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Plasma Phospholipid Transfer Protein (PLTP): Quantitation, Biosynthesis, and Involvement in Hepatic Lipid Homeostasis

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Plasma Phospholipid Transfer Protein (PLTP): Quantitation, Biosynthesis, and Involvement in Hepatic Lipid Homeostasis

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Academic Dissertation

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I. ORIGINAL PUBLICATIONS

The original publications listed below form the content of this thesis and are referred to in the text by their corresponding Roman numerals. Additional unpublished results are also presented.

I. Siggins, S.*, Kärkkäinen, M.*, Tenhunen, J., Metso, J., Tahvanainen, E., Olkkonen, V.M., Jauhiainen, M., and Ehnholm, C. (2004). Quantitation of the active and low-active forms of human plasma phospholipid transfer protein by ELISA. *J Lipid Res*. 45: 387-395

II. Jänis, M., Siggins, S., Tahvanainen, E., Vikstedt, R., Silander, K., Metso, J., Aromaa, A., Taskinen, M-R., Olkkonen, V.M., Jauhiainen, M., and Ehnholm, C. (2004). Active and low-active forms of serum phospholipid transfer protein in a normal Finnish population sample. *J Lipid Res*. 45: 2303-2309

III. Siggins, S., Jauhiainen, M., Olkkonen, V.M., Tenhunen, J., and Ehnholm, C. (2003). PLTP secreted by HepG2 cells resembles the high-activity PLTP form in human plasma. *J Lipid Res*. 44: 1698-1704

IV. Siggins, S., Bykov, I., Hermansson, M., Somerharju, P., Lindros, K., Miettinen, T.A., Olkkonen, V.M., Jauhiainen, M., and Ehnholm, C. (2005). Altered hepatic lipid and apolipoprotein A-I metabolism in mice lacking phospholipid transfer protein. Submitted.

* The authors contributed equally to the study

II. ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
AD	Alzheimer's disease
ApoA-I	apolipoprotein A-I
BMI	body mass index
BPI	bactericidal/permeability increasing protein
bp	base pair
CDCA	chenodeoxycholic acid
C/EBP	CCAT/enhancer binding protein
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CSF	cerebrospinal fluid
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
$DxSO_4$	dextran sulfate
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FBS	foetal bovine serum
FFA	free fatty acid
FXR	farnesoid X-activated receptor
HA-PLTP	high-activity-PLTP
HDL	high-density lipoprotein
HL	hepatic lipase
HuPLTP	human PLTP
IDL	intermediate-density lipoprotein
kb	kilobase
kDa	kilodalton
KO	knockout
LA-PLTP	low-activity-PLTP

LBP	lipopolysaccharide binding protein
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
LXR	liver X-activated receptor
mRNA	messenger ribonucleic acid
NO	nitric oxide
PC	phosphatidylcholine
PAF-AH	platelet activating factor acetyl-hydrolase
PLTP	phospholipid transfer protein
PON-1	paraoxonase-1
PPAR	peroxisome proliferator-activated receptor
RCT	reverse cholesterol transport
ROS	reactive oxygen species
r-PLTP	recombinant phospholipid transfer protein
RXR	retinoid X receptor
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SM	sphingomyelin
SMC	smooth muscle cell
SR-B1	scavenger receptor B1
SREBP	sterol regulatory element binding protein
TG	triacylglycerol
TRL	triglyceride-rich lipoprotein
VLDL	very low-density lipoprotein
WHR	waist-to-hip ratio
WT	wild type

III. REVIEW OF THE LITERATURE

1. LIPOPROTEIN METABOLISM

1.1 OVERVIEW

Lipoproteins are soluble complexes of apolipoproteins and lipids that transport cholesterol and triglycerides in the circulation from one tissue site to another. Lipoproteins are synthesised from exogenous lipids by enterocytes in the intestine or then endogenously by hepatocytes in the liver, and can arise from the metabolic changes of circulating precursor lipoproteins. Most lipoproteins share a common spheroid structure consisting of a neutral lipid core of triacylglycerols and cholesteryl esters surrounded by a surface monolayer of phospholipids, unesterified cholesterol and apolipoproteins. The apolipoproteins solubilise the insoluble lipids and can act as cofactors for plasma enzymes and as ligands for cell surface receptors. Lipoproteins exist as a heterogeneous population of particles and the major classes are chylomicrons (CM), very low-density (VLDL), intermediate-density (IDL), low-density (LDL) and high-density (HDL) lipoproteins. Their relative contents of protein and lipid determines their hydrated density, size and mobility on agarose-gel electrophoresis and hence, their classification (Betteridge 1999).

Lipoproteins are highly dynamic particles that are constantly modified in the circulation via a number of processes, such as enzymatic reactions affecting lipid components, spontaneous and facilitated lipid transfers, and from the exchange of soluble apolipoproteins between lipoprotein particles. The interaction of these processes largely specifies the delivery of lipids to different tissues. Most lipoproteins, with the exception of the largest, triglyceride-rich particles, can cross the vascular bed or endothelial cell layer by transcytosis and be internalised by cells for energy utilisation, membrane biogenesis or for the synthesis of various lipid-derived bioregulators like steroidogenic hormones and D-vitamins. They can also be assembled with cellular lipids of peripheral tissues and extracellular lipoprotein particles or apolipoproteins. After having crossed the endothelium, the route for lipoprotein recirculation is via the interstitial fluid of the main

trunk lymph ducts to the plasma for transport to the liver or kidneys for catabolism and excretion (Vance 2002).

1.2. THE EXOGENOUS PATHWAY

The exogenous pathway is defined as the transport and processing of dietary fats such as cholesterol, plant sterols and dietary fatty acids. Dietary fats must first be hydrolysed into monoacylglycerols and fatty acids for transport across the intestinal microvillus membrane to the enterocytes where they are then re-esterified for packaging into nascent CMs. Large amounts of triacylglycerols (TGs), together with a small amount of cholesteryl esters, are incorporated into the core of CMs which are composed of a phospholipid monolayer with apolipoprotein B-48. Newly formed CMs are then secreted into the mesenteric lymph and enter the circulation via the thoracic duct. Intestinal apoA-I is also secreted and forms nascent HDL at the same time the CM reaches the circulation and undergoes TG lipolysis. A simultaneous movement of apoE and apoC proteins, from large spherical HDL reservoirs in the plasma, to the surface of the CM particle also occurs (Eisenberg 1984). The lipolytic enzyme, lipoprotein lipase (LPL), is predominantly bound to endothelial surfaces by heparin-like glycosaminoglycans and uses apoC-II as a cofactor for lipolysis (Goldberg et al. 1990). As the lipolytic reaction proceeds, apoC-II molecules are released, the TG core shrinks and the surface remnants are transferred to other lipoproteins, primarily to HDL. Free fatty acids (FFA) become bound to albumin as FFA/albumin complexes for transport to tissue sites of the body such as to muscle for energy use and to adipose tissue for storage. In cases of LPL deficiency, an alternative mechanism for FFA formation and uptake into adipose tissue is believed to be facilitated by endothelial lipase, a recently discovered phospholipase (Kratky et al. 2005). The residual CM remnant particle, retaining most of its original cholesteryl ester and retinyl ester content, is finally endocytosed by the hepatic receptors; LDL-receptor (Herz et al. 1988) or low-density lipoprotein receptor-related protein (LRP), via apoE, for clearance by the liver (Russel et al. 1983).

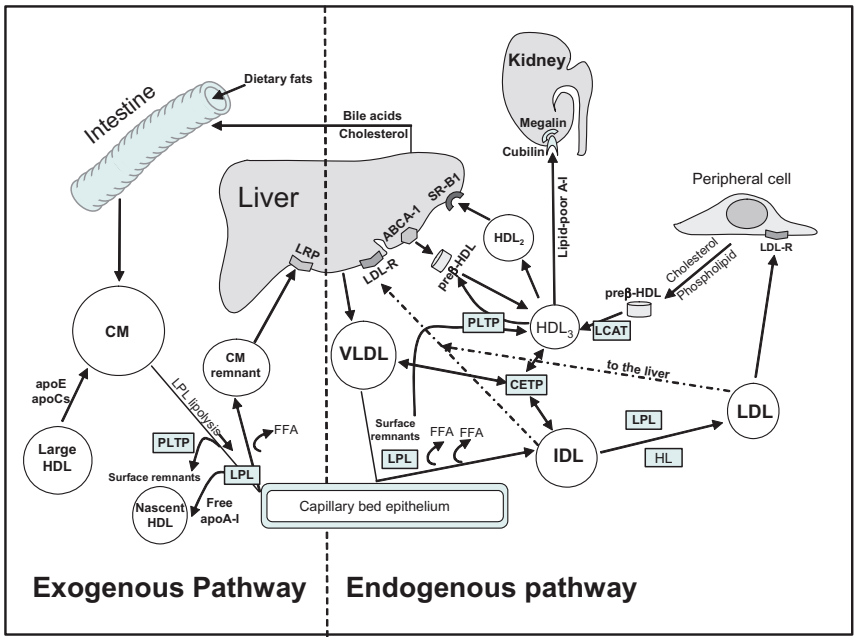

Figure 1. The pathways of lipoprotein transport and metabolism

1.3. THE ENDOGENOUS PATHWAY

The endogenous pathway begins with the production and secretion of triglyceride-rich lipoproteins by the liver in the form of VLDL. In contrast to CMs, VLDL particles contain a triacylglycerol-rich core comprised of a substantial amount of hepatic cholesteryl esters, the surface phospholipid monolayer is enriched in phosphatidylethanolamine, and the apolipoprotein constituent is apoB-100. The enrichment of VLDL with apoE and additional apoC proteins takes place in the plasma compartment, similar to that of CMs. When VLDL particles reach the capillary bed, their TGs are hydrolysed by LPL and the surface lipid/apolipoprotein remnants are subsequently transferred to HDL by phospholipid transfer protein (PLTP). LPL-mediated hydrolysis of VLDL occurs less efficiently than that of chylomicrons and this is most likely the cause for the slower plasma clearance rate of VLDL ($t_{1/2}$, days), as compared to chylomicrons ($t_{1/2}$, minutes to a few hours) (Vance 2002). The prolonged residency of

VLDL particles in the plasma compartment generates short-lived IDL particles. Approximately 50 - 70 % of the IDL particles are further modified into LDL by the action of hepatic lipase (HL) and LPL (Demant et al. 1988), and the remaining IDL portion is rapidly cleared by the liver via high affinity binding to the LDL-R. The cholesterol-rich LDL particles are then transported from the circulation to the liver and to peripheral cells and are consequently taken up by receptor-mediated endocytosis (Brown et al. 1986). The LDL-R interacts with apoB-100 on the particle surface to facilitate internalisation; the receptor is released and recycles to the cell surface, and the LDL particle is degraded in lysosomes, ultimately providing the cell with an extracellular source of cholesterol. A coordinated utilisation of both the intra-and extra-cellular cholesterol pools must therefore take place under highly stringent sterol regulatory feedback mechanisms in order to prevent excessive amounts of cholesterol accumulating in the cell.

1.4. REVERSE CHOLESTEROL TRANSPORT

The transfer of cholesterol from peripheral tissues to the liver for recycling or degradation and excretion into bile is denoted as Reverse Cholesterol Transport (RCT) (Fielding et al. 1995). This process is mediated by HDL particles that are formed when lipid-free apoA-I or lipid-poor preβ-HDL initiate the efflux of phospholipids and cholesterol from cell membranes via an interaction with the transmembrane ATP-binding cassette A1 (ABCA1) (Santamarina-Fojo et al. 2000). Cholesterol acquired by these nascent discoidal HDL particles is then esterified by lecithin-cholesterol acyltransferase (LCAT) and the newly formed cholesteryl esters move to the core of the particle, thus producing spherical α-HDL. The steady gradient of free cholesterol is maintained by LCAT and is essential for enabling HDL to accept cholesterol from various donors (Fielding et al. 1995). HDL also acquires phospholipids from the lipolytic surface remnants of triglyceride-rich lipoproteins that are transferred by PLTP (Tall et al. 1985). Also, a reciprocal exchange of cholesteryl ester from HDL for TG from triglyceride-rich lipoproteins (CM and VLDL) occurs via the activity of cholesteryl ester transfer protein (CETP). This lipid exchange shifts the bulk of cholesteryl esters to apoB-100-containing lipoprotein particles for clearance by the liver and, at the same time, enriches HDL with TG. Triglyceride-

enriched HDL consequently enhances HL-mediated lipolysis of the triglycerides and

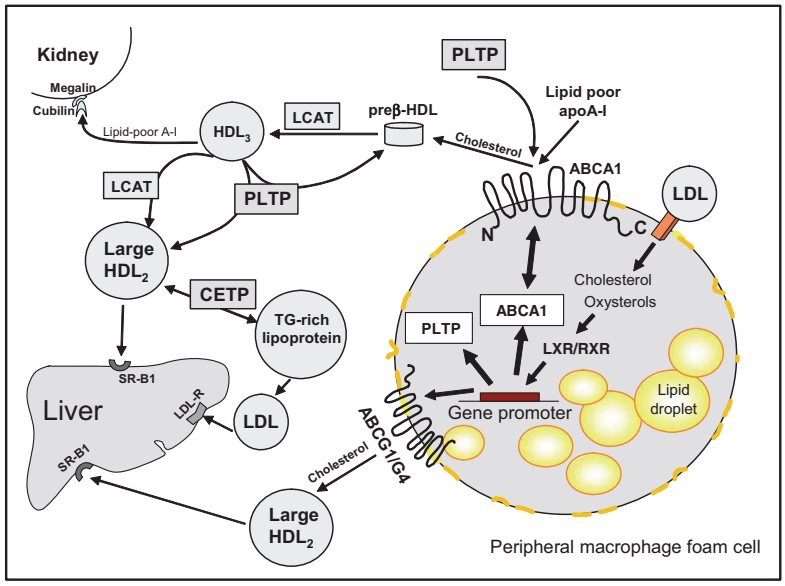

Figure 2. Reverse cholesterol transport

phospholipids thus assisting in the formation of smaller HDL_3 particles and preβ-mobile HDL. Cholesteryl ester-enriched HDL particles deliver cholesteryl esters to the liver via a selective uptake process activated upon binding to the scavenger receptor-B1 (SR-B1) (Acton et al. 1996). Once cholesterol enters the liver it can then be re-assembled into new lipoproteins, incorporated into membranes, secreted as such into bile, or converted into oxysterols for bile acid synthesis. In order for the cycle of RCT to persist, new acceptors of cellular cholesterol (apoA-I, preβ-HDL) must be continuously synthesised or regenerated in reactions that are catalysed by PLTP, CETP and HL, acting in conjunction with ABC transporters to enhance cellular cholesterol efflux.

1.5. ATHEROSCLEROSIS

Atherosclerotic vascular disease develops from complex multifactorial processes that contribute to the deposition and accumulation of cholesterol in focal areas of the arterial wall. The pathobiology behind the progression of the disease involves a series of

oxidative and enzymatic modifications of subendothelial lipoproteins, endothelial alterations, multifaceted inflammatory responses such as T cell recruitment, cytokine secretion and monocyte chemotaxis, and most profoundly, the receptor-mediated cellular cholesterol uptake and accumulation in macrophages and also smooth muscle cells (SMC) (Glass et al. 2001; Lusis et al. 2004). These events take years to develop and result in the formation of foam cells that aggregate into complex lesions containing vast amounts of extracellular lipid and calcium which are covered by a fibrous cap. When these plaques become unstable and rupture, an occlusion of the arterial lumen by the contents of the plaque leads to haemorrhage, thrombosis and ultimately, to acute vascular events such as heart attack, stroke or sudden death. Indeed, cardiovascular disease (CVD) represents the most frequent cause of death in the industrialised world (Glass et al. 2001). With an aging population, genetic risk factors such as gender, diabetes and hypertension, along with the environmental influences of a 'westernised' high-fat diet, smoking and inactivity, to name but a few, the implications of this disease will severely impact many societies world-wide. This societal burden will undoubtedly magnify in spite of the valiant advances in plasma lipid-lowering therapies achieved thus far.

2. HIGH DENSITY LIPOPROTEINS (HDL)

2.1. ANTI-ATHEROGENIC PROPERTIES OF HDL

The RCT pathway is believed to be one of the key mechanisms in the ability of HDL to provide a cardioprotective effect from the accumulation of non-hepatic peripheral cellular cholesterol. RCT entails the removal of cellular cholesterol by HDL at plasma membranes, and will therefore be described in more detail in section 2.4. Another mechanism by which HDL may impart its anti-atherogenic effects is through its ability to restore endothelial dysfunction by impacting on the bioavailability of nitric oxide (NO) of endothelial cells. During the early stages of atherosclerosis, a decreased bioavailability of NO, a potent vasodilator, as well as an increased affinity of the endothelial surface for leukocytes is observed (Voetsch et al. 2004). When HDL interacts with the surface of endothelial cells, it activates endothelial nitric oxide synthase (eNOS) through binding to

SR-B1 localised on caveolae (Yuhanna et al. 2001). HDL enhances eNOS activity by up-regulating eNOS gene expression (Kuvin et al. 2002) and by increasing the phosphorylation pathway via activating the Akt kinase and mitogen-activated protein kinase (MAPK) (Mineo et al. 2003). This, in turn, causes a release of NO, which induces vasorelaxative effects and inhibits apoptosis. HDL also functions to attenuate the expression of vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, and E-selectin, all of which recruit leukocytes, especially monocytes, to the endothelial surface (Dimayuga et al. 1999).

The anti-atherogenic mechanism of HDL in inhibiting LDL lipid oxidation, especially via the 12-lipoxygenase-induced pathway, is believed to be crucial for preventing the scavenger receptor-mediated uptake of modified LDL particles into macrophage cells located within the subendothelial intima (Navab et al. 2004). The oxidation of phospholipids is largely generated by the lipoxygenase and myeloperoxidase pathways in the formation of cell-derived reactive oxygen species (ROS) (Vance 2002). These ROS oxidise lipoprotein phospholipids containing arachidonic acid, which in turn, makes the particles proinflammatory. The ability of HDL to inhibit oxidation and inflammation is by way of the high levels of antioxidants inherent in this lipoprotein, such as apoA-I, and the enzymes; paraoxonase (PON)-1 (Shih et al. 1998; Shih et al. 2000) and platelet activating factor acetyl-hydrolase (PAF-AH) (Watson et al. 1995). These prevent oxidation reactions and catalyse the breakdown of oxidised phospholipids on the LDL particle.

2.2. HDL STRUCTURE AND SUBPOPULATIONS

Plasma HDL is a heterogeneous population of particles differing in size, density, surface charge, and in the apolipoprotein, enzyme and lipid composition (von Eckardstein et al. 1994). The major apolipoproteins in HDL are apoA-I and apoA-II, as well as a minor portion of the metabolically important apoE, apoA-IV, apoD, apoJ, apoM and apoC (C-I, C-II and C-III) proteins (Xu et al. 1999; Bottcher et al. 2000). A subdivision into HDL_1, HDL_2 and HDL_3, by ultracentrifugal and gel-filtration separations, represents three major subclasses separated in decreasing order of particle size and increasing hydrated density. HDL_1 is the least abundant and least dense subclass in plasma, whereas the HDL_2 and

HDL_3 subclasses are the most abundant and are therefore further subdivided into HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c} (Anderson et al. 1977). The density range for HDL_1 is from 1.050-1.090 g/ml; a region overlapping with LDL and HDL_2. The density of HDL_2 is from 1.063 to 1.125 g/ml, and for HDL_3 the range spans from 1.125 to 1.21 g/ml. The approximate size diameter for HDL_2 and HDL_3 is 11 to 7 nm, respectively, and the core volume of HDL_2 is 50 % larger than that of HDL_3, containing 3 - 4 fold more cholesteryl ester and triglyceride molecules (Eisenberg 1984). Separation of HDL on the basis of surface charge by agarose gel electrophoresis and two-dimensional non-denaturing gradient gel electrophoresis has assisted in the separation of minor HDL subclasses from the most abundant α-HDL subclass (HDL_2 and HDL_3), and these are the preα-, preβ- and γ-migrating particles that are made of a single lipid bilayer, as determined by sectional electron microscopy (Hamilton et al. 1976). Furthermore, these particles are lipid-poor, composed of apoA-I (preα, preβ), apoA-IV (α, preβ) or apoE (γ-E) as the only protein constituents, and are good acceptors of cellular-derived cholesterol (von Eckardstein et al. 1995; von Eckardstein et al. 1998). Likewise, preβ-HDL are present at higher levels in interstitial fluid than in plasma and are the main nascent forms of HDL that take up cholesterol from cells ($preβ_1$-HDL), and undergo cholesterol esterification by LCAT (to form $preβ_2$- and $preβ_3$-HDL). $Preβ_1$-HDL contains two molecules of apoA-I, which represents 90 % protein as particle mass, 7 % phospholipid (mostly phosphatidylcholine and sphingomyelin in equimolar ratio), 0.3 % esterified cholesterol and 1.8 % free cholesterol (Kunitake et al. 1985). $Preβ_2$-HDL contains three apoA-I molecules, additional free cholesterol plus cholesteryl esters and an increased PC/SM ratio compared to that of $preβ_1$ (Fielding et al. 1995).

Table 1. Physical properties, lipid and apolipoprotein composition of human plasma lipoproteins

				Chemical Composition (% of dry mass)				
				CORE		SURFACE		
Class	Density (nm)	Density (g/ml)	Electrophoretic Mobility	TG	CE	FC	PL	Protein[a]
CM	25-1200	0.93	At origin	86	3	2	7	2
VLDL	30-80	0.95-1.006	pre-β	55	12	7	18	8
IDL	25-35	1.006-1.019	slow pre-β	23	29	9	19	19
LDL	18-25	1.019-1.063	β	6	42	8	22	22
HDL_2	9-12	1.063-1.125	α	5	17	5	33	40
HDL_3	7-9	1.125-1.210	α	3	13	4	25	55

CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid; TG, triglyceride
[a]Protein does not include bound carbohydrate
(Adapted from: Lipoproteins in Health and Disease, 1999. Betteridge, Illingworth and Shepherd)

2.3. HDL APOLIPOPROTEINS

All of the apolipoproteins that associate with HDL, primarily apoA-I and apoA-II, along with the minor components mentioned in section 2.2, are defined as being exchangeable by their ability to transfer readily between lipoprotein particles, by being able to acquire lipids in the circulation and most importantly, by solubilising and transporting neutral lipids (cholesteryl ester and triacylglycerol) via a spontaneous binding to phospholipid/water interfaces (Alaupovic et al. 1972). Moreover, the exchangeable apolipoproteins are capable of altering their conformation according to changes in lipid content, compositions, and metabolic states of the lipoproteins, as well as to modulate enzymatic activities and to serve a role in receptor recognition (Jänis et al. 2005).

ApoA-I is the major protein constituent of HDL and comprises 70 % of its total protein mass. It is synthesised by the liver and the intestine as a pre-proprotein (267-aa), processed co-translationally to pro-apoA-I (249-aa) and secreted into the circulation (Law et al. 1984). A metalloenzyme rapidly cleaves the six amino acid-long propeptide from the amino terminus to form mature apoA-I (Edelstein et al. 1983). Mature apoA-I exists as a single polypeptide composed of 243 amino acids (molecular mass is 28 kDa). Exon 3 encodes the N-terminal domain and the remaining region, which contains eight 22-mer and two 11-mer homologous repeats spanning residues 44-243, is encoded by exon 4 (Li et al. 1988). The amphipathic helical bundles contain a hydrophobic face that mediate

binding to phospholipid surfaces so that the α-helical content increases upon lipid binding. ApoA-I for example, increases from a 50 % helical state when lipid-free to a 60-85 % helical state when lipid bound and this occurs primarily at the C-terminal 187-243 region (Davidson et al. 1996). When apoA-I is lipid-bound it adopts a 'belt-like' arrangement of a continuous series of α-helices that are aligned perpendicular to the phospholipid fatty acyl chains (Klon et al. 2002). In spherical HDL, apoA-I is most likely flexible with its conformation, being governed by the size and core lipid composition of the particles. The C-terminal end of apoA-I is also required for the activation of LCAT (Pyle et al. 1996).

ApoA-II is synthesised predominantly by the liver and is secreted into the plasma either as a propeptide, which is rapidly cleaved, or directly as mature apoA-II (Gordon et al. 1983). Human apoA-II is a disulfide-linked dimer composed of identical subunits and has a molecular mass of 17 kDa. ApoA-II normally represents 20 % of the total HDL protein content and is found in association with apoA-I in the plasma of normolipidemic subjects; however, apoA-I can also be found in particles not associated with apoA-II (Cheung et al. 1984).

The gene for apoE is polymorphic for three common alleles; ε2, ε3, and ε4, that code for three major isoforms which result in six common genotypes. Each isoform differs by cysteine and arginine at positions 112 and 158 such that apoE3, the most common isoform, contains cysteine residues at both sites, apoE4 contains only arginines and apoE2 contains both cysteine and arginine (Weisgraber et al. 1981). Human apoE is synthesised as a single polypeptide chain of 299 amino acids displaying similar primary and secondary structures to apoA-I, and having a molecular mass of 34 kDa. ApoE is a minor constituent of HDL, however, the profound effect of apoE on plasma HDL levels is demonstrated by an association of apoE4 with lower HDL-cholesterol concentrations and an increased risk for CVD (Kataoka et al. 1996; Wilson et al. 1996).

ApoA-IV, a 46 kDa protein also associated with HDL, is able to mimic certain roles of apoA-I such as mediating cholesterol efflux and activating LCAT (Steinmetz et al. 1985; Steinmetz et al. 1990). Recently, apoM, a 26 kDa apolipoprotein expressed in the liver and kidneys, has been found to be associated with HDL particles and to be a modulating factor for the formation of preβ-HDL. In apoM-deficient mice, a complete

absence of preβ-HDL is observed, together with a reduced capacity for cholesterol efflux from macrophage cells to apoM-deficient HDL, compared with normal HDL (Wolfrum et al. 2005). Finally, the apoC proteins (apoC-I; 6.6 kDa, apoC-II; 8.9 kDa and apoC-III; 8.8 kDa) of HDL display either inhibitory or stimulatory effects on various enzymes and lipid transfer proteins. For instance, apoC-II activates LPL whereas apoC-III inhibits it, and the apoC-I protein has been shown to inhibit CETP activity (Gautier et al. 2000; Shachter 2001).

2.4. FORMATION OF HDL

The formation of HDL begins with the synthesis of monomolecular lipid-poor apoA-I displaying preβ mobility in agarose gel electrophoresis and containing only a small amount of phospholipid. Lipid-poor apoA-I in plasma is derived from; synthesis in the liver and the intestine (Eisenberg 1984), release from triglyceride-rich lipoproteins during LPL-mediated lipolysis (Schaefer et al. 1982), and during the remodelling of mature, spherical HDL particles in plasma (Rye et al. 1999). For the formation of discoidal preβ-HDL, lipid-poor apoA-I must initially accept phospholipid and cholesterol from cell membranes provided by ABCA1. The ABCA1 transporter was originally identified as one of the key regulators of cellular cholesterol efflux after the mutated form of the gene was revealed in human fibroblast cells of Tangier patients (Bodzioch et al. 1999; Brooks-Wilson et al. 1999). These patients display a deficiency of plasma HDL due to rapid degradation of lipid-poor apoA-I, as well as an accumulation of macrophage foam cells in various tissues and moderately accelerated atherosclerosis. The function of ABCA1 is to increase the availability of cholesterol and phospholipid at the cell surface with which apoA-I directly interacts (Chambenoit et al. 2001). Binding of apoA-I either occurs on the plasma membrane at sites interrupted by ABCA1 or otherwise directly to the two large extracellular domains of the transporter (Wang et al. 2000). Moreover, ABCA1 is not restricted to the cell surface as it shuttles between early and late endosomes and the plasma membrane, recycling apoA-I and transporting cholesterol to the cell surface for export (Neufeld et al. 2001). Early hepatic lipidation events occur within endocytic compartments during recycling, or as has recently been reported, within the secretory pathway upon apoA-I synthesis (Chisholm et al. 2002; Zheng et al. 2005). As apoA-I

acquires enough free cholesterol and phospholipid to remodel into a discoidal structure, further growth and maturation is dependent on the enzymatic activity of LCAT, which consumes unesterified cholesterol and phosphatidylcholine to produce insoluble cholesteryl esters (Nakamura et al. 2004). Cholesteryl esters move to the core, thus forming a spherical particle and a concentration gradient that enables more free cholesterol and phospholipids to move from the cell and lipoprotein surfaces to the growing HDL particle.

Two other members of the ABC transporter family, ABCG1 and ABCG4, have recently been shown to promote net cholesterol efflux to HDL particles, primarily to HDL_2 and HDL_3 (Wang et al. 2004). This is in contrast to ABCA1 which mediates efflux mainly to lipid-poor apolipoproteins, such as to apoA-I, and not to HDL particles that constitute the bulk of plasma HDL. As ABCG1 is highly expressed in macrophage foam cells it may explain the inverse relationship between large-sized HDL_2 and atherosclerosis risk (Lorkowski et al. 2001).

2.5. HDL CATABOLISM

Clearance of plasma HDL by the liver is an essential process for the catabolism of cholesterol into bile acids for excretion. The hepatic uptake of cholesteryl esters in HDL is facilitated by SR-B1, a multi-ligand cell surface transmembrane protein, that may be responsible for the selective sorting of cholesterol to the bile canaliculus (Acton et al. 1996). The SR-B1 pathway is a high-capacity system in which cells selectively internalise cholesteryl esters, but not the apolipoprotein component of the HDL particle (Ji et al. 1997). Another pathway independent of SR-B1 that has been proposed is the catabolic pathway for HDL protein through the high-affinity binding of the particle to a novel receptor, the β-chain of ATP synthase, a protein complex of the mitochondrial inner membrane (Martinez et al. 2003). Ectopic expression of the β-chain of ATP synthase has been shown to facilitate endocytosis of *holo*-HDL particles by a mechanism that depends on the generation of adenosine diphosphate (Martinez et al. 2003). However, the extent to which this receptor plays a role in HDL metabolism *in vivo* remains to be shown. Finally, hepatic uptake of HDL-derived cholesteryl esters also occurs via the action of CETP which transfers cholesteryl esters from HDL particles to apoB-100-

containing lipoproteins that are then internalised by the liver through LDL-receptor (R)-mediated endocytosis (Tall 1993).

In addition to the catabolism of HDL and its components in the liver, degradation of HDL also occurs in the kidneys. The mechanism for HDL binding and endocytosis is through the cubilin-megalin pathway which involves the cubilin receptor, a peripheral membrane protein that is assembled with the amnionless transmembrane protein, and its co-receptor megalin (Christensen et al. 2002; Strope et al. 2004). The cubilin/amnionless complex is expressed along the apical surfaces of kidney proximal tubule cells and clears lipid-poor apoA-I, as well as lipid-depleted HDL_3 particles via binding to its ligand, apoA-I, for internalisation and lysosomal degradation (Hammad et al. 1999; Kozyraki et al. 1999; Fyfe et al. 2004).

2.6. LIPID TRANSFER PROTEINS

In human plasma, the transfer of lipid species between lipoprotein particles occurs via the action of two distinct lipid transfer proteins; cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). CETP is a hydrophobic glycoprotein that is secreted primarily by the liver and circulates in plasma, bound mainly to HDL (Tall et al. 1983). The function of CETP is to promote the redistribution of cholesteryl esters from HDL to triglyceride-rich lipoproteins and of triglycerides from triglyceride-rich lipoproteins to LDL and HDL. PLTP mediates the transfer of phospholipids from triglyceride-rich lipoproteins to HDL as well as the remodelling of HDL so that, together with CETP, it plays an important role in the pathway of RCT by modifying the metabolic properties and size distribution of HDL (Huuskonen et al. 2000).

CETP was originally designated Lipid Transfer Protein-I (LTP-I) and PLTP was designated LTP-II upon isolation and purification of these two proteins in human plasma (Tollefson et al. 1988). When human CETP and PLTP were subsequently cloned, sequence analysis revealed a 21.7 % amino acid identity between the two proteins. In addition, CETP and PLTP belong to the same gene family; the lipid transfer/lipopolysaccharide binding protein family (Day et al. 1994). The other family members include bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP). BPI and LBP mediate anti-bacterial and pro-

inflammatory activities via binding to lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria (Gray et al. 1989; Schumann et al. 1990). In addition to humans, homologous CETP and PLTP genes have been found in other mammalian species. PLTP activity has been detected in all species studied thus far but, in contrast, substantial CETP activity is found only in man, rabbit, chicken and trout (Guyard-Dangremont et al. 1998). Importantly, CETP is not present in the mouse, the most commonly used experimental animal model of atherosclerosis (Breslow 1996). On the basis of the available crystal structure of the closely related BPI, computational models of CETP and PLTP have since been constructed (Bruce et al. 1998; Huuskonen et al. 1999). These models from both proteins predict an elongated, boomerang-like structure containing two highly conserved, hydrophobic lipid binding pockets. The amino terminal domains in CETP and PLTP are highly homologous whereas the carboxyl terminal domains vary in hydrophobicity and hence, functional specificity.

The possibility that CETP and PLTP might act as independent risk factors in the development of atherosclerosis has raised the question of whether they are protective against or detrimental to the disease progression. As PLTP will be discussed in detail in the following chapter, only CETP and its role in atherogenesis will be outlined below.

The pro-atherogenic nature of CETP and the possibility to inhibit its activity to favour an anti-atherogenic lipoprotein profile was raised over a decade ago (Brown et al. 1989). The potential for CETP as a pro-atherogenic factor relates to its ability to transfer cholesteryl esters from anti-atherogenic HDL particles to pro-atherogenic VLDL and LDL fractions. CETP also functions to mediate the selective uptake of cholesteryl esters by human adipose tissue (Benoist et al. 1997). Adipocytes synthesise and secrete CETP, which is immunoreactive on the plasma membrane, and selectively acquire cholesteryl esters from HDL. CETP also facilitates the uptake of HDL-cholesteryl esters (CE) by hepatic cells expressing SR-B1 (Collet et al. 1999). The effect of CETP activity on the development of atherosclerosis, however, is not straightforward. For example, the development of atherosclerosis in CETP-transgenic mice depends both on diet and on other genes that affect lipoprotein metabolism. When the human CETP gene is expressed in mice, a species naturally deficient in CETP, a dose-related reduction in HDL cholesterol levels and a small increase in VLDL and LDL cholesterol occurs (Agellon et

al. 1991). More strikingly, in transgenic mice expressing the simian CETP gene driven by the mouse metallothionein promoter, the development of atherosclerosis occurs rapidly and lesion morphology is severe (Marotti et al. 1993). Conversely, circumstances in which CETP gene expression in mice have been found to be anti-atherogenic have also been reported. This includes mice that, in addition to CETP, have been engineered to over express the human apoC-III gene, the human LCAT gene as well as in mice that have hypertriglyceridemia (Hayek et al. 1995; Berard et al. 1997; Kako et al. 2002). Such conflicting reports illustrate the complexity and inconsistencies arising from studies in CETP transgenic mice. Rabbits on the other hand, are highly susceptible to diet-induced atherosclerosis and display naturally high levels of CETP (Quinet et al. 1990). When rabbits are injected with antisense oligonucleotides against CETP to inhibit expression, atherosclerosis is substantially reduced and HDL cholesterol levels are elevated (Sugano et al. 1998). Small CETP inhibitors have recently been shown to not only increase HDL cholesterol levels, but also to affect the size distribution of HDL subpopulations and the apolipoprotein and enzyme composition in rabbits (Okamoto et al. 2000; Rittershaus et al. 2000; Brousseau et al. 2004; Clark et al. 2004; Zhang et al. 2004).

CETP deficiency in humans has been attributed to several missense, nonsense and splicing mutations in the CETP gene. This invariably results in elevated levels of HDL cholesterol, apoA-I, apoA-II and apoE as well as a substantial reduction in the concentration of LDL cholesterol and apoB (Inazu et al. 1990). Therefore, despite the inconsistent results obtained from transgenic mice, the atherogenic effects of CETP expression in rabbits and the anti-atherogenic profile of CETP deficiency in humans strongly support a therapeutic potential for CETP inhibition (Clark et al. 2004; Brousseau et al. 2005).

3. PHOSPHOLIPID TRANSFER PROTEIN (PLTP)

3.1. CHARACTERISTICS OF PLTP

The human PLTP gene has been mapped to chromosome 20q12-q13.1, which is in close proximity to the genes for LBP and BPI located on 20q11.23-q12 (Gray et al. 1993; Whitmore et al. 1995). The intron positioning as well as the exon size and distribution for PLTP, BPI and LBP are almost identical and with the structural organisation of the entire LPS-binding/lipid transfer protein family members being highly conserved, the presence of a common ancestral gene is evident (Hubacek et al. 1997; Kirschning et al. 1997). The PLTP gene is approximately 13.3 kilobases (kb) in length and comprises 16 exons that range in size from 43 base pairs (bp) (exon 13) to 304 bp (exon 16), along with 15 intervening introns (Tu et al. 1995). The first exon of the PLTP gene contains the 5'-untranslated region with an interruption by intron 1 occurring 11 bp upstream of the translation initiation site, a feature characteristic of most apolipoprotein genes (Li et al. 1988). The second exon of the PLTP gene contains the entire signal peptide, which is cleaved from the protein upon secretion, and also contains 16 amino acids of the mature protein. Exons 3 to 15 encode mainly the mature protein and exon 12 encodes a segment of 21 amino acids (residues 372-392) that consists of seven basic amino acids that form a heparin binding site on PLTP. Exon 16 contains partly coding as well as the entire 3'-untranslated region (Tu et al. 1995).

A gene named human protective protein has been found to partially overlap by 58 nucleotides at the 3'-untranslated region with the PLTP gene (Shimmoto et al. 1996). This overlap results in the convergent transcription of both genes from opposite DNA strands and is found to exist also in the mouse genome. PLTP and human protective protein mRNA expression patterns do not, however, coincide, nor are they mutually affected by individual gene expression levels. This indicates that both genes, despite their close proximity, are not cooperatively regulated (Jiang et al. 1996). The mouse PLTP gene which is localised to chromosome 2, corresponding to human chromosome 20, exhibits intron-exon junctions that are highly conserved with the human PLTP gene and shares an 81.1 % nucleotide sequence identity in the promoter region (LeBoeuf et al. 1996; Tu et al. 1997). Considering that the human PLTP promoter region responsible for

transcription has been determined to be between -230 and -72 relative to the transcription initiation site, contains a TATA box, a high GC region and several consensus sequences for the potential binding of transcription factors such as Sp1, AP-2, AP-3, C/EBP, NF-kB, it is reasonable to assume that the mouse PLTP promoter is located at the same region and is subject to similar transcriptional regulation mechanisms (Tu et al. 1995; Tu et al. 1997).

PLTP complementary DNA (cDNA) is 1750 bp in length and encodes a 17 amino acid hydrophobic signal peptide and a 476 amino acid mature protein (Day et al. 1994). The messenger RNA (mRNA) of PLTP is ubiquitously expressed and the presence of a single transcript of 1.8 kb of mRNA occurs at the highest levels in the ovary, thymus and placenta, and at moderate levels in the pancreas, small intestine, testes, lung, and prostate. Relatively low levels of the transcript have been detected in the kidney, liver, and spleen, as well as very low levels in the heart, colon, skeletal muscle, leukocytes and brain (Day et al. 1994; Albers et al. 1995). This indicates that PLTP gene expression has a tissue-specific regulation and this appears to differ between human and mouse tissues (Albers et al. 1995). Although moderate levels of PLTP mRNA have been found in the liver, the relatively large mass of this organ may still make it a major contributor to plasma PLTP levels.

The mature PLTP protein has a predicted molecular mass of 55 kDa, yet the observed 81 kDa by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified plasma PLTP under reducing conditions is due to extensive glycosylation (Day et al. 1994; Huuskonen et al. 1998). For example, the major secreted forms of PLTP carry complex N-glycans at six potential N-glycosylation sites (Huuskonen et al. 1998). When PLTP is expressed in a baculovirus/insect-cell system, which is capable of performing only high-mannose type glycosylation, efficient secretion and full activity of the protein is still observed (Huuskonen et al. 1998b). PLTP also has numerous potential O-glycosylation sites and this may account for the increased mass estimation by SDS-PAGE. PLTP contains four cysteine residues with the potential to form two intra-chain disulfide bonds, whereby the bridge that is formed between cysteine residues 146 and 185 is found to be important for the structural integrity of the protein (Huuskonen et al. 1999). According to homology modelling, PLTP displays a two-

domain architecture composed of two symmetrical barrels with hydrophobic pockets. Site-directed mutagenesis of conserved amino acid residues has revealed that the N-terminal pocket is critical for PLTP transfer activity and that the C-terminal pocket is required for HDL binding (Huuskonen et al. 1999). The final 30 C-terminal amino acid

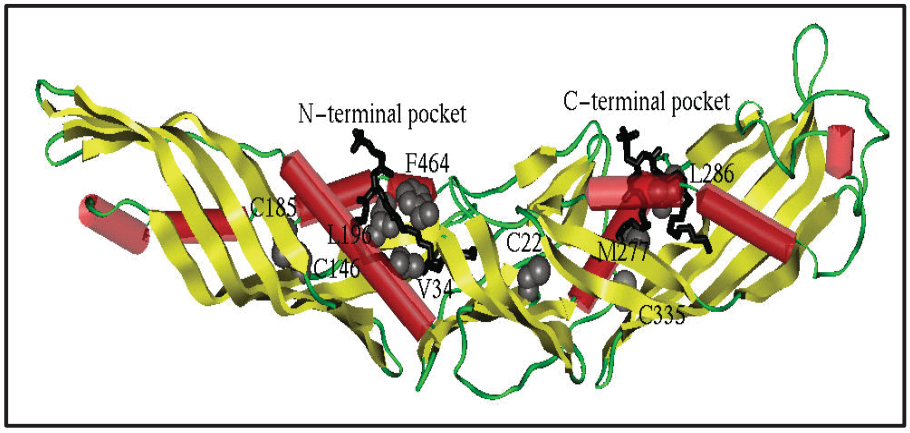

Figure 3. Molecular model of phospholipid transfer protein
(Huuskonen et al. 1999. J Lipid Res 40:1123-30)

residues are not required for secretion or activity (Huuskonen et al. 1999). Based on secondary structure predictions, PLTP is also thought to contain two potential transmembrane regions spanning from residues 169 through 181, and residues 288 through to 304 (Albers et al. 1996b). However, there is no evidence that these would anchor PLTP integrally to membranes. Finally, PLTP displays a high content of hydrophobic residues that are scattered throughout the polypeptide representing more than 40 % of the total amino acids.

3.2. FUNCTIONS OF PLTP

3.2.1. PHOSPHOLIPID TRANSFER ACTIVITY

PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL during lipolysis (Tall et al. 1985). This function was discovered via an *in vitro* method in which

phospholipid vesicles were incubated with HDL or plasma (Tall et al. 1981). By using this method it was discovered that the rate of transfer of radiolabelled phosphatidylcholine from unilamellar egg phosphatidylcholine vesicles to HDL was sufficiently enhanced by the activity of LTP-II, nowadays termed PLTP (Tall et al. 1985). The phospholipid substrate specificity of plasma PLTP has since been determined from the acyl chain length and polar head groups of different pyrene-labelled phospholipids (Huuskonen et al. 1996; Rao et al. 1997). These studies demonstrate that PLTP is capable of transferring all common phospholipid classes non-specifically, except for phosphatidylethanolamine which is transferred more slowly, and this transfer is unaffected by the acyl chain length (from 6-12 carbons) or the acyl chain position at sn-1 or sn-2. As no PLTP-phospholipid intermediates have been detected, it appears unlikely that PLTP would form a tight complex with its lipid substrate. Accordingly, instead of being a true lipid carrier it is more probable that PLTP forms a ternary complex between donor and acceptor particles to facilitate the transfer of phospholipids. The exact mechanism behind PLTP-facilitated phospholipid transfer, which is responsible for approximately half of the overall exchange activity between lipoproteins, the other half being mediated by CETP, has not been fully resolved (Brown et al. 1990). However, the physiological importance of PLTP *in vivo* is evident in PLTP-deficient mice, which show a total absence of transfer of phospholipids from VLDL to HDL in plasma, along with markedly decreased HDL levels and a subsequent accumulation of surface remnants in apoA-IV-rich lamellar lipoproteins (Jiang et al. 1999; Qin et al. 2000).

The non-specificity of PLTP-mediated phospholipid transfer is also extended to its ability to transfer diacylglycerol (Rao et al. 1997). Diacylglycerols are not only released by LPL in the capillary endothelium and during lipolysis in adipose tissue, but diacylglycerol is also transported in HDL particles (Vieu et al. 1996). Furthermore, PLTP enhances the transfer of other lipophilic substances such as α-tocopherol (vitamin E) (Kostner et al. 1995), LPS (Hailman et al. 1996; Levels et al. 2005), and cholesterol between cells and lipoproteins (Nishida et al. 1997). The importance of α-tocopherol as an anti-oxidant of plasma lipoproteins and cell membranes is its role in inhibiting LDL oxidation and the subsequent uptake of these particles by vascular cells. Originating from the intestine, α-tocopherol is firstly incorporated into chylomicrons, transported to the

liver and then packaged into nascent VLDL particles for release into the circulation. PLTP removes α-tocopherol from atherogenic lipoproteins *in vitro* and PLTP over-expression reverses the beneficial phenotype of the decreased susceptibility to atherosclerosis observed in PLTP-knockout (KO) mice (Jiang et al. 2002; Yang et al. 2003). Hence, the deleterious loss of α-tocopherol from apoB-containing lipoproteins can be verified by the concomitant increase in the oxidizability of these lipoproteins (Yang et al. 2003). On the other hand, PLTP deficiency in the brain, an organ displaying a rich source of α-tocopherol in the body, results in decreased levels of α-tocopherol and an increase in oxidative brain injury that subsequently leads to neurological dysfunctions (Desrumaux et al. 2005). Such findings prompted the authors of this study to suggest a novel function for PLTP as a local transporter of α-tocopherol, offering a putative route of transfer of α-tocopherol across the blood-brain barrier.

3.2.2. HDL CONVERSION

In addition to transferring lipophilic substances between lipoproteins, PLTP also functions to maintain the heterogeneity of plasma HDL via a conversion process that modifies HDL_3 into a population of enlarged HDL_2 particles, together with a concomitant release of apoA-I molecules termed small dense preβ_1-HDL (Jauhiainen et al. 1993; Tu et al. 1993). This process involves particle fusion rather than a net lipid transfer or particle aggregation for the enlargement of HDL and relies on the interaction of PLTP with apoA-I on HDL (Lusa et al. 1996; Korhonen et al. 1998; Huuskonen et al. 2000c). The fusion of unstable particles is suggested to be a response to the phospholipid transfer process that increases the surface pressure of HDL which releases apoA-I from the surface (Lusa et al. 1996). HDL conversion is a process dependent on both PLTP concentration and time and proceeds as a continuum of intermediate particle formation with the transfer of phospholipids preceding the change in particle size (Rao et al. 1997). In addition to human PLTP, mouse recombinant PLTP also facilitates HDL conversion as well as pig PLTP which can convert not only homogeneous pig HDL but also human HDL_3 into larger and smaller particles (Albers et al. 1995; Pussinen et al. 1995). With a continued interest in HDL remodelling by PLTP, further studies have shown that an enrichment of HDL with triglycerides enhances the rate of conversion, an interesting observation

considering that hypertriglyceridemic patients contain higher amounts of small HDL than normolipidemic controls due to accelerated remodelling (Murakami et al. 1995; Rye et al. 1998). The process of remodelling is believed to be most important for the formation of small preβ$_1$-HDL conversion products as they are rich in apoA-I, devoid of triglycerides and have an approximate equimolar PC/SM ratio; the preferred lipoprotein species that serves as the initial acceptor of cellular unesterified cholesterol (Sviridov et al. 2002). Furthermore, HDL$_2$ can serve as a substrate for PLTP in generating preβ-HDL (Marques-Vidal et al. 1997).

3.2.3. CELLULAR CHOLESTEROL EFFLUX

In addition to PLTP's role in lipoprotein metabolism in the plasma compartment, increasing evidence points to a function for PLTP in hepatic and extra-hepatic tissues. A positive correlation between human serum PLTP activity and cellular cholesterol efflux was initially demonstrated in Fu5AH rat hepatoma cells by the ability of PLTP to augment the removal of cellular phospholipids and cholesterol into HDL (Syvanne et al. 1996). Furthermore, exposure of cholesterol-enriched human skin fibroblasts to PLTP enhanced cholesterol efflux to HDL, but not to albumin and this did not occur in fibroblasts from Tangier patients who lack the ABCA1 transporter (Wolfbauer et al. 1999). It was later suggested that the enhancement of lipid efflux by PLTP occurs via interplay with ABCA1. In the presence of HDL acceptor particles, PLTP efficiently stimulates phospholipid and cholesterol efflux and stabilises ABCA1 from calpain-mediated degradation and, together with apoA-I, binds to the same or closely related sites on ABCA1 at the cell surface (Oram et al. 2003). In the plasma of ABCA1-KO mice, PLTP and LCAT activities are decreased by more than 80 %, suggesting that the maturation of HDL is substantially affected, and thereby provides a plausible explanation for the low HDL levels observed when ABCA1 is absent (Francone et al. 2003). It may also be possible that PLTP promotes cellular lipid efflux via an indirect mechanism, such as by increasing the concentration of plasma preβ$_1$-HDL (von Eckardstein et al. 1996). For example, plasma from huPLTP transgenic mice is more efficient at preventing the acetylated LDL-induced accumulation of intracellular cholesterol in cultured macrophages so that the increased plasma concentration of preβ$_1$-HDL by elevated PLTP

may be contributing more to the RCT pathway (van Haperen et al. 2000). Additionally, chymase, a neutral protease secreted by mast cells in human atherosclerotic lesions, can proteolytically degrade PLTP and so irreversibly inhibit the PLTP-dependent formation of preβ_1-HDL with ensuing impairment of cholesterol efflux from macrophage cells (Lee et al. 2003). Alternatively, PLTP could function as an atherogenic molecule by mediating cellular lipid retention and this may be explained by the high PLTP mRNA expression levels in macrophage foam cells of atherosclerotic plaques which correlate with increased cellular cholesterol levels. For instance, when macrophage cells are loaded with cholesterol *in vitro* a 2- to 3-fold increase in PLTP steady-state mRNA levels, protein expression and activity is observed (O'Brien et al. 2003).

3.3. REGULATION OF PLTP GENE EXPRESSION

Even though the PLTP gene promoter was found not to display homology to steroid hormone response elements, it does contain response elements to non-steroidal receptors such as farnesoid X-activated receptor (FXR), peroxisome proliferator-activated receptor α (PPARα), and the liver X-activated receptor (LXR) (Tu et al. 2001; Laffitte et al. 2003). These nuclear receptors bind DNA as heterodimers with their obligate partner, retinoid X receptor (RXR) (Chawla et al. 2001). Transcriptional regulation of many genes that are involved in bile acid and cholesterol metabolism occurs via the pivotal role of various members of the orphan nuclear hormone receptor superfamily. As PLTP is one such target gene of the nuclear receptors it contains, in addition to several transcriptional binding sites (sterol regulatory element-binding protein [SREBP], CCAT/enhancer-binding protein [C/EBP]), several response elements in its promoter region such as one for FXR (Tu et al. 1999). Within the PLTP promoter region is an inverted repeat-1 response element (IR-1) to which FXR binds once it has formed a heterodimer with RXRα. Chenodeoxycholic acid (CDCA), a bile acid and potent activator of FXR, is capable of inducing human PLTP promoter expression 8-10 fold in the presence of FXR-RXRα (Urizar et al. 2000). CDCA is also capable of inducing mouse PLTP gene expression (Tu et al. 2001), and the effect is lost in FXR-KO animals (Kast et al. 2001). It is proposed that when bile acids increase due to a higher cholesterol concentration,

elevated PLTP gene expression would subsequently raise or otherwise maintain HDL levels (Urizar et al. 2000).

In addition to FXR response elements, the PLTP promoter region also contains PPARα-responsive element-like sequences, to which PPARs bind after heterodimerising with RXRα upon fatty acid or fibrate-induced activation. Fibrates constitute a class of normolipidemic drugs that efficiently decrease triglycerides and increase HDL plasma levels in humans (Brown 1987). Fenofibrate profoundly affects the promoter activity of the human PLTP gene by lowering its expression (Tu et al. 1999). Furthermore, as C/EBP and PPARα share the same binding region on the PLTP gene, C/EBP may compete for PPARα binding, subsequently modifying gene promoter function in response to fibrate. It has been documented that the ratio of PPARα and C/EBP proteins within cells seems to dictate PLTP gene transcription in response to fibrate (Tu et al. 1999). Interestingly, when fenofibrate is applied to the mouse, PLTP gene expression significantly increases, the opposite effect being observed for PLTP in humans (Tu et al. 2001). This indicates that, despite the mechanisms for basic transcription of the PLTP gene in human and mouse being highly conserved, the regulatory pathways in response to environmental stimuli for PLTP gene expression appears to be species-specific.

LXRs, also belonging to the orphan nuclear receptor superfamily, act as master transcription factors for the regulation of cholesterol absorption in the intestine, cholesterol catabolism in the liver, and cholesterol efflux from peripheral tissues (Schultz et al. 2000). The LXR subfamily of nuclear receptors are bound and transcriptionally activated by oxidised forms of cholesterol (oxysterols) (Lehmann et al. 1997), and the LXR target genes include; apoE (Laffitte et al. 2001), LPL (Zhang et al. 2001), ABCG1 (Kennedy et al. 2001), ABCG5 and ABCG8 (Repa et al. 2002), FAS (Joseph et al. 2002), ABCA1, CETP, rodent Cyp7A and SREBP1c (Edwards et al. 2002). PLTP is also a direct target gene for LXR as the PLTP promoter region contains a high-affinity LXR response element that is bound by LXR/RXR heterodimers (Laffitte et al. 2003). LXR/RXR heterodimers are known to bind to direct repeats of a hexanucleotide repeat spaced by 4 bp (DR-4). The functional LXR-response elements in the PLTP gene that are necessary for maximal induction correspond to DR-4A and DR-4B sequences and show significant similarity to other known LXR-response elements (Laffitte et al. 2003).

Oxysterols and synthetic LXR ligands strongly regulate the expression of murine and human PLTP genes in the liver and in macrophage cells in an LXR-dependent manner (Cao et al. 2002; Mak et al. 2002; Laffitte et al. 2003). When specific LXR agonists are administered to mice, hepatic PLTP mRNA and plasma PLTP activity increases. This occurs alongside HDL cholesterol and phospholipid elevation, which generates enlarged HDL particles that are enriched in cholesterol, apoA-I, apoE and phospholipid (Cao et al. 2002). Such HDL enlargement may also involve the induction of ABCA1 and apoE; the genes that are also involved in HDL metabolism and regulated by LXRs. Short term administration of synthetic LXR ligands in animals has been shown to induce ABCA1 and increase plasma HDL cholesterol and phospholipid levels, yet plasma triglycerides also rise (Schultz et al. 2000; Joseph et al. 2002). In order to overcome the hypertriglyceridemia mediated by LXR activation but without losing the advantageous effects of LXR-mediated HDL enlargement and hepatic PLTP mRNA induction, a simultaneous activation of LXRs and PPARα by synthetic ligands has been shown to dissipate triglycerides in the liver and plasma without hindering the favourable formation of enlarged HDL and increased PLTP (Beyer et al. 2004). It is interesting that PLTP is also highly expressed in macrophage cells within atherosclerotic lesions, inducible by LXR, especially considering that the pharmacological activation of LXR has been shown to inhibit the formation of atherosclerotic lesions in mouse models (Joseph et al. 2002b). With FXR also regulating PLTP in the liver but not being expressed in macrophage cells, it is clear that PLTP can be regulated independently by either receptor and that LXR appears to play a crucial role in PLTP expression in macrophage foam cells.

3.4. PLTP IN HUMAN PLASMA AND TISSUES

Human genetic PLTP deficiency has not been unequivocally described thus far, apart from one case reported in an abstract form (Mallow et al. 1994), and the effects of PLTP polymorphisms on lipoprotein metabolism have not as yet been clearly delineated. Plasma PLTP activity levels do not significantly differ between males and females and the genetic variation of the PLTP gene within a normal Finnish population, identified by six intragenic and two neutral polymorphisms, showed no significant associations with plasma PLTP activity (Tahvanainen et al. 1999). Plasma PLTP activity increases with age

and correlates positively with body mass index, serum cholesterol and triglycerides (Tahvanainen et al. 1999). In premenopausal women, PLTP activity correlates positively with IDL and buoyant LDL particles, but not with dense LDL fractions, whilst in non obese subjects of both sexes, PLTP activity correlates positively with HDL (Murdoch et al. 2000; Cheung et al. 2002; Murdoch et al. 2002b). Infusion of apoA-I/PC discs into healthy male subjects causes an increase in plasma PLTP activity and an increase in small preβ-HDL (Kujiraoka et al. 2003). Furthermore, when an increase in HDL triglyceride content coincides with high plasma triglycerides, preβ-HDL formation is enhanced (Rye et al. 1998; Dullaart et al. 2001). Thus, high plasma triglycerides and PLTP activity levels possibly act together in promoting HDL conversion. Likewise, various studies have reported relationships between plasma PLTP activity, elevated plasma triglycerides and indexes of obesity (Dullaart et al. 1994b; Riemens et al. 1998b; Murdoch et al. 2000; Kaser et al. 2001b). In a clinical setting, plasma PLTP activity rises following a 24 h intravenous fat load and conversely, it decreases with diet-induced weight loss accompanied by reductions in abdominal subcutaneous fat in males and females (Riemens et al. 1999; Murdoch et al. 2003; Kaser et al. 2004).

Plasma PLTP activity has also been associated with diabetes mellitus. Thus, in type 1 diabetes PLTP activity is increased and related to HDL subclass distribution (Colhoun et al. 2001; Colhoun et al. 2002). Furthermore, PLTP activity has been reported to be elevated in type 2 diabetic patients in association with high plasma triglycerides and obesity (Riemens et al. 1998; Riemens et al. 1998b). Likewise, plasma PLTP activity is not elevated in type 2 diabetic subjects with a relatively normal body mass index (Riemens et al. 1998b; Riemens et al. 1999). Attempts to decrease plasma FFA and triglycerides such as by insulin infusion and the administration of the nicotinic acid derivative, Acipimox, consequently decreases plasma PLTP activity, yet this decrease is blunted in obese type 2 diabetic patients (Riemens et al. 1998b; Riemens et al. 1999b; Dullaart et al. 2001b). Hence, high plasma PLTP activity in insulin-resistant states could, in part, be due to the diminished lowering by insulin. When comparing insulin-resistant with insulin-sensitive subjects without diabetes mellitus, a higher plasma PLTP activity is related to insulin resistance and appears associated with altered triglyceride metabolism (Riemens et al. 1998b; Jonkers et al. 2003). The suppressive effect of insulin on PLTP

activity has been demonstrated *in vitro* using HepG2 cells, and a suppression of PLTP by leptin; the adipocyte-derived hormone which also increases with body fat mass, has also been demonstrated (Kaser et al. 2001).

Recently, plasma PLTP activity was found to be related to cardiovascular disease (CVD). Patients within the highest quintile of PLTP activity revealed a 1.9-fold increased risk for CVD compared to patients within the lowest quintile (Schlitt et al. 2003). The authors from this study concluded that PLTP activity is an independent predictive value for CVD. Whether this is the case or not, PLTP activity has at least been shown to increase when in conjunction with other independent risk factors for CVD such as cigarette smoking (Dullaart et al. 1994), excessive alcohol intake (Lagrost et al. 1996; Liinamaa et al. 1997), and the over consumption of diterpenes present in filter coffee (van Tol et al. 1997; De Roos et al. 2000).

In atherosclerotic lesions, especially foam cell macrophages and SMCs, extracellular and cellular PLTP immunostaining is widespread suggesting that cellular cholesterol accumulation might increase PLTP expression (Desrumaux et al. 2003; O'Brien et al. 2003). However, the notion that PLTP may be a novel risk factor for CVD and a therapeutic target should be treated with extreme caution on the basis that PLTP may also function beneficially in certain tissues and organs. For example, PLTP mRNA is widely distributed throughout all regions of the central nervous system at levels comparable to other organs, and PLTP activity in cerebrospinal fluid (CSF) represents 15 % of the plasma activity, showing that PLTP is synthesised in the brain. Interestingly, a significant increase in PLTP levels in brain tissue homogenates from patients with Alzheimer's disease (AD) has been documented (Vuletic et al. 2003). Yet, when PLTP activity and mass are measured from the CSF of AD patients an overall reduction in PLTP levels compared to the control subjects is observed. PLTP in the CSF of patients with multiple sclerosis is also lower (Vuletic et al. 2005), and in the brains of patients with Down syndrome, there is a down-regulation of PLTP mRNA (Krapfenbauer et al. 2001). This indicates that alterations in brain lipid metabolism and the pathogenesis of neurological diseases are closely linked. Human seminal plasma as well as human tear fluid also contain high levels of PLTP activity (Masson et al. 2003; Jauhiainen et al. 2005). Notably, lung tissue displays the highest levels of PLTP compared to other tissues.

This suggests that PLTP may serve an important role in maintaining the normal function of this organ, such as in the transport of surfactant components, primarily phospholipid. PLTP gene is highly expressed in alveolar type II epithelial cells and is induced during hypoxia and in emphysema (Jiang et al. 1998).

3.5. LOW ACTIVITY (LA) AND HIGH ACTIVITY (HA) PLTP

One limiting factor in evaluating the physiological significance of PLTP has been the absence of a suitable method allowing for the quantification of PLTP concentration in biological samples from various human populations. In order to simultaneously measure PLTP mass and transfer activity from human samples, three separate groups developed specific ELISA methods (Desrumaux et al. 1999; Oka et al. 2000; Huuskonen et al. 2000b). As each assay employed a different combination of polyclonal and/or monoclonal anti-PLTP antibodies, subsequent discrepancies in the correlations of PLTP mass and activity were observed. An explanation for the lack of association between PLTP mass and transfer activity is that two forms of PLTP exist in human plasma; one being catalytically active (HA-PLTP) and the other inactive (LA-PLTP) (Oka et al. 2000). Adenovirus-mediated overexpression of human PLTP in mice also revealed the presence of two PLTP forms (Jaari et al. 2001). In human plasma, these two forms are associated with macromolecular complexes of different size so that the apparent size of LA-PLTP is 520 kDa and that of HA-PLTP is 160 kDa (Kärkkäinen et al. 2002). Following this, a partial characterisation of the two forms revealed that LA-PLTP is complexed with apoA-I while HA-PLTP co-purifies with apoE (Kärkkäinen et al. 2002). Using surface plasmon resonance analysis, PLTP was shown to bind apoA-I, apoE and also apoA-IV. Furthermore, the activation of LA-PLTP into an active form was achieved with proteoliposomes containing either apoE or apoA-IV, but not with those containing apoA-I (Jänis et al. 2005). Based on these findings, the authors have suggested 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 rendered inactive. This hypothesis was, in part, fashioned from the calculated concentration of circulating LA-PLTP which represented approximately 70 % of the total PLTP amount under

normolipidemic conditions (Oka et al. 2000). Soon after, Murdoch and co-workers questioned the above approximation of HA- and LA-PLTP distribution by demonstrating that differences in the reactivity of the antibodies toward HA- and LA-PLTP exist (Murdoch et al. 2002). For instance, they showed that the monoclonal antibody, against full-length recombinant PLTP, is more reactive to LA-PLTP and thereby overestimates its amount, consequently underestimating the amount of HA-PLTP. An explanation for the inability of the monoclonal antibody to quantitatively detect HA-PLTP is from a failure to form an immunocomplex due to the potential masking of specific PLTP epitopes and that the conformation of PLTP may differ when associated with macromolecular complexes of dissimilar size. It has therefore been deemed essential to improve the ELISA method so that both forms of PLTP can be measured precisely.

4. PLTP AND ATHEROSCLEROSIS

INSIGHTS FROM MOUSE MODELS

The mouse has become a widely accepted model of human disease such as atherosclerosis, cardiovascular disease and hyperlipidemia (Paigen et al. 1994; Breslow 1996). Studies relating to these cholesterol-related diseases have escalated since the advent of molecular techniques allowing for the creation of transgenic (Jaenisch 1988) and gene knockout mice (Capecchi 1989). Human PLTP transgenic mice, first created in 1996 (Jiang et al. 1996), have resulted in a complicated phenotype. Transgenic mice that express moderate levels (~30 % increase) of human PLTP do not exhibit marked changes in lipoprotein metabolism, unless these mice are crossed into a human apoA-I background, and only after that, increases in α-HDL and preβ-HDL are observed (Albers et al. 1996; Jiang et al. 1996). An over-expression of huPLTP by adenovirus-mediated infection increases preβ-HDL levels, as well as decreases α-HDL and enhances the hepatic uptake of HDL-CE, thereby implying that the fractional catabolic rate of these particles is increased (Foger et al. 1997; Ehnholm et al. 1998). Transgenic mice expressing high, stable PLTP levels (2.5 - 4.5-fold increase in activity) display a 30 – 40 % decrease in plasma HDL cholesterol and a concomitant rise in preβ-HDL formation, as

compared to wild type controls (van Haperen et al. 2000). Similar findings can also be seen from transient adenovirus-mediated over-expression of human PLTP (Jaari et al. 2001).

When intracellular cholesterol accumulation in macrophages became inhibited in PLTP transgenic mice it was concluded that PLTP behaves as an anti-atherogenic factor (van Haperen et al. 2000). However, further analysis of PLTP over-expression in mice heterozygous for the LDL-R revealed an increased potential for atherosclerosis due to a moderate elevation in VLDL secretion (1.5-fold) (Van Haperen et al. 2002). In order to study the contribution of CETP and PLTP in the metabolism of apoB-100-containing lipoproteins, mice transgenic for CETP and PLTP were created. These mice display a similar moderate 1.5-fold elevation in VLDL secretion (Lie J 2002). Conversely, when CETP and PLTP are over-expressed in the LDL-R heterozygous background (LDLR-KO/huCETPtg/huPLTPtg), a strong PLTP dose-dependent decrease in VLDL and LDL cholesterol is observed and additionally, the activities of the anti-atherogenic enzymes PON-1 and PAF-AH are also PLTP-dose-dependently reduced (Lie et al. 2004). As HDL cholesterol is also diminished in these animals, the authors of this study concluded that these mice had an increased risk for atherosclerosis.

Numerous insights into the role of plasma PLTP have also evolved from studies with PLTP deficient mice that were originally generated by homologous recombination in embryonic stem cells (Jiang et al. 1999). In PLTP-KO mice the *in vivo* phospholipid transfer from triglyceride-rich lipoproteins to HDL is completely abolished and when on a chow diet, a marked decrease in HDL phospholipid (60 %), cholesterol (65 %), and apoA-I (85 %) is observed. On a high-fat diet, the HDL levels in these mice are similarly decreased, but there is also an increase in VLDL and LDL phospholipids (210 %), free cholesterol (60 %) and cholesteryl ester (40 %), without changes in apoB-100 levels, thus suggesting an accumulation of surface components of triglyceride-rich lipoproteins (Jiang et al. 1999). In addition, the surface material deriving from the triglyceride-rich lipoproteins is a fraction enriched in lamellar structures composed of primarily apoA-IV (55 %) and apoE (25 %) and is enriched in phospholipid and free cholesterol (Qin et al. 2000). The small HDL pool in these animals has been suggested to be the result of hypercatabolism of HDL protein and CE, an impairment that may possibly lead to the

condition of hypoalphalipoproteinemia. When the CETP transgene is expressed in the PLTP-KO background it is unable to compensate for the PLTP deficiency and causes a further lowering of plasma HDL concentrations (Kawano et al. 2000). Kawano and colleagues later showed that HL and SR-B1 play a major role in the clearance of free cholesterol and phospholipid surface remnants in the plasma of PLTP-deficient mice (Kawano et al. 2002).

To address the involvement of PLTP in the turnover of apoB-100-containing lipoproteins and dyslipidemia, PLTP-KO mice were bred with different hyperlipidemic mouse strains. In this study, PLTP deficiency in apoB-100 transgenic and apoE-deficient backgrounds resulted in a reduced production and levels of apoB-100-containing lipoproteins, as well as reduced atherosclerosis. A diminished secretion of apoB-100 from the hepatocytes of these animals was also observed and could be corrected when PLTP was reintroduced by adenovirus (Jiang et al. 2001). The apparent defect in apoB-100 secretion appeared to be not only a result of the lack of PLTP protein *per se* but also by the substantial reduction in the α-tocopherol content and elevated lipid peroxides in the livers of these mice, as compared to wild type. By replenishing the isolated primary hepatocytes with α-tocopherol, the hepatic oxidant tone was lowered which subsequently reduced the cellular ROS-dependent destruction of newly synthesised apoB-100 via a post-ER process. As the defect in apoB-100 secretion was fully restored by α-tocopherol alone, this new study bolstered the concept that PLTP inhibitors might be beneficial for increasing the α-tocopherol content also in plasma LDL (Jiang et al. 2005). Previous reports by the same authors demonstrated that the α-tocopherol content of VLDL and LDL was significantly increased in PLTP-KO mice (Jiang et al. 2002). Moreover, PLTP deficiency produced a dramatic delay in the generation of conjugated dienes in copper oxidised apoB-100-containing lipoproteins, and lower titres of autoantibodies to oxidised LDL. Thus, in mice, the bioavailability of α-tocopherol in atherogenic lipoproteins appears to be down-regulated by PLTP and PLTP deficiency could reduce their atherogenicity. When PLTP removes α-tocopherol from atherogenic lipoproteins, the propensity for atherosclerotic lesions to form is shown to increase (Yang et al. 2003). PLTP deficiency was also found to improve the anti-inflammatory properties of HDL as

well as to reduce the ability of LDL to induce monocyte chemotactic activity in human artery cell wall cocultures exposed to human LDL (Yan et al. 2004).

Genetic mouse models have played a crucial role in elucidating the role of PLTP in lipoprotein metabolism and atherosclerosis whereby PLTP-transgenic and PLTP-KO mice have provided the first *in vivo* evidence for the vital role of PLTP in the maintenance of circulating HDL levels, in lipolytic phospholipid transfer from triglyceride-rich lipoproteins to HDL, and in the development of atherosclerosis. However, it still remains to be shown whether the same mechanisms operate in humans.

IV. AIMS OF THE STUDY

1. To quantitatively determine the high-activity (HA) and low-activity (LA) PLTP forms in human plasma by modifying the ELISA method
2. To correlate HA- and LA-PLTP with lipid and carbohydrate parameters in a normal Finnish population sample using the modified ELISA
3. To characterise PLTP secreted by HepG2 cells, and to establish the association of PLTP with apolipoproteins in the secreted macromolecular complexes
4. To investigate the effect of PLTP deficiency on hepatic lipid status, serum lipoprotein distribution, and on the biosynthesis of apoA-I in primary hepatocytes of PLTP-KO and control mice.

V. MATERIALS AND METHODS

1. LIST OF PUBLISHED METHODS

All of the methods that are listed in the table below have been used in this thesis and are sited according to the original publications in which they appear. Some essential methods that have contributed to this thesis as well as those not published will be explained in detail below.

Method	**Original Publication**
Purification of PLTP	I, II
Detergent treatment of PLTP	I
Calibration and optimization of PLTP ELISA	I
Dextran-sulfate-$CaCl_2$ precipitation of LA-PLTP	I
ApoE genotyping	II
Enzymatic lipid analysis	II, IV
Statistical Analysis	I, II, IV
Heparin-Sepharose Affinity Chromatography	I, III
Size-Exclusion Chromatography	I, III, IV
Immunoaffinity Chromatography	I, III
SDS-PAGE	I, III,IV
Western Blotting	I, III
Enhanced Chemiluminescence detection	I, III
Autoradiography	IV
Lowry Protein Determination	I, II, III, IV
PLTP activity assay	I, II, III, IV
PLTP ELISA	I, II, III
ApoA-I ELISA (human and mouse)	III, IV
ApoB-100 ELISA	III
ApoE ELISA	III
Cell Culture	III
Non-denaturing GGE	III
Immunoprecipitation of PLTP	III
Immunoprecipitation of apoA-I	IV
Whole liver perfusion	IV
Primary hepatocyte preparation and culture	IV
Metabolic labeling	IV
Lipid Extraction	IV
Liquid chromatography/mass spectrometry	IV

2. PLTP EXPRESSION IN MAMMALIAN CELLS (UNPUBLISHED)

The cDNA of full-length human PLTP lacking the stop codon was engineered by PCR and cloned into the pEGFP-N1 expression vector (BD Biosciences Clontech), linearised with ApaL1 (New England Biolabs) and transfected into the human hepatocellular liver carcinoma ATCC cell line, HepG2, using Fugene 6 transfection reagent (Roche). For the control cell lines, the pEGFP sequence was excised from pEGFP-N1 using Not1/EcoR1 (New England Biolabs) and the remaining vector was transfected into cells. Single colonies expressing the PLTP-EGFP fusion protein or containing the empty vector were selected for the production of stable cell lines. As the pEGFP-N1 expression vector contains the neomycin gene, the selection antibiotic chosen was Geneticin (G-418 sulphate; Life Technologies).

The HepG2 stably transfected cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal bovine serum (FBS) (Life Technologies), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.6 mg/ml Geneticin. The cells were seeded onto 6 cm dishes and incubated in serum-free culture medium for up to 48 h and the PLTP-EGFP fusion protein was characterized. The secreted PLTP-EGFP protein was measured for activity and mass (methods described in publications I-III) and EGFP fluorescence was measured with a Victor Spectrophotometer at 488 nm for excitation and 535 nm for emission. The accumulation of apolipoproteins secreted into the culture medium was measured by ELISA (method described in I, III).

Live cell microscopy was employed for identifying the intracellular dynamics of the PLTP-EGFP fusion protein whereby 50 µg/ml of cycloheximide in serum-free medium was added to the cells and then followed for 4 h. Brefeldin A (Epicentre Technologies) was added at 5 µg/ml to the cells in serum-free medium and followed for 30 min post-treatment at 10 min intervals.

For immunofluorescence microscopy, the cells were fixed for 20 min at room temperature with 4 % paraformaldehyde, 250 mM Hepes, pH 7.4 and permeabilised for 5 min with 0.1 % TX-100. The primary antibodies, diluted in 5 % FBS/PBS, were incubated for 30 min at 37 °C and the bound antibodies were detected with Alexa568-conjugated goat anti-rabbit or anti-mouse IgG (Molecular probes).

VI. RESULTS AND DISCUSSION

1. QUANTITATION OF HA- AND LA-PLTP BY ELISA

Human plasma PLTP exists in two forms that are distinguished by their ability to transfer phospholipids and are thereby identified as high-activity (HA) and low-activity (LA) PLTP (Oka et al. 2000). Characterisation of each form demonstrates that they are associated with distinct macromolecular complexes of different size and of specific apolipoprotein composition (Kärkkäinen et al. 2002). In order to concurrently measure PLTP concentration with phospholipid transfer activity, three methods based on the ELISA principle have been established (Desrumaux et al. 1999; Oka et al. 2000; Huuskonen et al. 2000b). When HA- and LA-PLTP are separated from human plasma by chromatographic methods and the concentrations measured by ELISA, each form displays a dissimilar immunological reactivity with the antibodies used, and for this reason, is inaccurately detected (Murdoch et al. 2002). With accumulating evidence that each form distributes disproportionately in human plasma (Oka et al. 2000; Kärkkäinen et al. 2002), the biased overestimation of LA-PLTP and the subsequent underestimation of HA-PLTP has since called for a re-evaluation of the ELISA methods in use. In this thesis, we sought to improve the detection technique by modifying the conditions of the ELISA so that we could measure HA- and LA-PLTP precisely and thereby determine the true distribution of each form in normal human plasma.

To improve the accuracy of PLTP mass detection, modification of the reported ELISA method required a denaturation step before antibody binding so that the detectability of the protein, especially that of HA-PLTP, would be improved. The denaturation capacity of five detergents in a sample pre-treatment step and whether they would improve the reactivity of human PLTP was evaluated. The detergents that were applied included anionic (SDS), cationic (CTAB), nonionic (OG and Tween 20), and zwitterionic (CHAPS) species. Each detergent was assayed at concentrations of 0.1 % and 0.5 %, and the critical micellar concentration differed for each detergent (I, Table 1). We found that from all of the detergents tested, 0.5 % SDS resulted in the largest increase in the immunoreactivity of human plasma PLTP (I, Table 1). Once SDS treatment was identified as the most suitable for plasma PLTP, we set out to re-calibrate the ELISA

using two primary standards: purified recombinant (r)-PLTP produced in the baculovirus/insect cell expression system, and PLTP purified from human plasma. We selected r-PLTP as the primary calibrator because i) the production and purification procedures are relatively straightforward and highly reproducible, and ii) no contamination bias from other plasma proteins is possible, which makes this recombinant protein a widely applicable calibration tool. Each calibrator was pre-treated with 0.5 % SDS and standard curves within the range of 25-100 ng PLTP/ml were obtained (I, Fig. 2.). Each curve was linear throughout the entire concentration range and was suitable for the quantification of PLTP to as low as 0.0125 mg/l. When 70 % FBS was added to PLTP purified from human plasma, there was no matrix effect from the bovine serum since a total mass recovery of the primary calibrator was still fully obtainable.

Having calibrated the ELISA, we used normolipidemic plasma from a healthy male volunteer to produce the secondary standard. When plasma was first treated with SDS and then diluted to cover a PLTP mass range of 25-200 ng PLTP/ml, a comparable slope to that of the primary standard curve was obtained (I, Fig. 3.). In order to determine the PLTP concentration in human serum, we assayed 80 serum samples from normolipidemic Finnish individuals and calculated the mean PLTP mass. The value obtained was 5.81 ± 1.33 mg/l (mean ± SD) and the mean phospholipid transfer activity was 5.84 ± 1.39 μmol/ml/h. This is in contrast to our previously reported mass value of 15.6 ± 5.1 mg/l (Huuskonen et al. 2000b). An explanation for such a broad difference is that highly purified active plasma PLTP was used as the calibrator in the previous ELISA for PLTP and it obviously reacted poorly to the monoclonal capture antibody in the absence of a denaturing pre-treatment. This resulted in a low absorbance value for the calibrator, and correspondingly, to very high values for the serum samples. This was also the case for the active r-PLTP that was used in a similar ELISA method where a monoclonal capture antibody was applied (Oka et al. 2000).

In order to study the reactivity of the two PLTP forms in human plasma with our modified ELISA, it was essential that we separate HA- and LA-PLTP first for analysis. This was achieved by utilising four separate methods, with three being chromatographic separations that have previously been reported (Kärkkäinen et al. 2002). From size-exclusion, Heparin-Sepharose affinity and immunoaffinity chromatographies, we

successfully separated HA-PLTP from LA-PLTP. When we applied the HA-PLTP fraction, as separated by the three chromatographic methods, to the non-SDS ELISA, we detected only negligible PLTP mass. A significant increase in immunoreactivity for HA-PLTP was only obtainable with SDS-denaturation and thereby enabled us to confirm, following all three methods, that in human plasma HA-PLTP represents approximately 35 % of the total PLTP mass and LA-PLTP represents approximately 65 % (I, Fig. 4,5,6).

Chromatographic methods used for separating HA- and LA-PLTP are somewhat laborious and too cumbersome for analysing PLTP distributions in a large clinical setting. Taking this into account, we introduced a fast new method for separating plasma HA- and LA-PLTP. This method applies dextran sulfate ($DxSO_4$)-$CaCl_2$ precipitation and involves the selective precipitation of LA-PLTP. We modified this method from previous studies that have used $DxSO_4$-$CaCl_2$ as a purification step for PLTP (Tu et al. 1993). In this approach, LA-PLTP is first precipitated from the sample, the concentration of HA-PLTP in the supernatant is measured and the value is then subtracted from the total plasma PLTP concentration to deduce a value for LA-PLTP. When applying this method to the sera of 80 randomly selected Finnish subjects, the mean concentration of HA-PLTP was 1.87 ± 0.85 mg/l and LA-PLTP in the precipitate was 3.94 ± 1.4 mg/l. Hence, the mass distribution of HA-PLTP was calculated to be 32 % and that of LA-PLTP 68 %. These values are in accordance with the values obtained by the traditional chromatographic separations mentioned above. Furthermore, HA-PLTP mass correlated positively with serum PLTP activity, while LA-PLTP mass did not correlate at all (I, Fig. 7). The total serum PLTP concentration also correlated with total serum PLTP activity, albeit weakly, and this may be explained by the different apolipoprotein complexes that each form associates with and that the specific activity of HA-PLTP in the circulation may vary considerably during different metabolic stages.

2. CORRELATION OF HA-PLTP AND LA-PLTP WITH LIPID AND CARBOHYDRATE PARAMETERS IN A NORMAL FINNISH POPULATION SAMPLE

In this thesis, we modified the PLTP ELISA method so that it would serve as a useful tool for measuring the two PLTP forms in human plasma (I). It would also enable us to address the mechanisms by which each form interacts with lipoproteins and how this influences the progression of atherosclerosis. We applied our modified ELISA to a Finnish population sub-sample (as part of a large Health 2000 Health Examination Survey) to analyse the distribution of serum HA- and LA-PLTP and to identify whether lipid and carbohydrate connections between the two PLTP forms exist. We measured the total PLTP concentration from the sera of 250 Finnish individuals and then determined the specific concentration of each PLTP form separated by $DxSO_4$-$CaCl_2$ precipitation. The total serum PLTP concentration was 6.56 ± 1.45 mg/l (mean ± SD), and approximately 46 % was in a catalytically active form; 3.00 ± 1.21 mg/l (range, 0.92-7.63 mg/l). The mean concentration of LA-PLTP was 3.56 ± 1.14 mg/l (range, 1.20-8.43 mg/l). These values are similar to what we obtained after we modified the ELISA (I).

In order to study the correlation of PLTP transfer activity with the mass of HA- and LA-PLTP, we performed two separate radiometric assays on the serum samples: the exogenous assay ($PLTP_{exo}$), measuring phospholipid transfer to exogenously added HDL (Damen et al. 1982), and the endogenous assay ($PLTP_{endo}$), measuring phospholipid transfer to endogenous serum HDL (Lagrost et al. 1999). In both assays exogenously added radiolabeled PC-liposomes served as the phospholipid donor. The serum $PLTP_{exo}$ activity was 6.59 ± 1.66 μmol/ml/h, whereas the serum $PLTP_{endo}$ activity was 1.37 ± 0.29 μmol/ml/h (II, Table 2). This difference in activity can be explained by the dependency of the $PLTP_{endo}$ assay on endogenous HDL levels which most likely reflect the activity of PLTP modulated by the composition of circulating lipoproteins. The two radiometric assays also resulted in different correlations to lipoprotein parameters and serum glucose (II, Table 4). $PLTP_{endo}$ activity correlated with total cholesterol, TG, and LDL-C, suggesting that lipoproteins other than HDL also significantly affect the outcome of the assay and therefore the endogenous PLTP activity level does not reflect the true amount of the active form of PLTP in serum. The exogenous assay, however, reveals more

directly the amount of active PLTP in serum. $PLTP_{exo}$ activity correlated with body mass index (BMI), waist-to-hip ratio (WHR) and VLDL, but not with LDL-C or HDL-C.

As each of the two PLTP forms associates with different macromolecular complexes of varying apolipoprotein and lipid compositions, it is plausible that the phospholipid transfer activity of PLTP may be mediated by or be dependent on the apolipoprotein/lipid constituents of the macromolecular complexes. The observation that plasma HA-PLTP co-purifies with apoE and that apoE-containing liposomes are capable of activating LA-PLTP into a catalytically active form (Kärkkäinen et al. 2002; Jänis et al. 2005), prompted us to measure the apoE concentrations and isoform distributions in our sample population. The apoE allele frequencies were similar to those reported previously for Finns (Ehnholm et al. 1986). The ε2-carrying genotypes displayed the highest serum levels of apoE and had the highest serum $PLTP_{endo}$ activity. Both activity assays correlated weakly with total apoE, however, neither $PLTP_{exo}$ activity nor PLTP concentration correlated separately with the apoE isoforms. Therefore, the $PLTP_{endo}$ activity levels that were detected in the ε2-carrying genotypes were most likely a reflection of the serum lipid/lipoprotein profile of the sample that had in some way affected the outcome of the endogenous assay. An explanation for the lack of any correlation between HA- or LA-PLTP concentration, the exogenous PLTP activity and the apoE isoforms may be due more or less to the normolipidemic sample population that was used and that different individual lipid/lipoprotein profiles were not distinguished from one another. A possibility also remains that the different apoE isoforms simply do not affect the PLTP parameters.

In clinical approaches, PLTP has been linked to carbohydrate metabolism and indexes of obesity (Dullaart et al. 1994b; Riemens et al. 1998b; Murdoch et al. 2000). In addition, elevated PLTP activity has been associated with type 1 diabetes (Colhoun et al. 2002), as well as with type 2 diabetic patients who have high plasma TG and are obese (Riemens et al. 1998b). We were interested in investigating whether any associations of insulin and/or glucose with HA- and LA-PLTP existed. $PLTP_{exo}$ activity correlated with glucose and with the index for insulin resistance (HOMA IR), supporting previous reports that high PLTP activity is related to insulin resistance and therefore high insulin levels (Jonkers et al. 2003). This data, in addition, suggests that $PLTP_{exo}$ activity could be

elevated in metabolic syndrome. $PLTP_{endo}$ activity did not correlate with these parameters at all, whereas LA-PLTP did correlate with insulin while HA-PLTP did not (II, Table 4). Therefore, the distribution of the two PLTP forms appears to be influenced by lipid and glucose metabolism suggesting that, in the future, each form could serve as an independent marker for CVD risk evaluation.

3. PLTP SECRETED BY HEPG2 CELLS RESEMBLES HA-PLTP IN HUMAN PLASMA

One approach towards understanding the source of HA- and LA-PLTP in human plasma is to study the tissue- and cell-specific expression and secretion of nascently formed PLTP. Keeping in mind the expression pattern of human PLTP mRNA as well as the different size of each PLTP-expressing tissue, the liver is invariably one of the major sources of circulating PLTP, and is especially important when considering lipoprotein research. Therefore, we set out to characterise PLTP secreted from HepG2 cells, a human hepatoma cell line, and were interested in elucidating the interaction of PLTP with hepatic apolipoproteins. We chose HepG2 cells as the model system because they closely resemble lipid and lipoprotein metabolism in human liver; such as fatty acid uptake, lipid synthesis, apolipoprotein synthesis, and lipolytic enzyme and transfer protein synthesis (LCAT, CETP, and HL), along with apoB-100-lipoprotein assembly and secretion (Thrift et al. 1986; Arrol et al. 1991).

We found that HepG2 cells secrete apoA-I, apoE and apoB-100 in appreciable amounts, and also PLTP displaying activity (0.30-0.35 μmol/ml/h at 48 h) (III, Fig. 1.). The concentration of PLTP was only measurable with our SDS-ELISA method as the mass was below immuno-detection levels with the previously reported ELISA (II), despite Western blot analysis indicating abundant PLTP amounts. At 48 h, the specific activity (an activity to mass ratio) of HepG2-derived PLTP was calculated to be 2.8-3.9 μmol/μg PLTP protein/h. Surprisingly, this value is very close to the specific activity of HA-PLTP in human plasma (2.5-3.3 μmol/μg PLTP protein/h) (Kärkkäinen et al. 2002), suggesting that HepG2-derived PLTP resembles the high-activity form. We were not able to measure any LA-PLTP mass with the SDS-ELISA, nor could we separate any inactive PLTP from a Heparin-Sepharose affinity column, the method which is reported to

separate both PLTP forms (Kärkkäinen et al. 2002). HA-PLTP displays affinity for heparin and is retained on the column while LA-PLTP does not bind heparin and is therefore eluted immediately.

Characterisation of the secreted PLTP entailed studying the size of nascent PLTP complexes secreted into the culture medium from HepG2 cells. Using non-denaturing gradient gel electrophoresis, we identified PLTP within the size range of 150-450 kDa and concluded that PLTP is secreted not as a monomer but instead, as part of a large complex. Additional properties of HepG2 PLTP were then established by using Heparin-Sepharose affinity and size-exclusion chromatography. This data demonstrated that PLTP displays affinity for Heparin and that PLTP activity and mass co-elute with apoE in a position corresponding to the molecular mass of 160 kDa. All of these properties were akin to those of HA-PLTP isolated from human plasma, and as the derivation of HA- and LA-PLTP is suggested to involve an interaction with apolipoproteins, we decided to study the possible association of HepG2 PLTP with apoE. Polyclonal antibodies specific against human apoE were found to inhibit PLTP activity in a concentration-dependent manner, reaching 87 % inhibition at an IgG concentration of 0.5 mg/ml. Furthermore, PLTP quantitatively bound to an anti-apoE affinity column, and eluted at low pH with the portion of apoE that had also bound to the column. This suggests that high-activity HepG2 PLTP associates with apoE and this can be supported by recent data that plasma LA-PLTP is activated by apoE-containing liposomes (Jänis et al. 2005). Even though the present data suggests a functional role for apoE in PLTP activity, apoE deficient mice display normal levels of PLTP activity (M. Jauhiainen and K. Aalto-Setälä, unpublished observations). In addition, human tear fluid devoid of apoE contains only active PLTP eluting at the apparent molecular mass of 160 – 170 kDa in size-exclusion chromatography (Jauhiainen et al. 2005). A compensatory mechanism by other apolipoproteins such as apoA-IV, or possibly lipocalin in tear fluid, may be important for keeping the protein functionally active. This is supported by the recent finding that also apoA-IV proteoliposomes activate LA-PLTP (Jänis et al. 2005). Finally, the present study demonstrates that HepG2 cells are a relevant model system to study the high-activity form of human plasma PLTP secreted by hepatocytes.

4. STABLE EXPRESSION OF A PLTP-EGFP FUSION PROTEIN IN HEPG2 CELLS

In order to study the intracellular localisation and possible function of PLTP, we utilised the human hepatoma cell line, HepG2, to develop a line that constitutively over-expresses a fluorescently tagged PLTP-EGFP fusion protein. In accordance with the observation that PLTP is translocated into the ER lumen and secreted by HepG2 cells (III), the PLTP-EGFP fusion protein was found to be localised within the secretory pathway. PLTP-EGFP fluorescence was dominant in the Golgi apparatus and the fusion protein was also detected in reticular ER compartments [Fig 4].

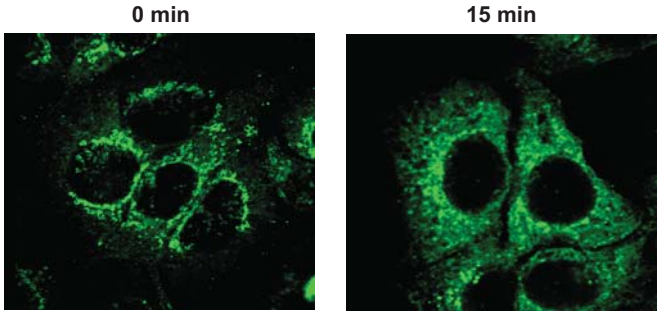

Fig. 4. PLTP-EGFP HepG2 cells in 10 % FBS +/- Brefeldin A

A co-localisation of PLTP-EGFP with the Golgi marker antibodies, cis-Golgi marker (GM)-130, ER Golgi Intermediate Compartment (ERGIC)-53, and Trans Golgi Network (TGN)-46, was demonstrated, hence confirming a localisation of PLTP-EGFP to the Golgi complex [Fig 5]. When the Golgi stack was dissociated with the fungal toxin Brefeldin A, PLTP-EGFP staining was dispersed to a more ER-like pattern, thus further confirming localisation of the protein within Golgi compartments [Fig 4]. When the more peripheral parts of the cells were studied using a higher photomultiplier gain, co-localisation with the ER marker Protein Disulphide Isomerase (PDI) was also observed [Fig. 5]. When protein synthesis was inhibited with 50 µg/ml cycloheximide, PLTP-EGFP was chased out from the secretory pathway within 3 h [Fig 6], an observation well

within the range of secretion rates determined for several other plasma proteins (Ledford et al. 1983; Lodish et al. 1983; Fries et al. 1984).

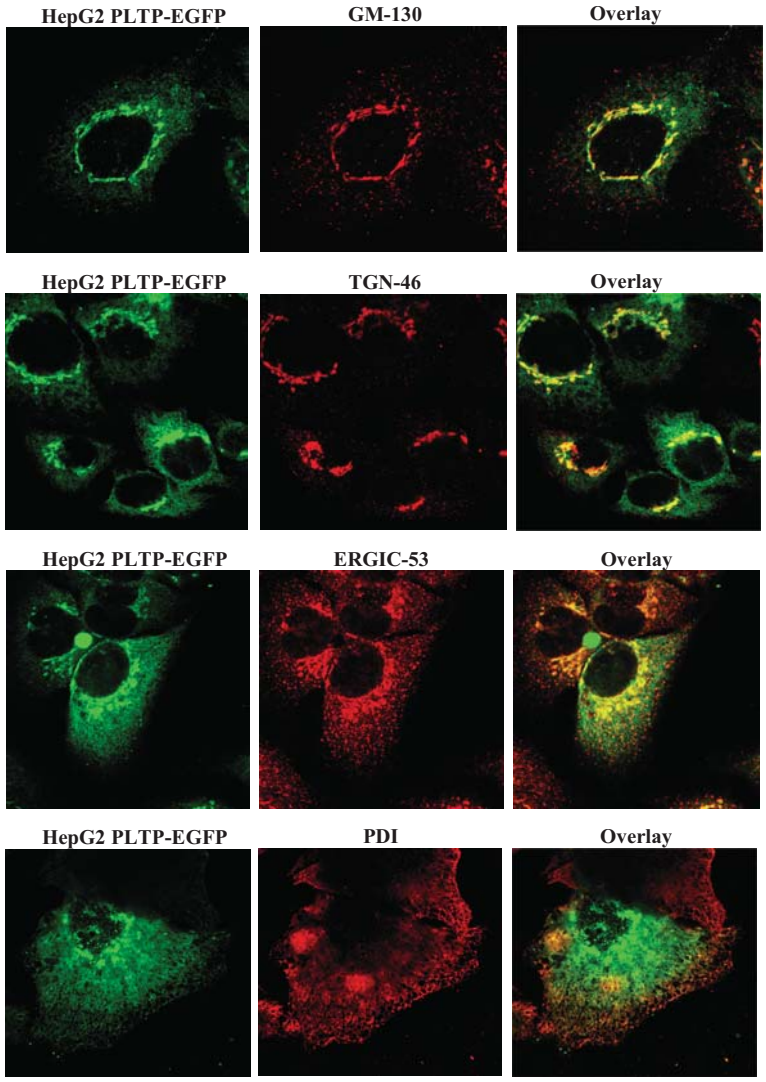

Fig. 5. Immunofluorescence image of PLTP-EGFP and different Golgi markers

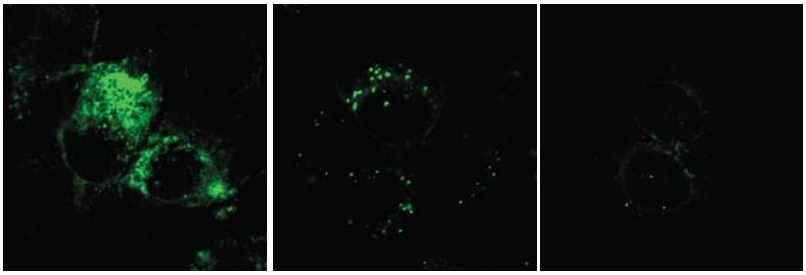

Fig. 6. PLTP-EGFP HepG2 cells + 50µg/ml cycloheximide

Given that PLTP-EGFP was detected within the secretory pathway, the next step was to measure PLTP-EGFP secreted into the culture medium. This was performed by incubating the cells in serum-depleted medium for up to 48 h, collecting the medium and radiometrically assaying the phospholipid transfer activity (Damen et al. 1982; Jauhiainen et al. 1993). PLTP mass was also measured using the SDS-ELISA (I), and fluorescence was measured with a Victor Spectrophotometer at 488 nm for excitation and 535 nm for emission. From these measurements, the secreted PLTP-EGFP fusion protein displayed a low specific activity (SA = 0.07 µmol/µg per h) [Fig 7.1]. In contrast, the mock transfected cell line secreted endogenous PLTP which displayed a high specific activity (SA = 1.7 µmol/µg per h) [Fig 7.2]. Analysis of EGFP fluorescence in the medium revealed a time-dependent increase of fluorescence by the PLTP-EGFP cells that greatly exceeded background levels observed in the medium of mock-transfected cells. The size and presence of the proteins that were secreted into the culture medium and within the cells were confirmed by Western blotting with anti-PLTP full-length and anti-GFP peptide antibodies. The bands that were resolved from the cells were the PLTP-EGFP fusion protein representing the intracellular ER and cis-Golgi PLTP forms (approximately 92 kDa) [Fig 8.1], and in the culture medium representing the fully glycosylated PLTP-EGFP form (approximately 110 kDa) [Fig 8.2].

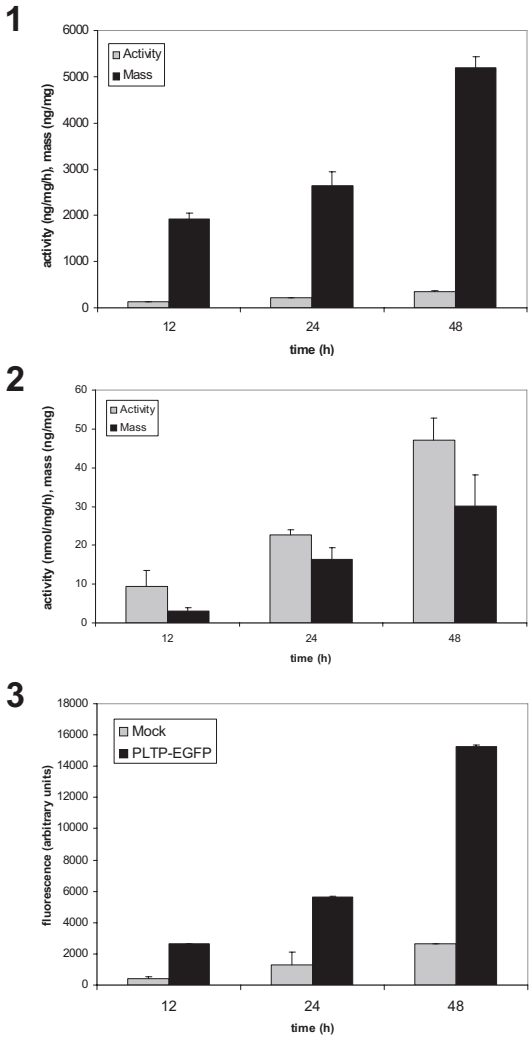

Fig. 7. Characterization of the secreted PLTP-EGFP fusion protein. 1) PLTP-EGFP secreted from HepG2 stable cell line. 2) Endogenous PLTP secreted from the mock transfected cell line. 3) Fluorescence measured from mock transfected and PLTP-EGFP expressing cells.

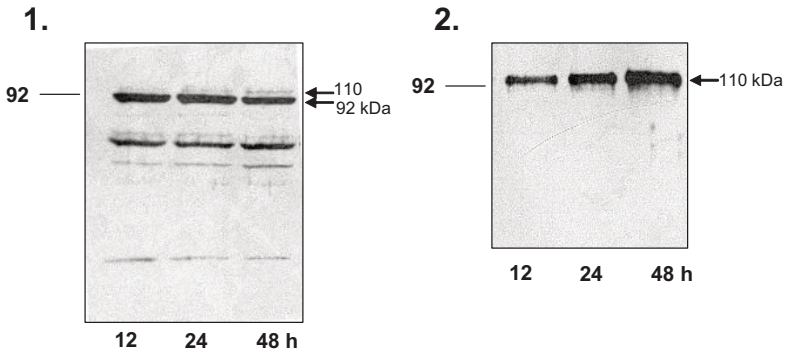

Fig. 8. Western blot of PLTP-EGFP from stable-transfected HepG2 cells. 1) Anti-GFP antibody detecting the fusion proteins: 92 kDa band represents intracellular ER and cis-Golgi PLTP, 110 kDa form represents the fully glycosylated form. 2) Anti-PLTP mAb59 monoclonal antibody detecting the fusion protein secreted into the culture medium with one 110 kDa band .

When studying the effect of PLTP-EGFP over-expression on the secretion of certain apolipoproteins, we found that the accumulation of apoA-I into the culture medium from the stable cell line was significantly diminished compared to the mock-transfected cells [Fig 9.]. Even though PLTP-EGFP is secreted from the stable-transfected cells, the low-specific activity suggests that the fusion protein is defective and may interfere with the secretion of apoA-I. This suggests that active PLTP could be involved in the secretion of apoA-I or in its stabilization in the culture medium and may therefore play a role in the early steps of HDL biosynthesis. Evidence from ABCA1 studies suggest a role for PLTP in cholesterol efflux at the extracellular face of plasma membranes whereby PLTP may interact directly with either apoA-I or ABCA1 or both during cholesterol efflux (Wolfbauer et al. 1999; Oram et al. 2003). It is also possible that PLTP facilitates the early phospholipidation of apoA-I within the secretory pathway post-synthesis in the Golgi region (Mendez et al. 1996; Bhat et al. 2004), or is involved in the recycling of ABCA1 and apoA-I through endocytic compartments (Neufeld et al. 2004). Furthermore, the possibility that PLTP might be directly involved in the phospholipidation of apoA-I or

in its secretion from the Golgi complex was raised by the finding that PLTP-EGFP and the endogenous apoA-I synthesised in the HepG2 stable cells extensively co-localised in the Golgi complex [Fig 10]. In order to investigate these hypotheses using a more physiological setting, we decided to study the role of PLTP in the biosynthesis of apoA-I from primary hepatocytes by applying an available PLTP gene knock-out mouse model.

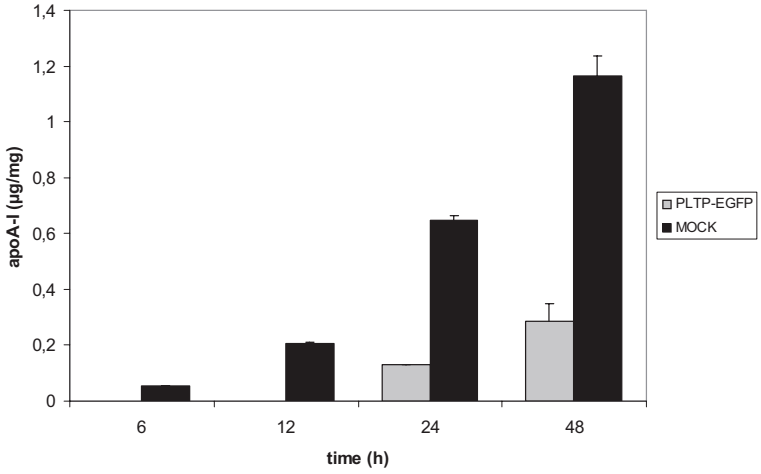

Fig. 9. Accumulation of apoA-I in the culture medium from PLTP-EGFP HepG2 cells

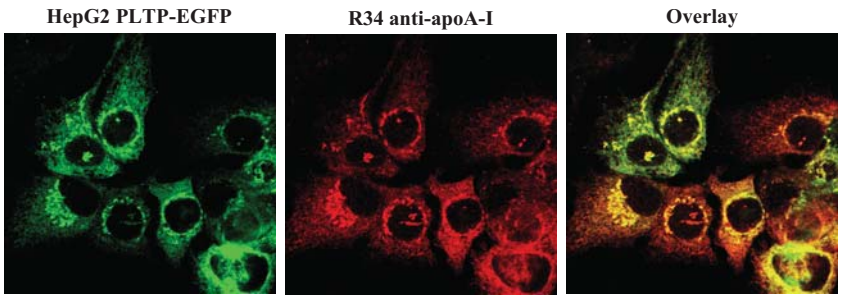

Fig. 10. Immunofluorescence image of PLTP-EGFP co-localising with apoA-I in the Golgi

5. LIPOPROTEIN AND LIPID LEVELS IN PLTP-KO MICE

PLTP deficiency in mice has been reported to result in markedly decreased HDL phospholipid (60 %), cholesterol (65 %), and apoA-I (85 %) levels (Jiang et al. 1999). PLTP deficiency has also been implicated in decreased secretion and levels of apoB-100-containing lipoproteins as demonstrated by crossing the PLTP deficiency trait into apoE-deficient and apoB-100 transgenic backgrounds (Jiang et al. 2001). Increasing evidence that PLTP may play a role in hepatic lipoprotein biosynthesis is also demonstrated from findings that human PLTP is associated with apolipoproteins *in vitro* (Pussinen et al. 1998; Kärkkäinen et al. 2002)(III), and *in vivo* (Barlage et al. 2001). In order to investigate the impact of PLTP deficiency on lipoprotein biosynthesis, we measured mouse hepatic tissue lipid status, the serum lipoprotein profiles, and apolipoprotein A-I secretion from primary hepatocytes in culture. The animals we used were female, they were grouped according to age; 6, 12 and 18 weeks, and were produced in a C57Bl/6 background (wild type, WT; PLTP-knock out, PLTP-KO).

With data on serum and hepatic lipid levels in PLTP-KO mice at different age groups being limited, we designed a study that would enable us to identify lipid level changes, that if present, would occur with age. Comparing each age group, all animals at the onset of sample collection were of similar body mass, liver mass, and displayed similar liver to body mass ratios (IV, Table1). As expected, PLTP-KO mice at all ages displayed decreased serum total cholesterol and apoA-I levels when compared to the WT

controls, and at 6 weeks of age serum choline phospholipids were also reduced in the PLTP-KO mice (IV, Table 1, 2). We also measured serum TG and FFAs and found no significant differences between the groups. To analyse serum lipoprotein profiles we pooled serum from four mice within each group at the age of 12 and 18 weeks. Lipoproteins were separated by size-exclusion chromatography and the elution profile for the PLTP-KO mice revealed a TG, choline phospholipid (PL) and cholesterol peak distribution predominantly within fractions corresponding to the elution position of VLDL (IV, Fig 1). In the 12 week old PLTP-KO sera, TG levels in the position of VLDL were elevated in comparison to WT (IV, Fig 1A, B).

The elevated TG in serum VLDL fractions may be connected with increased liver TG that was also observed in the PLTP-KO animals, an elevation of up to 30 % at 6 and 12 weeks of age. There were no differences in hepatic lipid peroxidation levels and since we found no increase in serum FFA, the most obvious cause for liver TG accumulation, the molecular mechanisms leading to increased TG still remain unclear. It is possible that the increased levels of hepatic TG are due to intracellular regulatory changes in TG synthesis (Farese et al. 2000), FA β-oxidation (Grefhorst et al. 2005), or alterations in hepatic glucose metabolism (Bandsma et al. 2001; McDevitt et al. 2001). The analysis of hepatic lipids also revealed an elevation in PL and cholesterol in the PLTP-KO mice, while bile acid synthesis appeared normal, if not elevated (IV, Table 2, 3). The general view is that cholesterol carried in HDL is the major precursor for hepatic bile acid synthesis (Botham et al. 1995). This suggests that the lack of PLTP activity in these mice, resulting in diminished circulating HDL levels, may lead to hepatic uptake of plasma LDL cholesterol and/or subsequent alterations in cellular cholesterol and PL homeostasis. Increased cholesterol biosynthesis would be a likely pathway to investigate for future studies.

As recent reports suggest that of all newly synthesised apoA-I a substantial portion is lipidated intracellularly (Chisholm et al. 2002; Zheng et al. 2005), and with PLTP-EGFP in HepG2 cells co-localising with apoA-I in the Golgi compartment, we thought it would be interesting to study the involvement of PLTP in the secretion of apoA-I or nascent HDL by the liver. We isolated primary hepatocytes from PLTP-KO and WT mice (8-12 weeks of age) by whole liver collagenase perfusion and examined the

synthesis and secretion of apoA-I from these cells. The accumulation of apoA-I into serum-free culture medium up to 24 h, as measured by ELISA, revealed that at the latest time points the PLTP-KO hepatocyte culture medium contained 66 % lower apoA-I levels when compared to that of the WT hepatocytes (IV, Fig 2). This indicated to us that either (i) there was a secretion defect of apoA-I in the hepatocytes, or (ii) increased proteolytic degradation occurring in the cells or culture media. To address the question of synthesis and secretion, we carried out metabolic labeling of newly synthesised apoA-I, with or without a 24 h pre-incubation in serum-free medium, followed by a 3 h chase period. This revealed that the synthesis of apoA-I in both cell types is similar (IV, Fig 3). The 24 h pre-incubation in serum-free medium was designed to eliminate any possibilities that the apoA-I defect would arise after a delayed response by the PLTP-KO cells to reduce their synthesis of apoA-I following serum depletion. Having established that PLTP deficiency incurs no impairment on the biosynthesis or secretion of apoA-I, we considered that an instability of the protein would be the likely cause for the progressive reduction in the accumulation of the protein over time.

Degradation of exogenously added lipid-free apoA-I by PLTP-KO and WT hepatocytes was detected upon a 24 h incubation in conditioned medium from both cell types so that the trichloroacetic acid (TCA) precipitable non-degraded fraction represented 42 ± 0.5 % (SD) from PLTP-KO and 50 ± 5.7 % from WT media. When exogenous apoA-I was incubated with the cells, degradation from the PLTP-KO cells was 35 ± 5.0 % and from WT, 41 ± 11 %; values slightly higher than from conditioned medium alone, but comparable for both cell types. This demonstrates that PLTP-KO and WT cells degrade lipid-free apoA-I to a similar extent. Therefore, with degradation being similar in both cell types, we consider it most likely that the increased instability of the apoA-I produced by the PLTP-KO cells is caused by a difference in protease susceptibility, probably due to a difference in lipidation.

To address the likelihood that apoA-I secreted from PLTP-KO cells is lipidated differently to WT apoA-I, we employed liquid chromatography/mass spectrometry (LC/MS). We found that apoA-I from the PLTP-KO cells contained a lower total amount of PL (32 % decrease; IV, Fig 4A), and the PC/SM ratio was also significantly reduced (IV, Fig 4B). Detailed analysis of the apoA-I PL species composition for each PL class

revealed a substantially different composition in the PLTP-KO apoA-I compared to WT (IV, Fig 5A). This difference was also evident in the PLTP-KO cells (IV, Fig 5B-F), whereby a relative decrease in the long-chain arachidonic acid (20:4)-containing species was evident and seemed to be compensated for by a relative increase in medium-chain FA-containing PL species. In addition, a relative increase of certain docosahexaenoic acid (22:6)-containing species in the PLTP-KO cells was observed. These differences in PL molecular species composition of the PLTP-KO hepatocytes and PLTP-KO apoA-I were almost identical, whereas the PL species of the WT cells and apoA-I produced by them showed distinct differences (IV, Fig 5A,B). This suggests that in PLTP deficiency, newly synthesised apoA-I is phospholipidated differently either within the secretory pathway or at the plasma membrane, which leads to destabilization and ultimately impaired accumulation of the protein into the culture medium. A suggested role for PLTP in the partial lipidation of newly synthesised apoA-I during nascent HDL formation from the liver is now presented as an essential process for the effective stabilization of the protein. Therefore, an altered hepatic lipid status and impaired biosynthesis of nascent HDL in the PLTP-KO mice reveals a role for PLTP in hepatic lipid homeostasis.

VII. SUMMARY AND CONCLUSIONS

This doctoral thesis consists of a number of studies in which different aspects of PLTP research are tackled so that the role of how PLTP influences hepatic lipoprotein metabolism in the context of dyslipidemias and atherosclerotic disease progression can be further understood. The approaches used in this thesis stem from studies performed in human serum, in cellular model systems, and in genetically modified mice that display the PLTP deficiency trait. The objectives were (i) to develop a new and applicable laboratory assay for measuring the two forms of PLTP, (ii) to apply this assay in a clinical setting, and (iii) to study the characteristics and likely role of PLTP in lipoprotein and lipid metabolism in the liver.

The first part of this thesis aimed at developing an accurate ELISA method that would quantitatively measure the HA and LA forms of human plasma PLTP. By applying a simple, yet effective pre-treatment of the samples, we discovered that the detectability of HA-PLTP dramatically improved via exposure of epitopes that were otherwise concealed for antibody detection. Calibration of the new ELISA, that would be applicable to most laboratories, as well as the introduction of a fast and effective method ($DxSO_4$-$CaCl_2$ precipitation) to separate HA-PLTP from LA-PLTP, provides new tools that have previously been unavailable. This innovative approach for PLTP analysis has since allowed for accurate and comparable measurements of total PLTP protein concentration in plasma or serum samples and for determining the distribution of PLTP between the LA-PLTP and HA-PLTP forms. In doing so, this methodology will be useful for establishing the relationship that PLTP mass and activity have with associated lipoproteins, plasma factors, and lipid metabolism in general.

This leads to the second part of this thesis in which the methodology for PLTP mass determination was applied to a random Finnish population sample (n = 250). The association of PLTP activity and the concentration of both forms with lipid and carbohydrate parameters were investigated. Of the total serum PLTP concentration, approximately 46 % was in a catalytically active form and this HA-PLTP form correlated positively with serum $PLTP_{exo}$ activity, HDL cholesterol and apoA-I. The role for PLTP in PL transfer activity and in HDL conversion has been well established, and with the positive associations of HA-PLTP with HDL parameters identified in this study, we now

await large, prospective studies on PLTP to assess whether concentration, activity, or both combined can be used as independent markers for cardiovascular risk evaluation.

When the importance of HA- and LA-PLTP in human plasma in disease progression is better understood, the source of each form and the properties of each must be established. As most studies on PLTP have been performed in human plasma, we decided to direct our studies to the tissue- and cell-specific expression and secretion of nascently formed PLTP. We set out to characterise PLTP secreted from HepG2 cells, a human hepatoma cell line, and were interested in elucidating the interaction of PLTP with hepatic apolipoproteins. We established that PLTP secreted from HepG2 cells closely resembles the high-activity PLTP form isolated from human plasma, and in addition, associates with apoE. ApoE is a powerful regulator of lipoprotein metabolism in human serum and has recently been shown to activate human plasma LA-PLTP (Jänis et al. 2005). Therefore, this study presents the HepG2 cell model as a tool (i) for studying the mechanisms by which active PLTP becomes converted into the low-activity form associated with apoA-I, and (ii) for studying the intracellular function of newly synthesised PLTP. In this thesis, we also utilized HepG2 cells for developing a stable cell line that would constitutively over-express a PLTP-EGFP fusion protein. This fusion protein was efficiently secreted and within the secretory pathway it was found to concentrate in the Golgi compartment, to co-localize with apoA-I, and also to inhibit the accumulation of apoA-I in the culture medium. Consequently, these cells provided us with the clue that PLTP could serve an intracellular role in the biosynthesis and/or stabilization of apoA-I and may be a regulator of HDL formation.

The final study of this thesis utilizes the PLTP gene knockout mouse model to investigate the hypothesis that PLTP is required for the biosynthesis of apoA-I and may be an important regulator of hepatic lipid metabolism. In the situation of PLTP deficiency, we confirmed previous findings that plasma HDL cholesterol and apoA-I in these mice are severely reduced and that the cholesterol distribution is mostly in the fractions eluting in the VLDL and LDL region, also found to contain elevated TG. Furthermore, the hepatic lipid composition was drastically altered in PLTP-KO animals with cholesterol and choline phospholipid being elevated in each age group, along with increased TG in the younger mice. We concluded that without any increases in lipid

peroxidation or impairment in bile acid secretion, the observations would most likely be attributed to cellular alterations in cholesterol biosynthesis or increased uptake of apoB-100-containing lipoproteins as a result of a decreased amount of HDL cholesterol available for hepatic uptake. We also studied the effect of PLTP deficiency on HDL biosynthesis in the primary hepatocyte cultures of these mice and found that apoA-I is phospholipidated differently to WT, which presumably affects the stability of the protein in the culture medium upon secretion. Such lipid-related outcomes of PLTP deficiency in mice demand further investigation and illustrate the magnitude of consequences that manipulation of HDL populations by PLTP inactivation would cause on lipid metabolism as a whole.

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