cell system, which is capable of performing only high-mannose type glycosylation, efficient secretion and full activity of the protein were still observed (24). Two conservative amino acids Arg218 and Arg245 of PLTP might mediate the electrostatic interaction of PLTP with lipoprotein negative charges (25). Desrumaux et al., indicated that a cluster of hydrophobic residues (Trp91, Phe92 and Phe93) in PLTP is critical for PLTP activity. Substitution of all three amino acids to alanine drastically reduced the HDL size conversion and PL transfer (from vesicle to HDL) activities of PLTP. Based on the results, the authors proposed that a functional solvent-exposed hydrophobic cluster in the PLTP molecule specifically contributes to the PLTP transfer activity on HDL substrates (26).

In terms of lipid transfer activity, PLTP has its own characteristics. PLTP has no neutral lipid transfer activity (27-31). 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 phospholipid into HDL results in the formation of larger, less dense species (27,31). In human plasma, PLTP is responsible for about half of the activity mediating phospholipid exchange between lipoproteins (28,31), and CETP accounts for the other half (28,31). Plasma PLTP is also a non-specific lipid transfer protein. Several studies have indicated that PLTP is capable of transferring all common phospholipids (29-32). Besides phospholipids, diacylglycerol, α-tocopherol, cerebroside and lipopolysaccharides are also transferred efficiently (33). The PLTP-mediate transfer of phosphatidylcholine molecules varying in their fatty acyl chain between the sn-1 and sn-2 positions in the phospholipid molecules does not affect the phospholipid-transfer rate, indicating that PLTP is unable to discriminate between positional isomers (29,31). Although, CETP also can transfer phospholipid, there is no redundancy in function of PLTP and CETP in mouse model (34).

Two forms of PLTP exist in human plasma, one catalytically active (high activity form, HA-PLTP) and the other inactive (low activity form, LA-PLTP) (35, 36). The two forms are associated with macromolecular complexes of different size. The apparent size of LA-PLTP is 520 kDa and that of HA-PLTP 160 kDa. Of the circulating PLTP mass only a minor portion is in the HA-PLTP form in normolipidemic subjects. Both LA- and HA-PLTP bind to Heparin-Sepharose and can be separated by elution with 0-0.5 M NaCl gradient, with HA-PLTP displaying higher affinity for the matrix. Analysis of the two forms of PLTP by SDS-PAGE, Western blotting, immunoprecipitation and gel filtration demonstrates that LA-PLTP is complexed with apoA-I while HA-PLTP is not. Instead, HA-PLTP copurified with apoE. Based on these findings the author suggest a model in which nascent PLTP enters the circulation as a high specific activity form not associated with apoA-I. During or after the transfer of lipolytic surface remnants to HDL, PLTP is transferred to apoA-I-containing HDL particles and thereby becomes part of the low-activity complex (37).

3. PLTP REGULATION

PLTP activity and mRNA can be regulated by many factors. A high fat, high cholesterol diet caused a significant increase (35%) in PLTP activity and this increased activity was accompanied by approximately 100% in PLTP mRNA in lung (13). After lipopolysaccharide injection, plasma PLTP activity was decreased by approximately 66%. This decrease in activity was associated with a similar decrease in PLTP mRNA in lung, adipose tissue, and liver (13). PLTP expression and activity can be upregulated by glucose (38, Jiang et al unpublished observation) and downregulated by insulin (39,40). It is reported that diacylglyceroles (DAG) can modulate the PLTP-dependent phospholipid transfers, both by competition with phospholipid and by increasing the viscosity of the particle surfaces (41).

How PLTP expression is regulated? PLTP promoter shows no strong homology to known steroid response elements (SREs), but contains farnesoid X-activated receptor (FXR) and peroxisome proliferator-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 (42,43). The transcriptional activity of this gene was significantly increased by chenodeoxycholic acid and fenofibrate, suggesting that transcription FXR and PPAR are likely involved in the transcriptional regulation (44). DNA sequence analysis suggests that DNA sequences from -407 to -395 and from -393 to -381 are homologous to the recognition motifs of FXR (44), and those from -859 to -847 and from -309 to -297 are similar to the potential

binding motif for PPAR (44). Significant rises in plasma PLTP activity were observed after 2 weeks of fenofibrate, a PPAR activator, treatment in both wild-type and human apoAI transgenic mice. Simultaneously, hepatic PLTP mRNA levels increased in a dose-dependent fashion (45). Fenofibrate significantly increased HDL size, an effect that was more pronounced in human apoAI transgenic mice than in wild-type mice. This effect in wild-type mice was completely abolished in PLTP-deficient mice. Moreover, fenofibrate treatment did not influence PLTP activity or hepatic mRNA in PPAR deficient mice indicating that fenofibrate treatment increases plasma phospholipid transfer activity as the result of up-regulation of PLTP gene expression through a PPARα-dependent mechanism. (45). Mice fed a chow diet supplemented with bile acid showed increased hepatic PLTP mRNA levels indicating that FXR may also play a role in high density lipoprotein metabolism via the regulation of PLTP gene expression (46). Although both mouse and human PLTP promoter regions do not contain typical LXR element (DR-4) (47), the administration of an LXR agonist (48) in mouse upregulated both PLTP mRNA and activity in a dose-dependent manner (Jiang X.C., and Cao G., unpublished observation). The treatment of oxidized steroids, ligands for LXR, also induced PLTP mRNA levels in macrophage (Tall, AR, personal communication). Earlier reports show that various degenerate forms of IR-1, DR-3, or DR-4 elements were bound by FXR/RXRα in vitro (18,22). It is conceivable that some degenerate DR-4 element(s), which is (are) responsible for the induction of PLTP expression by LXR agonist, may exist. The physiologic relevance of PLTP upregulated by both LXR and FXR deserves further studies.

Among non-insulin-dependent diabetes mellitus patients, PLTP levels were positively correlated with fasting glycemia and glycohemoglobin levels but not with plasma lipid parameters. It is proposed that plasma PLTP mass levels are related to glucose metabolism rather than to lipid metabolism (49). One report indicated that the glucose-responsive elements are located between -759 and -230 of the PLTP 5'-flanking region, within which two binding motifs (-537 to -524 and -339 to -327) for either PPAR and FXR are involved in this glucose-mediated transcriptional regulation. This finding suggests that high glucose upregulates the transcription of human PLTP gene via nuclear hormone receptors (38).

4. PLTP OVEREXPRESSION

Mouse PLTP transgenic approaches resulted in a complicated phenotype. 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 apoA-I showed a significant increase in the plasma levels of α-HDL (50,51) and preβ-HDL (50). The minor 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 preβ-HDL particles are in part generated by the PLTP reaction.

Overexpression of PLTP in mice was also achieved by adenovirus and adenovirus associated virus mediated infection. The former resulted in a 10- to 40-fold increase in plasma PLTP activity (52,53). These mice were characterized by the increased preβ-HDL levels, but decreased α-HDL levels due to an increased fractional catabolic rate of HDL, and enhanced hepatic uptake of HDL-CE compared with the wild-type 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 (more than 6 months) and resulted a dramatic decrease of total cholesterol and HDL cholesterol in C57BL/6 mice (Jiang et al., unpublished observation).

Recently, transgenic mice that overexpress human PLTP at high levels were generated. Compared with wild-type mice, these mice show a 2.5- to 4.5-fold increase in PLTP activity in plasma. This results in a 30% to 40% decrease of plasma levels of HDL cholesterol. Incubation of plasma from transgenic animals at 37^0C reveals a 2- to 3-fold increase in the formation of preβ-HDL compared with plasma from wild-type mice. Although preβ-HDL is normally a minor subfraction of HDL, it is known to be a very efficient acceptor of peripheral cell cholesterol and a key mediator in reverse cholesterol transport. Further experiments show that plasma from transgenic animals is much more efficient in preventing the accumulation of intracellular cholesterol in macrophages than plasma from wild-type mice, despite lower total HDL concentrations. It is concluded that PLTP can act as an antiatherogenic factor preventing cellular cholesterol overload by generation of preβ-HDL (54).

HDL are considered anti-atherogenic because they mediate peripheral cell cholesterol transport to the liver for excretion and degradation. An important step in this reverse cholesterol-transport pathway is the efflux of cellular cholesterol by a specific subclass of small, lipid-poor apolipoprotein A-I particles designated preβ-HDL. The two lipid-transfer proteins present in human plasma (CETP and PLTP) have both been implicated in the formation of preβ-HDL. However, both lipid transfer proteins have non-overlapping function *in vivo* (34, also see below). In order to investigate the relative contribution of each of these proteins, Lie et al. (55) used transgenic mouse models. Comparisons were made between human CETP transgenic mice (huCETPtg), human PLTP transgenic mice (huPLTPtg) and mice transgenic for both lipid-transfer proteins (huCETPtg/huPLTPtg). These animals showed elevated plasma levels of CETP activity, PLTP activity or both activities, respectively. The authors evaluated the generation of preβ-HDL in mouse plasma by immunoblotting and crossed immuno-electrophoresis. Generation of preβ-HDL was equal in huCETPtg and wild-type mice. In contrast, in huPLTPtg and huCETPtg/huPLTPtg mice, preβ-HDL generation was 3-fold higher than in plasma from either wild type or huCETPtg mice. The findings demonstrate that, of the two plasma lipid-transfer proteins, PLTP rather than CETP is responsible for the generation of preβ-HDL. These data

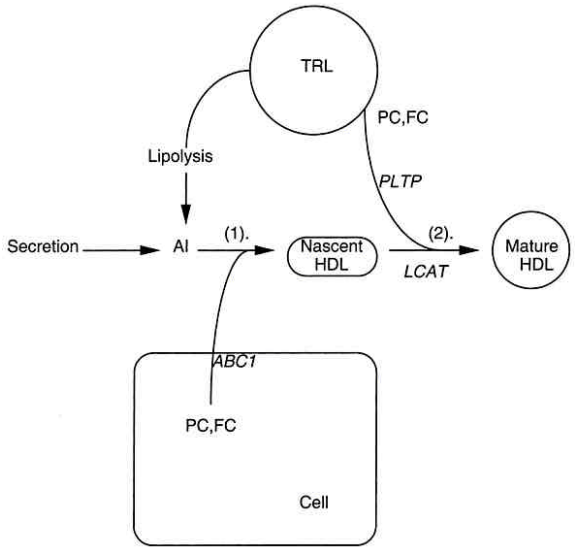

Figure 1. A hypothetical scheme for the role of PLTP in the formation of mature HDL particles.

support the hypothesis of a role for PLTP in the initial stage of reverse cholesterol transport.

5. PLTP DEFICIENCY

So far there is no PLTP deficiency found in human. The allelic frequencies of six intragenic polymorphisms, -79G/T, -56G/A, -37T/C, -31A/G, Phe2Leu, Arg121Trp, and two neutral polymorphisms, located in the immediate vicinity of the PLTP gene were determined and there were no significant associations between these polymorphisms and serum PLTP activity (56). The most informative knowledge about PLTP deficiency was obtained from PLTP gene knockout mice. The *in vivo* phospholipid transfer from triglyceride rich lipoprotein to HDL was completely blocked in the PLTP knockout mice (57). On a chow diet, the mice showed a marked decrease in HDL phospholipid, cholesterol and apo A-I, demonstrating the important role of PLTP-mediated transfer of surface components of TG-rich lipoproteins in the maintenance of HDL levels (57). Furthermore, the HDL of the PLTP knockout mice was enriched with protein and poor in phosphatidylcholine, and turnover studies showed a 4-fold increase in the catabolism of HDL protein and CE compared with that of wild-type mice (58). Thus, the impairment of phospholipid transfer from TG-rich lipoproteins into HDL may lead to hypoalphalipoproteinemia characterized by hypercatabolism of HDL protein.

Besed on the results from PLTP deficient mice, we proposed a hypothetical scheme for the role of PLTP in the formation of mature HDL particles (58). In step 1, phospholipids and free cholesterol efflux from cells as a result of ABCA1 activity (59,60), combine with apoA-I, generating nacent HDL. In step 2, nascent HDL acquires additional phospholipids and free cholesterol as a result of transfer from triglyceride-rich lipoprotein (TRL) by PLTP, and is acted on by LCAT (61), generating mature HDL (Figure 1). If this hypothesis is correct, it is conceivable that plasma PLTP activity not only influence HDL but also TRL metabolism.

PLTP may interact with other factors in lipoprotein metabolism. PLTP and CETP are homologous molecules that mediate neutral lipid and phospholipid exchange between plasma lipoproteins. Biochemical experiments suggest that only CETP can transfer neutral lipids but that there could be overlap in the ability of PLTP and CETP to transfer or exchange phospholipids. To see whether CETP can compensate for PLTP deficiency *in vivo*, we bred the human CETP transgene into the PLTP0 background. Our results indicate 1) that there is no redundancy in function of PLTP and CETP *in vivo* and 2) that the combination of the CETP transgene with PLTP deficiency results in an additive lowering of HDL levels, suggesting that the phenotype of a human PLTP deficiency state would include reduced HDL levels (34). A very interesting observation in PLTP deficient mice is that the accumulation of excess phospholipids and free cholesterol ("surface remnants") in plasma of the mouse on a high saturated fat diet (57,58). In order to study the role of diet, hepatic lipase (HL) and scavenger receptor BI (SRBI) in determining the accumulation of these particles, we crossed PLTP and HL deficient mice. Accumulation of phospholipid and free cholesterol was dramatically increased in double knockout mice compare to the single ones. Turnover studies indicated that the high saturated fat diet was associated with delayed catabolism of the particles. Incubation of these particles with primary hepatocytes in the presence and absence of SRBI neutralizing antibody indicated that SRBI was primarily responsible for removal of phospholipid and free cholesterol from mice on the Western diet. In hepatocytes of high saturated fat fed mice, removal of free cholesterol and phospholipid from these particles by SRBI was markedly reduced, even though SRBI protein expression levels was unchanged. These studies indicate that HL and SRBI both have major role in the clearance of phospholipid and free cholesterol of surface remnants in PLTP deficient mice. SRBI appears to be dysfunctional in high saturated fat fed animals, possible related to changes in hepatocyte membrane fatty acid composition (62).

Increased secretion and levels of ApoB-containing lipoproteins (BLp) commonly occur in familial hyperlipidemia, obesity and diabetes. The plasma PLTP is known to mediate transfer of phospholipids between BLp and HDL during their intravascular metabolism. To address a possible role of PLTP in dyslipidemia, PLTP-deficient mice were bred with different hyperlipidemic mouse strains. In ApoB-transgenic and ApoE-deficient backgrounds, PLTP deficiency resulted in reduced production and levels of BLp. BLp secretion was diminished in hepatocytes from ApoB-transgenic PLTP-deficient mice, a defect that was corrected when PLTP was reintroduced in adenovirus (63).

Vitamin E is one of the substrates of PLTP (64). To determine whether the plasma PLTP regulates lipoprotein vitamin E content in vivo, we measured the α-tocopherol content and the oxidation parameters of

lipoproteins from PLTP deficient mice crossed into the apoE-deficient, LDL receptor-deficient, or apoB/CETP transgenic backgrounds. In all three backgrounds, the vitamin E content of VLDL and/or LDL was significantly increased in PLTP deficient mice, compared to controls. Moreover, PLTP deficiency produced a dramatic delay in generation of conjugated dienes in copper-oxidized apoB-containing lipoproteins, as well as markedly lower titers of both circulating oxidized lipid epitopes and IgG auto-antibodies to oxidized LDL. The addition of purified PLTP to deficient plasma lowered the vitamin E content of V/LDL and normalized the generation of conjugated dienes. The data indicate that PLTP regulates the bioavailability of vitamin E in atherogenic lipoproteins. (65,66, Jiang et al unpublished observation).

6. PLTP IN DYSLIPIDEMIA AND ATHEROSCLEROSIS

The physiological role of PLTP in lipoprotein metabolism and atherosclerosis development is still far from resolved. PLTP activity has been measured in several different human pathological settings, which are closely related to dyslipidemia and atherosclerosis. The PLTP activity was increased in aging (56), obesity (67,68) NIDDM (69) and higher plasma PLTP activity is also associated with insulin resistance in conjunction with altered non-esterified fatty acids and triglyceride metabolism (68). Tahvanainen et al. measured serum PLTP activity in 400 healthy individuals and reported that the activity correlated positively with body mass index, total cholesterol and triglyceride (56). In terms of plasma HDL cholesterol (HDL-C) levels which have powerful antiatherogenic properties (70), the existing reports are contradictory. In one set of studies, plasma PLTP activity was positively related with HDL-C (71) and in patients with low HDL and cardiovascular disease, plasma PLTP activity is positively correlated with the concentration of HDL particles containing apoAI but not apoAII (72). However, in another study, Huuskonen et al. reported that the activity of PLTP in human plasma showed negative correlation with HDL-C and apoAI (73). The conflicting results might be due to 1) the small sample size, 2) the subjects were not controlled very well, and 3) the method for PLTP measurement was not precise enough.

Genetic mouse models have played a crucial role in elucidating the role of PLTP in lipoprotein metabolism and atherosclerosis. PLTP deficient mice have recently provided the first in vivo evidence of crucial roles for PLTP-mediated lipid transfer in the maintenance of lipoprotein levels and the atherosclerosis development. PLTP deficiency resulted in markedly decreased atherosclerosis (63) owing to at least two mechanisms, 1) decreased production and levels of apoB-containing lipoproteins (63); and 2) increased antioxidation potential (65,66, Jiang et al., unpublished observation).

Animal and human studies suggest that plasma PLTP levels are an important factor for the lipoprotein metabolism and atherosclerosis development. However, plasma PLTP levels have never been systematically assessed as a risk factor for atherosclerosis in humans. Since there are two forms of PLTP in human plasma (35-37), one is catalytically active and the other is inactive, PLTP activity measurement is more relevant than PLTP mass measurement. Recently, we utilized a novel, highthroughput method to test the hypothesis that PLTP activity levels are associated with coronary artery disease (CAD) in an angiographic case-control study (cases, n=1102, controls, n=444). We found plasma PLTP activity in CAD patients was higher than in control subjects ($p<0.0001$). Using multivariate logistic regression analysis, plasma PLTP activity was found to have independent predictive value for CAD after adjusting for age, plasma lipids, smoking, diabetes, hypertension, homocystein and c-reactive protein. The finding indicate that human plasma PLTP activity is positively and independently related to CAD in the studied population and suggest that 1) prospective studies to evaluate this relationship are warranted, and 2) PLTP is a therapeutic target for atherosclerosis (Blackenberg and Jiang, unpublished observation).

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