The Role of Plasma Phospholipid Transfer Protein (PLTP) in the Development of Atherosclerosis

Studies in Genetically Modified Mice

De rol van plasma *phospholipid transfer protein* (PLTP) in de ontwikkeling van atherosclerose

Studies in genetisch gemodificeerde muizen

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Jessica Theresa Lie
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Promotor:
Prof.dr. H. Jansen

Overige leden:
Prof.dr. D.J.G.M. Duncker
Prof.dr. F.G. Grosveld
Prof.dr. F. Kuipers

Copromotoren:
Dr. M.P.G. de Crom
Dr. A. van Tol

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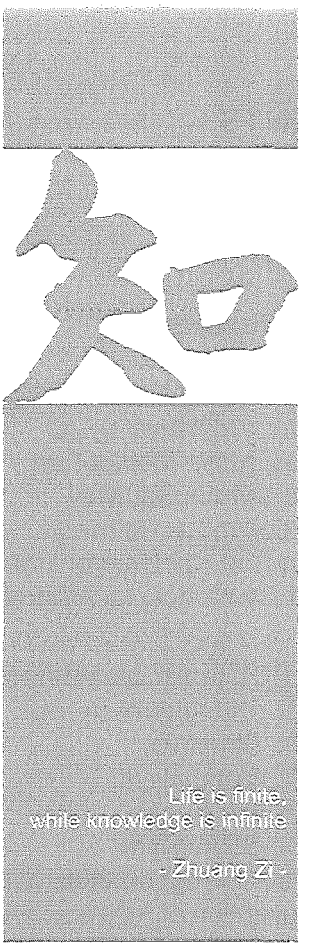

In remembrance of my father
Lie Han-Tong

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Abbreviations

ABCA1	ATP binding cassette A1
apo	apolipoprotein
CETP	cholesteryl ester transfer protein
EL	endothelial lipase
ELISA	enzyme linked immunosorbant assay
FXR	farnesoid liver X-activated receptor
HDL	high density lipoproteins
HL	hepatic lipase
IDL	intermediate density lipoproteins
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoproteins
LDL-R	low density lipoprotein receptor
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LXR	liver X activated-receptor
oxLDL	oxidized LDL
PAF-AH	platelet-activating factor acetyl hydrolase
PLTP	phospholipid transfer protein
PON	paraoxonase
PPAR	peroxisome proliferator activated-receptor
RXR	retinoid X activated-receptor
SRBI	scavenger receptor class B type I
VLDL	very low density lipoproteins

CHAPTER I

General Introduction

1 Atherosclerosis and Lipoproteins

1.1 Atherosclerosis

Complications of atherosclerosis are the most common cause of death in the Western society. Atherosclerosis is a disease of the large arteries caused by a long-term process of an accumulation of lipids combined with an inflammatory response. The process starts with fatty streaks at the luminal side of the vascular wall. The development of atherosclerotic lesions accelerates at about ages 25 to 30 in men and 40 to 45 in woman according to autopsy examinations in Western populations (1). The clinical manifestations of atherosclerosis often begin to occur in the 5th or 6th decade of life. The ultimate result of atherosclerosis may be a heart attack (Figure 1) or stroke. The notion that cholesterol plays a crucial role in the development of atherosclerosis is based on strong experimental and clinical evidence (2, 3). Low density lipipoproteins (LDL), the major carriers of plasma cholesterol in man, were shown to be a causal factor. Many randomized, placebo-controlled, clinical trials have shown that lowering of LDL cholesterol results in a substantial reduction in cardiovascular events (4-6).

In the past two decades, research was increasingly focused on the pathobiology of the arterial wall during atherosclerosis (3). The arterial wall consists of three layers, the intima, media and adventitia (Figure 1). The intima is situated at the luminal side of the vessel wall, and mainly comprises endothelial cells. The media mainly consists of layers of smooth muscle cells and elastic fibres. The adventitia surrounds the media, and comprises a layer of loose connective tissue that merges with surrounding connective tissue, and elastic fibres that are less abundant than in the media.
Atherogenesis can be viewed as an inflammatory, or healing, response of the endothelial cells to injury (7, 8). This response to injury hypothesis is the most widely accepted hypothesis for the initiation of atherosclerosis and postulates that the atherosclerotic process is triggered by a local dysfunction of the endothelial lining of the vessels. This event leads to subsequent changes in the vascular permeability, expression of adhesion molecules and release of growth factors. Physical damage, metabolic changes, toxicological damages, lipoproteins or other risk factors are seen as the injurious agents (2, 9).
The development of atherosclerosis can be described by a sequence of events generating the fatty streak lesions or atherosclerotic plaques (3, 10, 11). The initiation of lipid accumulation starts with LDL, which infiltrate in the sub-endothelial space of the arterial wall. LDL can be retained in the intima, and may undergo modification by oxidation or other processes. The oxidized lipoprotein particles and their constituents can then elicit the production of various cytokines, inflammatory mediators and growth factors that affect

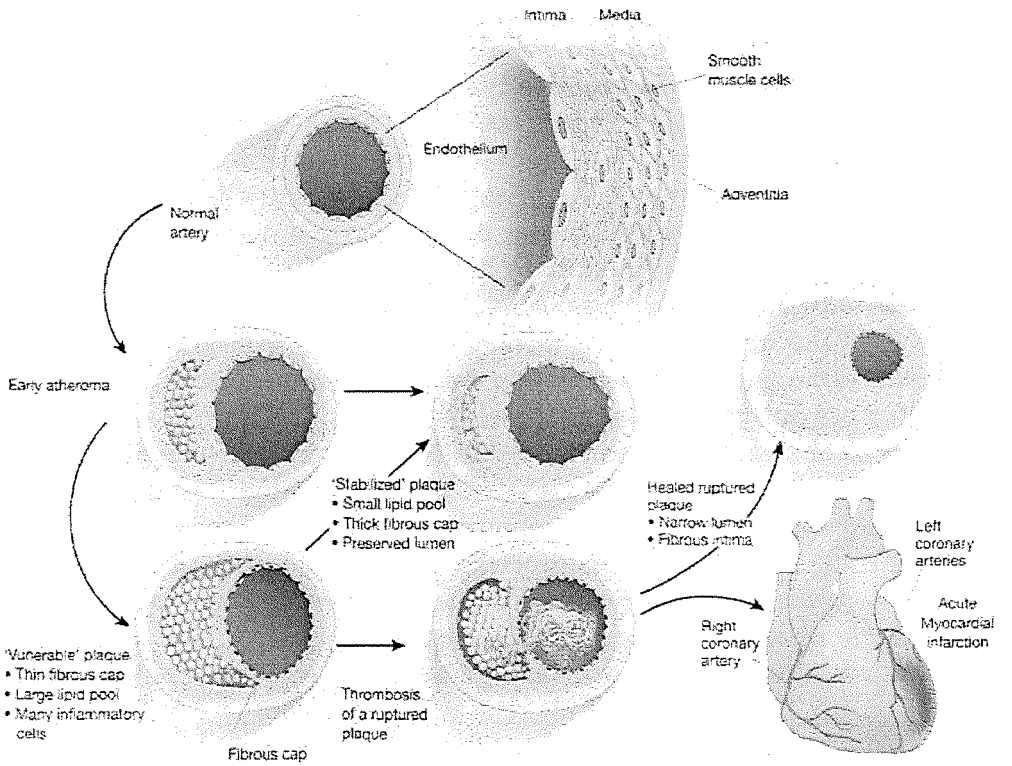

Figure 1. A schematic representation of atherosclerosis. Reprinted from Nature Medicine (8) by kind permission from Prof. P. Libby and Nature Publishing Group. The normal human coronary arterial wall consists of three layers, the intima, media and adventitia. The intima is situated at the luminal side of the vessel wall, and comprises a single layer of endothelial cells that separate the vessel wall from the blood stream. The internal elastic lamina forms the barrier between the tunica intima and the underlying tunica media. The media consists of multiple layers of smooth muscle cells embedded in an interstitial matrix, mainly composed of elastin as well as collagen. The adventitia surrounds the media, and comprises a layer of loose connective tissue composed of collagen, elastin fibres and fibroblasts. In early atherogenesis, recruitment of inflammatory cells and the accumulation of lipids lead to an early atheroma which can grow if inflammatory conditions prevail and persist. The fibrous cap may become thinner and susceptible to rupture under the influence of proteinases and pro-inflammatory cytokines yielding a 'vulnerable plaque'. Lipid lowering can reduce lipid content and calm the intimal inflammatory response, yielding a 'stabilized plaque'. But under persistent dyslipidemic conditions, the plaque may rupture and thrombus formation is instigated by the coagulation cascade. If the thrombus occludes the vessel persistently, an acute myocardial infarction may occur. However, a wound healing response is triggered during blood coagulation which can eventually lead to the expansion of the intima in an inward direction yielding a constriction of the lumen.

the behavior of the endothelial cells in the vascular wall. Recruitment and expression of pro-inflammatory cytokines promote thrombosis, a complication of atherosclerosis. Studies in genetically modified mice showed that expression of adhesion molecules that promote monocyte and T-lymphocyte recruitment into the vascular wall can be triggered by high-cholesterol diet (12). After transmigration of the monocytes into the sub-endothelial space, they are transformed into macrophages under the influence of various growth factors and cytokines. Macrophages start to accumulate cholesterol from modified LDL and eventually become 'foam cells'. Lesion progression proceeds with the migration of smooth muscle cells from the media into the intimal fatty streaks. The proliferation and migration of arterial smooth muscle cells, stimulated by growth factors and cytokines, generates a fibrous cap lesion. A lipid core is formed as a consequence of further foam cell accumulation and macrophage death by necrosis or apoptosis. Other features of further lesion progression are: continued accumulation of macrophages and T-lymphocytes, loss of smooth muscle cells and calcification. Eventually, plaque rupture may occur at sites where the concentration of lipid laden macrophages is high and the fibrous cap is weak. After rupture, the coagulation cascade and accumulation of platelets will lead to the formation of a thrombus which can occlude the vessel.

Atherosclerosis is in fact a multi-factorial process in which may risk factors are involved, including age, gender, smoking, obesity, diabetes, lack of physical activity, hypertension and genetic predisposition. In the past decades much research was focused on lipoprotein metabolism. In 1985 Brown and Goldstein received the Nobel Price for discovering the involvement of the LDL receptor in familial hypercholesterolemia. Familial hypercholesterolemia is the first mono-genetic disorder found to cause coronary heart disease and provides strong evidence for a causal relation between elevated LDL cholesterol and atherosclerosis (13). With the discovery of the LDL receptor, effective cholesterol lowering therapies evolved slowing down the atherosclerotic process (see section 2.5). However, many aspects of the relation between atherosclerosis and lipoprotein metabolism remain to be clarified.

1.2 Lipoprotein Metabolism

As the name implies, plasma lipoproteins are complexes of lipids and proteins. Cholesterol, phospholipids and triglycerides are quantitatively the most important compound lipids present in the circulation. Cholesterol is an essential constituent of all cellular membranes and is a precursor for the synthesis of steroid hormones and bile acids. Phospholipids are the

major class of lipids in cellular membranes. Phospholipids are also metabolic intermediates in the formation or degradation of other compound lipids. As second messengers, these intermediates regulate a broad spectrum of cellular activities. Triglycerides are used as an energy source for cardiac and skeletal muscle and are stored in adipose tissue. During the process of lipolysis, *i.e.* the breakdown of fat stored in adipose tissue, free fatty acids are released into the bloodstream and circulate throughout the body bound to albumin. Free fatty acids have a high metabolic turnover rate, are constantly recycled in biological systems and are also oxidized in many tissues for energy production. Cholesterol, phospholipids and triglycerides are not soluble in water and need to be packaged into lipoproteins for transport through the body via aqueous compartments such as lymph and blood.

Lipoproteins contain a central hydrophobic core of cholesteryl esters and triglycerides and a surface layer of phospholipids, free cholesterol and apolipoproteins. Apolipoproteins are proteins associated with lipoproteins that have several functions, including stabilization of the lipoprotein structure, enzyme activation or inhibition and binding to cell membrane receptors.
Lipoproteins in plasma can be divided into five main subclasses: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), which are traditionally isolated by density ultracentrifugation. Lipoproteins can also be divided according to electrophoretic mobility. Lipoprotein classes also differ profoundly in size, lipid composition and apolipoprotein moiety (Table 1) (14).

LDL is the major cholesterol carrier in the blood. If too much LDL cholesterol circulates in the blood, it can accumulate in the walls of various arteries, including the coronary arteries feeding the heart. Together with other substances it can form plaque, a thick firm deposit that may clog these arteries, which describes the condition of atherosclerosis (see Figure 1). High levels of LDL cholesterol reflect an increased risk for atherosclerosis. Apolipoprotein (apo)B is the structural protein of all atherogenic lipoproteins (Table 1). Each of these atherogenic lipoproteins contains one apoB molecule and therefore the apoB concentration is a direct reflection of the number of atherogenic particles in the blood (15). Roughly 25% of blood cholesterol in normolipidemic subjects is carried by HDL. It is believed that HDL removes excess cholesterol from atherosclerotic plaques and thus slows down or even prevents their growth. In general, high levels of HDL protect against atherosclerosis and low HDL levels (< 0.9 mM) indicate an increased risk.

Table 1. Physical properties, lipid and apolipoprotein composition of human plasma lipoproteins, adapted from (14).

Source	Chylomicrons Intestine	VLDL Liver	IDL VLDL	LDL VLDL	HDL Liver+Intestine
Physical properties					
Diameter (nm)	75 - 1200	30 - 80	25 - 35	18 - 25	5 - 12
Density (g/ml)	< 0.96	0.96 - 1.006	1.006 - 1.019	1.019 - 1.063	1.063 - 1.210
Mobility*	origin	pre-β	slow pre-β	β	α
Composition**					
Triglycerides	88	53	28	9	6
Phospholidpids	7	18	23	22	22
Cholesteryl esters	3	14	30	38	17
Free cholesterol	1	7	8	10	5
Protein	1 - 2	6 - 10	11	21	45 - 55
Apolipoproteins	AI, AIV B48 CI, CII, CIII E	- B100 CI, CII, CIII E	- B100 CI, CII, CIII E	- B100 - -	AI, AII, AIV - CI, CII, CIII E

* According to electrophoretic mobility of plasma α- and β-globulins on agarose gel electrophoresis.
**Expressed as a percentage of total weight.

The metabolism of lipoproteins can be conceptually discerned into two major pathways: 1) the exogenous pathway, concerning the uptake and transport of dietary lipids from the intestine to peripheral tissues, and 2) the endogenous pathway, covering the metabolism of lipoproteins which are synthesized in the liver (Figure 2). Reverse cholesterol transport involves the transport of cholesterol from peripheral tissues to the liver (16, 17).

1.2.1 Exogenous Pathway

Dietary cholesteryl ester, phospholipids and triglycerides are hydrolysed in the intestinal lumen and subsequently packaged in the intestinal cells into chylomicrons, which contain the apolipoproteins apoB48, apoAI and apoAIV. The chylomicrons are secreted into the intestinal lymph and enter the circulation via the thoracic duct upon which they loose apoAI and apoAIV and acquire apoCI apoCII, apoCIII and apoE from HDL. These apolipoproteins regulate the further metabolism of the chylomicrons. Since chylomicrons are too large to enter the endothelial barrier, they need to be metabolized in the blood. The enzyme lipoprotein lipase, which is present at the surface of endothelial cells in the capillary beds of adipose tissue, heart and skeletal muscle, hydrolyzes

the triglyceride core of chylomicrons (18, 19). The resulting free fatty acids are stored as triglycerides in adipocytes or can be used as an energy source by muscle. As a consequence of triglyceride hydrolysis, the chylomicrons shrink to chylomicron remnants. During lipolyses of chylomicrons and VLDL, surface remnants, containing phospholipids and free cholesterol , are formed as well (20). These surface remnants fuse with HDL. The chylomicron remnants are removed from the circulation by the liver through apoE-mediated binding to lipoprotein receptors, *i.e.* the LDL-receptor (LDL-R) (21) and the LDL-receptor related protein (22, 23), which are briefly outlined below.
The LDL-R controls plasma LDL levels and recognizes lipoproteins containing either apoB100 (present on IDL and LDL) or apoE (present on chylomicron remnants, IDL and a specific HDL subfraction). The LDL-R is located in membrane indentations of the hepatic cell membrane, called coated pits. After binding, the receptor-LDL complex is internalized by the hepatocyte. Via receptor-mediated endocytosis, the entire LDL particle is delivered to lysosomes, wherein it is disassembled by hydrolysis of its components, releasing cholesterol for subsequent cellular metabolism. mRNA expression of the LDL-R is regulated by cellular cholesterol by a feedback mechanism. The cellular LDL-R level regulates, together with the biosynthesis of cholesterol, cellular cholesterol homeostasis (24). The LDL-R related protein has a number of protein domains and motifs that are also present in the LDL-R (22). It is a multiligand receptor which recognizes apoE (25) and therefore is able to bind and internalize chylomicron remnants.

1.2.2 Endogenous Pathway

The endogenously synthesized lipids are secreted from the liver in the form of VLDL, containing apoB100. Upon entering the circulation, VLDL acquire apoE and apoCs from HDL. In addition, apoE may be obtained from the surface of the hepatocyte in the space of Disse (26). By the action of the enzyme lipoprotein lipase, which needs apoCII as a cofactor, VLDL is hydrolyzed into IDL, which can be further hydrolyzed or taken up by the liver via the LDL-R. A second lipolytic enzyme, hepatic lipase, which may function as a triglyceride hydrolase as well as a phospholipase, contributes to the further hydrolyses of IDL into LDL. During the conversion to LDL, the majority of the apoCs and apoE dissociate from IDL to be reassociated with HDL. The end product of the cascade is LDL which protein moiety consists of only apoB100. IDL, containing apoE, are removed by hepatic uptake trough the LDL-R and LDL-R related protein as described in the previous paragraph. The apoB100 containing LDL may undergo receptor-mediated endocytosis by the LDL-R in hepatocytes and in peripheral cells.

Alternatively, LDL may become modified or oxidized in the vessel wall, perhaps due to local inflammatory reactions (27). This modified or oxidized LDL (oxLDL) binds to scavenger receptors on the surface of macrophages. These include SRA and CD36 (3, 11). The scavenger receptors then mediate endocytosis and lysosomal degradation of the modified lipoprotein (28). Unlike the LDL-R, macrophage scavenger receptor activity is not regulated by intracellular cholesterol pools. Therefore the scavenger receptors can mediate massive accumulation of cholesterol, resulting in the formation of lipid-laden foam cells. Incubation of foam cells with HDL leads to efflux of cellular cholesterol (29), which is indicative for an anti-atherogenic role of HDL.

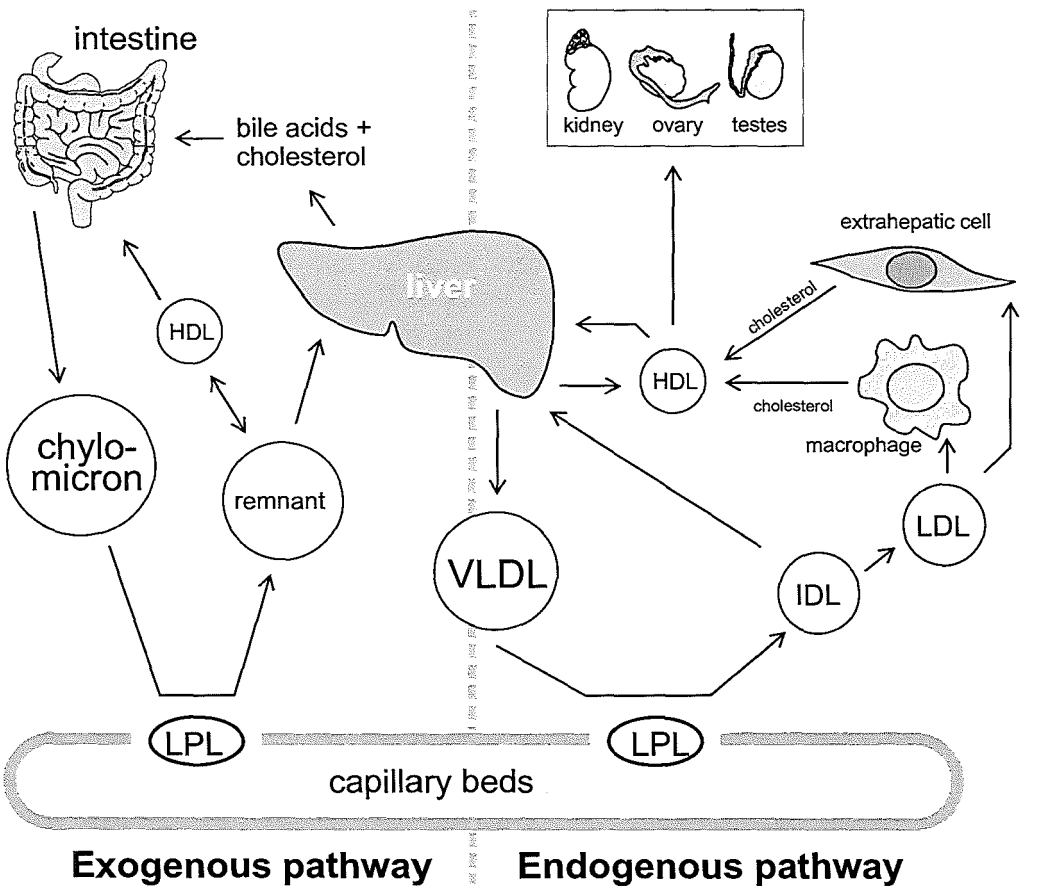

Figure 2. The exogenous and endogenous pathways of plasma lipoproteins. Adapted figure, kindly provided by Dr. R. de Crom. In the exogenous pathway the lipoproteins are transported from the intestine to the liver, and in the endogenous pathway from the liver to the peripheral tissues. See text for details.

1.2.3 Reverse Cholesterol Transport

Reverse cholesterol transport is a process in which cholesterol is moved from peripheral cells via plasma HDL to the liver for recycling or degradation and secretion in bile. Reverse cholesterol transport involves several identifiable steps (Figure 3): 1) Peripheral formation of discoidal HDL by an ATP binding cassette A1 (ABCA1)-dependent process. The nascent preβ-HDL particles efficiently remove cholesterol from cells in peripheral tissues (30). ABCA1 has been proposed to facilitate the efflux of cellular cholesterol and phospholipids to lipid poor apolipoproteins (31-33). 2) Esterification of cholesterol in the HDL discs results in spherical HDL by the enzyme lecithin:cholesterol acyl transferase (LCAT). LCAT plays a central role in reverse cholesterol transport by catalyzing the esterification of free cholesterol to cholesteryl esters on plasma lipoproteins. LCAT matures the nascent preβ-HDL into spherical α-HDL by the transfer of newly synthesized cholesteryl esters to the hydrophobic core. LCAT maintains a concentration gradient for cholesterol efflux from peripheral cells to HDL. HDL also acquires cholesterol, phospholipids and apolipoproteins from the surface remnants formed during hydrolysis of triglyceride-rich lipoproteins. 3) HDL cholesteryl esters can be transferred to apoB-containing lipoproteins by the action of cholesteryl ester transfer protein (CETP), partly in exchange for triglycerides (34). Subsequently, the LDL cholesteryl ester is taken up via the LDL-R (35). Thus, HDL can transport cholesterol indirectly to the liver following transfer to apoB-containing lipoproteins. 4) HDL can

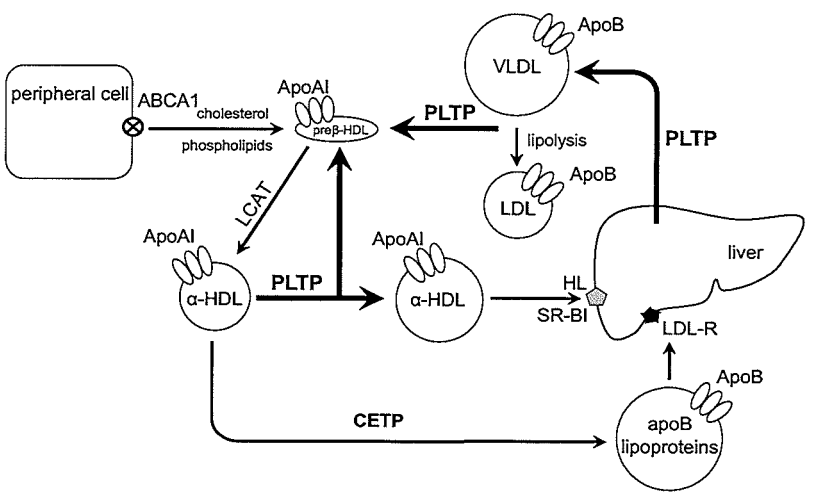

Figure 3. Reverse cholesterol transport. Adapted reprint from Hart Bulletin (2002; 33:127-129) by kind permission from Drs. R de Crom, A. van Tol and Mediselect bv. In this process the cholesterol from peripheral tissues is transported to the liver. See text for details. In this scheme the actions of PLTP are indicated with bold arrows. PLTP is able to transfer phospholipids from VLDL to HDL during lipolysis. PLTP is involved in the maturation of HDL by fusing α-HDL particles to form larger HDL followed by shedding off preβ-HDL. In addition, PLTP is also involved in hepatic VLDL secretion.

also directly deliver cholesterol to the liver via an endocytotic pathway or via selective uptake (36). In the endocytotic pathway, putative HDL receptors may be involved in HDL uptake (37). The selective uptake of cholesteryl esters from HDL is mediated by SRBI receptors (38). Once cholesterol enters the liver it can be re-used for lipoprotein assembly, used for bile acid synthesis, or secreted directly into the bile (39, 40). HDL is involved in all steps of reverse cholesterol transport, but its concentration in plasma is not per se the key determinant of the rate of reverse cholesterol transport. It is likely that processes in peripheral tissues, like the action of ABCA1, that make cholesterol available for uptake by HDL are most important (41, 42). Groen *et al.* showed in ABCA1 knockout mice that HDL levels do not always control cholesterol transport from the periphery via the liver into bile (43). Biliary cholesterol in ABCA1 knockout mice was similar to that in control mice which is surprising because one might have expected a decreased delivery of cholesterol to the liver due to the lower HDL levels in plasma. Studies in other mice with low HDL levels, *e.g.* CETP or SRBI overexpression or apoAI deficiency (44), show no change in centripetal flow of cholesterol from the periphery to the liver. These data indicate that HDL concentration is not always the rate limiting step in reverse cholesterol transport.

1.3 Mouse Models for Atherosclerosis Research

In humans, investigation of the effects of certain lipoprotein metabolism mediators on the development of atherosclerosis is complicated, because genetic variability and inter-individual differences in environmental factors and life style often affect the development of atherosclerosis. These factors may be reduced to a minimum in mice, because inbred strains are used, and similar housing and dietary conditions are applied. In addition, *in vivo* quantification of atherosclerosis in humans is difficult, whereas in mice atherosclerosis can be quantified at any time under standardized conditions (45, 46). However, wild-type mice are highly resistant to the development of atherosclerosis. In response to a low-fat, low-cholesterol (normal chow) diet, mice have low plasma levels of cholesterol and the main lipoprotein class in their plasma is the anti-atherogenic HDL. In contrast, the predominant lipoproteins in humans are the atherogenic VLDL and LDL. This discrepancy between the species may be due to several differences in lipoprotein metabolism, including the absence of CETP in mouse plasma, the higher efficiency of murine hepatic LDL receptors, leading to faster clearance of IDL and LDL, different locations of hepatic lipase, which is 100% hepatic in humans and partly in plasma in mice, and reduced synthesis of apoB100 in mice due to a higher degree of editing of the apoB mRNA in the liver (45). Atherosclerosis in the relatively

atherosclerosis susceptible mouse strain C57BL6 is only developed when they are fed a cholesterol containing diet with cholate for several months (47). However, with the development of genetically modified mice, several mouse models have been generated which are more suitable for the study of atherosclerosis, by overexpression or knocking out specific genes (45). Mice deficient in apoE (48, 49) or LDL-R (50) develop advanced lesions and are the models most commonly used in studying atherosclerosis.

2 Role of HDL in Atherosclerosis

2.1 HDL and Atherosclerosis

In many clinical studies HDL cholesterol levels are shown to correlate inversely with the risk of atherosclerosis and it is established that both high levels of LDL and low levels of HDL are important independent risk factors for atherosclerosis (51, 52). Although high levels of HDL have been shown to be beneficial, the clinical significance of targeting HDL therapeutically is unknown. One of the targets to raise HDL cholesterol is inhibition of CETP activity, which indeed raises HDL cholesterol in humans (53) and protects against atherosclerosis in cholesterol fed rabbits (54). In addition, data from a multicenter pilot trial showed that five weekly infusions of a synthetic HDL can remove significant amounts of plaque from coronary arteries in patients with acute coronary syndromes (55).
Therapies for lipid lowering focus on lowering LDL. Trials with statins,

Table 2. HDL subclasses.

Classification	Separation	Designation	
density	ultracentrifugation	HDL_2 HDL_3	1.063 - 1.125 g/ml 1.125 - 1.21 g/ml
diameter	polyacrylamide gradient gel electrophoresis	HDL_{2a} HDL_{2b} HDL_{3a} HDL_{3b} HDL_{3c}	9.7 - 12.9 nm 8.8 - 9.7 nm 8.2 - 8.8 nm 7.8 - 8.2 nm 7.2 - 7.8 nm
NMR signals converted to size	NMR spectroscopy	H1 H2 H3 H4 H5	for more information: www.liposcience.com
charge	agarose gel electroporesis	α-HDL preβ-HDL →	 $preβ_1$-HDL $preβ_2$-HDL $preβ_3$-HDL
apolipoprotein-composition	sequential immuno-affinity chromatography	LpA-I LpA-I/A-II	

which are drugs primarily lowering LDL cholesterol, have revealed that LDL reduction reduces morbidity and mortality (5, 56). However, the reduction in event rates in these trials was by about one third, meaning that despite LDL reduction, most cardiovascular events still occur. It is therefore necessary to look beyond LDL cholesterol and consider other risk factors that might explain the mortality in landmark trials. One of these risk factors is a low level of HDL. This is supported by strong evidence from the Framingham Heart Study, which revealed that low levels of HDL predict an increased incidence of cardiovascular diseases independent from LDL levels (51). Another landmark study, the VA-HIT study intervening with gemfibrozil, which increase HDL cholesterol, demonstrated that increasing HDL, even in the absence of LDL changes, is associated with a decrease in coronary events among patients with low HDL levels (57). The association of low levels of HDL cholesterol with an increased incidence of cardiovascular events implies a critical role of HDL in the protection against atherosclerosis. The interest in HDL is emerging and further clinical and fundamental research is needed since our understanding of the metabolism of HDL and its anti-atherogenicity is far from complete. In the following section HDL is introduced and the potential anti-atherogenic roles of HDL are discussed.

2.2 HDL Structure

HDL consist primarily of protein (50%), with very small amounts of triglyceride (6%) and moderate amounts of phospholipids (22%) and cholesterol (22%), accounting for approximately 25% of cholesterol in the blood in humans (58). The HDL fraction is a very heterogeneous lipoprotein class (59) which can be separated by different techniques showing differences in density, diameter, nuclear magnetic resonance (NMR) signal, electrophoretic mobility, shape and apolipoprotein content (Table 2).

Density and *diameter*: The main particle populations are HDL_2 and HDL_3, which both are spherical in shape (39). These particles can be interconverted by hepatic lipase (60), endothelial lipase (EL) (61) LCAT (62), CETP and phospholipid transfer protein (PLTP) (36). HDL_2 particles are less dense ($1.063 < d < 1.125$ g/ml) and larger (8.8-12.9 nm diameter) than HDL_3 particles (1.125-1.21 g/ml; 7.2-8.8 nm diameter).

NMR signal: NMR spectroscopy can measure levels and HDL subpopulations in plasma (63, 64). The amplitude of spectral signals emitted by HDL subclasses, depending on the different chemical composition of the subfractions, are converted to size by sophisticated calculations. The HDL subclasses H5, H4,

H3, H2, and H1 are closely related to the gradient gel electrophoresis subclass designations HDL2a, HDL2b, HDL3a, HDL3b, and HDL3c, respectively. This technique is mostly performed at LipoScience Inc. and its true place in HDL analysis remains to be determined.

Charge: A subtle classification of the HDL population is achieved by using native polyacrylamide gradient gel electrophoresis: HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b} and HDL_{3c} are separated. Agarose gel electrophoresis allows the separation of α-HDL (the majority of HDL particles), which are spherically shaped, and preβ-HDL, which are discoidally shaped (65). These subfractions are believed to be functionally important in reverse cholesterol transport. The amount of preβ-HDL in human plasma constitutes 4 to 14% of total apoAI (66). Preβ-HDL can be subdivided into three classes : $preβ_1$-HDL, $preβ_2$-HDL and $preβ_3$-HDL (30, 59, 66). $Preβ_1$-HDL particles contain only apoAI and phospholipids. $Preβ_2$-HDL have additional cholesterol and an increased phosphatidyl choline: sphingomyeline ratio compared to $preβ_1$-HDL. Due to extremely low amounts of $preβ_3$-HDL in plasma their characteristics are not well defined.

Apolipoprotein composition: HDL containing only apoAI (LpA-I), only apoAII (LpA-II) or both apoAI and AII (LpA-I/A-II) have been identified. In normal human plasma LpA-I and LpA-I/LpA-II particles are most abundant, constituting 25% and 65% respectively of circulating apoAI (67). LpA-I and LpA-I/LpA-II may have different physiological functions, but it is not exactly clear at present how the function of LpA-I differs from LpA-I/LpA-II with respect to atherosclerosis development (68-70).

2.3 HDL Metabolism

HDL metabolism is extremely complex, due to multiple pathways for synthesis and degradation of its components. It also includes the extracellular modification or interconversion of HDL which occurs in the plasma compartment and involves various enzymes and the lipid transfer proteins, CETP and PLTP.

2.3.1 HDL Synthesis

HDL is synthesized *de novo* in the liver and small intestine as protein-rich disc-shaped particles. The exact origin of HDL is not clear because there is no reliable non-transferable marker for HDL. As a consequence, several hypotheses for the first step of HDL processing have been proposed. One

of these is the release of lipid free apoAI from the liver and intestine which associates with phospholipids (71), a second possibility is the secretion of nascent HDL particles containing apoAI, phospholipids and cholesterol from the liver and intestine (72), and a third hypothesis is the release of surface components of triglyceride-rich lipoproteins during lipolysis which form nascent HDL (73). In all cases, nascent discoidal preβ-HDL is formed. Recent studies have unambiguously shown that ABCA1 is crucial for HDL-synthesis (31, 32, 74). ABCA1 is present in all tissues examined to date and facilitates the efflux of phospholipids and cholesterol from cells onto apoAI, thus forming preβ-HDL. These particles are processed into mature α-HDL through the activity of the HDL bound enzyme LCAT. LCAT requires apoAI for activation and catalyses the formation of cholesteryl esters from unesterified cholesterol and the fatty acyl chain at the sn2 position of phosphatidyl choline. The lipid transfer proteins CETP and PLTP both exchange phospholipids between lipoproteins, replenishing HDL with unsaturated phospholipids needed for an optimal LCAT reaction (36). Plasma from ABCA1 knockout mice shows profound changes in phospholipid composition, possibly due to the very low levels of PLTP activity. This altered phospholipid composition impairs the plasma activities of LCAT and as a consequence the maturation of HDL, thereby contributing to the near absence of HDL (75). During HDL maturation HDL increases in size, ultimately forming HDL_2. The phospholipids and triglycerides of HDL_2 are hydrolyzed by hepatic lipase, resulting in smaller HDL_3 particles (60, 76, 77).

2.3.2 HDL Catabolism

Although in some genetic disorders changes in HDL levels can be explained by adjustments in the rates of HDL synthesis, clearance rather than synthesis appears to be the major mechanism managing the plasma levels of HDL (78). Because all components of HDL are exchangeable through protein mediated or spontaneous transfer mechanisms, the proteins and lipids of HDL can be catabolized independently of one another and of the HDL particle. Thus, plasma HDL is catabolized through different pathways.
In humans, the half-time for the removal of apoAI from plasma is 3-5 days (79). The kidneys and the liver are the primary sites for catabolism of apoAI, the major component of HDL (80, 81). Other HDL proteins degraded in kidney and liver are apoAIV (82) and apoE (81). ApoAI dissociates from cholesterol depleted HDL and is probably internalized and degraded by the cubilin-megalin pathway. Cubilin functions as a holoparticle HDL endocytosis receptor, and megalin functions as a co-receptor because cubilin has no transmembrane domain (83).

HDL cholesteryl esters can be transferred into triglyceride-rich lipoproteins by CETP (34). These lipoproteins are subsequently taken up by the liver through LDL-R-mediated endocytosis as described in section 1.2.1. In addition to the CETP-mediated pathway for HDL cholesteryl ester catabolism, which is present in some mammalian species, *e.g.* man, rabbit and hamster, but not in others, *e.g.* pig, rat and mice, HDL particles are also a direct source of cholesteryl esters by selective HDL uptake via SRBI (84, 85). This involves cholesteryl ester uptake from HDL after binding to the cell surface followed by re-entry into the circulation. This process occurs predominantly in the liver and steroidogenic tissues (86, 87). A series of genetically modified mouse models have definitively shown the relevance of SRBI in controlling plasma HDL levels (88, 89). These mouse studies established that HDL levels are inversely correlated with the level of SRBI expression. Macrophage studies showed that the expression of SRBI results in a reduction in ABCA1-mediated efflux of cholesterol to HDL (90). It was proposed that SRBI expression produces a re-uptake pathway in which cholesterol, released from the macrophage by ABCA1, is incorporated back into the cell by SRBI-mediated influx. However, it is possible that in hepatocytes SRBI and ABCA1 could be expressed on different sides of the cell, and together might promote movement of HDL-derived cholesterol across the cell (90).
Direct catabolism of HDL particles might also be exerted by other putative HDL receptors (37, 91). It is not clear to what extent these HDL receptors play a role in HDL metabolism *in vivo*. Alternatively, HDL might be catabolized after binding to proteoglycans which participate in hepatic lipase and apoE-mediated binding and uptake of HDL (92).

2.4 Anti-Atherogenic Role of HDL

Cholesterol cannot be degraded in peripheral tissue, including the artery wall. Reverse cholesterol transport provides a pathway for the transport of cholesterol to the liver, where it can be degraded (39, 40). A large number of *in vivo* studies support the hypothesis that HDL is anti-atherogenic due to its key function of HDL in reverse cholesterol transport. However, there may be other mechanisms by which HDL also protect against atherosclerosis, including protecting LDL from oxidation, reducing the inflammatory response of endothelial cells, promoting the availability of nitric oxide, and inhibiting the coagulation pathway. Thus HDL might protect against atherosclerosis through a variety of beneficial effects on other plasma lipoproteins, coagulation and vasculature.

HDL and its role in reverse cholesterol transport

Below, factors involved in this process will be presented in the order of the distinctive steps.

ApoAI is the major apolipoprotein of HDL. Mice that have been genetically modified to overexpress the human apoAI gene show increased HDL cholesterol. Elevation of HDL in these animals is accompanied by a protection against atherosclerosis (93). Further, overexpression of human apoAI in apoE knockout mice, which are prone to atherosclerosis, significantly decreased the marked atherosclerotic lesions that would otherwise develop (94). Complete deletion of the apoAI gene in humans results in very low levels of HDL cholesterol and premature cardiovascular disease (95, 96).

ABCA1 deficiency in man causes Tangier disease, a rare disorder characterized by reduced HDL levels (31, 32, 97) . Fibroblasts from patients with Tangier disease have markedly reduced cholesterol and phospholipid efflux to apolipoproteins (98) that is corrected by expression of ABCA1 (99). ABCA1 deficiency in mice (100) causes a defect in cellular lipid efflux and the absence of lipid rich α-HDL in plasma. As a consequence, foam cells accumulate in many tissues. Mutations in ABCA1 are a cause of low HDL cholesterol levels in some families (101, 102). It is not clear whether all patients with Tangier disease are at increased risk for atherosclerosis.

LCAT deficiency in humans is not always associated with atherosclerosis despite the virtual HDL deficiency (103). LCAT knockout mice also have markedly reduced levels of HDL cholesterol (104). Transient LCAT overexpression in dyslipidemic obese mice decreases atherosclerosis (105).The relation of LCAT to reverse cholesterol transport is not very well established *in vivo*. For instance, no increase in reverse cholesterol transport could be observed as assessed by centripetal cholesterol flux in mice overexpressing LCAT (44), and studies of reverse cholesterol transport in LCAT deficiency have not been performed.

SRBI deficiency in humans has not been reported. SRBI deficiency in mice causes increased HDL cholesterol levels (89). SRBI overexpression (106) and gene transfer (107) of SRBI in LDL-R knockout mice fed a high-fat, high-cholesterol diet resulted in decreased atherosclerosis. In both cases, HDL cholesterol levels were significantly decreased. However, disruption of the SRBI gene in apoE knockout mice accelerated the onset of atherosclerosis despite higher HDL cholesterol levels (108).

Hepatic lipase, lipoprotein lipase, endothelial lipase, CETP and PLTP, which may also be involved in reverse cholesterol transport, will be outlined in later sections.

In summary, the exact molecular mechanism by which altered gene expression is pro- or anti-atherogenic has not been elucidated for any of the genes involved in HDL metabolism. This mechanism might include enhanced reverse cholesterol transport via HDL or facilitated uptake by the liver of

plasma cholesterol transported in apoB-containing lipoproteins. Moreover, atherosclerosis studies in humans and genetically modified mouse models indicate that the relationship between HDL levels and reverse cholesterol transport might not always be operative. Some data indicate that the flux of HDL-cholesterol, rather than the absolute HDL levels, might be more indicative of the activity of the reverse cholesterol pathway (59).

HDL acts as an anti-oxidant

Another explanation for the anti-atherogenic role of HDL is that HDL is protective against the harmful effects of oxidized LDL. *In vitro* experiments showed that HDL impedes LDL oxidation by metal ions (109). It has been demonstrated that HDL abolishes the transmigration of monocytes induced by modified LDL in co-cultures of human aortic wall cells (110). The anti-oxidant effect of HDL may be due to apoAI and the enzymes platelet activating factor acetylhydrolase, paraoxonase (111, 112) and LCAT (105, 111, 112). Plasma from mice transgenic for the human apoAI gene inhibits the oxidation of LDL *in vitro* to a greater extent than plasma from control mice (111, 112).

HDL reduces the inflammatory response of endothelial cells

HDL may have anti-atherogenic properties by inhibiting oxidized LDL-induced up-regulation of adhesion molecules and cytokines (113), and thereby inhibiting the initiation and progression of vascular plaque formation. Studies have shown that cytokine-induced expression of cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin is inhibited by HDL (113, 114). The most pronounced inhibition of the expression of these proteins was observed at physiological HDL concentrations. *In vivo,* elevation of HDL concentration reduces IL-1 induced expression of E-selectin (115).

HDL promotes the availability of nitric oxide

HDL cholesterol was shown to be an independent predictor of the flow-induced vasodilatation in patients with cardiovascular disease (116). Nitric oxide induces endothelium-dependent vasodilatation. Nitric oxide release is diminished in persons with risk factors for atherosclerosis, indicating that inhibition of vasodilatation may be contributing to the disease (117). A mechanism to explain the beneficial effect of HDL on endothelial function may be the increased expression of endothelial nitric oxide synthase, which increases nitric oxide bioavailability, which in turn contributes to the observed enhancement in endothelial-dependent vasodilatation (118, 119).

HDL shows antithrombotic properties

Plaque disruption and subsequent thrombus formation is important for the onset of cardiovascular diseases. It has been shown that HDL inhibits the coagulation pathway (120). Naqvi *et al.* demonstrated that low HDL cholesterol is an independent predictor of acute platelet-dependent thrombus formation (121). HDL also inhibits thrombin-induced binding of fibrinogen on platelets (122).

As yet, it is not clear to what extent the anti-atherogenic effect of HDL is the result of its modulating activity on the efficiency of reverse cholesterol transport, compared with the other HDL mediated protective effects. In addition, the functional properties of HDL subfractions rather than the quantity of HDL subfractions may determine HDL function and thereby cardiovascular risk (52, 59, 123).

2.5 Prevention of Atherosclerosis: Lipid Regulating Drugs and Impact on HDL

Drug treatment to lower plasma (V)LDL and total plasma cholesterol is usually considered as an option only if non-pharmacological interventions (altered diet and exercise) have failed. The following classes of lipid regulating drugs are the most widely used.

HMG-CoA reductase inhibitors or statins: atorvastatin, simvastatin, pravastatin, rosuvastatin, block the synthesis of cholesterol by the liver. Diminished concentrations of cholesterol in the liver in turn give rise to enhanced levels of LDL-R on the surface of liver cells by enhancing LDL-R synthesis. Increased LDL-R activity leads to increased hepatic uptake of LDL. The intracellular synthesis of cholesterol is inhibited and cells are therefore dependent on extracellular sources of cholesterol. Most statins increase HDL cholesterol by approximately 5-10%. In addition, statins possess an array of anti-inflammatory properties, which may limit atherosclerosis development (124).

Nonabsorbable resins that bind bile acids: cholestyramine, in the gut after oral intake binds bile acids, which therefore cannot be reabsorbed, resulting in enhanced excretion in the faeces. The drop in hepatic bile acids causes the release of the feedback inhibitory mechanism of bile acid synthesis. A greater amount of cholesterol is then converted into bile acids to maintain a steady level in the gastrointestinal tract. Additionally, the synthesis of LDL-R is stimulated to allow increased hepatic cholesterol uptake, which leads to reduced plasma cholesterol levels.

Fibrates: gemfibrozil, fenofibrate, bezafibrate, may promote VLDL lowering, via activation of lipoprotein lipases. They also induce the diversion of hepatic free fatty acids from esterification to oxidation, thereby decreasing the liver's secretion of triglyceride-rich VLDL. Fibrates increase HDL cholesterol by about 10%. No consistent negative correlation between effects on HDL levels and coronary artery disease was evident when comparing several fibrate trials (125-127). This may be a reflection of the different study designs, *e.g.* use of different fibrates and populations studied. Fibrates regulate HDL metabolism as ligands and activators of the transcription factor PPARα. This factor binds to PPARα responsive elements which are found in several genes involved in HDL metabolism. Binding of PPARα increases the transcription of apoAI and therefore increases the synthesis and turnover of HDL (128).

Nicotinic acid (derivatives, like acipimox) reduce the plasma levels of both VLDL and LDL by lowering hepatic VLDL secretion. These drugs suppress the release of free fatty acids from adipose tissue by inhibiting lipolysis, thereby decreasing VLDL and LDL and more specifically the number of small dense LDL particles. Niacin also increases the HDL half-life by reducing its degradation. Niacin increases HDL by more than 20% (129).

It is often not exactly known how HDL is increased by the lipid regulating drugs. The elevation of HDL is partly related to VLDL and LDL lowering which results in less CETP-mediated lipid transfer. In addition it is not always clear whether the contribution of the increase in HDL by these drugs has a direct effect on coronary events. Modulation of HDL metabolism to reduce the risk of atherosclerosis has just begun (130), but is an appealing subject for basic and clinical research. These HDL modulators will be introduced in the next section.

2.6 Remodeling of HDL by Plasma Factors

HDL particles are continuously remodeled during their life span by factors that affect their size, structure and function and therefore their role in atherosclerosis (131). These factors include the enzymes hepatic lipase (HL), lipoprotein lipase (LPL), endothelial lipase (EL), LCAT, paraoxonase 1 (PON), platelet activating factor acetyl hydrolase (PAF-AH) as well as the lipid transfer proteins CETP and PLTP. The HDL remodeling proteins are annotated below. The properties of both lipid transfer proteins CETP and PLTP and their relation to atherosclerosis will be outlined in more detail in the next two chapters respectively.

HL is a lipolytic enzyme that is synthesized and secreted exclusively by the liver (132, 133). In steroid hormone synthesizing tissues a lipase activity similar to HL has been found. In rat adrenals and ovaries full-length HL mRNA could not be detected (134), suggesting that the enzyme is not locally synthesized and presumably originates from the liver and is subsequently transported to the steroid hormone synthesizing organs (134). However, HL activity is virtually undetectable in human and rat plasma. Thus, the origin of HL in the steroid hormone synthesizing organs is uncertain. HL in steroidogenic tissues stimulates the selective uptake of HDL cholesteryl esters via SRBI (135). HL may also act as a ligand for the removal of apoB-containing lipoproteins (136, 137). HL functions as an acylglycerol hydrolase, hydrolyzing triglycerides in chylomicron remnants, IDL and HDL (138). In addition, HL functions as a phospholipase, converting phospholipid-rich HDL_2 into HDL_3 (60). The release of surface fragments during HL-mediated HDL remodeling may participate in the initial step of reverse cholesterol transport by generating preβ-HDL (139). It has not been unambiguously shown whether HL is anti or pro-atherogenic (140).

LPL is a key player in hydrolysis of triglycerides in triglyceride-rich lipoprotein chylomicrons and VLDL (141). After lipolysis, surface phospholipids, cholesterol and apolipoproteins dissociate and are acquired by HDL. LPL is widely expressed in a variety of tissues (142). Transgenic overexpression of LPL in mice results in increased HDL cholesterol levels (143). Conversely, the mouse without genes coding for LPL has severe hypertriglyceridemia and very low HDL cholesterol levels (144). In humans, deficiency of LPL is associated with severe hypertriglyceridemia and very low LDL and HDL levels (145).

EL is located in the vascular endothelial cells and its expression is highly regulated by cytokines and physical forces, suggesting that it may play a role in the development of atherosclerosis (146). In *in vitro* assays the enzyme is most active on lipids present in HDL, although it will release fatty acids from all classes of lipoproteins (147). Consistent with this finding, adenovirus-mediated overexpression of EL in LDL-R knockout mice reduced plasma concentrations of VLDL and LDL cholesterol by about 50% whereas HDL cholesterol decreased to almost zero in these animals (148). Inhibition of EL in mice results in increased HDL cholesterol and apoAI levels (149). EL knockout mice have increased HDL phospholipids, HDL cholesterol and HDL-associated apolipoproteins. In addition, EL knockout mice have large HDL particles and absence of EL delays HDL clearance *in vivo* (61).

LCAT is a hydrophobic plasma enzyme which catalyzes the transfer of the sn-2 acyl group from phosphatidylcholine to cholesterol, generating cholesteryl esters and lysophosphatidylcholine (16). This reaction occurs on the surface of HDL and accounts for the generation of cholesteryl esters in human plasma. LCAT converts discoidal HDL into spherical HDL. Plasma from patients with familial LCAT deficiency show abnormal lipoprotein levels with markedly reduced plasma HDL cholesterol (103). A minor part of HDL is disc shaped. LCAT deficiency syndromes are characterized by severe HDL deficiency.

PON1 is one of the three genes of the paraoxonase family. The antioxidant activity of HDL is largely due to PON1. PON2 and PON3 may also have antioxidant properties, but are less well understood than PON1. PON1 is exclusively located on HDL. PON1 was initially identified for its ability to hydrolyze organophosphate pesticides, and is believed to inactivate phospholipid hydroperoxides formed during early events of LDL oxidation. The immunoreactivity of PON1 is increasingly present in the arterial wall as atheroma advances (150). There is no way at present of knowing whether this is part of a protective response, but a recent study has shown that PON1 has the ability *ex vivo* to hydrolyse lipid peroxides within human carotid and coronary atheromatous lesions (151).

PAF-AH plays a role in the degradation of pro-inflammatory oxidized phospholipids and in the formation of lysophosphatidylcholine and oxidized fatty acids (152). Oxidized phospholipids are formed during the oxidative modification of LDL and play key roles in several aspects of atherogenesis. Consequently, by degrading these phospholipids, PAF-AH could act as a potent anti-atherogenic enzyme. PAF-AH in humans is associated primarily with LDL (153). About 15% is found in the HDL range. In mice however, this enzyme is transported almost exclusively on HDL (154). Recently, it was shown that PON1 also exhibits PAF-AH activity. It was observed that PAF-AH protein is lacking in HDL, suggesting that the HDL-associated PAF-AH activity is due to PON1 (155). HDL-associated PAF-AH activity, although much lower than that in LDL, may contribute substantially to protection of LDL from oxidation and to the HDL-mediated inhibition of cell stimulation induced by oxidized LDL.

2.6.1 Lipid Transfer Proteins

Both CETP and PLTP were shown to be important factors in the reverse cholesterol transport pathway by regulating the size and composition of HDL and are therefore implicated in the pathophysiology of atherosclerosis (36). Both lipid transfer protein activities have been measured in various vertebrate

species (156, 157). In contrast to CETP activity, PLTP activity could be detected in all species studied. This preservation of PLTP activity among the different species might indicate a fundamental role of PLTP *in vivo*. In a comparative study, PLTP activity in rats and mice was higher when compared to humans (158).

CETP and PLTP belong to a gene family that also includes lipopolysaccharide-binding protein and bactericidal/permeability-increasing protein that are involved in the binding of lipopolysaccharides (LPS) of the outer membrane of Gram-negative bacteria (159). Conservation of intron-exon organization of the corresponding genes suggests that they might be derived from a common ancestral gene. In addition, the conservation of charged amino acids in this gene family suggests that they are required for structural integrity, lipid binding, and transfer activity. On basis of the crystal structure of the related BPI, computational models of CETP (36) and PLTP were built (160). They appear as boomerang shaped molecules that exhibit two apolar lipid binding sites on their concave surface. However, in spite of structural homologies, significant differences exist in hydrophobicities of the C-terminal domains of CETP and PLTP that might account for the functional specificity, whereas the N-terminal regions are strongly homologous. This observation suggests that the C-terminal region may carry the specific properties of CETP and PLTP.

3 Cholesteryl Ester Transfer Protein (CETP)

3.1 Characteristics and Functions of CETP

CETP is a hydrophobic glycoprotein with a molecular weight that varies from 66 kDa to 74 kDa as determined by SDS PAGE analysis. This variation is due to four potential N-linked glycosylation sites. The human CETP gene exists in a single copy, comprising 16 exons and encompassing about 25 kbp of genomic DNA. CETP contains 476 amino acid residues and the gene has been localized to chromosome 16q12-16q21 near the LCAT locus (161).
CETP is mainly associated with small HDL particles (162) and facilitates the transfer of cholesteryl esters from HDL to triglyceride-rich particles (VLDL, LDL and chylomicrons), partially in exchange for triglycerides. *In vitro* experiments showed that CETP is responsible for all plasma cholesteryl ester and triglyceride transfers, and for about one third of the plasma phospholipid transfer activity (163). The remaining of the phospholipid transfer activity is mediated by PLTP. However, *in vivo* experiments showed that there is no redundancy in the function of CETP and PLTP. This was based on the transfer of ^{3}H-phosphatidylcholine from VLDL into HDL with plasma samples of mice transgenic for human CETP (huCETPtg) compared with huCETPtg mice in a PLTP knockout background (huCETPtg/PLTP KO). In plasma from huCETPtg mice there was a rapid transfer of a substantial portion of phosphatidylcholine radioactivity, in contrast to huCETPtg/PLTP KO mice in which the phosphatidylcholine transfer was absent, indicating that PLTP is responsible for all phospholipid transfer (164).
One consequence of the CETP-mediated transfer of cholesteryl esters and triglycerides between lipoproteins is a reduction in cholesterol content and the size of HDL (130). The magnitude of the net flux of cholesteryl esters and triglycerides between lipoproteins is dependent on the sizes of the VLDL, LDL and HDL pools (165).
Thus, CETP is responsible for neutral lipid exchange activity in plasma and can play a determinant role in the process of HDL remodeling.

3.2 CETP Activity in Humans

It has been demonstrated that variation in CETP mass and/or activity is closely associated with lipoprotein phenotype, and notably with HDL cholesterol levels (166). Plasma CETP levels are about 2 μg/ml in normolipidemic subjects (167). Under normal conditions, CETP-mediated transfer of HDL cholesteryl esters is directed preferentially to LDL.
Various missense, nonsense and splicing mutations causing CETP deficiency in

plasma have been reported in humans (36, 168, 169). Taq1B is the best studied restriction fragment length polymorphism, for which over 10.000 individuals have been genotyped and HDL levels determined. Many of the restriction fragment length polymorphism studies demonstrate an association between the CETP single-nucleotide polymorphism and changes in plasma CETP and HDL levels. However, the relation between these polymorphisms and susceptibility to atherosclerosis is variable (169-172), which could be caused by a multiplicity of factors such as life style and environmental influences (173, 174).
CETP activity measured by cholesteryl ester transfer from HDL to VLDL and LDL was shown to be higher in patients with familial hypercholesterolemia (175), and type 2 diabetes (176, 177). In patients with type 2 diabetes and hypertriglyceridemia, HDL cholesteryl esters are preferentially transferred by CETP to large VLDL particles that become richer in cholesterol and thus, more atherogenic (178). However, in type 1 diabetics without complications, CETP activity was not increased compared with the non-diabetic control group (176).
In morbidly obese, middle-aged, female subjects it has been shown that weight loss is associated with a pronounced decrease in CETP mass and activity (179). Exercise training effectively reduces the plasma concentration CETP in a population of normolipidemic subjects (180). The CETP change was observed both in subjects who lost weight and in those who were weight stable. In line with these observations, lower plasma CETP mass (181) and CETP activity (182) were measured in a group of marathon runners during training periods. Male runners also had significantly lower apoB-containing lipoproteins, and significantly higher concentrations of HDL and apoAI than male controls (182).

3.3 Regulation of CETP Gene Expression

In humans CETP mRNA is widely expressed, with the highest level found in the liver, spleen and adipose tissue (183). In addition, an isoform of CETP mRNA exists in all human tissues. Its proportion ranges from 20 to 50% of total CETP mRNA among the various tissues (184). Transfection studies showed that expression of the alternatively spliced mRNA leads to a protein that is poorly secreted (185), which was confirmed with studies in transgenic mice which in addition showed no alteration in their lipopoprotein profile compared with control mice (186).
Control of the expression of the CETP gene constitutes a major component in the regulation of plasma CETP mass in humans (187, 188). Dietary cholesterol is a major factor which influences CETP gene expression (189). Mice which

are transgenic for human CETP with its natural promoter showed a marked increase in CETP mRNA and protein in response to a high-fat, high-cholesterol diet, whereas the same transgene under control of the mouse metallothionein promoter did not exhibit a significant change in CETP mRNA or protein in response to an atherogenic diet (190). Thus, the sequences necessary for the increment of CETP mRNA in response to dietary cholesterol are located in the natural flanking regions. This region was further examined by using transgenic mice expressing constructs that contain different lengths of natural flanking sequence of the CETP gene (191). Deletion of the downstream sequences showed no change in the response of the transgene to dietary cholesterol, whereas deletion of upstream sequences indicate that the region between -138 to -370 bp upstream of the transcription site contains elements responsive to sterols (191). This region has a tandem repeat of a sequence with identity to the sterol regulatory element (SRE) in the HMG-CoA reductase promoter responsible for the sterol-mediated regulation of the HMG-CoA reductase gene. Gel shift assays showed that this element binds SRE binding protein (SREBP1) (192) and Red25 (193) which was identical to the transcription factor Yin Yang-1 (YY-1). By using CETP transgenic mice with point mutations in the SRE-like element, Chouinard *et al.* showed that SREBP1 activates the CETP gene *in vivo* (194).

It has been shown that plasma CETP levels and hepatic CETP mRNA are also markedly increased in response to endogenous hypercholesterolemia in mice with a deficiency in LDL-R or apoE (189, 195). This is due to an enhanced transcription of the CETP gene in the liver and some peripheral tissues. Because plasma cholesterol levels are positively correlated with plasma CETP in huCETPtg mice as well as in humans, it has been speculated that CETP gene expression is driven by a mechanism which senses high levels of cholesterol independent of apoE and LDL-R (196). Another study revealed that the positive sterol response of the human CETP gene is mediated by a nuclear hormone receptor DR-4 element (*i.e.*, with a spacing of 4 nucleotides between 2 direct repeats) (197). This element is activated by the orphan nuclear receptor liver X activated-receptor (LXR), which acts as a heterodimer with retinoid X receptor α (RXRα). LXR has been shown to mediate the upregulation of the cholesterol 7α-hydroxylase (Cyp7a) promoter by dietary cholesterol. Cyp7a is the first rate-limiting enzyme in the pathway converting cholesterol into bile (198). Taken together, LXR may coordinate the regulation of HDL cholesteryl ester catabolism and bile acid synthesis in the liver. It has been demonstrated that nuclear receptor LRH-1 also transactivates the CETP promoter by binding to a proximal promoter element distinct from the DR-4 site (199).

In normolipidemic subjects, the percentual decrease in CETP activity induced by a monounsaturated fatty acid diet was higher than that induced by a polyunsaturated fatty acid diet (200). In HepG2 cells it has been demonstrated

that the degree of unsaturation of the acyl carbon chain in fatty acids regulate the gene expression of CETP (201).
It has also been shown that CETP expression is decreased following LPS administration in huCETPtg mice (202). Furthermore, transfection studies in HepG2 cells have shown that several regulatory elements in the CETP promoter are involved. These include: the CCAAT/enhancer binding protein (203), which may preserve the CETP gene promoter activity; the orphan nuclear hormone receptor apoAI regulatory protein1 (204), which was shown to play a dual role, *i.e.* it can act both as a transcriptional repressor and a transcriptional activator, and recently it has been shown that the nuclear transcription factor Sp1/Sp3 ratio is a critical factor in regulating CETP expression (205).

3.4 Role of CETP in the Development of Atherosclerosis in Animal Models

Because mice and rats by nature are deficient in CETP, CETP transgenic mice and rats have provided models for the assessment of CETP expression on lipoprotein metabolism and atherosclerosis (Table 3).
Introduction of the human CETP gene into mice results in a dose-related reduction in HDL levels and a small increase in VLDL and LDL cholesterol levels (190, 206). Transgenic mice expressing human CETP under the control of the mouse metallothionein promoter showed a 1.4 to 1.6-fold increase in plasma CETP concentration with concomitantly increased CETP activity levels after zinc induction (190). In addition, these mice showed a reduction in cholesterol levels due to a 20-30% decrease in serum HDL cholesterol concentration and HDL particle size (190).
Transgenic mice expressing cynomolgus monkey CETP driven by the mouse metallothionein promoter develop severe atherosclerosis (207). These cynomolgus monkey CETP transgenic mice showed the same trend in lipoprotein changes compared to those of human CETP transgenic mice (208). In line with these results, an inverse correlation between CETP concentration, total cholesterol and HDL levels has been demonstrated in a series of mice overexpressing the human CETP gene to various levels driven by the human β-actin promoter (209).
Enhanced atherosclerosis was observed in human CETP transgenic mice with apoE knockout (apoE KO) and apoE KO/apoAI transgenic backgrounds fed a chow diet, and in a LDL-R knockout background fed a high-fat, high-cholesterol diet (210). CETP expression in these mouse models redistributed cholesterol from HDL to the VLDL/LDL pool. The atherogenicity of CETP overexpression was also demonstrated in transgenic Dahl salt-sensitive hypertensive rats expressing human CETP, which show spontaneous atherosclerosis, combined hyperlipidemia and decreased survival (211).

Rabbits have a high level of plasma CETP. Studies in these animals also demonstrated that CETP has pro-atherogenic properties: 1) CETP anti-sense oligodeoxynucleotide injections in rabbits resulted in reduced aortic cholesterol content (212). 2) Immunizing of these rabbits against their own CETP resulted in reduced activity of CETP, increased HDL cholesterol levels, decreased LDL cholesterol levels and protection against diet induced atherosclerosis (213). 3) Finally, a CETP inhibitor JTT-705 has been proven to increase HDL cholesterol, decrease non-HDL cholesterol and inhibit the progression of atherosclerosis in rabbits (54). Interestingly, a 4-week treatment with this CETP inhibitor, at a dose of 900 mg/day, in healthy volunteers led to a decrease in CETP activity (-37%), a large increase in HDL cholesterol (34%) and a decrease in LDL cholesterol (-7%) (53).

In summary, all these results show that CETP may be atherogenic, possibly by reducing the concentration of HDL and redistributing cholesterylesters from HDL to apoB-containing lipoproteins.

The expression of CETP has also been reported to be anti-atherogenic rather than pro-atherogenic. For instance, mice engineered to overexpress human LCAT have an increase in plasma HDL levels, but paradoxically also an increased susceptibility to atherosclerosis (214). When simian CETP is expressed in these animals, a reduction of atherosclerosis is observed (215). HDL which accumulate in the LCAT transgenic mice are enriched in cholesteryl esters, and in the absence of CETP, may become dysfunctional in terms of their ability to promote cell cholesterol efflux. If so, CETP expression leads to the transfer of excess cholesteryl esters out of HDL, thereby restoring their efficiency as acceptors of cholesterol and decreasing atherosclerosis development. Another example of an anti-atherogenic effect of CETP is demonstrated in mice transgenic for human apoCIII and in mice transgenic for both human apoCIII and human apoAI, which have high levels of triglyceride rich lipoprotein remnants and develop atherosclerosis. Introduction and expression of the human CETP gene into these mice reduces the extent of atherosclerosis (216). An increase in reverse cholesterol transport may provide an anti-atherogenic balance to the remnant particles and hence reduce atherosclerosis.

The results from these animal studies reflect the complexity of CETP function in lipoprotein metabolism. It is still uncertain whether high HDL levels induced either by genetic deficiency of CETP or by therapeutic inhibition of CETP would be beneficial or not (165). Controlled clinical trials on CETP inhibition are necessary to answer this question.

Table 3. CETP transgenic animals.

promoter	CETP construct	background	phenotype	atherogenicity*	reference
mouse metallothionein	human minigene	WT	HDL↓	–	190
mouse metallothionein	cynomolgus monkey cDNA	WT	VLDL↑, HDL↓	increased	207
mouse metallothionein	cynomolgus monkey cDNA	WT	apoB↑, apoAI↓	–	208
human β-actin	human cDNA	WT	HDL↓	–	209
natural flanking regions	human minigene	apoE KO	HDL↓	increased	210
natural flanking regions	human minigene	apoE KO/hu-apoAI	HDL↓	increased	210
natural flanking regions	human minigene	LDL-R KO	HDL↓	increased	210
apoCIII	human cDNA	Dahl S rat**	HDL↓	increased	211
mouse metallothionein	cynomolgus monkey cDNA	hu-LCAT	HDL↓	decreased	215
mouse metallothionein	human minigene	hu-apoCIII	HDL↓	decreased	216
mouse metallothionein	human minigene	hu-apoCIII/hu-apoAI	HDL↓	decreased	216

WT, wild-type; hu-, human transgene; *determined by quantifying aortic lesion areas; **transgenic rat

4 Phospholipid Transfer Protein (PLTP)

4.1 Characteristics of PLTP

PLTP shares structural and functional characteristics with CETP (36). The human PLTP gene is located in chromosome 20q12-q13, spans approximately 13.3 kb and comprises 16 exons (217). The second exon encodes for the entire signal sequence as well as 16 amino acids of the mature protein. The PLTP gene overlaps with the gene for human protective protein (218) but the two genes are not cooperatively regulated because they are located on opposite DNA-strands (219). The murine PLTP gene is located on chromosome 2 (220) and shows perfect exon/intron conjunction conservation with the human gene (221).
The reported human PLTP cDNA is 1750 bp in length with an open reading frame of 1518 nucleotides, which encodes a signal sequence of 17 amino acids and a mature protein of 476 residues (159). A large part of these residues are hydrophobic giving PLTP its special character. PLTP has a predicted molecular weight of 55 kDa, but due to glycosylation the mature PLTP circulating in blood has a mass of approximately 81 kDa (222). Human PLTP contains four cysteine residues and six potential N-glycosylation sites. Site-directed mutagenesis and cell culture studies revealed that the cysteine residues 146 and 185 form a disulphide bridge in PLTP which is necessary for the correct folding and secretion of the protein (160).
The amino acid sequences of PLTP from different species show a high degree of homology, *e.g.* mouse PLTP is 83% and pig PLTP is 93% identical to the human sequence (223-225), suggesting that PLTP plays a crucial role *in vivo*.
PLTP activity is associated with the HDL fraction in human plasma (162). Human PLTP expressed in transgenic mice is also associated with HDL particles (226, 227). It has been demonstrated that PLTP is associated with apoAI and apoAII (228).

4.2 Functions of PLTP

Although phospholipids are transferred spontaneously between lipoproteins, the process is too slow to be of physiological importance (229). Several studies have shown that PLTP facilitates the transfer and exchange of phospholipids but not neutral lipids (230-232). PLTP is unable to discriminate between positional isomers (233, 234). The electrostatic charge of lipoproteins influences the PLTP-mediated phospholipid transfer process (235).
During LPL-mediated hydrolysis of triglycerides from the cores of chylomicrons and VLDL particles, surface remnants containing phospholipids

are released. PLTP circulates bound to HDL and mediates the net transfer of these phospholipids into HDL (236, 237). The physiological importance of PLTP *in vivo* was demonstrated in PLTP knockout mice, which show a total absence of transfer of phospholipids in plasma. Using this mouse model, it was demonstrated that PLTP facilitates the transfer of phospholipids from VLDL to HDL upon triglyceride-rich lipoprotein lipolysis (238). In these mice compositional analysis indicated that the HDL was enriched in protein, and specifically depleted in phosphatidylcholine. These changes presumably reflect the defect in the transfer of phosphatidylcholine from triglyceride-rich lipoproteins into HDL in plasma from PLTP knockout mice compared to plasma from wild-type mice (239).
Although CETP is also able to transfer phospholipids, only PLTP was shown to promote the transfer of phospholipids from phosphatidylcholine liposomes to HDL (162, 230, 237). It is concluded that only PLTP is responsible for the net mass transfer of phospholipids from triglyceride-rich lipoproteins towards HDL. This was confirmed with *in vivo* studies using the PLTP knockout model, in which CETP expression could not compensate for PLTP deficiency. Assays determining radiolabeled phosphatidylcholine transfer from vesicles to HDL *in vitro* and from VLDL to HDL *in vivo,* showed that phospholipids transfer activity was absent in either PLTP knockout (PLTP KO) or in PLTP KO/huCETPtg mice (164).
Besides transferring phospholipids, PLTP is also able to transfer other lipophilic substances of which cholesterol, α-tocopherol and LPS are of most interest with respect to lipoprotein metabolism and atherosclerosis.
α-tocopherol is the most potent anti-oxidant form of vitamin E and may prevent the oxidative damage of atherogenic lipoproteins. In human serum PLTP catalyzed exchange of α-tocopherol between different lipoproteins (240). Desrumeaux *et al.* demonstrated that PLTP accelerates the spontaneous transfer of α-tocopherol between lipoproteins and endothelial cells (235). Studies with rabbit aortic segments showed that PLTP, in combination with α-tocopherol-albumin complexes, is able to counteract the harmful effect of oxidized LDL. It was suggested that the PLTP-mediated α-tocopherol transfer may be anti-atherogenic by providing LDL with antioxidants and by preserving normal endothelial cells. In contrast, PTLP knockout mice were protected against atherosclerosis, presumably due to accumulation of vitamin E in VLDL and/or LDL (241). It has been shown that adenoviral overexpression of murine PLTP in apoE knockout mice increases the oxidizability of LDL and increases atherosclerosis. A PLTP induced decrease in vitamin E content of VLDL and LDL could contribute to atherogenesis (242).
PLTP also transfers LPS which could have consequences *in vivo*. LPS is a membrane lipid of Gram-negative bacteria able to evoke inflammatory responses. Incubation of LPS with purified recombinant PLTP resulted in the

inhibition of the ability of LPS to stimulate adhesive responses of neutrophils, and addition of recombinant PLTP to blood impaired cytokine production in response to LPS (243).
Next to the transfer of lipophilic components, PLTP is able to promote the conversion of HDL into larger and smaller particles, thereby generating preβ-HDL. HDL conversion has been studied *in vitro* and *in vivo*. PLTP purified from plasma converts isolated HDL_3 into both larger and smaller populations (244). This activity is accompanied by the release of apoAI and is inhibited by polyclonal rabbit anti-PLTP antibodies (232). Similar observations have been made with PLTP from pigs or mice (224, 245). The conversion process is dependent on both time and PLTP concentration. The mechanism of HDL conversion has been addressed in several studies which all conclude that particle fusion accounts for the observed HDL conversion (233, 246, 247). Enrichment of HDL with triglycerides enhances the rate of conversion (247, 248). When human plasma is incubated in the presence of PLTP, the amount of immuno-detectable preβ-HDL can be increased. This may be of physiological importance, because preβ-HDL was proposed to be the primary acceptor of cellular cholesterol (249), and thus may play a role in cholesterol efflux from cells. PLTP has been shown to enhance the removal of cholesterol and phospholipids from cholesterol loaded cells (250). This is probably by interaction with ABCA1 (251). Oram *et al.* suggested that PLTP removes cellular lipids by the ABCA1 pathway and acts as an intermediary in the transfer of cellular lipids to lipoprotein particles (252). Phospholipid transfer activity is also positively correlated with cholesterol efflux from Fu5AH hepatoma cells (253) and macrophages (227).

4.3 PLTP Activity in Humans

PLTP activity can be measured by different methods, which may explain the variable PLTP activity levels observed in healthy subjects from several studies (176, 254, 255). The physiological roles of PLTP in lipoprotein metabolism and the development of atherosclerosis are still far from resolved (256). Until today, no PLTP deficiency has been found in humans. In the immediate vicinity of the PLTP gene six intragenic and two neutral polymorphisms were determined, but showed no associations with serum PLTP activity (257).
Several reports described studies on the relationship between PLTP mass and PLTP activity (254, 255, 258). A study by Oka *et al.* used a double monoclonal sandwich ELISA system for detection of PLTP mass and reported no correlation between PLTP mass and activity in a group of 132 Japanese subjects (258). This is caused by the presence of inactive PLTP which is approximately 70% of the total PLTP protein in human plasma (259). However, PLTP mass measurement

is dependent on the antibody used (260). Based on these results Kärkkäinen *et al.* suggest a model in which nascent PLTP enters the circulation as a high specific activity form not associated with apoAI (259, 261). During or after the transfer of lipolytic surface remnants to HDL, PLTP is transferred to apoAI containing HDL particles and thereby becomes part of the low activity complex (259, 261). No function of inactive PLTP has been described at present.
Various habits, such as consumption of coffee, alcohol or fat and smoking, may affect plasma PLTP activity. Unfiltered coffee contains the diterpenes cafestol and kahweol, which induce a more atherogenic lipoprotein profile by increasing VLDL and LDL cholesterol and lowering HDL cholesterol. These changes in lipoprotein profile coincided with increased plasma PLTP activity (262). In addition, it has been shown that a long-term consumption of unfiltered coffee causes an increase in PLTP activity in a group of healthy normolipidaemic subjects (263). In male alcohol abusers plasma PLTP activity was increased by 33% compared to controls (264). This result is in agreement with a French study, in which alcoholic patients on a cessation program show a decrease in HDL cholesterol levels and a reduction in plasma PLTP activity (265). However, moderate alcohol intake does not affect plasma PLTP activity despite increased HDL levels (266). PLTP activity levels have also been studied in healthy human subjects upon the intake of experimental diet containing stearic or *trans* fatty acids (267). These were compared with PLTP activity levels measured in subjects on a dairy fat-based baseline diet. Minor changes in PLTP activity between the *trans* fatty acid group and the stearic acid group were observed, which did not correlate with the decrease in HDL cholesterol observed in both groups. Another diet study in normolipidemic subjects showed that PLTP activity was higher in subjects fed a lauric acid diet than in those fed a palmitic acid diet. In addition, there was no difference in PLTP activity when comparing the saturated diets with an oleic acid diet (268). Cigarette smoking is associated with insulin resistance an atherogenic lipoprotein profile. In normolipidemic cigarette smoking men PLTP activity was 8% higher compared with non-smokers (269). Another study showed no differences in fasting PLTP activity levels in smokers and controls, but postprandially, PLTP activity was decreased in smokers (270).
PLTP activity has also been measured in different human pathological settings, which are closely related to dyslipidemia and atherosclerosis, such as obesity and diabetes. PLTP activity was shown to correlate positively with age (257, 271), body mass index, plasma cholesterol and triglyceride levels (257). In familial combined hyperlipidemia, affected family members frequently have reduced levels of HDL cholesterol in addition to elevated levels of total cholesterol and triglycerides. PLTP activity measured in affected family members is only slightly higher than in unaffected family members (272). PLTP activity is elevated in obese individuals (273-276) while it decreases

with weight loss (277). Increased PLTP activity has been associated with diabetes mellitus. In plasma from patients with type 1 diabetes PLTP activity is substantially increased and related with altered HDL subclass distribution. In addition, PLTP activity was positively associated with apoB-containing lipoproteins in these patients (176, 278). Plasma PLTP activity is also increased in patients with type 2 diabetes (177). Administration of insulin or Acipimox, a nicotinic acid drug, both decreased plasma PLTP activity in type 2 diabetics (279). Furthermore, increased plasma PLTP activity could also be observed in normolipidemic patients with peripheral vascular disease (280).
Recently, a relationship between PLTP activity and coronary artery disease was established in an angiographic case control study with 1102 cases and 444 controls. Using multivariate logistic regression analysis, PLTP activity was found to be related to coronary artery disease after adjusting for age, plasma lipids, smoking, diabetes, hypertension, homocystein and C-reactive protein. Patients within the highest quintile of PLTP activity revealed a 1.9-fold increase in risk for coronary artery disease compared with patients within the lowest quintile (281). The findings suggest that PLTP is a novel risk factor for atherosclerosis and may be considered as a therapeutic target. However, better standardized and validated methods for measuring PLTP activity and PLTP mass are necessary to unravel the role of PLTP in atherosclerosis.

4.4 Regulation of PLTP Gene Expression

The mRNA of PLTP is widely expressed in a variety of tissues. Differences in the mRNA levels in various tissues suggest that PLTP may also have local tissue specific functions. For instance, it has been speculated that the high PLTP expression in the lungs plays a role in regulating the alveolar surfactant metabolism (282), but the lungs in PLTP knockout mice showed no abnormalities. An increase in PLTP expression in the brain of patients with Alzheimer disease possibly indicate that PLTP has a specific function in brain lipid and lipoprotein metabolism (283). It has been demonstrated that PLTP is highly expressed by macrophages within human atherosclerotic lesions (284, 285), suggesting a potential role in lipid-loaded macrophages. Taking into account the mass of tissues, the liver and adipose tissue probably are the major sites for plasma PLTP production in humans (36).
PLTP expression is regulated at the transcriptional level. Characterization of the promoter region of the PLTP gene showed that it consists of a TATA box, a high GC region, and several consensus sequences for the potential binding of transcription factors (286). Both promoters of the human and mouse PLTP genes show five consensus sequences for the binding sites for transcription factors Sp1 and AP2 that have been shown to be necessary for PLTP transcription,

residing between -245 and -69 in the murine PLTP gene (287). The fact that these binding sites are highly conserved and essential for transcription for both human and murine PLTP genes, indicate that the transcription of the PLTP gene in mouse and human is regulated by similar mechanisms.
Analysis of PLTP promoter activity by using cultured cells and transfection experiments revealed that there is no strong homology to known steroid response elements (221), but contains reponse elements for non-steroidal receptors such as farnesoid X- activated receptors (FXR), peroxisome proliferator activated receptor α (PPARα) and liver X-activated receptors (LXR). These nuclear receptors bind DNA as heterodimers with the obligate partner 9-cis retinoic acid receptor α (RXRα), which subsequently bind to the hormone response elements of the PLTP in order to initiate the transcription of PLTP. Urizar *et al.* reported that the PLTP promoter is responsive to FXR. They used CV-1 cells, showing that the human PLTP promoter is sensitive through an FXR-dependent mechanism by administrating chenodeoxycholic acid, a bile acid which is a ligand for FXR. When C57BL6 mice were fed a chow diet supplemented with bile acid, the increase in hepatic PLTP mRNA levels increased 1.6-fold (288). This effect was lost in FXR-knockout animals (289). The sensitivity for the transcription of PLTP by an FXR-dependent mechanism was also observed by using HepG2 cells (287). DNA sequence analysis suggests that DNA sequences in the mouse from -407 to -395 and from -393 to -381 are homologous to the recognition motifs of FXR. In addition, Tu *et al.* found that DNA sequences in the mouse from -859 to -847 and from -309 to -279 are similar to the potential recognition motifs for PPARα. The lipid lowering drug fenofibrate is a ligand for PPARα. Mice transgenic for human apoAI fed fenofibrate showed increased PLTP activity (up to 3-fold) and increased hepatic mRNA expression in a dose dependent manner. Concomitantly, HDL size was increased and the presence of human apoAI augments this phenomenon. This increase in HDL size is abolished in PLTP knockout mice. The induction of PLTP mRNA and activity was reduced in PPARα -knockout animals (290). Thus after fenofibrate feeding, upregulated PLTP activity through PPARα may contribute to HDL enlargement.
Mak *et al.* demonstrated that PLTP mRNA was induced 6-fold as quantified by Affymetrix GeneChip® analysis or Northern blot assays when either murine or human macrophages were incubated in the presence of ligands for LXR and RXR (291). Although both the human and murine PLTP promoter region do not contain a typical LXR-responsive element (DR4), the administration of an LXR agonist in mice up-regulated PLTP mRNA and activity in a dose dependent fashion (292). Mouse macrophages treated with the synthetic LXR ligands T0901317 or GW3965, or treated with oxysterols, which are thought to be endogenous ligands for LXR, also induced PLTP mRNA (285, 292), which implies that PLTP can be regulated by LXR agonists.

4.5 Mouse Models for PLTP Research

Although *in vitro* studies suggest that PLTP influences HDL size, composition and transfers phospholipids between the different lipoproteins classes, its physiological role *in vivo* is poorly understood. To study its function, several animal models have been generated. These models include knockout and transgenic models (Table 4).

4.5.1 PLTP Knockout Mice

To evaluate the *in vivo* role of PLTP, a mouse model without PLTP expression (PLTP KO) was generated by gene targeting (239). In plasma from these mice the transfer of all major plasma phospholipids classes from VLDL to HDL was completely blocked. On a chow diet, plasma cholesterol was markedly decreased (-65%), as well as HDL phospholipids (-60%) and apoAI (-85%), but there was no change in VLDL/LDL levels compared with control mice. On a high- fat, high-cholesterol diet, the HDL levels were similarly decreased, in contrast to VLDL/LDL phospholipids which were significantly increased (+210%), as well as free cholesterol (+60%) without change in apoB levels, suggesting an accumulation of surface components from VLDL/LDL (239). Turnover studies in these knockout mice, using autologous HDL, revealed a 4-fold increase in catabolism of HDL protein and cholesteryl esters compared with control mice, whereas there were minor differences in synthesis rates of apoAI (238). Thus, the impairment of phospholipid transfer from apoB-containing lipoproteins into HDL could lead to hypoalphalipoproteinemia characterized by hypercatabolism of HDL protein. These data suggest that PLTP may be crucial for HDL maturation.
The involvement of PLTP in atherosclerosis and VLDL synthesis was demonstrated in PLTP knockout mice crossbred into various hyperlipidemic strains with increased susceptibility to atherosclerosis (293). In mice with apoB transgenic and apoE knockout backgrounds, PLTP deficiency resulted in reduced production of apoB-containing lipoproteins and a markedly decrease in atherosclerosis. The decrease of VLDL synthesis provided an explanation for the decrease found in atherosclerosis in these PLTP deficient mice with apoB transgenic or apoE knockout backgrounds compared with control mice. *In vitro* experiments with hepatocytes from PLTP knockout mice also revealed the defect in VLDL secretion, which was corrected when PLTP was reintroduced by adenovirus. Another explanation for the decreased susceptibility to atherosclerosis of PLTP KO mice may rely on the increased in α-tocopherol (vitamin E) content in VLDL and LDL compared to wild-type mice. Vitamin E is one of the substrates of PLTP and is believed to protect VLDL/LDL against oxidation which can trigger atherosclerosis (241).

4.5.2 Mice Overexpressing PLTP

Albers *et al.* generated PLTP transgenic mice which showed no significant difference in PLTP activity compared with non-transgenic mice (226). Although small differences in HDL cholesterol were found, the physiological relevance of these differences is uncertain. Jiang *et al.* generated human PLTP transgenic mice which showed an increase of 29% in PLTP activity, but analysis of plasma lipoproteins revealed no marked changes in their lipid or apolipoprotein content compared with non-transgenic mice (219). However, when these transgenic mice were crossbred with mice transgenic for human apoAI, PLTP activity was increased by about 47% (219). Concomitantly there was an increase in HDL phospholipids (+26%), apoAI levels (+22%) and preβ-HDL formation (+56%). These large HDL are not functioning in prevention of cellular cholesterol accumulation, probably due to their limited capacity to stimulate cholesterol efflux. Van Haperen *et al.* generated transgenic mice expressing higher levels of human PLTP showing a 2.5 to 4.5 fold increase compared with wild-type mice, resulting in a 30 to 40% decrease in plasma HDL cholesterol and apoAI (227). In addition, it has also been determined that plasma from huPLTPtg mice was more efficient in preventing accumulation of intracellular cholesterol in macrophages (227). Despite the lower total plasma HDL concentration, plasma from huPLTPtg mice had increased potential for preβ-HDL formation. Jiang *et al.* also found increased preβ-HDL formation (219). These data suggested an anti-atherogenic role for PLTP, as preβ-HDL is considered a very efficient acceptor during efflux of cellular cholesterol (30).
Besides PLTP, the other lipid transfer protein, CETP, has also been implicated in the formation of preβ-HDL (294, 295) as outlined in this thesis. We compared the ability of PLTP and CETP to generate preβ-HDL (296). PLTP rather than CETP is responsible for the generation of preβ-HDL. In plasma from PLTP overexpressing mice the formation of preβ-HDL was 3-fold higher compared with plasma from control and CETP transgenic mice, indicating that PLTP may play a role in the initial stage of reverse cholesterol transport.
The same trend was found in earlier studies employing adenoviral overexpression of human PLTP (297, 298). Föger *et al.* showed that low and high dose adenoviral expression of PLTP resulted in an increase in PLTP activity (13 and 40-fold) leading to decreased HDL cholesterol (-54% and -91%) and apoAI (-64% and -98%) levels in mouse plasma. These mice were characterized by the increased formation of preβ-HDL levels, but decreased α-HDL due to an increased fractional catabolic rate of HDL and enhanced HDL hepatic uptake compared with control mice (298). This suggests that PLTP may have a stimulating role in reverse cholesterol transport *in vivo*. Moreover, Post *et al.* found that PLTP overexpression results in enhanced disposal of cholesterol from the body via increased bile acid excretion (299).

However, in a series of transgenic mice with increasing PLTP expression, a dose-dependent relation with a reduction in plasma HDL levels and an increased susceptibility to diet-induced atherosclerosis was found, indicating a potential pro-atherogenic role for PLTP (300, 301). In addition, elevation of PLTP in transgenic mice increases VLDL secretion as outlined in this thesis (302).The potential pro-atherogenic role for PLTP is in line with the findings from atherosclerosis studies in PLTP knockout mice, and in apoE knockout mice with adenoviral overexpression of mouse PLTP which respectively showed decreased and increased atherosclerotic lesion areas (241, 242).

Table 4. PLTP mouse models.

Promoter	PLTP construct	background	phenotype	atherogenicity*	reference
	KO	WT	HDL↓	–	239
	KO	hu-apoB	HDL↓, apoB↓	decreased	293
	KO	apoE KO	apoB↓	decreased	293
	KO	LDL-R KO	HDL↓	decreased	293
natural flanking regions	human gene	WT	HDL/non-HDL↑	–	226
natural flanking regions	human gene	WT	no change	–	219
natural flanking regions	human gene	hu-apoAI	HDL↑	–	219
natural flanking regions	human gene	WT	TC↓, apoAI↓	–	227
natural flanking regions	human gene	hu-CETP	HDL↓	–	296
natural flanking regions	human gene	WT	apoAI↓	–	300
natural flanking regions	human gene	LDL-R+/-	HDL↓	increased	300
mouse albumin	human gene	WT	apoAI↓	–	300
mouse albumin	human gene	LDL-R+/-	HDL↓	increased	300
natural flanking regions	human gene	hu-CETP/LDL-R+/-	TC↓, VLDL↓, HDL↓	increased	301
mouse albumin	human gene	hu-CETP/LDL-R+/-	TC↓, VLDL↓, HDL↓	increased	301
CMV	human cDNA**	WT	HDL↓	–	297
CMV	human cDNA**	WT	HDL↓	–	298
mouse albumin	mouse cDNA**	apoE KO	HDL↓	increased	242

KO, knockout; WT, wild-type; hu-, human transgene; *determined by quantifying aortic lesion areas; **adenovirus mediated.

5 Outline of the Thesis

PLTP is an important regulator of HDL and lipoprotein metabolism. The exact role of PLTP in HDL and lipoprotein metabolism is far from resolved. Aim of the performed studies described in this thesis was to answer the question whether PLTP is anti- or pro-atherogenic, and thus to clarify the role of PLTP in HDL metabolism and in the development of atherosclerosis by using genetically modified mouse models.

One of the mechanisms by which HDL may protect against atherosclerosis is their role in reverse cholesterol transport. An important step in this reverse cholesterol transport pathway is the uptake of cellular cholesterol by preβ-HDL. The two lipid transfer proteins present in human plasma, CETP and PLTP, have both been implicated in the formation of preβ-HDL. In **chapter 2** the relative contribution of each of these proteins is investigated.

Studies in PLTP knockout mice have shown a reduction in atherosclerosis which was explained by a reduction of hepatic VLDL secretion and the resulting decreased LDL concentration. However, until today PLTP deficiency has not been found in humans and in addition mice by nature do not have plasma CETP activity. Thus, total deficiency of both CETP and PLTP represent an extreme situation from which the effects on lipoprotein metabolism cannot be simply extrapolated to humans. Therefore we evaluate the influence of PLTP on VLDL secretion in a mouse model with appreciable CETP activity in **chapter 3**.

In **chapter 4** we study the susceptibility of PLTP to diet induced atherosclerosis in LDLR$^{+/-}$/huCETPtg mice with moderately and highly elevated plasma PLTP activity. These mouse models are more suitable to evaluate the role of PLTP in atherosclerosis than PLTP knockout mice.

It is not clear by which mechanism PLTP affects diet induced atherosclerosis. From experiments in PLTP knockout mice, it was concluded that PLTP may stimulate hepatic VLDL secretion. This was confirmed in PLTP overexpressing mice. In this model the atherogenic effect of PLTP was attributed to a decrease in HDL cholesterol levels. In **chapter 5** we study which of these two pro-atherogenic effects are related to atherosclerosis in PLTP overexpressing mice, with appreciable plasma CETP activity.

Fibrates are well known to lower the plasma concentration of triglycerides and increase HDL cholesterol levels. In **chapter 6** we study the influence of fenofibrate on PLTP in several mouse lines.

The results obtained in these studies are discussed in **chapter 7**.

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CHAPTER II

Evaluation of PLTP and CETP as Contributors to the Generation of Preβ-HDL

Evaluation of Phospholipid Transfer Protein and Cholesteryl Ester Transfer Protein as Contributors to the Generation of Preβ-High Density Lipoproteins

Jessica Lie*[1], Rini de Crom†,‡[1], Matti Jauhiainen§, Teus van Gent*, Rien van Haperen†, Leo Scheek*, Hans Jansen*, Christian Ehnholm§, Arie van Tol*

*Department of Biochemistry, Cardiovascular Research Institute COEUR, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

†MGC-Department of Cell Biology and Genetics, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

‡Department of Vascular Surgery, Academic Hospital "Dijkzigt", Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

§Department of Biochemistry, National Public Health Institute, Mannerheimintie 166, FIN-0300 Helsinki, Finland.

[1]These authors have equally contributed to this paper

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Abstract

High-density lipoproteins (HDLs) are considered anti-atherogenic because they mediate peripheral cell cholesterol transport to the liver for excretion and degradation. An important step in this reverse cholesterol transport pathway is the uptake of cellular cholesterol by a specific subclass of small, lipid-poor apoA-I particles designated preβ-HDL. The two lipid-transfer proteins present in human plasma, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), have both been implicated in the formation of preβ-HDL. In order to investigate the relative contribution of each of these proteins, we used transgenic mouse models. Comparisons were made between human CETP transgenic mice (huCETPtg), human PLTP transgenic mice (huPLTPtg) and mice transgenic for both lipid-transfer proteins (huCETPtg/huPLTPtg). These animals showed elevated plasma levels of CETP activity, PLTP activity or both activities, respectively. We evaluated the generation of preβ-HDL in mouse plasma by immunoblotting and crossed immuno-electrophoresis. Generation of preβ-HDL was equal in huCETPtg and in wild-type mice. In contrast, in huPLTPtg and huCETPtg/huPLTPtg, preβ-HDL generation was 3-fold higher than in plasma from either wild-type or huCETPtg mice. Our findings demonstrate that of the two plasma lipid transfer proteins PLTP, rather than CETP, is responsible for the generation of preβ-HDL. These data support the hypothesis of a role for PLTP in the initial stage of reverse cholesterol transport.

Introduction

Plasma levels of high-density lipoprotein (HDL) cholesterol correlate negatively with the risk of coronary artery disease, and raising HDL-cholesterol levels by drug treatment results in prevention of coronary artery disease (1-4). HDL is considered to protect against atherosclerosis via its role in reverse cholesterol transport (5). This pathway involves the uptake of cholesterol from peripheral cells and subsequent transport and delivery to the liver for degradation and excretion. Recent data indicate that the ATP-binding cassette transporter A1 (ABCA1) plays a key role in the efflux of cholesterol from cells (6-8). Patients suffering from Tangier disease, with a mutated form of the ABCA1 gene, show premature atherosclerosis, providing compelling evidence for a substantial protective role of reverse cholesterol transport in the development of atherosclerosis. The initial extracellular acceptor of cellular cholesterol is a specific subclass of HDL, designated preβ-HDL (9). Cellular cholesterol taken up by preβ-HDL is subsequently esterified by the action of lecithin: cholesterol acyltransferase (LCAT). As a consequence preβ-HDL matures

into α-HDL. Esterified cholesterol from α-HDL can be taken up in the liver either directly by selective uptake, or indirectly via low-density lipoproteins (LDLs). The indirect pathway follows transfer of cholesteryl esters to very low-density lipoproteins (VLDLs) and LDLs by cholesteryl ester transfer protein (CETP) (9, 10). Thus, preβ-HDL is a key factor in reverse cholesterol transport. Although the plasma lipid-transfer proteins phospholipid transfer protein (PLTP) and CETP have non-overlapping functions (11), studies both *in vitro* and *in vivo* have demonstrated that CETP and PLTP may both contribute to the formation of preβ-HDL (12-18).
PLTP is involved in the transfer of phospholipids between lipoproteins and in the remodeling of HDL (19-21). Its physiological function has been studied in several mouse models (12, 22-24). PLTP deficient mice show an impaired transfer of post-lipolytic VLDL surface remnant phospholipids to HDL and, as a consequence, decreased plasma HDL levels (21). In PLTP transgenic mice with slightly elevated PLTP activity levels minor effects on plasma lipids and lipoprotein patterns were found (15, 23). However, in double transgenic mice expressing both PLTP and human apolipoprotein (apo) A-I, preβ-HDL levels were increased (23). We generated transgenic human PLTP mice which show a moderate, though substantial overexpression of PLTP (2.5-3 fold in hemizygous transgenic mice) (12). Plasma from these mice has an increased ability to generate preβ-HDL and prevents cholesterol accumulation in macrophages to a greater extent than plasma from wild-type animals. From these studies, we concluded that PLTP could have anti-atherogenic properties via its ability to generate preβ-HDL. Both CETP and PLTP belong to a family of lipid-transfer/lipopolysaccharide-binding proteins (10, 25). Although CETP has profound effects on lipoprotein metabolism in humans, some species including mice, lack CETP activity (26). *In vitro* studies demonstrate that CETP transfers cholesteryl esters from HDL to apoB-containing lipoproteins in exchange for triglycerides, whereas PLTP does not transfer neutral lipids (5). In addition to PLTP, CETP is able to transfer phospholipids *in vitro*, but it is unknown whether they share this ability *in vivo*. In the present study we evaluated the relative contributions of CETP and PLTP in the formation of preβ-HDL. To this end, we made comparisons between various lines of transgenic mice expressing human CETP, human PLTP, or both lipid-transfer proteins.

Material and Methods

Breeding and treatment of transgenic mice

Human CETP transgenic mice (line 5203; huCETPtg) were kindly donated by Dr A.R. Tall (Columbia University, New York) (27) and have a C57BL/6 background. Human PLTP transgenic mice (huPLTPtg) were generated as described previously (12) and were backcrossed to C57BL/6 background for at least 7 generations. Mice expressing the human CETP and PLTP transgenes (huCETPtg/huPLTPtg) were obtained by crossbreeding huCETPtg and huPLTPtg mice. Both transgenes had the natural flanking sequences, including the autologous promoters. Animals were housed in a temperature-controlled room operating under a 12 h: 12 h light/dark cycle. Animals were fed regular chow and water *ad libitum*. Blood samples were collected after fasting overnight from the orbital plexus by using Vitrex™ sodium-heparinized micropipettes (80 IU) (Modulohm A/S, Copenhagen, Denmark) and were immediately stored on ice. Blood was centrifuged at 1500 g_{max} for 15 min at 4 °C. Plasma was either used directly or stored in small aliquots at -80°C before analysis. All animal experiments were done in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Erasmus University Rotterdam (prot.nr. 120.99.05) and with the European Committee Standards on the care and use of laboratory animals (Ministry of Welfare, Health and Cultural Affairs, The Netherlands).

DNA analysis

Genomic DNA was isolated from tail clips of 10-day-old mice and analyzed for the presence of the huCETP transgene and/or huPLTP transgene by PCR analysis; huCETP: sense primer 5'-CACTAGCCCAGAGAGAGGAGTGC-3', antisense primer 5'- CTGAGCCCAGCC GCACACTAAC-3', 28 cycles (94 °C, 1 min; 65 °C, 1 min; 72 °C, 1.5 min); huPLTP: sense primer 5'-GCCACAGCAGGAGCTGATGC-3', antisense primer 5'-GCGGATGGAC ACACCCTCAGC-3', 28 cycles (94 °C, 1 min; 65 °C, 1 min, 72 °C, 2 min).

Plasma activity assays

CETP and PLTP assays were performed according to Speijer *et al.* (28). CETP activity was determined by measuring the rate of transfer/exchange of radiolabeled cholesteryl oleate between exogeneously added human LDL and HDL. PLTP activity was determined by measuring the transfer of radiolabeled dipalmitoyl phosphatidyl choline (DPPC) from liposomes {composed of egg lecithin, Amersham, Beckinghamshire U.K. and [^{3}H]-DPPC as a tracer} to exogenously added human HDL (density, 1.063 g/ml < d < 1.21 g/ml). CETP and PLTP activities are expressed as percentage of human reference plasma: 100% is equivalent to the following activities; CETP, 215.6 nmol/ml per h; PLTP 13.9 μmol/ml per h.

Hepatic lipase activity was measured as described by Jansen *et al.* (29). LCAT activity was determined by measuring the formation of radiolabeled cholesteryl ester after addition of 10 or 20 µl of mouse plasma to excess heat-inactivated plasma containing [^{3}H]-cholesterol (Amersham, Bucking ham shire, U.K.) (30). Hepatic lipase and LCAT activities are expressed as percentage of plasma from wild-type mice (C57BL/6).

Quantitation of plasma lipids and apolipoproteins

Plasma lipids were determined enzymatically with commercially available kits: total cholesterol with the F-chol kit of Boehringer Mannheim (Mannheim, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer Mannheim). Phospholipids were measured with the PAP150 kit from BioMérieux (Lyon, France). Mouse apoA-I was quantitated by sandwich ELISA as reported (12).

Gel filtration chromatography of mouse plasma lipoproteins

Lipoprotein profiles were determined by gel filtration of freshly isolated plasma on two HR10/30 FPLC columns in tandem (Superose 6 prepgrade and Superdex 200 prepgrade; Pharmacia Biotech, Uppsala, Sweden). The columns were equilibrated with 2 mM NaH_2PO_4/Na_2HPO_4, pH 7.4 containing 0.9% NaCl (w/v), 0.02% NaN_3 (w/v) and 5 mM EDTA. Combined plasma samples from four to seven mice were passed through 0.45 µm filters from Millipore S.A. (Molsheim, France), and 0.5 ml was subjected to gel filtration. The columns were run at 4 °C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected. Recoveries were > 90% for all analyses.

Quantitation of preβ-HDL

Mouse plasma samples were either frozen directly or incubated in the presence of iodoacetate in order to measure preβ-HDL formation. Incubation conditions were as reported in (12). Plasma samples were separated by agarose-gel electrophoresis under non-reducing, non-denaturing conditions and low ionic strength according to the manufacturer's instructions (Paragon Lipo system; Beckman, Fullerton, CA, U.S.A) to separate preβ- and α-migrating HDL. Proteins were transferred by capillary blotting to nitrocellulose membranes (Schleicher & Schuell NY 13N 6214/95, Dassel, Germany). Membranes were blocked for 1 h in 5% (w/v) skimmed milk in 20 mM Tris/HCl pH 7.4/0.5 M NaCl. Membranes were subsequently incubated with an affinity-purified rabbit anti-mouse apoA-I polyclonal antibody. ^{125}I-labeled donkey anti rabbit $F(ab')_2$ fragment (17 Ci/g; Amersham) was used as the detection antibody. The relative abundance of apo-AI among the α- or preβ-HDL species was calculated by quantitative scanning using a PhosphorImager (GS-363; BioRad). Crossed immuno-electrophoresis was performed as described in (12).

Statistical analysis

Data are expressed as means ± SD. Differences between two genotypes were analysed by two sample Wilcoxon rank-sum tests. Differences between three genotypes were analysed by ANOVA followed by Bonferroni correction.

Results

Plasma activities of CETP and PLTP

CETP activies were measured in plasma from huCETPtg mice and huCETPtg/huPLTPtg mice (Figure 1A). CETP activity in wild-type mice was virtually absent (< 4 % of human reference plasma). HuCETPtg mice had a CETP activity of 144 ± 34% of human reference plasma whereas CETP activity in huCETPtg/huPLTPtg mice was about 20% lower ($P < 0.01$).

PLTP activity levels were measured in plasma samples of huCETPtg mice and huCETPtg/huPLTPtg mice (Figure 1B). The PLTP activity in plasma of huCETPtg mice was somewhat higher than human PLTP activity levels: 136 ± 16% of human reference plasma, which is in agreement with measurements in wild-type mice (12). The PLTP activity in the huCETPtg/huPLTPtg was 4-fold higher (Figure 1B). This activity level closely resembles the level reported previously for huPLTPtg mice (12).

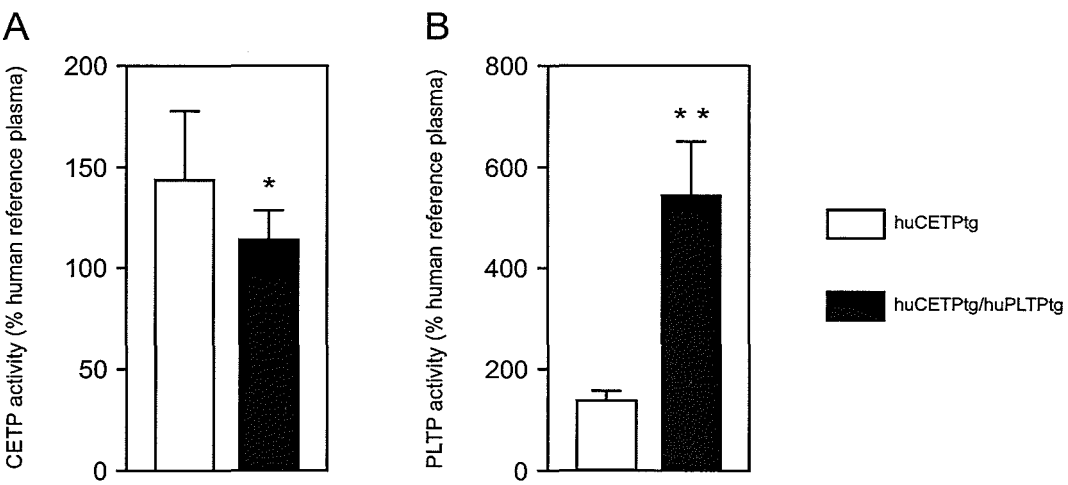

Figure 1. Plasma CETP and PLTP activities in transgenic mice. Plasma activities were determined in individual plasma samples from huCETPtg mice (n = 12) and huCETPtg/huPLTPtg mice (n = 10). Values are expressed as percentage of human reference plasma (mean ± SD). (A) CETP activity: 100% human reference plasma is equivalent to 215.6 nmol/ml/h CETP activity. *$P < 0.01$ (rank-sum test). (B) PLTP activity: 100% human reference plasma is equivalent to 13.9 μmol/ml/h PLTP activity. **$P < 0.0001$ (rank-sum test).

Plasma hepatic lipase activity and LCAT activity

Mice express hepatic lipase activity in plasma, even without intravenous injection of heparin (31). We investigated whether any differences in the activity of hepatic lipase in plasma between huCETPtg mice and huCETPtg/huPLTPtg mice could be observed. As shown in Figure 2A, the huCETPtg/huPLTPtg mice have lower levels of hepatic lipase activity than huCETPtg mice ($P<0.05$). Hepatic lipase activity in the huPLTPtg mice was 91 ± 27% of wild-type mouse plasma. In plasma, preβ-HDL matures into α-HDL by the action of LCAT. The levels of LCAT activity were not significantly different between the three tested genotypes (Figure 2B).

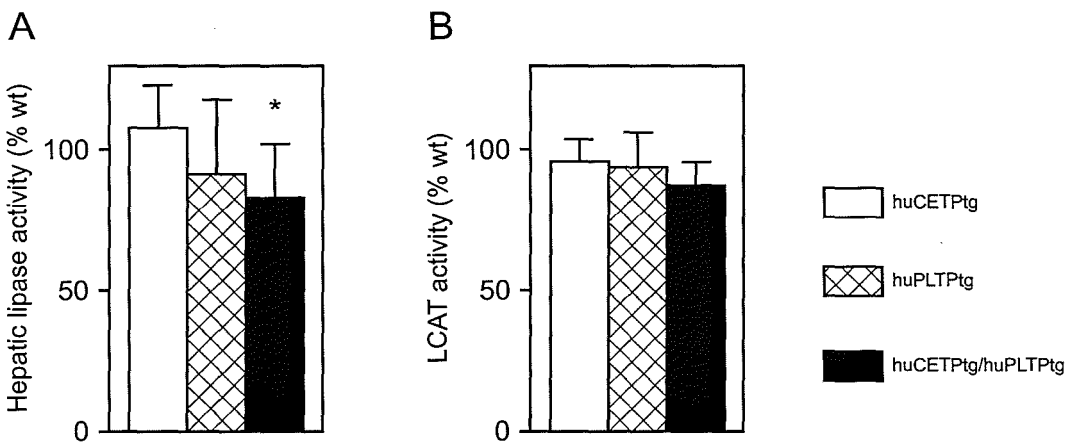

Figure 2. Plasma hepatic lipase and LCAT activities in transgenic mice. Plasma activities were measured in individual plasma samples from wild-type mice (wt; $n = 16$), huCETPtg mice ($n = 12$), huPLTPtg mice ($n = 8$) and huCETPtg/huPLTPtg mice ($n = 10$). Values are mean ± SD and are expressed as percentage of wt. (A) Hepatic lipase activity: 100% hepatic lipase activity of wt is equivalent to 56.1 nmol/ml/h. *significantly different from huCETPtg mice ($P < 0.05$, ANOVA followed by Bonferroni correction). (B) LCAT activity: 100% LCAT activity of wt is equivalent to 71.3 nmol/ml/h. Transgenic mice show similar LCAT activities (not significantly different).

Lipid and lipoprotein analysis

Plasma samples from either huCETPtg or huCETPtg/huPLTPtg animals were analysed by gel-filtration chromatography in order to examine the lipoprotein profile. Cholesterol and phospholipid contents as well as CETP and PLTP activities were determined in all fractions (Figure 3). In unfractionated plasma, huCETPtg/huPLTPtg mice had lower levels of plasma cholesterol than

huCETPtg mice (1.09 ± 0.15 mM, $n = 9$ *versus* 1.43 ± 0.16 mM, $n = 11$; $P < 0.01$), whereas triglyceride levels were similar (huCETPtg mice, 0.38 ± 0.14 mM, $n = 5$; huCETPtg/huPLTPtg mice, 0.48 ± 0.25 mM, $n = 5$, not statistically significant). As evident from Figure 3A, which shows the cholesterol profile, this is due to a decrease in HDL cholesterol. The phospholipids have a similar profile, showing a decrease in HDL phospholipids due to PLTP overexpression (Figure 3B). The phospholipids present in fractions 20-25 represent lysophosphatidylcholine bound to albumin. In both groups of mice CETP activity eluted in fractions 14-18, corresponding to the position of HDL particles (Figure 3C). PLTP activity eluted in fractions 11-15, the position of relatively large HDLs (Figure 3D), a situation also found in humans.

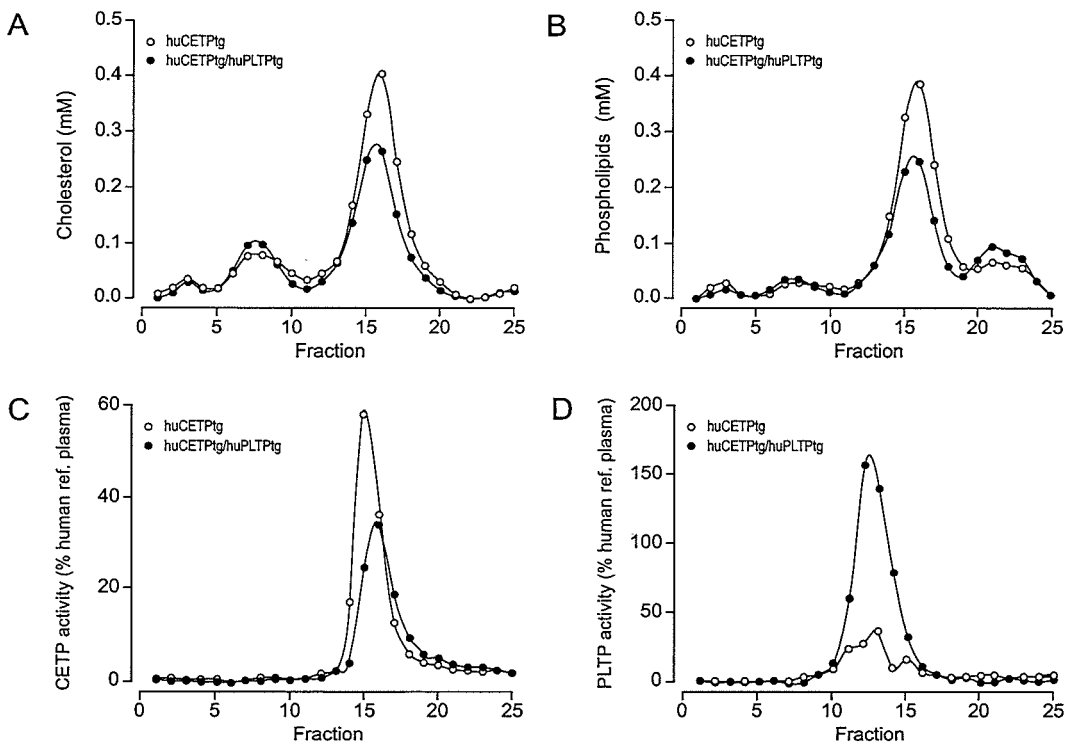

Figure 3. Lipoprotein profiles of plasma from transgenic mice. Equal amounts of plasma from either huCETPtg mice ($n = 4$) or huCETPtg/huPLTPtg mice ($n = 7$) were pooled and subjected to gel filtration on Superose 6 and Superdex 200 columns connected in tandem as described in the Materials and Methods section. Fractions were analyzed for (A) cholesterol, (B) phospholipids, (C) CETP activity and (D) PLTP activity. Fractions 1-4 contain VLDL, 5-10 contain LDL, 11-19 contain HDL and 20-25 contain lysophosphatidyl choline bound to albumin.

Effects of huPLTP and huCETP expression on preβ-HDL formation

To investigate the formation of preβ-HDL, incubated plasma samples from wild-type, huCETPtg, huPLTPtg and huCETPtg/huPLTPtg mice were subjected to agarose-gel electrophoresis followed by immunoblotting. Upon incubation, the levels of preβ-HDL are increased in mice expressing human PLTP, whereas neither wild-type nor huCETPtg mice showed appreciable levels of preβ-HDL formation (Figure 4). In order to quantify preβ-HDL formation, crossed immuno-electrophoresis experiments were performed (Figure 5). The results corroborated the findings from the immunoblotting experiments, since incubated samples from mice expressing human PLTP showed relatively higher preβ-HDL peaks. Measurements of preβ-HDL in plasma without incubation revealed no significant differences between the genotypes tested (Table 1). However, in incubated samples, significant differences were observed in both relative and absolute concentrations of preβ-HDL between huPLTPtg mice on the one hand and wild-type mice or huCETPtg mice on the other (Table 1). Using the data in Table 1, the formation of preβ-HDL during incubation of plasma was calculated (see Figure 6). Preβ-HDL formation in plasma from huCETPtg/huPLTPtg and huPLTPtg mice was at least 3-fold higher than in plasma from wild-type mice and huCETPtg mice. Thus CETP does not seem to make a substantial contribution towards the formation of preβ-HDL.

Table 1. Preβ-HDL levels in plasma of transgenic mice.

Genotype	total plasma apoA-I	Relative preβ-HDL (% of plasma apoA-I)		Absolute preβ-HDL (µg apoA-I/ml)	
		Pre-incubation	Post-incubation	Pre-incubation	Post-incubation
wild type	1.0 ± 0.07	3.7 ± 0.9	7.3 ± 0.5	36.7 ± 12.1	73.5 ± 10.2
huCETPtg	0.88 ± 0.05	5.0 ± 0.8	7.3 ± 0.5	44.1 ± 10.9	64.5 ± 8.6
huPLTPtg	0.73 ± 0.24	6.0 ± 0.8	16.7 ± 0.9*	44.2 ± 14.8	125.1 ± 49.4*
huCETPtg/huPLTPtg	0.7 ± 0.09	7.3 ± 1.7	17.3 ± 1.3*	52.3 ± 20.2	121.2 ± 22.7*

Plasma samples were incubated as described in the legend of Figure 4. Values represent the relative and absolute amounts of preβ-HDL in plasma before and after incubation at 37 °C for 3h. These were calculated as described in the legend of Figure 6, from the percentage of apoA-I present in preβ-HDL and the total plasma apoA-I concentrations respectively. Values are mean ± SD (n = 3). *Significantly different from wild type mice, $P < 0.05$ (rank-sum test).

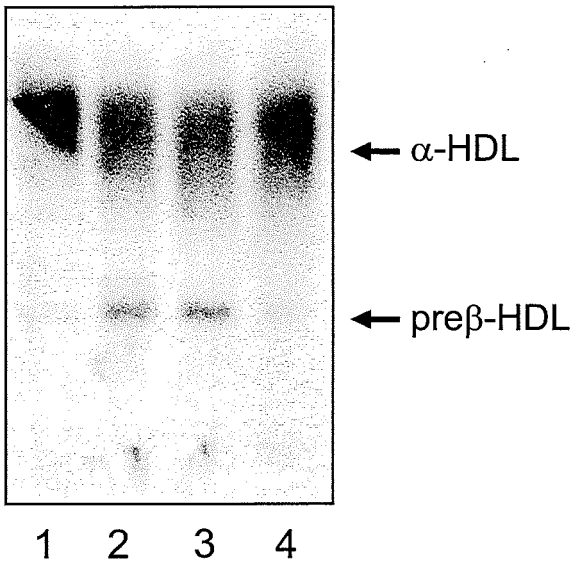

Figure 4. HDL subfractions of incubated plasma samples from transgenic mice. Analysis of HDL subfractions was performed in plasma from huCETPtg (lane 1), huCETPtg/huPLTPtg (lane 2), huPLTPtg (lane 3) and wild-type (lane 4) mice. Mouse plasma (10 μl) was incubated in the presence of an LCAT inhibitor (1 mM iodoacetic acid) at 37 °C for 3 h. Equal amounts of plasma were run on a 0.5% agarose gel, transferred to nitrocellulose membranes and subjected to immunoblotting using rabbit anti-mouse apoA-I IgG.

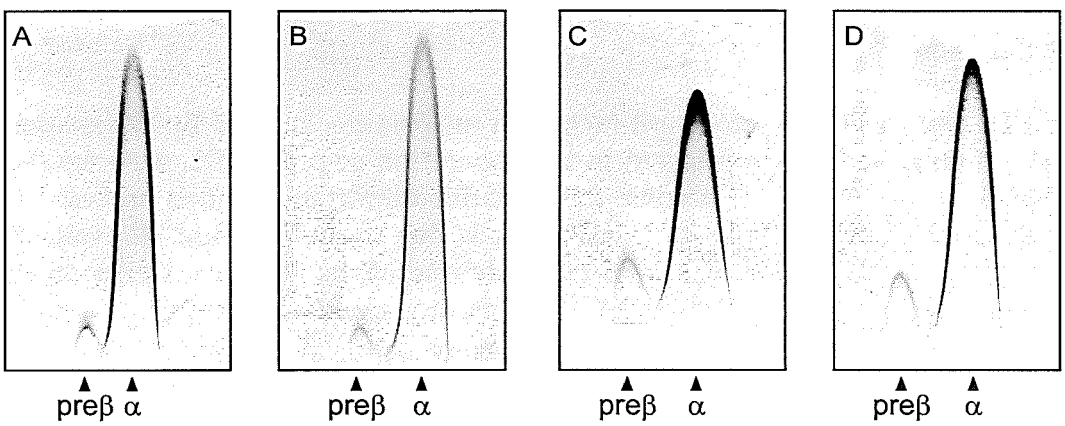

Figure 5. Crossed immuno-electrophoresis of incubated plasma samples from transgenic mice. Plasma samples from transgenic were incubated as described in the legend of Figure 4 and in the Materials and Methods section. Subsequently, 5 μl aliquots were applied to agarose gels for the first dimension, followed by electrophoresis into an anti-mouse apoA-I containing gel for the second dimension. (A) Wild-type mice, (B) huCETPtg mice, (C) huPLTPtg mice, and (D) huCETPtg/huPLTPtg mice.

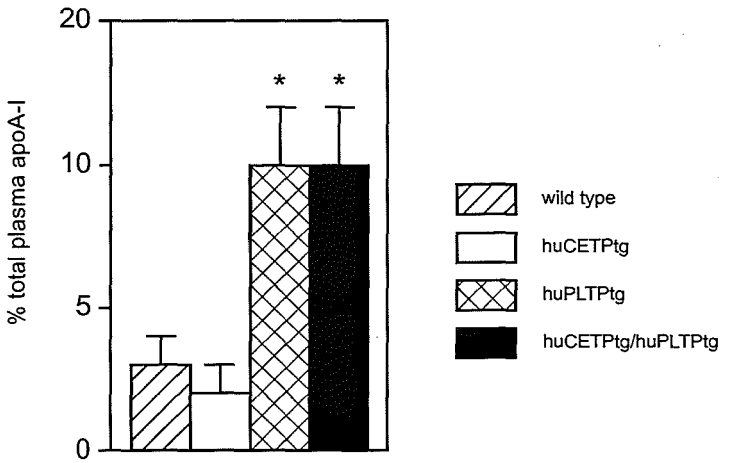

Figure 6. Preβ-HDL formation in plasma samples from transgenic mice. Plasma samples from wild-type, huCETPtg, huPLTPtg and huCETPtg/huPLTPtg mice were incubated as described in the legend of Figure 4. Values represent the differences in the relative amounts of preβ-HDL, expressed as percentage of total plasma apoA-I before and after incubation. These were calculated based on the areas underneath the peaks of preβ-HDL and α-HDL following crossed immuno electrophoresis. Values are mean ± SD, $^*P < 0.001$ compared with both wild-type and huCETPtg mice (ANOVA followed by Bonferroni correction).

Discussion

CETP and PLTP are important factors in modulating plasma lipid transport and both have been implicated in the generation of preβ-HDL particles (12, 13, 15, 16, 18). However, to date their relative importance to this process has not been established unequivocally. This is due to the fact that all available data originate from studies in which only one of the lipid transfer proteins was investigated. Besides, the use of different models, either *in vivo* or *in vitro*, as well as the use of different techniques, hampers a comparative evaluation. For this reason we have used transgenic mouse models with relatively modest overexpression of the human PLTP and/or CETP proteins, enabling us to make direct comparisons of preβ-HDL formation. The human PLTP mice that we used as the parental strain to generate huPLTPtg/huCETPtg mice have been described previously (12). These mice have approximately 3-fold higher plasma PLTP activity levels than wild-type mice. This results in more pronounced effects on plasma lipids, compared to those reported by other groups using transgenic mice with low levels of PLTP expression (15, 23). Strong overexpression, as

found in mice expressing human PLTP after transfection with adenoviral constructs, was avoided since this causes extreme effects on lipoprotein metabolism, resulting in near absence of plasma cholesterol (22, 24). The huCETPtg mice that were used in the present study have been created and characterized by Jiang *et al.* (27). In addition to a human CETP mini-gene, these mice have the natural flanking DNA sequences of the human CETP gene (3.2 kb upstream and 2.0 kb downstream sequence). This construct results in CETP plasma activity levels that are much lower than other CETP-overexpression models (32, 33). Moderate expression of CETP in the huCETPtg mice used in the present study was confirmed by measuring CETP mass by ELISA. Concentrations ranged from 2.7 to 4.1 μg/ml in plasma samples from huCETPtg mice (T. van Gent, J.T. Lie and A. van Tol, unpublished work), which is about 1.5-fold higher than values found in human plasma (34). Thus this mouse model shows modest CETP expression and resembles the human condition in terms of plasma CETP activity and mass. In both models, expression of the transgene resulted in distinct effects on plasma lipids/lipoproteins. In order to evaluate possible interactions between the two lipid transfer proteins, in terms of either their respective transfer activities or on the formation of preβ-HDL, we extended our studies with double transgenic animals, huCETPtg/huPLTPtg mice. This model resembles the situation in humans since, in contrast to mice, humans express both plasma transfer proteins. huCETPtg and huCETPtg/huPLTPtg mice showed a small difference in plasma CETP activity levels. The expression of the human PLTP gene is associated with a decrease in CETP activity of about 20%. This decrease is confirmed on analysis of the CETP activity profiles after gel filtration (Figure 3C). This effect could be the result of a direct effect of overexpression of human PLTP on the level of expression of the CETP transgene. An alternative explanation is that PLTP expression causes a lower plasma HDL concentration. Since HDL is the carrier of CETP, this could eventually result in a decrease in CETP levels. The profiles show that CETP activity elutes at a position corresponding to that of HDL particles. PLTP activity measured in huCETPtg/huPLTPtg mice equals the PLTP activity in the huPLTPtg mice described previously (12). PLTP activity in huCETPtg mice is comparable with that in wild-type mice. Therefore CETP does not seem to affect the activity of the endogenous mouse PLTP in plasma. In comparison with the lipoprotein profile found in huPLTPtg mice (12), huCETPtg/huPLTPtg mice have somewhat smaller HDLs, which is in agreement with previous studies in transgenic mice expressing human CETP (35). The activity profiles of PLTP in huCETPtg mice and huCETPtg/huPLTPtg mice were measured after gel filtration of plasma (Figure 3D). The profiles show that PLTP activity elutes at the position corresponding to relatively large-sized HDL particles in both groups of mice. Thus CETP and PLTP co-elute with particles of different sizes, a situation also found in humans (28).

The main purpose of the present study was to determine the relative contribution of PLTP and CETP to preβ-HDL generation. We evaluated the formation of preβ-HDL in plasma using two different methods and demonstrated that preβ-HDL formation is increased in plasma from transgenic mice expressing human PLTP, whereas expression of human CETP did not affect this process. These findings cannot be attributed to differences in preference towards human *versus* mouse HDL, since incubations of plasma from huCETPtg mice with freshly isolated human HDL did not show formation of preβ-HDL (J.T. Lie, R. de Crom, M. Jauhiainen, T. van Gent, R. van Haperen, L.M. Scheek, C. Ehnholm and A. van Tol, unpublished work). LCAT activity was similar in plasma of the various transgenic mice, which implies that a difference in maturation of preβ-HDL into α-HDL is unlikely. Therefore differences in LCAT activity cannot explain the observed distinctions in preβ-HDL formation. The involvement of hepatic lipase in the formation of preβ-HDL has been suggested (36). In humans, virtually all hepatic lipase is bound to liver endothelial cells, whereas in mice hepatic lipase circulates in plasma (31). It was therefore important to know whether plasma from huCETPtg/huPLTPtg mice display higher hepatic lipase activity than plasma from huCETPtg mice. Hepatic lipase activity was found to be lower in plasma from huCETPtg/huPLTPtg compared with plasma from huCETPtg mice, so this activity cannot explain the observed differences in preβ-HDL generation. Other groups have reported a role for CETP in preβ-HDL formation (13, 17, 18). Kunitake *et al.* (13) showed that preβ-HDL formation is stimulated by CETP *in vitro*. Francone *et al.* (18), using double transgenic animals that overexpressed CETP (driven by the metallothionein promoter) as well as human apolipoprotein A-I, observed increased levels of preβ-HDL in these mice. In the mouse models used here, CETP expression is driven by its native promoter and CETP levels and activities were comparable with those in human plasma. An increase in the generation of relative and absolute plasma levels of preβ-HDL formation was only observed in huPLTPtg mice. Despite lower HDL concentrations in huCETPtg/huPLTPtg mice compared with huCETPtg mice, plasma of huCETPtg/huPLTPtg mice had a greater ability to produce preβ-HDL. This finding of a combination of low HDL cholesterol levels and increased preβ-HDL formation was also observed in huPLTPtg mice (12), as well as in mice using adenoviral overexpression of PLTP (22, 24). Although CETP and PLTP share similar structural properties and belong to the same gene family, it has been found that they have non-overlapping functions *in vivo* (11). The roles of the HDL modulators CETP and PLTP in atherosclerosis have not yet been elucidated. CETP participates in reverse cholesterol transport, a function that may mean it has an anti-atherogenic potential (10). On the other hand, CETP mediates the exchange of cholesteryl

esters in HDL for triglycerides in VLDL. This process leads to a decrease of HDL cholesterol and to an increase of potentially pro-atherogenic VLDL and LDL cholesterol (37). Although substantial overexpression of human CETP was reported to cause increased susceptibility to diet-induced atherosclerosis (32), CETP has been proved to be anti-atherogenic in other (compound) transgenic mouse models (38, 39). In rats, high level expression of human CETP is highly atherogenic (33), whereas in rabbits inhibition of CETP proved to be anti-atherogenic (40). The impact of CETP on atherosclerosis in humans remains inconclusive (37). The present study supports the notion that PLTP, rather than CETP, is responsible for the generation of preβ-HDL. The impact of PLTP on diet-induced atherosclerosis will be the subject of future investigations.

Acknowledgements

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CHAPTER III

Elevation of Plasma PLTP in Transgenic Mice Increases VLDL Secretion

Elevation of Plasma Phospholipid Transfer Protein in Transgenic Mice Increases Very Low Density Lipoprotein Secretion

Jessica Lie[1], Rini de Crom[2,3], Teus van Gent[1], Rien van Haperen[2], Leo Scheek[1], Inge Lankhuizen[1], Arie van Tol[1]

[1]Department of Biochemistry, Cardiovascular Research Institute COEUR, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.
[2]MGC-Department of Cell Biology and Genetics, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.
[3]Department of Vascular Surgery, Academic Hospital "Dijkzigt", Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

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Abstract

Two lipid transfer proteins are active in human plasma, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). Mice by nature do not express CETP. Additional inactivation of the PLTP gene resulted in reduced secretion of VLDL and subsequently in decreased susceptibility to diet-induced atherosclerosis. The aim of this study is to assess possible effects of differences in PLTP expression on VLDL secretion in mice that are proficient in CETP and PLTP. We compared human CETP transgenic (huCETPtg) mice with mice expressing both human lipid transfer proteins (huCETPtg/huPLTPtg).
Plasma cholesterol in huCETPtg mice was 1.5-fold higher compared with huCETPtg/huPLTPtg mice ($P < 0.001$). This difference was mostly due to a lower HDL level in the huCETPtg/huPLTPtg mice, which subsequently could lead to the somewhat decreased CETP activity and concentration that was found in huCETPtg/huPLTPtg mice ($P < 0.05$). PLTP activity was 2.8-fold increased in these animals ($P < 0.001$). The human PLTP concentration was 5 μg/ml.
Moderate overexpression of PLTP resulted in a 1.5-fold higher VLDL secretion compared with huCETPtg mice ($P < 0.05$). The composition of nascent VLDL was similar in both strains. These results indicate that elevated PLTP activity in huCETPtg mice results in an increase in VLDL secretion. In addition, PLTP overexpression decreases plasma HDL cholesterol as well as CETP.

Introduction

Phospholipid transfer protein (PLTP) is an important modulator of plasma HDL levels, size and composition (1-5). HDL is considered to protect against atherosclerosis by transporting cellular cholesterol from cells in the arterial wall to the liver for further excretion via the bile, as well as by exerting anti-inflammatory and anti-oxidant effects (6-8).
The role of PLTP in atherosclerosis was recently evaluated in PLTP deficient mice (5). PLTP deficiency in hyperlipidemic mouse models resulted in decreased atherosclerosis. *In vitro* experiments with cultured hepatocytes from PLTP deficient mice revealed a defect in VLDL secretion. These effects on VLDL secretion provided an explanation for the decreased atherosclerosis found in PLTP deficient mice (5).
Earlier we reported anti-atherogenic properties in mice overexpressing human PLTP (huPLTPtg). Despite lower HDL levels, plasma from these mice is more effective in preventing *in vitro* accumulation of cholesterol by macrophages and is able to generate more preβ-HDL (3, 9). Studies in mice with adenovirus-mediated overexpression of human PLTP showed similar effects on HDL subclass distribution (10, 11).

Thus, depending on the metabolic setting, PLTP may have anti- or pro-atherogenic properties that require further investigation. Presently, we aimed to evaluate whether VLDL secretion is affected by variations in PLTP activity. For this purpose, we crossbred transgenic mice for human CETP (huCETPtg) with huPLTPtg mice (9) and obtained huCETPtg/huPLTPtg mice. These mice provide a unique model to study the role of PLTP in VLDL metabolism in the presence of CETP, which by nature is the situation in humans. Plasma lipoproteins, plasma CETP, and PLTP activities, as well as human CETP and human PLTP mass were also measured to study the impact of PLTP in huCETPtg mice.

Materials and Methods

Breeding and treatment of transgenic mice

The huCETPtg mice were kindly provided by Dr. A.R. Tall (Columbia University, New York) and are in C57BL6 background (9). HuPLTPtg mice (3) were backcrossed to C57BL6 background for at least seven generations. Mice expressing both human CETP and human PLTP (huCETPtg/huPLTPtg) were obtained by crossbreeding homozygous huCETPtg with homozygous huPLTPtg mice. Both transgenes have the natural flanking sequences, including the native promoters. Female mice were used in further experiments. Animals were housed under standard conditions with free access to water and regular chow diet.

After fasting overnight blood samples were collected from the orbital plexus by using Vitrex™ sodium-heparinized micropipettes (80 IU) (Modulohm A/S, Copenhagen, Denmark) and immediately stored on ice. Blood was centrifuged at 2700 rpm for 15 min at 4 °C. Plasma was either used directly or stored in small aliquots at -80 °C before analysis. All experiments were approved by institutional and national guidelines (protocol nr.120.99.05).

In vivo hepatic VLDL secretion and composition

VLDL secretion experiments were performed according to Jong *et al.* (12). VLDL secretion was measured in overnight fasted mice which were injected intravenously with 15% (w/v) Triton WR1339 (Sigma, St Louis, MO, USA)(500 mg/kg body weight) dissolved in 0.9% NaCl. After injection of Triton WR1339, blood samples were drawn at appropriate time points (up to 90 min) and triglyceride content was measured as described below. The triglyceride accumulation in plasma was linear during this time period. Hepatic triglyceride secretion rate was calculated from the slope of the line and expressed as μmol/h per kg body weight.

From the blood samples obtained at t = 90 min after Triton WR1339

administration, plasma was taken and centrifuged at d = 1.006 g/ml in a Beckman 42.2 Ti rotor (34200 rpm, 3 h, 12 °C). The top fraction containing VLDL was isolated by tube slicing.

Quantification of plasma lipids and protein

Cholesterol was determined enzymatically with the Free Cholesterol C kit no. 274-47109 (WAKO, Neuss, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer, Mannheim, Germany). Triglycerides were measured with the Sigma GPO-Trinder kit no.337-B (Sigma, St Louis, MO, USA) and free fatty acids were measured with the NEFA C kit no. 994-75409 (WAKO, Neuss, Germany). Phospholipids were measured with the PAP150 kit from Bio Merieux (Lyon, France). Protein was measured with a modification of the Lowry assay (13).

Separation of plasma lipoproteins by gelfiltration

Plasma from transgenic mice was analyzed by gelfiltration on two HR10/30 FPLC columns in tandem (Superdex 200 prepgrade, Superose 6 prepgrade, Pharmacia Biotech., Uppsala, Sweden). The columns were equilibrated with 2 mM NaH_2PO_4/Na_2HPO_4, pH 7.4 [containing 0.9% NaCl (w/v), 0.02% NaN_3 (w/v) and 5 mM EDTA]. Combined plasma samples from seven to ten mice were passed through 0.45-µm filters from Millipore S.A. (Molsheim, France), and 0.5 ml was subjected to gelfiltration. The columns were run at 4 °C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected. Recoveries were > 90% for all analyses.

Plasma activity assays

CETP and PLTP activity assays were performed according to Speijer *et al.* (14) as described before (9). The activities are expressed as percentage of human reference pool plasma. One hundred percent is equivalent to the following activities: CETP 215.6 nmol/ml/h; PLTP 13.9 µmol/ml/h.

Human CETP and human PLTP mass determinations by ELISA

The CETP ELISA assay was essentially performed as described by Mezdour *et al.* (15). CETP mass was measured by a two-site antibody immunoassay using a combination of two specific monoclonal antibodies for CETP: TP1 and TP2 (Ottawa Heart Institute, Canada), which are able to inhibit the transfer of cholesterylesters as well as triglycerides and recognize a similar epitope localized in the carboxyterminal region of the CETP molecule (16). Both antibodies (10 µg/ml) were coated overnight by incubation in phosphate buffered saline (PBS, pH 7.4) at 4 °C on microwell plates (Immunoplate MaxiSorp; Nunc, Roskilde, Denmark). After washing the plates 5 times with

washing buffer [PBS containing 6% (v/v) methanol and 0.2% (w/v) Thesit (Boehringer, Mannheim, Germany)], the plates were incubated for 1 h at 37 °C with PBS containing 1% of bovine serum albumin (BSA; ICN, Ohio, USA) to prevent non-specific binding. After incubation, the wells were washed 5 times and standard solution or plasma samples, diluted in assaybuffer [washing buffer containing 1% (w/v) BSA], were added and incubated for 2 h at 37 °C. After washing 5 times, antibody TP20 labeled with digoxigenine (Ottawa Heart Institute, Canada) was added and the plates were incubated for 2 h at 37 °C followed by 5 times washing and incubation with a solution containing anti-digoxigenine Fab fragments for another 2 h at 37 °C. Then, after washing 5 times, a solution with anti-digoxigenine coupled to peroxidase (Boehringer, Mannheim, Germany) was added followed by incubation for 45 min at 37 °C. Tetramethylbenzidine (TMB, Merck, Darmstadt, Germany) was freshly prepared in DMSO (6 mg/ml). 500µl of the TMB/DMSO solution and 3 µl of H_2O_2 were added to 30 ml citrate buffer (35 mM, pH 5.5). 100 µl of this TMB solution was added to the wells after washing the plates 5 times. After 30 min, the reaction was stopped by the addition of 100 µl 2 N H_2SO_4. Absorbance was read at 450 nm. The intra- and inter-assay coefficients of variation were 3.6% and 8.0% respectively. In the present study CETP activity correlated with human CETP mass ($r = 0.93$, $P < 0.001$).
Human PLTP mass was measured with a sandwich-type ELISA essentially as described previously (17) with minor modifications: the washing buffer contained 6% methanol (v/v) with 0.2% Thesit (w/v) (Boehringer, Mannheim, Germany) and 0.1% (w/v) Tween 20. The substrate solution used and the absorbance measurements were performed as described for the CETP mass determination. PLTP antibodies and standards were a generous gift from Dr. H. Hattori (BML incorporated, Saitama, Japan). In the present study PLTP activity correlated with human PLTP mass ($r = 0.84$, $P < 0.05$).

Statistical analysis

Data are expressed as mean ± SD. Differences between huCETPtg mice and huCETPtg/huPLTPtg mice were analyzed by two sample Wilcoxon rank-sum tests.

Results

Plasma activities of CETP and PLTP

The activities of CETP and PLTP were measured in plasma samples from huCETPtg mice and huCETPtg/huPLTPtg mice. CETP activity was 1.15-fold higher in huCETPtg mice compared with huCETPtg/huPLTPtg mice ($P < 0.05$) (Table 1). PLTP activity in plasma of huCETPtg mice was equal to activity levels found in wild-type mice (3). In huCETPtg/huPLTPtg mice PLTP activity was 2.8-fold higher ($P < 0.001$).

Table 1. Plasma levels of lipids, CETP and PLTP activities as well as human CETP and human PLTP concentrations in transgenic mice.

	n	huCETPtg	*n*	huCETPtg/huPLTPtg
Cholesterol (mM)	10	1.9[a] ± 0.3	11	1.3 ± 0.1
Triglycerides (mM)	9	0.3 ± 0.4	5	0.2 ± 0.1
CETP activity (%)	12	126[b] ± 13	11	110 ± 22
Human CETP (µg/ml)	6	4.4[b] ± 1.9	7	2.9 ± 0.5
PLTP activity (%)	13	148[a] ± 24	11	418 ± 54
Human PLTP (µg/ml)	4	ND	4	5.1 ± 0.7

CETP and PLTP activities are expressed as percentage of human reference pool plasma values (%). One hundred percent of the human reference pool plasma is equivalent to a CETP activity of 215.6 nmol/ml/h and to a PLTP activity of 13.9 µmol/ml/h. Differences between huCETPtg and huCETPtg/huPLTPtg mice were analyzed by two sample Wilcoxon rank-sum tests. Values are means ± SD. *n* , individual mouse plasma samples measured. [a]$P < 0.001$, [b]$P < 0.05$. ND, not detectable.

Lipoprotein analyses

Plasma samples from either huCETPtg or huCETPtg/huPLTPtg mice were analyzed by gelfiltration chromatography to examine their lipoprotein profiles. Phospholipid contents as well as CETP and PLTP activities were determined in all fractions (Figure 1). The HDL phospholipids in huCETPtg/huPLTPtg mice show a decrease due to PLTP overexpression (Figure 1A). The phospholipids in fractions 20-25 represent lysophosphatidylcholine bound to albumin. A similar profile, apart from the peak in fractions 20-25, was obtained for cholesterol (not shown). CETP activity eluted in fractions corresponding to the size of HDL (Figure 1B) and PLTP activity eluted in the fractions 9-15 corresponding with relatively large HDL particles (Figure 1C), a situation also found in humans (14).

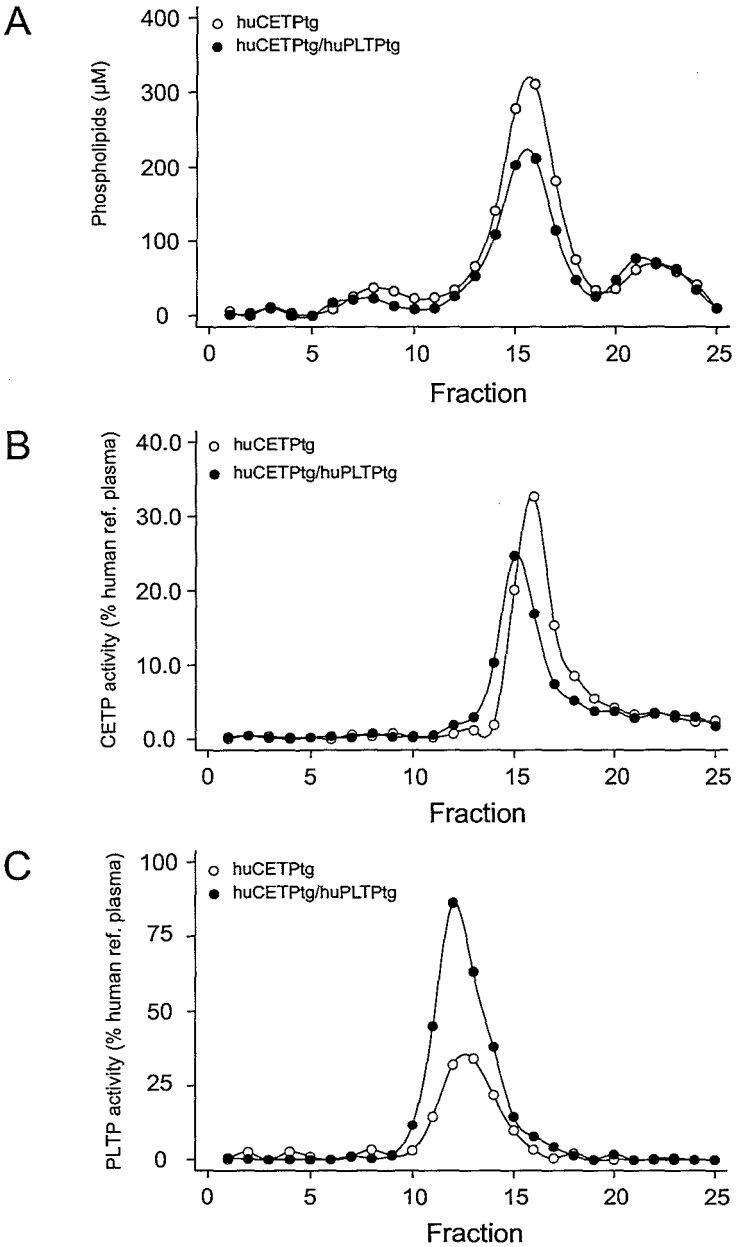

Figure 1. Lipoprotein profiles of plasma from transgenic mice. Equal amounts of plasma from either huCETPtg mice ($n = 7$) or huCETPtg/huPLTPtg mice ($n = 10$) were pooled and subjected to gel filtration on Superose 6 and Superdex 200 columns connected in tandem as described in Materials and Methods. Fractions were analyzed for (A) Phospholipids, (B) CETP activity: 100% human reference pool plasma is equivalent to CETP activity of 215.6 nmol/ml/h. (C) PLTP activity: 100% human reference pool plasma is equivalent to PLTP activity of 13.9 µmol/ml/h. Fractions 1-5 contain VLDL; 6-11 contain LDL; 12-20 contain HDL and; 21-25 contain lysophosphatidylcholine bound to albumin.

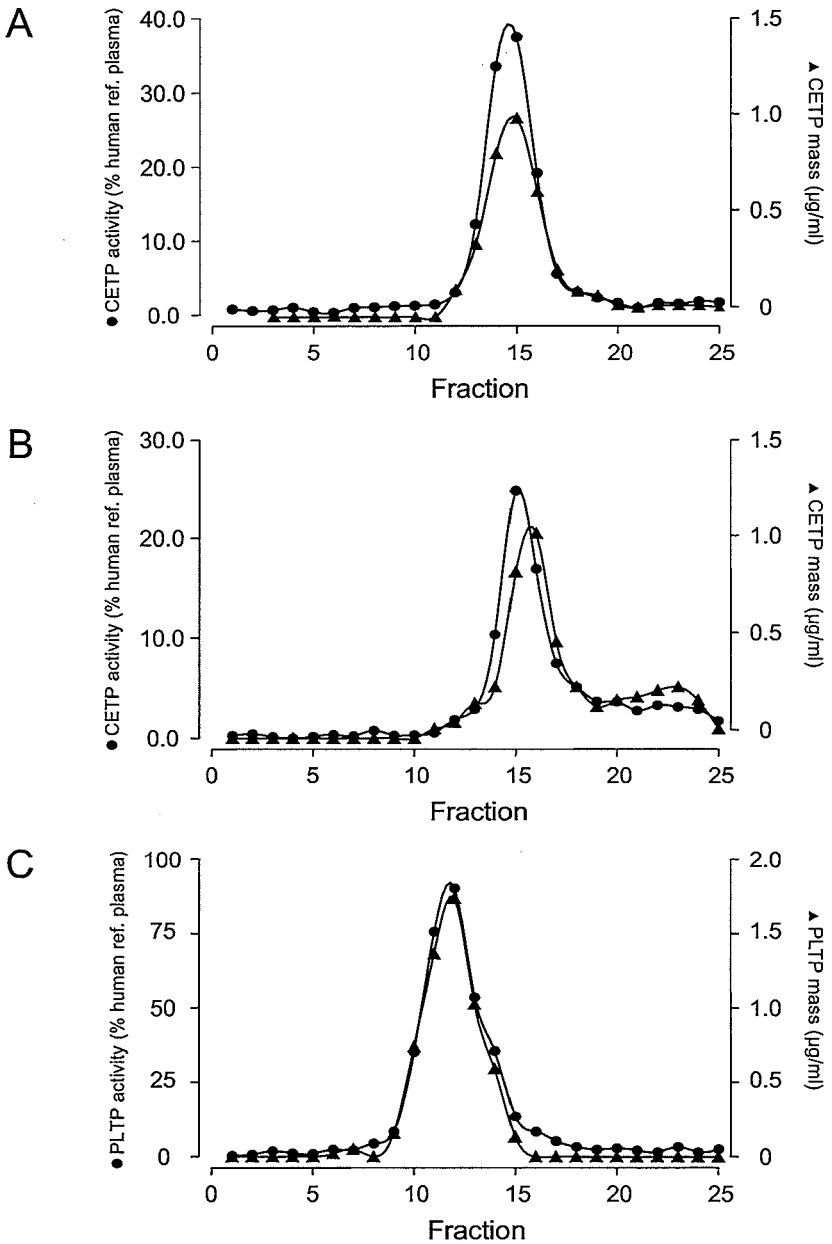

Figure 2. Human CETP and PLTP concentrations with their corresponding activities measured in gelfiltration fractions of plasma from transgenic mice. (A) Human CETP concentrations and CETP activities in huCETPtg mice ($n = 7$, $r = 0.99$, $P < 0.001$). (B) Human CETP concentrations and CETP activities in huCETPtg/huPLTPtg mice ($n = 10$, $r = 0.92$, $P < 0.001$). (C) Human PLTP concentrations and PLTP activities in huCETPtg/huPLTPtg mice ($n = 13$, $r = 0.97$, $P < 0.001$).

Determination of human CETP and human PLTP concentrations

Human CETP and human PLTP mass were analyzed in the plasma of huCETPtg and huCETPtg/huPLTPtg mice. CETP concentration was higher in huCETPtg mice compared with huCETPtg/huPLTPtg mice ($P < 0.05$). Human CETP mass co-eluted with CETP activity in the lipoprotein profiles of both huCETPtg and huCETPtg/huPLTPtg mice (Figure 2A and 2B respectively). PLTP concentration in huCETPtg/huPLTPtg mice was 5.1 ± 0.7 μg/ml. In huCETPtg/huPLTPtg mice human PLTP mass co-eluted with PLTP activity in the lipoprotein profile (Figure 2C).

Cholesterol, triglyceride and free fatty acid measurements

Before studying the influence of PLTP on VLDL metabolism, we measured cholesterol, triglyceride and free fatty acid content in plasma of huCETPtg mice and huCETPtg/huPLTPtg mice. Plasma cholesterol in huCETPtg mice was 1.5-fold higher than in huCETPtg/huPLTPtg mice ($P < 0.001$) (Table 1). The triglyceride and free fatty acid levels were similar in both groups of mice (Table 1).

Effect of human PLTP on hepatic VLDL secretion

After intravenous injection of Triton WR1339, the rate of plasma triglyceride accumulation was measured by determining triglycerides in plasma at appropriate time points (Figure 3A). The triglyceride accumulation rates calculated for each individual mouse were related to their body weights. There was no difference in body weight between the mice from the two groups (huCETPtg mice 19.6 ± 1.3 g; huCETPtg/huPLTPtg mice 19.2 ± 0.9 g). The secretion rate was calculated from the slope of the individual lines and is expressed as μmol/kg/h. As shown in Figure 3B, the VLDL secretion rate was 1.5 fold higher in huCETPtg/huPLTPtg mice. HuCETPtg mice showed a rate of 140 ± 49 *versus* 208 ± 41 μmol/kg/h in huCETPtg/huPLTPtg mice ($P < 0.05$). The chemical composition of VLDL was analyzed at 90 min after Triton WR1339 injection. The composition of nascent VLDL (Figure 3C) was similar in the two groups of mice. Others have verified that catabolism of VLDL is completely blocked by Triton WR1339 and therefore the composition of the accumulated VLDL is a direct measure of the composition of nascent VLDL).

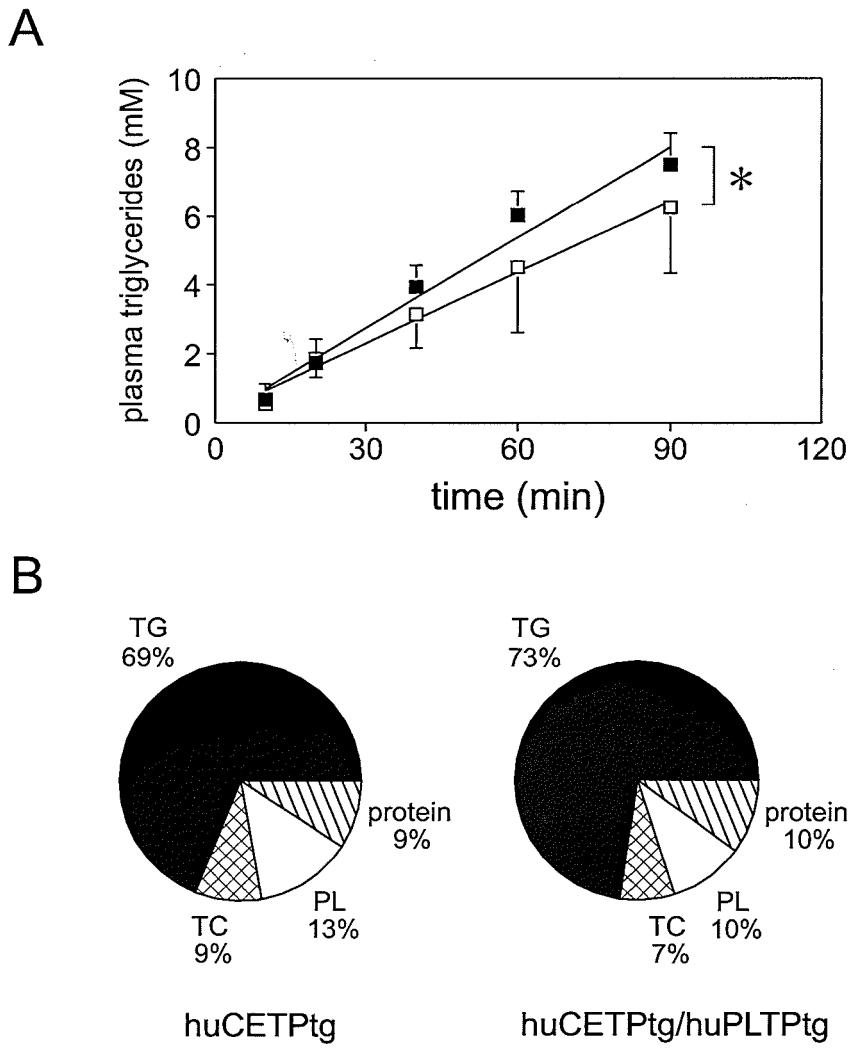

Figure 3. *In vivo* hepatic VLDL secretion in transgenic mice. (A) Triglyceride production rates in transgenic mice were measured after Triton WR1339 administration. HuCETPtg mice are represented by open squares ($n = 7$) and huCETPtg/huPLTPtg mice are represented by closed squares ($n = 6$). Differences between huCETPtg and huCETPtg/huPLTPtg mice were analyzed by two sample Wilcoxon rank-sum tests. The lines represent the mean triglyceride rates ± SD of individual mice within huCETPtg and huCETPtg/huPLTPtg mice (*$P < 0.05$). (B) Lipoprotein composition in nascent VLDL. VLDL was isolated from transgenic mouse plasma 90 min after Triton WR1339 administration by density ultracentrifugation at $d = 1.006$ g/ml (34200 rpm, 3 h, 12°C). Lipids and protein were determined and expressed in percentage of total weight. Black segments represent the percentage of triglycerides, hatched segments represent the percentage of protein, white segments represent the percentage of phospholipids, and cross-hatched segments represent the percentage of cholesterol in nascent VLDL.

Discussion

The exact role of PLTP in lipoprotein metabolism is not clear. Earlier studies have shown that PLTP has both anti- and pro-atherogenic effects on lipoproteins (3, 5, 9). It was demonstrated that mouse models lacking PLTP are less prone to diet-induced atherosclerosis. In two out of three models studied (5), this could be attributed to a reduced secretion of VLDL. PLTP deficiency or functional gene polymorphisms have not been found in humans. Moreover, unlike humans, mice do not have plasma CETP activity (19). Thus, total deficiency of both CETP and PLTP represent an extreme situation from which the effects on lipoprotein metabolism cannot be simply extrapolated to humans.
The purpose of the present study is to evaluate the influence of PLTP on VLDL metabolism in a mouse model with appreciable CETP activity. In this study we demonstrate that elevated expression of PLTP increases hepatic VLDL secretion in mice in the presence of human CETP, without affecting the composition of the secreted VLDL. Despite the increase of VLDL secretion in the huCETPtg/huPLTPtg mice, we did not observe an increase in plasma VLDL levels. This may be due to an increased turnover of VLDL, or to increased receptor-mediated uptake by the liver. It is unlikely that the augmented VLDL secretion by PLTP is caused by an increased provision of free fatty acids from plasma to the liver, since no difference was found between the free fatty acid content in plasma of huCETPtg and huCETPtg/huPLTPtg mice (Table 1). Additional experiments showed that the elevation of VLDL secretion can be specifically attributed to the PLTP transgene. Using mice solely transgenic for human PLTP, we observed a 1.6-fold increase in VLDL secretion, if compared to wild-type mice. As recently demonstrated (5), PLTP plays a possible intracellular role in the liver. Because PLTP activity has been found in the Golgi, PLTP could be involved in the process of adding lipids to nascent VLDL particles. Plasma cholesterol content was 1.5-fold higher in huCETPtg mice compared with huCETPtg/huPLTPtg mice (Table 1). Thus, overexpression of human PLTP in huCETPtg mice results in a decrease in total plasma cholesterol, mostly due to HDL lowering, as HDL is the major component of the plasma lipoproteins in mice. Raised PLTP activity in other transgenic mouse models also give rise to low HDL cholesterol levels (3, 10, 11). The decrease in plasma HDL is explained by an enhanced uptake of HDL cholesteryl esters by the liver (10). Interestingly, PLTP deficient mice also show markedly reduced HDL, probably due to HDL hypercatabolism (20, 21). Kawano *et al.* demonstrated that CETP overexpression could not compensate PLTP deficiency and caused an additional lowering of HDL (22). Plasma CETP activity measured in huCETPtg mice is higher than in huCETPtg/huPLTPtg mice (Table 1). The lower CETP activity found in huCETPtg/huPLTPtg mice may be a result from the lower plasma HDL concentrations caused by PLTP

overexpression (9). Because CETP is carried on HDL (14), lower plasma HDL concentrations could eventually give rise to lower CETP levels. The CETP concentrations in both groups of mice (Table 1) are within the range of human values, indicating that the mouse models used resemble the human situation both in terms of CETP activity and mass (23).
PLTP activity levels in plasma of huCETPtg were equal to the activities found in wild-type mice (3). As expected, PLTP activity was higher in huCETPtg/huPLTPtg mice than in the huCETPtg mice (Table 1). The human PLTP mass measured in huCETPtg/huPLTPtg mice is within the range of reported plasma PLTP concentrations in humans (17, 24, 25). In contrast to what has been reported in humans (26, 27), in our mice PLTP activity correlates with human PLTP mass. In human plasma, such a correlation is not found due to the presence of inactive PLTP mass. In our studies, we found no evidence of inactive forms of PLTP in mice, since PLTP activity and PLTP mass elute in the same fractions in the lipoprotein profile (Figure 2C). At present, the physiological importance, if any, of inactive PLTP is unknown. Studies showed that type II diabetic patients have higher PLTP mass and activity compared with nondiabetic subjects (24, 28). Increased PLTP activity has also been reported in type I diabetic patients (29) and in the obese (30, 31). Both diabetes and obesity have been associated with an increased risk of coronary artery disease, indicating a possible pro-atherogenic potential for PLTP.
By comparing huCETPtg mice with huCETPtg/huPLTPtg mice we observed that elevated PLTP leads to increased hepatic VLDL secretion. This is in line with experiments by Jiang *et al.* performed with cultured hepatocytes isolated from PLTP deficient mice, which showed a defect in VLDL secretion (5), and strengthens their suggestion that PLTP has an intracellular function in liver cells.
In the present study we showed for the first time that elevation of plasma PLTP in transgenic mice increases VLDL secretion. Concomitantly with increased VLDL secretion, elevated PLTP resulted in lower levels of plasma HDL, both effects resulting in a more atherogenic lipoprotein profile. The huCETPtg/huPLTPtg mice used in this study provide a unique model because it resembles the human condition in terms of CETP activity and mass. In future studies we will test the impact of PLTP overexpression on diet induced atherosclerosis in huCETPtg mice.

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CHAPTER IV

Elevation of PLTP Increases the Risk of Atherosclerosis despite Lower ApoB-Containing Lipoproteins

Elevation of Plasma Phospholipid Transfer Protein Increases the Risk of Atherosclerosis despite Lower Apolipoprotein B- Containing Lipoproteins

Jessica Lie*, Rini de Crom*‡, Teus van Gent*†, Rien van Haperen†, Leo Scheek*, Farah Sadeghi-Niaraki*, Arie van Tol*†

Departments of *Biochemistry, †Cell Biology & Genetics, and ‡Vascular Surgery, Erasmus University Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

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Abstract

Plasma phospholipid transfer protein (PLTP) transfers phospholipids between lipoproteins and mediates HDL-conversion. PLTP overexpressing mice have increased atherosclerosis. However, mice do not express cholesteryl ester transfer protein (CETP), which is involved in the same metabolic pathways as PLTP. Therefore, we studied atherosclerosis in heterozygous LDL-receptor deficient ($LDLR^{+/-}$) mice expressing both human CETP and human PLTP. We used two transgenic lines with moderately and highly elevated plasma PLTP activity. In $LDLR^{+/-}$/huCETPtg mice, cholesterol is present in both LDL and HDL. Both are decreased in $LDLR^{+/-}$/huCETPtg/huPLTPtg mice (> 50%). An atherogenic diet resulted in high levels of VLDL+LDL cholesterol. PLTP expression caused a strong PLTP-dose dependent decrease in VLDL+LDL cholesterol (–26% and –69%) and a decrease in HDL cholesterol (–70%). Surprisingly, atherosclerosis was increased in the two transgenic lines with moderately and highly elevated plasma PLTP activity (1.9-fold and 4.4-fold, respectively), indicating that the adverse effect of the reduction in plasma HDL outweighs the beneficial effect of the reduction in apolipoprotein B (apoB)-containing lipoproteins. The activities of the anti-atherogenic enzymes, paraoxonase (PON) and platelet activating factor acetyl hydrolase (PAF-AH), were both PLTP dose dependently reduce d (about -33 and –65%, respectively). We conclude that expression of PLTP in this animal model results in increased atherosclerosis in spite of reduced apoB-containing lipoproteins, by reduction of HDL and of HDL-associated anti-oxidant enzyme activities.

Introduction

Plasma levels of high density lipoproteins (HDL) are inversely correlated with the risk for development of atherosclerosis (1). HDL are considered to have various atheroprotective effects, including anti-inflammatory and anti-oxidant properties and the potential to transport excess cholesterol from peripheral cells to the liver for degradation and excretion via the bile (1-4).

Phospholipid transfer protein (PLTP) plays several key roles in HDL metabolism (5-8). PLTP facilitates the transfer of phospholipids, α-tocopherol and possibly unesterified cholesterol from triglyceride rich lipoproteins to HDL particles during lipolysis (9, 10). PLTP is able to modulate HDL size and composition (11-13) and may also be involved in HDL cellular mediated efflux of phospholipids and cholesterol (14).

Elevation of PLTP in transgenic mice results in a PLTP-dose dependent decrease in plasma HDL levels, coinciding with an increased susceptibility to diet-induced atherosclerosis (15). These results are in agreement with the previous study by Jiang *et al.* (16), who demonstrated that PLTP deficient mice

are less prone to atherosclerosis. However, the explanation was different in both cases, as the changes in athero-susceptibility in PLTP deficient mice were not explained by an HDL effect, but by a decrease of hepatic VLDL secretion. Elevation of PLTP results in a stimulation of VLDL secretion (17), but this effect was found to be PLTP-dose independent. Therefore, it was considered to be a contributor but not the main cause for the atherogenic effect of elevated PLTP (15). Mice do not have cholesteryl ester transfer protein (CETP). Thus, the previously described genetically modified mice might be considered poor models to study the relation between PLTP and atherosclerosis, inasmuch as CETP is thought to be critically involved in atherogenesis (18). Moreover, although PLTP and CETP function independently (19, 20), CETP affects the metabolism of both HDL and of apoB-containing lipoproteins (18). Therefore, we decided to study the effect of elevated PLTP activity on atherosclerosis using mice genetically modified to express CETP. We found that also in this context, PLTP increases the susceptibility to diet-induced atherosclerosis. Surprisingly, this effect was found in spite of lowered levels of apolipoprotein apoB-containing lipoproteins.

Materials and Methods

Animals

The human PLTP transgenic mice were obtained as described before (15). In this study we used mice with moderately and highly elevated PLTP activity (lines huPLTPtgP4 and huPLTPtgA2, respectively). LDL-receptor (LDLR) knockout mice were purchased from Jackson Laboratory. The huCETPtg mice were kindly provided by Dr. A.R. Tall (Columbia University, New York). All mice were in C57BL/6J background. We created LDLR$^{+/-}$/huCETPtg mice, which were used as control mice. By crossbreeding these mice with the PLTP transgenic mouse lines expresssing various levels of PLTP, we obtained LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. Male mice were used in further experiments.

After weaning, animals were kept on a standard chow diet (Hope Farms, The Netherlands). At 4 months of age all groups of mice were put on a high fat, high cholesterol (HFHC) diet for 14 weeks which contained 40% w/w sucrose 15% w/w fat, 1% w/w cholesterol and 0.5% w/w cholate to increase uptake of fat and cholesterol leading to high plasma cholesterol levels. Animals had free access to water and food. After fasting overnight blood samples were collected from the orbital plexus by using Vitrex™ sodium-heparinized micropipettes (80 IU) (Modulohm A/S, Copenhagen, Denmark) and immediately stored on ice. Blood was centrifuged at 2700 rpm for 15 min at 4 °C. Plasma was either used directly or stored in small aliquots at -80 °C before analysis. All experiments were approved by national and institutional guidelines.

Separation of plasma lipoproteins by gelfiltration

Plasma from transgenic mice was analyzed by gelfiltration on two HR10/30 FPLC columns in tandem (Superdex 200 prepgrade, Superose 6 prepgrade, Pharmacia Biotech., Uppsala, Sweden). The columns were equilibrated with 2 mM NaH_2PO_4/Na_2HPO_4, pH 7.4 (containing 0.9% NaCl (w/v), 0.02% NaN_3 (w/v) and 5 mM EDTA). Combined plasma samples were passed through 0.45-µm filters from Millipore S.A. (Molsheim, France), and 0.5 ml was subjected to gel filtration. The columns were run at 4 °C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected. Recoveries were > 90% for all analyses.

Separation of plasma lipoproteins by density gradient centrifugation

Lipoprotein fractions were obtained by density gradient ultracentrifugation of plasma samples in a Beckman SW60 Ti rotor (36500 rpm, 21 h 12 min, 12°C) (21). Alternatively, HDL and VLDL/LDL lipoprotein fractions were obtained by density gradient ultracentrifugation of plasma samples in a Beckman 42.2 Ti rotor (42000 rpm, 3h, 12 min, 12°C) at $d = 1.063$ g/ml. The lipoprotein fractions were collected by tube slicing.

Quantification of cholesterol

Cholesterol was determined enzymatically with the Free Cholesterol C kit no. 274-47109 (WAKO, Neuss, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer, Mannheim, Germany).

Plasma activity assays

CETP and PLTP activity assays were performed as described before (22). The activities are expressed as arbitrary units (AU); 1 AU is the activity found in human reference pool plasma. The activities are: CETP 216 nmol/ml/h; PLTP 14 µmol/ml/h.

PON activity towards paraoxon was quantified spectrophotometrically essentially as described (23). In short, 10-20 µl of serum or heparin-plasma was added to 1 ml incubation medium with 50 mM Tris-HCL buffer (pH 8.0), containing 1 mM $CaCl_2$ and 6.0 mM paraoxon. The reaction was monitored for up to 30 min at 25 °C by measuring the appearance of p-nitrophenol at 412 nm in a CARY 1E UV visible spectrophotometer. PON activity is expressed as Units/ml.

PAF-AH was determined as described by Tselepis *et al.* (24), using 2-[acetyl-^{3}H PAF] as substrate. In short, 80 nmoles of substrate (final concentration 0.33 mM) were incubated with 10 µl diluted (10-fold) plasma samples in incubation medium (total volume 0.24 ml) with 0.1 M Tris-HCl buffer pH 7.4, containing 0.9% NaCl and 1 mM EDTA. After incubation for 20 min at 37 °C, the reaction was stopped on ice and 300 µl $CHCl_3$ and 600 µl methanol were added. After mixing, another 300 µl of $CHCl_3$ was added with 300 µl 0.9% NaCl pH 2.0.

After after mixing again, phase separation was obtained by centrifugation for 30 min. 700 µl of the upper layer was used for counting radioactive acetid acid. PAF-AH activity is expressed as nmol/min/ml plasma or serum.

Histological assessment of atherosclerosis

After 14 weeks of feeding the HFHC diet, mice were sacrificed after collection of blood as described above. The hearts were dissected, stored in phosphate-buffered 4% formaldehyde until processed, and embedded in paraffin. Serial cross sections of 5 µm throughout the aortic valve area were used for histological analysis. Sections were stained with hematoxylin and eosin. Per mouse 5 sections with intervals of 60 µm were used for quantification of atherosclerotic lesions. To evaluate whether PLTP has an effect on accumulation of free cholesterol in atherosclerotic lesions, the amount of sections containing free cholesterol clefts was counted in each group as described by Delsing *et al.* (25).

Statistical analysis

Data are expressed as means ± SEM. Differences were analyzed by two sample Wilcoxon rank-sum tests by using Intercooled Stata 6.0 software (Stata Corporation, College Station, TX, USA).

Results

Elevation of PLTP activity lowers plasma cholesterol

Plasma levels of total cholesterol, VLDL+LDL cholesterol and HDL cholesterol were measured in LDLR$^{+/-}$/huCETPtg, LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice, respectively (Table 1). The PLTP overexpressing mouse lines were compared with LDLR$^{+/-}$/huCETPtg mice, which are referred to as control mice throughout the present study. Overexpression of PLTP resulted in a dramatic reduction of plasma levels of total cholesterol (-50% and -70% in LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice, respectively, compared to control). This reduction in plasma cholesterol was found in both VLDL+LDL and in HDL lipoprotein classes. On a chow diet, the majority of the plasma cholesterol in is HDL, in which class the strongest reduction in cholesterol was found.

Table 1. Plasma cholesterol levels in mice fed normal chow diet.

Transgenic mouse line	LDLR+/-/huCETPtg (n = 19)	LDLR+/-/huCETPtg/huPLTPtgP4 (n = 16 - 20)	LDLR+/-/huCETPtg/huPLTPtgA2 (n = 16-18)
TC (mM)	2.7 ± 0.1	1.4 ± 0.1 [a]	0.8 ± 0.1 [a]
HDL-TC (mM)	1.7 ± 0.1	0.7 ± 0.1 [a]	0.04 ± 0.01 [a]
VLDL/LDL-TC (mM)	0.8 ± 0.1	0.4 ± 0.1 [a]	0.44 ± 0.06 [a]

CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; TC, total cholesterol. [a]Significantly different from LDLR$^{+/-}$/huCETPtg, $P < 0.001$.

Table 2. Plasma cholesterol levels in mice fed HFHC diet.

Transgenic mouse line	LDLR+/-/huCETPtg (n = 17)	LDLR+/-/huCETPtg/huPLTPtgP4 (n = 18)	LDLR+/-/huCETPtg/huPLTPtgA2 (n = 13 - 17)
TC (mM)	11.3 ± 0.8	8.3 ± 0.6 [a]	5.5 ± 1.1 [b]
HDL-TC (mM)	1.0 ± 0.1	0.3 ± 0.02 [b]	0.3 ± 0.1 [b]
VLDL/LDL-TC (mM)	10.8 ± 0.7	8.0 ± 0.7 [a]	3.4 ± 0.3 [b]

HFHC, high fat, high cholesterol. [a]Significantly different from LDLR$^{+/-}$/huCETPtg $P < 0.05$. [b]Significantly different from LDLR$^{+/-}$/huCETPtg $P < 0.001$.

Overexpression of PLTP increases susceptibilty to atherosclerosis

After the animals had been fed an HFHC diet for 14 weeks, the areas of the atherosclerotic lesions were quantified in cross sections of the aortic valves from individual mice. As shown in Figure 1A, atherosclerosis was more severe in LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice as compared with control mice, while a further increase was found in LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice.

We also evaluated whether PLTP overexpression increased the number of sections containing cholesterol clefts (Figure 1B). In control mice, cholesterol clefts were not observed. However, in mice overexpressing human PLTP cholesterol clefts were present. Moreover, atherosclerotic lesions from LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice showed more cholesterol clefts than those from LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice ($P = 0.05$). Figure 1C shows representative photographs of the atherosclerotic lesions from mice of the various genotypes.

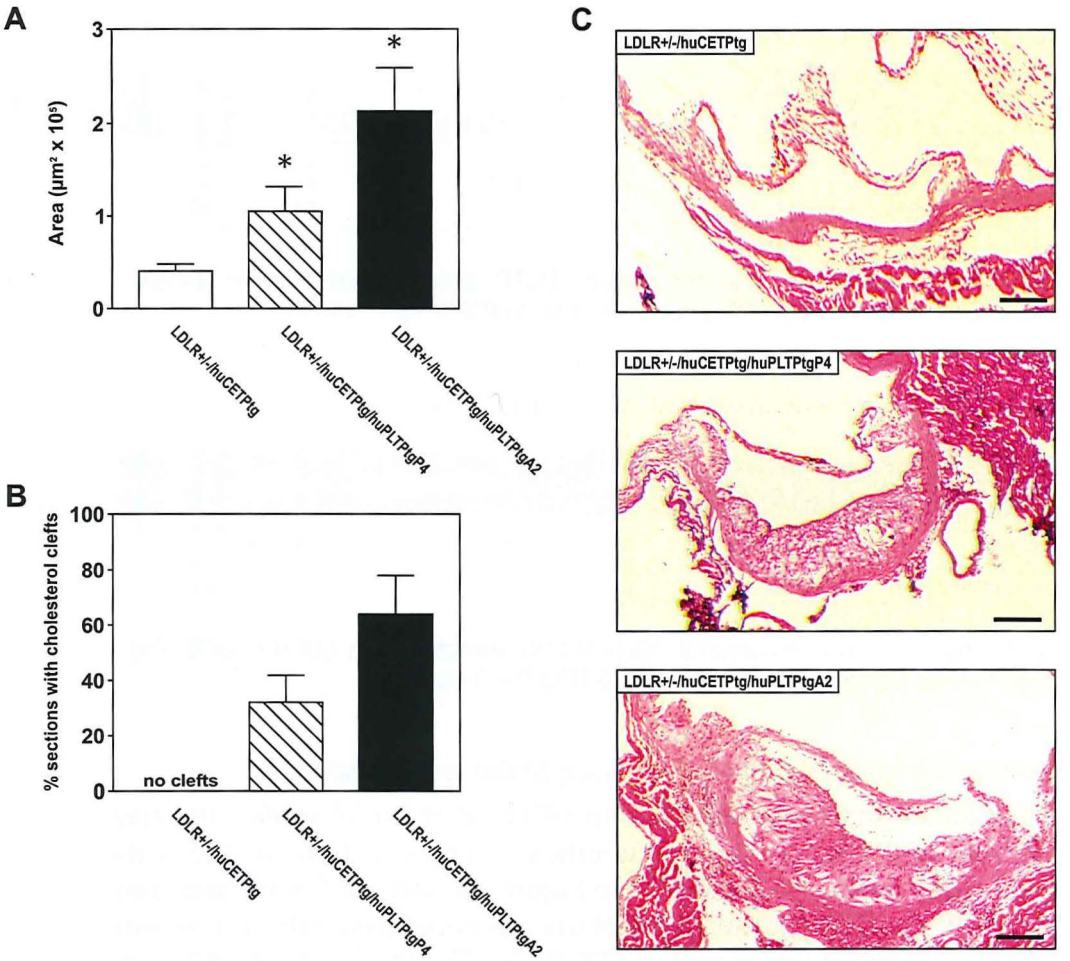

Figure 1. Atherosclerosis in transgenic mice after 14 weeks of a high-fat, high-cholesterol (HFHC) diet. Mice (n = 9 -13 per group) were sacrificed, and aortas were removed and atherosclerotic lesion areas in the aortic roots were determined as described in Materials and Methods. (A) aortic valve lesion areas; (B) percentage of sections containing free cholesterol clefts in the aortic lesions; (C) representative lesion areas of LDLR$^{+/-}$/huCETPtg, LDLR$^{+/-}$/huCETPtg/huPLTPtgP4, and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. Cholesterol clefts are particularly conspicuous in the lowest panel. Values represent means ± SEM. *$P < 0.05$ compared with LDLR$^{+/-}$/huCETPtg mice.

PLTP expression reduces diet-induced hypercholesterolemia

In order to find out why LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice have more atherosclerosis than control mice, we analyzed the levels of plasma cholesterol after feeding the animals a HFHC cholesterol diet for 14 weeks (Table 2). As expected, the diet resulted in a strong

increase of the plasma levels of total cholesterol in all three genotypes (compare Tables 1 and 2). However, overexpression of PLTP reduced total cholesterol in plasma compared with control mice. As a consequence of the diet, the bulk of the plasma cholesterol was in VLDL+LDL in all the three genotypes analyzed. A PLTP-dose dependent decrease in VLDL+LDL cholesterol was found in PLTP overexpressing mice compared to control mice (-26% and -69% in LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice, respectively). HDL-cholesterol was also strongly reduced in the PLTP overexpressing animals (-70% in both genotypes compared to control mice).
To examine the effects on lipoprotein classes in more detail, plasma samples from the various transgenic mouse lines were analyzed by gel filtration chromatography (Figure 2). The HFHC diet resulted in an increase of total plasma cholesterol in all lipoprotein fractions of all transgenic mouse lines compared with the cholesterol profiles found on chow diet (results not shown). The HFHC diet also induced a strong shift of the cholesterol profiles to the more atherogenic lipoproteins intermediate density lipoproteins and VLDLs. The effect of PLTP expression was a decrease in cholesterol in these lipoprotein fractions. The biggest effect was found in the mice with the highest plasma PLTP activity level, *i.e.* the LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. HDL cholesterol was almost absent after feeding the diet for 14 weeks in both PLTP expressing lines (Figure 2).
Therefore, overexpression of PLTP in the presence of CETP results in an elevation of diet-induced atherosclerosis in spite of markedly reduced levels of apoB-containing lipoproteins. This suggests that the effect on atherosclerosis is caused by the strong reduction in HDL. However, LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice hardly differ in HDL cholesterol levels after feeding the animals 14 weeks of the HFHC diet, while the atherosclerotic lesion size is considerably higher in the LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. Therefore, we proceeded with examining HDL associated lipid transfer and enzymatic activities.

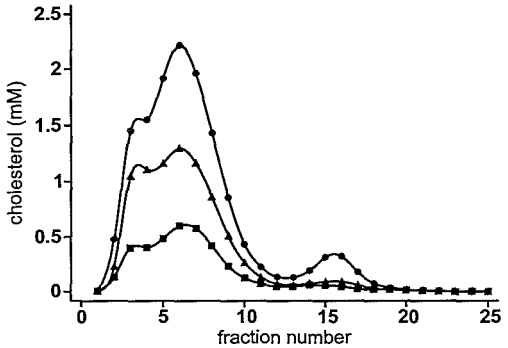

Figure 2. Distribution of lipoproteins in plasma from transgenic mice. Equal amounts of plasma from transgenic mice (n = 16-20) fed a HFHC diet for 14 weeks were pooled and subjected to gel filtration on Superose 6 and Superdex 200 columns connected in tandem as described in Materials and Methods. Fractions 1-5 contain VLDL, fractions 6-11 contain LDL, fractions 12-20 contain HDL and fractions 21-25 contain albumin. Circles, LDLR$^{+/-}$/huCETPtg mice; triangles, LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice; squares, LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice.

CETP and PLTP activities

After the animals had been fed a HFHC diet for 14 weeks, CETP activities were slightly decreased in PLTP overexpressing mice compared with control mice (Figure 3A). However, no difference was found in plasma CETP activity between mice with moderate and those with high PLTP expression. Differences in plasma PLTP activity levels were maintained after the diet period (Figure 3B).

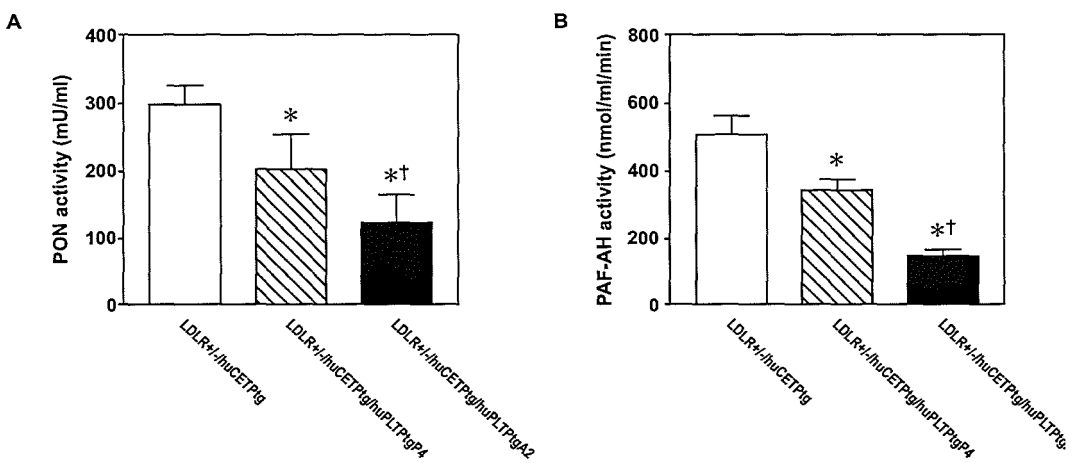

Figure 3. Cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities in plasma from transgenic mice. Plasma samples from the various transgenic mouse lines were analyzed for CETP (A) and PLTP (B) activities as described in Materials and Methods after the mice had been fed a HFHC diet for 14 weeks. Values shown are means ± SEM obtained from >13 mice per group. *P < 0.05 compared with LDLR$^{+/-}$/huCETPtg mice.

Effect of PLTP overexpression on PON and PAF-AH activities

PON and PAF-AH are HDL associated enzymes which may have anti-atherogenic properties (26, 27) . Because PLTP overexpression lowers the plasma HDL concentrations in our mice, we examined whether this might affect these enzymes as well (Figure 4). Indeed, we found that PLTP overexpression was associated with decreased activities of both PON and PAF-AH. In LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice, respectively, PON activity was 33% and 60% lower compared with control mice (Figure 4A). The reduction in PAF-AH activity was 33% and 70%, respectively (Figure 4B). The difference in activity between the two lines of PLTP expressing mice was statistically significant for both enzymes, suggesting PLTP dose-dependent lowering effects.

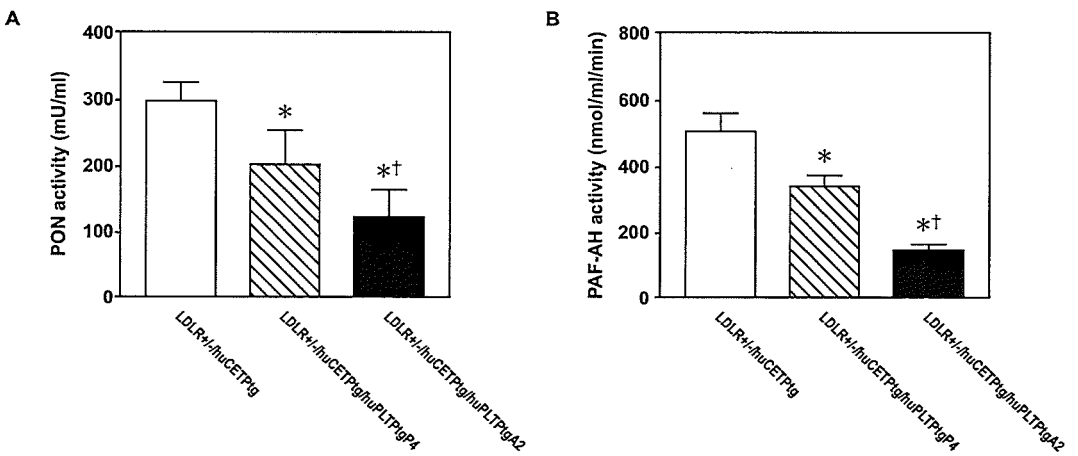

Figure 4. Anti-oxidant enzyme activities in plasma from transgenic mice. Plasma samples from the various transgenic mouse lines were analyzed for paraoxonase (PON) and platelet-activating factor acetyl hydrolase (PAF-AH) activities as described in Materials and Methods. Values shown are means ± SEM obtained from 4-7 mice per group. *$P < 0.05$ compared with LDLR$^{+/-}$/huCETPtg mice; †$P < 0.05$ compared with LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice.

Discussion

In the present study, we corroborated our previous finding that elevated PLTP activity in plasma results in increased atherosclerosis in mice (15). However, the crucial difference with the previous study is that we now used transgenic mouse models that express CETP in addition to PLTP. Increased CETP activity has been found to result in increased atherosclerosis in mice (28, 29), rats (30), and rabbits (31). We demonstrated that variations in PLTP activity modulate

the susceptibility to diet-induced atherosclerosis on top of the CETP effect. Surprisingly, the atherogenic effect of PLTP was found in spite of a decrease of plasma cholesterol in apoB-containing lipoproteins. Plasma levels of HDL were also lower in PLTP overexpressing mice. This is in agreement with what has been reported before in other mouse models with elevated PLTP activity from our as well as other laboratories (15, 32, 33). In the present study, we found that the decrease in plasma cholesterol in both lipoprotein classes studied, *i.e.* apoB-containing lipoproteins and HDL, persist after feeding the animals the HFHC diet for 14 weeks. These findings suggest that the reduction in plasma HDL affects the development of atherosclerosis to a greater extent than the reduction in apoB-containing lipoproteins.

We used two lines of transgenic mice overexpressing human PLTP to different levels. The LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice have the highest level of PLTP activity, and also have the highest level of atherosclerosis. However, the levels of plasma HDL cholesterol are similar between both lines at the end of the 14 weeks period of HFHC diet. On the other hand, on a chow diet, the LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice have lower levels of plasma HDL cholesterol than the LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice. Therefore, the variation in atherosclerosis could be caused by the difference in HDL cholesterol, even though this equals out during the diet period. The presence of a larger number of cholesterol clefts in LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice compared with LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice is indicative of a less efficient removal of cholesterol from the atherosclerotic lesions by reverse cholesterol transport, allowing the formation of extracellular cholesterol crystalline deposits, which are inert and cannot be mobilized (34). However, there could also be a qualitative difference between the HDL from the LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and the LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. Therefore, we analyzed the activity of transfer proteins and enzymes which have been found to be associated with HDL in various mouse models (17, 35, 36).

A small but statistically significant decrease in CETP activity was found in the mice expressing PLTP. This is in agreement with previous findings from our laboratory (20) and may be caused by the drop in HDL levels caused by PLTP expression, inasmuch as HDL is the carrier of CETP. As outlined above, CETP is atherogenic in various animal models. Therefore, a small decrease in CETP activity cannot explain the increase in atherosclerosis found in PLTP overexpressing animals. Moreover, CETP activity is not different between LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. As expected, the mice have different levels of plasma PLTP activity, which persist at the end of the diet period.

In addition, the activity of PON and PAF-AH were found to be decreased in PLTP overexpressing mice. Both enzymatic activities are lower in LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice than in LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 mice.

Elevation of the activity of either PON (37) or PAF-AH (38) results in decreased atherosclerosis in mice. Moreover, PON deficient mice have increased atherosclerosis (39), while decreased activities of both enzymes have been found in mouse models susceptible for atherosclerosis (36). It is likely that the decrease in PON and PAF-AH activities are related with the decrease in HDL, because in mice, these enzymes are mainly associated with HDL (35, 36), even in animals with extremely elevated plasma cholesterol in apoB-containing lipoproteins.

At present, it is not clear why overexpression of PLTP results in a decrease in VLDL+LDL cholesterol in plasma in LDLR$^{+/-}$/huCETPtg/huPLTPtgP4 and LDLR$^{+/-}$/huCETPtg/huPLTPtgA2 mice. In the absence of CETP, PLTP overexpression does not affect VLDL+LDL cholesterol levels (15). On the other hand, in mice that have no mutations in the LDLR (LDLR$^{+/+}$), VLDL+LDL cholesterol levels in huCETPtg mice and in huCETPtg/huPLTPtg mice are similar (20). Therefore, the LDLR seems to play a critical role in the effect by PLTP in CETP transgenic mice. An alternative explanation is that the effect of PLTP on apoB-containing lipoproteins exists only above a certain threshold level of these lipoproteins. Both heterozygous deficiency for the LDLR (15, 40) as well as the CETP transgene (20, 29) result in an increase in plasma levels of apoB-containing lipoproteins. Consequently, the levels of these lipoproteins are higher in the mice analyzed in the present study than in the earlier studies.

PLTP deficiency has been shown to increase the vitamin E content of VLDL and LDL, thereby protecting these lipoproteins from oxidation (41). Conversely, elevated PLTP expression might result in decreased vitamin E in VLDL+LDL and thus in a higher susceptibility to oxidative modification of these lipoproteins, resulting in a more rapid removal from the plasma compartment. This mechanism could contribute to the atherogenicity of PLTP overexpression, despite a decrease in plasma VLDL+LDL cholesterol concentration.

In conclusion, elevated expression of PLTP results in decreased plasma cholesterol in both atherogenic apoB-containing lipoproteins and in anti-atherogenic HDL. Inasmuch as the susceptibility to atherosclerosis is increased in a PLTP dose-dependent fashion, the effect on HDL apparently outweighs the effect on apoB-containing lipoproteins. The mechanism may include various atheroprotective properties of HDL, including the involvement in reverse cholesterol transport and the association with anti-oxidant enzymes.

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CHAPTER V

Elevated PLTP Causes Atherosclerosis Independent of Elevated HDL and Decreased Production

Elevated Phospholipid Transfer Protein Activity Causes Atherosclerosis through Decreased Plasma High Density Lipoproteins and not through Increased Production of Triglycerides

Jessica Lie[1], Matthijs Moerland[2], Teus van Gent[2], Rien van Haperen[2], Leo Scheek[1], Farah Sadeghi-Niaraki[1], Rini de Crom[2,3], Arie van Tol[1,2]

[1]Departments of Biochemistry, [2]Cell Biology & Genetics, and [3] Vascular Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands

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Abstract

We previously demonstrated that overexpression of human plasma phospholipid transfer protein (PLTP) in transgenic mice results in increased susceptibility to diet-induced atherosclerosis [J. Biol. Chem. 2002; 277:48938-48943]. This is in agreement with findings in PLTP deficient mice which have less atherosclerosis [Jiang *et al.*, Nat. Med. 2001; 7:847-852]. However, different mechanisms have been proposed in these two mouse models to explain the atherogenicity of PLTP. From experiments in PLTP deficient mice, it was concluded that PLTP stimulates hepatic secretion of triglycerides in apolipoprotein B-containing lipoproteins. This was confirmed in PLTP overexpressing transgenic mice, but in this model the atherogenic effect was attributed to the observed decline in high density lipoproteins (HDL). In order to find evidence to relate the increased atherosclerosis in PLTP transgenic mice to one of these atherogenic effects, a comparison was made between male and female mice with relatively modest overexpression of PLTP, as the development of atherosclerosis is known to be sex-dependent in most mouse models. Indeed, after feeding the animals an atherogenic diet atherosclerosis was about 7-fold higher in female mice than in male mice, while PLTP activity levels were virtually identical. Also, the rates of hepatic secretion of triglycerides were identical in male and female mice. In contrast, plasma levels of HDL were about 2-fold lower in female mice than in male mice after feeding an atherogenic diet. We conclude that overexpression of PLTP in transgenic mice causes atherosclerosis through a decrease in HDL, rather than through elevated hepatic secretion of triglycerides.

Introduction

Atherosclerosis is the leading cause of death and morbidity in industrialized countries (1-3). It is generally accepted that an elevated level of plasma cholesterol is an important risk factor for atherosclerosis. While the level of cholesterol in low density lipoproteins (LDL) correlates positively with the incidence of atherosclerotic disease, cholesterol in high density lipoproteins (HDL) appears to protect against the development of atherosclerosis (1, 2, 4). HDL is thought to have several anti-atherogenic properties. First, HDL is involved in the reverse cholesterol transport pathway, by which excess cholesterol is transported back to the liver for excretion (5-7). In addition, HDL has both anti-inflammatory and anti-oxidant properties (4, 8-10). One of the proteins involved in HDL metabolism is plasma phospholipid transfer protein (PLTP)(11). We previously demonstrated that transgenic mice overexpressing human PLTP to various activity levels are more prone to the

development of diet-induced atherosclerosis in a PLTP-dose dependent way (12). Concomitantly, the plasma level of HDL was decreased. We concluded that the increase in atherosclerosis is caused by this decrease in plasma HDL. However, in addition to an effect on HDL, PLTP has an effect on the production of very low density lipoproteins (VLDL), which was first described in PLTP deficient mice (13). The decreased atherosclerosis found in these mice was explained by decreased synthesis of apoB-containing lipoproteins by the liver. We examined whether elevated PLTP activity levels would affect hepatic VLDL secretion. Indeed, PLTP overexpressing mice were found to have an increase in the synthesis of apoB-containing lipoproteins (12, 14), which could contribute to the increased susceptibility to atherosclerosis found in these mice. Thus, elevated PLTP has two atherogenic effects on lipoprotein metabolism, *i.e.* increasing hepatic VLDL secretion and decreasing plasma levels of HDL. In order to find out which of these mechanisms is the most importa nt one for the development of atherosclerosis, we first determined whether male and female mice with similar levels of overexpression of PLTP differ in their susceptibility to atherosclerosis. Sex differences have been frequently found in mouse models, including the commonly used low density lipoprotein receptor (LDLR) deficient mice and apolipoprotein (apo) E deficient mice (15, 16). The present data show that the susceptibility to diet-induced atherosclerosis is sex-dependent in PLTP overexpressing mice, enabling us to relate these differences with the effects on apoB-containing lipoproteins versus the effects on HDL. We used transgenic mouse models which express also human cholesteryl ester transfer protein (CETP) (14, 17). CETP is normally not expressed in mice, but is involved in the same pathways of lipoprotein metabolism as PLTP and may be crucially involved in the process of atherosclerosis in man (18, 19).

Materials and Methods

Animals

The human PLTP transgenic mice (huPLTPtg) were described before (line P1;(12, 20)). LDL receptor knockout mice were purchased from Jackson Laboratory. Human CETP transgenic mice (huCETPtg) were kindly provided by Dr. A.R. Tall (Columbia University, New York). All mice were in C57BL/6J background for at least 8 generations. $LDLR^{+/-}$/huCETPtg and $LDLR^{+/-}$/huCETPtg/huPLTPtg mice were created by crossbreeding. After weening, animals were kept on a chow diet (Hope Farms, The Netherlands). For the induction of atherosclerosis, mice were fed a high fat high cholesterol (HFHC) diet for 14 weeks, which contained 40% w/w sucrose 15% w/w fat, 1% w/w

cholesterol and 0.5% w/w sodium cholate (Hope Farms, The Netherlands). Mice were 12 weeks old at the beginning of the diet studies. Animals were housed under standard conditions and had free access to water and food. After fasting overnight, blood samples were collected from the orbital plexus by using Vitrex™ sodium-heparinized micropipettes (80 IU) (Modulohm A/S, Copenhagen, Denmark) and immediately stored on ice. Blood was centrifuged at 2700 rpm for 15 min at 4 °C. Plasma was either used directly or stored in small aliquots at -80 °C before analysis. All experiments were performed according to national and institutional guidelines.

Separation of plasma lipoproteins by density gradient centrifugation

Lipoprotein fractions used in Table 1 were collected following ultracentrifugation in a Beckman 42.2 Ti rotor (42000 rpm, 3 h, 12 °C). Alternatively, the lipoprotein fractions used in Figure 2 were obtained by density gradient ultracentrifugation of plasma samples in a Beckman SW60 Ti rotor (36500 rpm, 21 h 12 min, 12 °C) as described (21).

Quantification of cholesterol

Total plasma cholesterol (TC) was determined enzymatically with the Free Cholesterol C kit no. 274-47109 (WAKO, Neuss, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer, Mannheim, Germany).

Plasma activity assays

CETP and PLTP activity assays were performed as described before (20). The activities are expressed as arbitrary units (AU); 1 AU is the activity found in human reference pool plasma. The activities are: CETP 216 nmol/ml/h; PLTP 14 μmol/ml/h.

In vivo hepatic VLDL secretion

VLDL secretion experiments were performed using the Triton WR1339 method, as described (14).

Histological assessment of atherosclerosis

After 14 weeks of HFHC diet feeding, mice were sacrificed after blood collection as described in the animal section. The hearts were dissected, stored in phosphate buffered 4% formaldehyde until processed. Atherosclerotic areas in the aortic valves were quantified by computer assisted measurements as described (12, 22). To evaluate whether PLTP has an effect on accumulation of free cholesterol in atherosclerotic lesions, the presence of cholesterol clefts in the sections was evaluated as described (23).

Statistical analysis

Data are expressed as means ± SEM. Differences between two groups of mice were analyzed by two sample Wilcoxon rank-sum tests by using Intercooled Stata 6.0 software (Stata Corporation, College Station, TX, USA).

Results

Levels of plasma cholesterol

Throughout the present study, comparisons are made between LDLR$^{+/-}$/huCETPtg and LDLR$^{+/-}$/huCETPtg/huPLTPtg mice of both sexes. As shown in Figure 1, there is no difference in the plasma level of cholesterol (total cholesterol) between male and female LDLR$^{+/-}$/huCETPtg mice. In contrast, in LDLR$^{+/-}$/huCETPtg/huPLTPtg mice plasma cholesterol is somewhat higher in females than in males. Plasma cholesterol is lower in the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice compared with LDLR$^{+/-}$/huCETPtg mice in either sex. HDL was separated from VLDL+LDL via ultracentrifugation in order to study the distribution of cholesterol between the lipoproteins (Table 1). Plasma levels of HDL-cholesterol are decreased in both male and female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice when compared to LDLR$^{+/-}$/huCETPtg mice. The difference is –35% for the male mice and –44% for the female mice. The concentrations of HDL-cholesterol are not different between males and females of either genotype. In mice fed a regular chow diet, a minor fraction of plasma cholesterol is in VLDL+LDL. Only small differences were found between the various groups. After feeding the mice a HFHC diet, the plasma levels of VLDL+LDL cholesterol are greatly increased (Table 1). There are no statistically significant differences between males and females of either genotype. The VLDL+LDL cholesterol levels are somewhat lower in plasma from LDLR$^{+/-}$/huCETPtg/huPLTPtg mice when compared to LDLR$^{+/-}$/huCETPtg mice, but this difference was not statistically significant for the females. In contrast, striking differences were found in HDL cholesterol levels. These were about 2-fold higher in male mice than in female mice in either genotype. In both sexes, LDLR$^{+/-}$/huCETPtg/huPLTPtg mice have lower levels of plasma HDL cholesterol than LDLR$^{+/-}$/huCETPtg mice. The lowest concentration of plasma HDL cholesterol was found in female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. In a separate experiment, the relative distribution of VLDL/IDL, LDL and HDL cholesterol was measured in larger samples of pooled plasma from mice fed a HFHC diet (Figure 2). This distribution is not different between male LDLR$^{+/-}$/huCETPtg versus LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. In female mice however, LDLR$^{+/-}$/huCETPtg/huPLTPtg mice have relatively less HDL and more apoB-containing lipoproteins in comparison with LDLR$^{+/-}$/huCETPtg mice.

Table 1. Plasma levels of lipoprotein cholesterol on chow and HFHC diet.

genotype	LDLR+/-/huCETPtg		LDLR+/-/huCETPtg/huPLTPtg	
sex	male	female	male	female
CHOW diet				
VLDL+LDL-C	0.8 ± 0.1	1.1 ± 0.1§	0.6 ± 0.1*	0.8 ± 0.1*§
HDL-C	1.7 ± 0.1	1.8 ± 0.1	1.1 ± 0.1*	1.0 ± 0.1*
HFHC diet				
VLDL+LDL-C	9.9 ± 1.3	8.2 ± 0.9	7.3 ± 0.8	6.9 ± 0.7
HDL-C	1.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.3 ± 0.03

*P < 0.001, LDLR$^{+/-}$/huCETPtg *versus* LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. §P < 0.001, male *versus* female. Cholesterol concentrations are in mM. n = 14-20 per group.

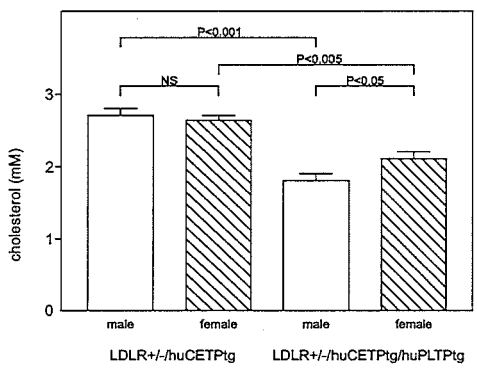

Figure 1. Plasma concentration of total cholesterol in transgenic mice on chow diet. Measurements represent means ± SEM of 14-20 mice.

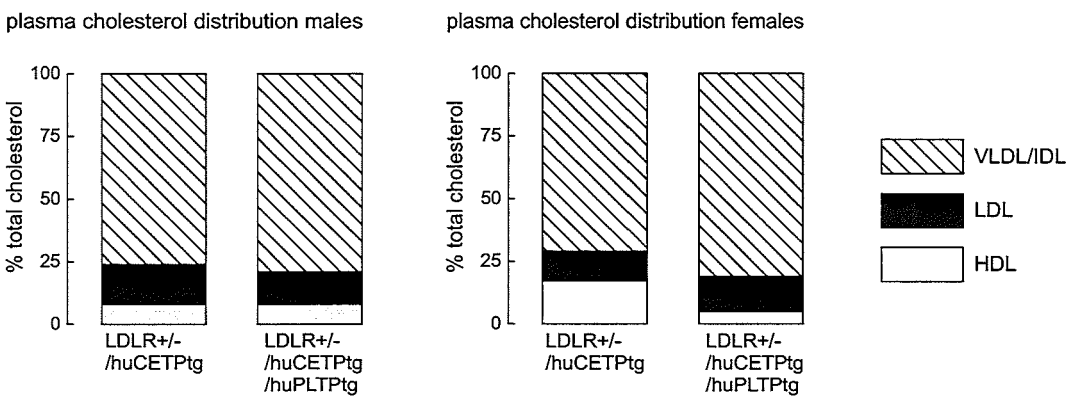

Figure 2. Distribution of plasma cholesterol between lipoprotein classes in transgenic mice fed a HFHC diet. Lipoproteins were by separated by density gradient ultracentrifugation of plasma samples in a Beckman SW60 Ti rotor (36500 rpm, 21 h 12 min, 12 °C). Data represent measurements in pooled plasma samples from 14-20 mice.

VLDL triglyceride secretion

PLTP is known to stimulate hepatic secretion of VLDL (13, 14). Therefore, a comparison was made between male and female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice in a set of experiments investigating the hepatic secretion of VLDL triglycerides by the Triton WR1339 method (Figure 3A). No significant differences between males and females are observed. After correction for body weight, the rates of VLDL secretion between these two groups are identical (males: 195 ± 40 μmol/kg/h; females: 195 ± 48 μmol/kg/h). In order to check whether the atherogenic diet could possibly induce a sex-specific difference, VLDL secretion was also measured in animals fed the HFHC diet. As shown in Figure 3B, the rate of VLDL secretion is considerably lower in these animals than in animals fed a chow diet. However, there is no statistically significant difference in the VLDL secretion rates after correction for body weight between males and females (males 69±9 μmol/kg/h; females 54±6 μmol/kg/h; $P = 0.18$).

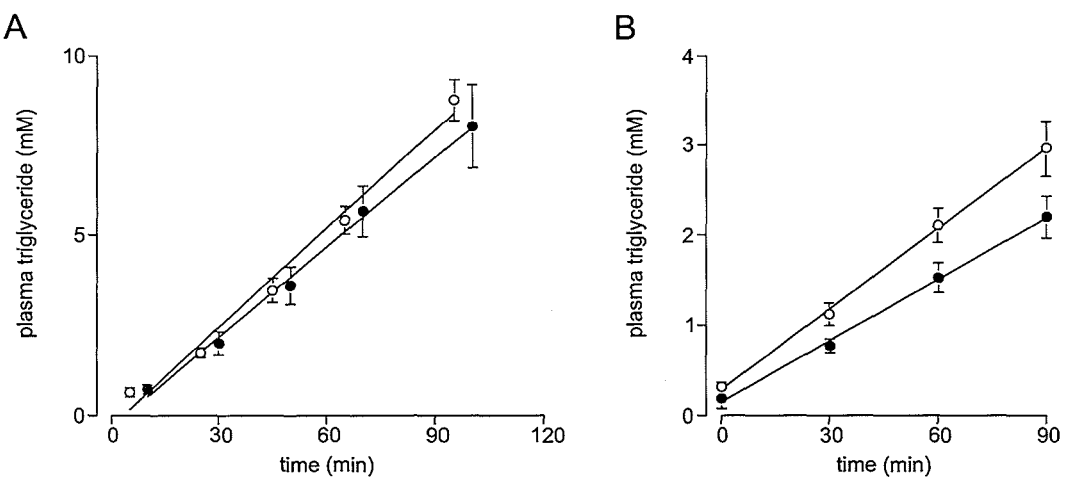

Figure 3. Hepatic secretion of triglycerides in transgenic mice fed a chow diet (A) or an HFHC diet (B). Plasma levels of triglycerides were measured at the indicated times in male (open circles) and female (closed circles) LDLR$^{+/-}$/huCETPtg/huPLTPtg mice after administration of Triton WR 1339. Measurements represent means ± SEM of 7 (A) or 10 (B) mice per group.

Plasma activities of CETP and PLTP

Figure 4 shows plasma CETP and PLTP activities in male and female mice fed a HFHC diet for 14 weeks. While the plasma activities of CETP and PLTP in mice expressing either only PLTP or the combination of the two proteins have been described in previous studies from our laboratory (14, 17), we did not investigate possible sex-related differences. As expected, PLTP activity is much higher in LDLR$^{+/-}$/huCETPtg/huPLTPtg mice than in LDLR$^{+/-}$/huCETPtg

mice. PLTP activity is not different between males and females from either genotype (Figure 4A). CETP activity is identical in male LDLR$^{+/-}$/huCETPtg mice and male LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. In female mice, CETP activity is slightly increased in LDLR$^{+/-}$/huCETPtg/huPLTPtg mice *versus* LDLR$^{+/-}$/huCETPtg mice. CETP activity is lower in females than in males of either genotype (Figure 4B). The HFHC diet causes a small increase in PLTP activity, but a strong induction of CETP activity in mice of both genotypes and of both sexes (PLTP activity: 124 ± 6%, $P < 0.05$; CETP activity: 344 ± 13%, $P < 0.001$; results not shown).

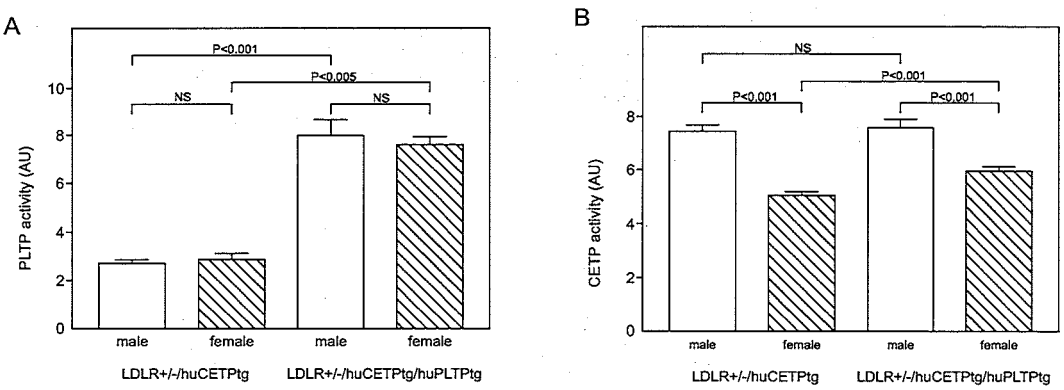

Figure 4. Activities of CETP and PLTP in plasma from transgenic mice fed a HFHC diet. (A) PLTP activities. 1 AU is equal to 14 μmol/ml/h. (B) CETP activities. 1 AU is equal to 216 nmol/ml/h. Data represent means ± SEM of 17-19 mice.

Atherosclerosis

Diet-induced atherosclerosis was evaluated by measuring the atherosclerotic area in the aortic root (Figure 5A). Male mice show small lesion areas that are similar in size between the two genotypes. In contrast, female mice develop substantial lesion areas in both genotypes. When comparing sexes, female mice show 3.4-fold LDLR$^{+/-}$/huCETPtg mice) and 7.0-fold (LDLR$^{+/-}$/huCETPtg/huPLTPtg mice) larger lesion areas than male mice (both $P < 0.001$) (Figure 5A). PLTP overexpression in female mice results in a 1.8-fold further increase ($P < 0.005$) in aortic lesion area. In addition, female mice overexpressing human PLTP show more severe plaques with larger numbers of cholesterol clefts (Figure 5B). In male mice, the severity of the plaque appears higher too, as no cholesterol clefts are observed in LDLR$^{+/-}$/huCETPtg mice, while a modest percentage of the plaques in the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice do contain cholesterol clefts. Representative images are shown in Figure 6.

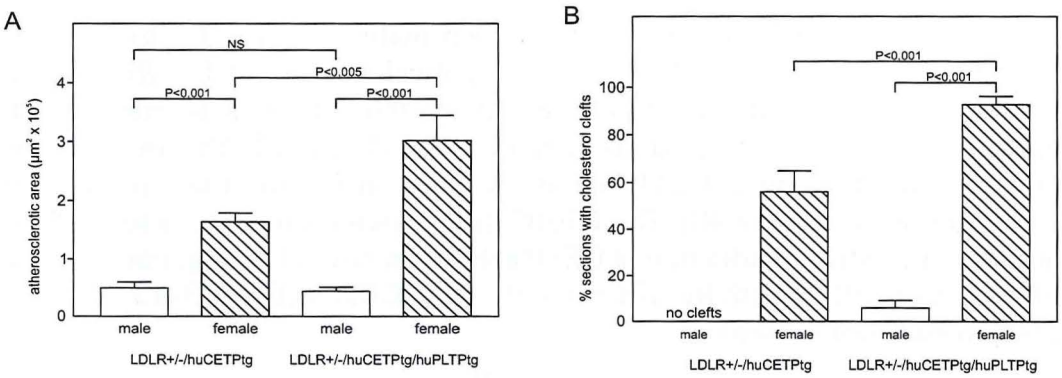

Figure 5. Atherosclerosis in transgenic mice fed a HFHC diet. (A) Atherosclerotic lesion areas were measured in 5 sections per animal from the aortic root. (B) Percentage of sections containing free cholesterol clefts in the aortic lesion. Data represent means ± SEM of 11-18 mice.

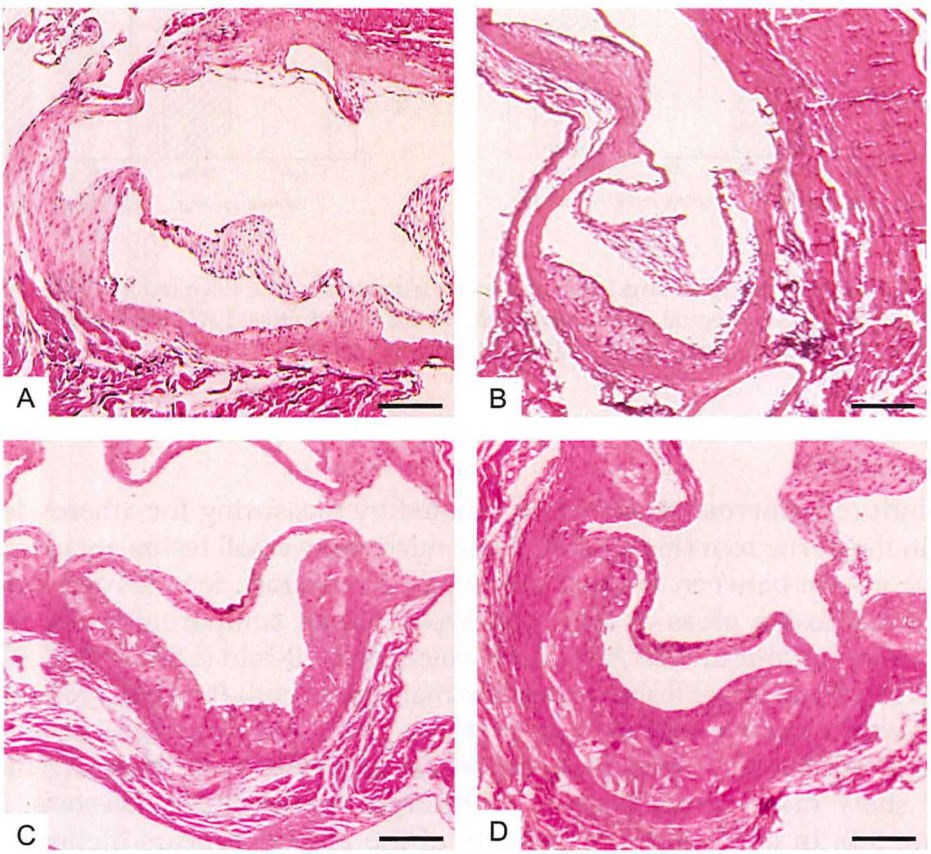

Figure 6. Atherosclerotic lesions in the aortic valves. Photomicrographs showing representative atherosclerotic lesions in aortic valves. (A) male LDLR$^{+/-}$/huCETPtg mice; (B) male LDLR$^{+/-}$/huCETPtg/huPLTPtg mice; (C) female LDLR$^{+/-}$/huCETPtg mice; (D) female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. Scale bars: 200 µm.

Discussion

In the present study we investigated whether a sex difference in susceptibility to diet-induced atherosclerosis exists in PLTP transgenic mice, and whether such a difference would be indicative for the mechanism involved in the increased development of atherosclerosis in PLTP overexpressing mice.
Indeed, more atherosclerosis was found in female than in male mice in the control group (LDLR$^{+/-}$/huCETPtg mice). These result are in agreement with those from Arai *et al.*(16), who compared atherosclerosis between males and females in LDLR$^{+/-}$ mice. A similar difference of about 3-fold in atherosclerotic lesion area was found in both studies.
Higher susceptibility to diet-induced atherosclerosis in female mice has been reported by several groups (15, 16, 24-28). The reason for this observation is unclear, but the genetic background of the mice is important (29). The mice used in the present study are all in C57BL/6J background, which is the most susceptible strain for diet-induced atherosclersosis.
When LDLR$^{+/-}$/huCETPtg mice are compared with LDLR$^{+/-}$/huCETPtg/huPLTPtg mice, the atherosclerotic lesion areas are not different in male mice. In a previous report studying male mice with various levels of PLTP overexpression, we demonstrated that the development of atherosclerosis was PLTP-dose dependent (12). However, in the line with the lowest level of PLTP overexpression the difference in atherosclerotic lesion formation with the controls did not reach statistical significance (12). This is in agreement with our current findings, in which the same line of PLTP transgenic mice has been used for the generation of the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. Both the stimulation of VLDL secretion and the decrease of plasma HDL-cholesterol levels were statistically significant in these mice (12). In the present study, the effect on plasma HDL is confirmed in the male mice. Although there is no obvious increase of the atherosclerotic area, the number of lesions with cholesterol clefts is increased. Taken together, these results suggest that a moderate overexpression of PLTP is atherogenic in male mice, although the effect is too small to be detected unambiguously in the mouse models used.
In contrast, the atherogenic effect of increased PLTP is very obvious in female mice (Figure 5A: $P < 0.005$ in LDLR$^{+/-}$/huCETPtg mice *versus* LDLR$^{+/-}$/huCETPtg/huPLTPtg mice). Thus, under circumstances where the susceptibility to atherosclerosis is increased, in this case by female sex, the atherogenicity of elevated PLTP becomes much more pronounced and therefore easier to detect.
This situation offered the opportunity to evaluate the contribution of two known atherogenic effects of PLTP on lipoprotein metabolism, *i.e.* the stimulatory effect on hepatic VLDL secretion and the lowering effect on plasma HDL. The

rate of VLDL secretion in LDLR$^{+/}$/huCETPtg/huPLTPtg mice was similar in male and female mice, either on chow or on HFHC diet. The female mice even tended to have a lower VLDL triglyceride secretion rate on HFHC diet, but this difference was not statistically significant. VLDL secretion is considerably less on HFHC diet when compared to the situation on chow diet, which is caused by the HFHC diet, which causes high hepatic cholesterol levels resulting in cholesterol-rich and triglyceride-poor VLDL (30). Female LDLR$^{+/}$/huCETPtg/huPLTPtg mice had the lowest levels of plasma HDL after feeding of the HFHC diet. This is true for both the absolute concentration and the relative amount of HDL cholesterol. Therefore, we conclude that the decrease in plasma HDL has a more substantial contribution to the atherogenicity of elevated PLTP than the stimulation of VLDL secretion.

In a comparison of the atherosclerotic lesion areas with published data (12, 16), it appears that in the present study the contribution of the CETP transgene to the development of atherosclerosis is not substantial. This is in agreement with previous findings from our group (31), and also with earlier reports from others in which the same line of CETP transgenic mice was used to study atherosclerosis in a background of either LDLR deficiency or apoE deficiency (32), showing that a moderate increase in atherosclerosis was found only when several points of time were taken into account, but not for any individual time. The HFHC diet resulted in a marked increase in CETP activity, as reported before (32, 33). However, it is likely that CETP activity levels have to be increased much stronger in order to find a clear atherogenic effect, as has been done in transgenic mice overexpressing simian CETP (34) and in transgenic rats overexpressing human CETP (35). Still, from all these studies CETP emerges as an atherogenic protein, which is further corroborated by the finding that CETP inhibition reduces atherosclerosis in rabbits (36). Therefore, the lower CETP activity in female mice compared to male mice after HFHC diet found in the present study is probably not related to the differences observed in the susceptibility to atherosclerosis.

In conclusion, we report that female mice with moderate overexpression of PLTP are more prone to the development of diet-induced atherosclerosis than male mice due to low levels of plasma HDL. HDL has several anti-atherogenic properties, including a key role in reverse cholesterol transport as well as anti-oxidant and anti-inflammatory properties. The relative importance of these different mechanisms is presently unkown.

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CHAPTER VI

Fenofibrate Restores the Decline in HDL in Mice Overexpressing PLTP

Fenofibrate Restores the Decline in HDL Cholesterol in Mice Overexpressing Human Phospholipid Transfer Protein

Jessica Lie[1], Inge M. Lankhuizen[1], Barbara Gross[2], Teus van Gent[1], Rien van Haperen[3], Leo Scheek[1], Bart Staels[2], Rini de Crom[3,4] and Arie van Tol[1,3]

[1]Department of Biochemistry, Erasmus University Medical Center, Rotterdam, the Netherlands.

[2]U545 INSERM – Dépt. d'Athérosclérose, Institut Pasteur de Lille, and Faculté de Pharmacie, Univ. de Lille II, Lille, France.

[3]Department of Cell Biology and Genetics, Erasmus University Medical Center, Rotterdam, the Netherlands.

[4]Department of Vascular Surgery, Erasmus University Medical Center, Rotterdam, the Netherlands.

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Abstract

Objective: Overexpression of human phospholipid transfer protein (PLTP) results in decreased plasma HDL levels and increased diet-induced atherosclerosis. Treatment with fibrates increases PLTP expression in mice. In humans, fibrates raise HDL cholesterol and lower plasma triglycerides. Our aim was to find out whether fibrate treatment affects human PLTP expression, plasma PLTP activity and HDL levels in human PLTP transgenic mice.
Methods and Results: Treatment with different fibrates did not influence PLTP mRNA levels in human hepatocytes. Hepatic human PLTP mRNA was not affected by fenofibrate treatment in two different human PLTP transgenic mouse lines tested, while PLTP activity was only slightly elevated. In wild-type mice however, fenofibrate elevated PLTP mRNA, resulting in a more than 4-fold increased PLTP activity. Plasma triglycerides were reduced in all mice by 48% or more by fenofibrate treatment. HDL-cholesterol concentrations were substantially increased by fenofibrate in PLTP overexpressing mice (+ 167 % in line A3 and +72 % in line P4), but unaffected in wild-type mice.
Conclusions: Fenofibrate treatment restores the HDL-lowering effect of PLTP overexpression in human PLTP transgenic mice. These results suggest that species differences in PLTP regulation contribute to the differences in HDL response upon fibrate treatment.

Introduction

A number of epidemiological studies have documented an inverse relationship between high density lipoprotein (HDL) levels and cardiovascular diseases (1, 2). Phospholipid transfer protein (PLTP) is a key player in HDL metabolism (3) and modulates HDL size and composition via remodelling of HDL particles (4-6). PLTP also facilitates the transfer of phospholipids, vitamin E and possibly unesterified cholesterol from triglyceride-rich lipoproteins to HDL particles during lipolysis (7, 8). In addition, PLTP may be involved in HDL-mediated cellular efflux of phospholipids and cholesterol (9, 10), an early step in reverse cholesterol transport, as well as in the excretion of cholesterol from the body (11). Although the latter effects are potentially anti-atherogenic, PLTP is atherogenic in mouse models and has been described as a risk factor for atherosclerosis in humans (12-15). The pro-atherogenic actions of PLTP may, at least in part, be attributed to its HDL-lowering effect (12, 14, 16).
Fibrates constitute a class of drugs which reduce plasma triglycerides and increase plasma HDL cholesterol in humans (17, 18). These effects are related to the activation of the transcription factor peroxisome proliferator-activated receptor alpha (PPARα) (18). PPARα mediates fibrate action on HDL levels directly through the transcriptional activation of apo AI and apo AII, the major apolipoproteins of HDL (19, 20). Fibrates also increase lipolytic activity via

PPARα resulting in lower plasma triglyceride levels (18), which may indirectly lead to increased HDL levels due to lower rates of cholesterylester transfer out of HDL as a consequence of the decreased plasma VLDL triglycerides (21). Several intervention trials have shown that fibrate treatment leads to a reduction of coronary events and a delay in the progression of coronary atherosclerosis (22, 23).
In mice, fenofibrate treatment does not result in an increase in plasma HDL cholesterol (24) and the activity of plasma PLTP is highly increased (25). The latter effect has to be considered atherogenic (14). However, *in vitro* studies, using the human PLTP promoter, have suggested that human PLTP promoter activity might be reduced rather than stimulated by fenofibrate (26). The effect of fibrates on the *in vivo* regulation of PLTP has never been studied in man. Because of the potential of fibrates to prevent atherosclerosis and coronary heart disease and of PLTP as a risk factor, it is important to understand the regulation of PLTP by these drugs. Among other factors, differences in PLTP regulation could contribute to the species differences in HDL response after fibrate treatment. To test this hypothesis, we first studied the effect of different fibrates on human PLTP gene expression in cultured primary human hepatocytes. Because effects on PLTP mRNA were not apparent in these *in vitro* studies, we subsequently studied PLTP regulation by fenofibrate *in vivo* in mice and compared the effects of fenofibrate feeding in wild-type mice with those in two lines of mice, one line expressing human PLTP under the control of its native promoter and one line with a heterologous, hepatocyte-specific promoter.

Materials and Methods

Mice

The human PLTP transgenic mice (C57BL/6J background) were described before (lines A3 and P4 (14)). C57BL/6J mice were used as control wild-type mice. Male mice were used for all experiments. The animals were 18 to 20 weeks old at the beginning of the study. The mice were fed either regular chow or fenofibrate-containing chow ad libitum for two weeks. Fenofibrate was mixed in the regular mouse chow at 0.2% (w/w) (25). Blood samples were collected by orbital bleedings after fasting the animals overnight. Plasma was obtained using a refrigerated centrifuge. All procedures in this study were approved by institutional and national guidelines.

Human hepatocytes

Human hepatocytes isolated from 5 independent donors were obtained as previously described (27). The cells were incubated with 200 μM of gemfibrozil or 200 μM of fenofibric acid for 48 h.

Plasma activity assays

PLTP activity assays were performed with exogenous substrates, using the liposome vesicles-HDL system, according to Speijer *et al.* (28). LCAT activity was determined by measuring the formation of radiolabeled cholesteryl ester after addition of mouse plasma to excess heat-inactivated plasma containing ^{3}H-cholesterol (29). Both activities are expressed as percentage of the activity in a reference pool of human plasma. 100% is equivalent to the following activities: PLTP: 17.7 µmol/ml/h, LCAT: 103.3 nmol/ml/h.

Gene expression analyses

RNA was isolated from the cultured hepatocytes and the expression of PLTP was measured by real time PCR on a MX4000 (Stratagene), as previously described (27), using specific primers for human PLTP : 5'-CAT GCT GCA GAT TGG GGT GAT-3' and 5'-ACG GTG TGG GGG CAG TGG AC-3' and human L-CPT1: 5'-ACA GTC GGT GAG GCC TCT TAT GAA-3' and 5'-TCT TGC TGC CTG AAT GTG AGT TGG-3'.
PCR amplification was performed in a volume of 25 µl containing 100 nM of each primer, and the Brilliant SYBR Green QPCR Master mix as recommended by the manufacturer (Stratagene). The conditions were 95°C for 10 min, followed by 40 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C. PLTP and CPT1 mRNA levels were subsequently normalized to 28S RNA.
Total RNA was prepared from frozen livers of mice by the Ultraspec RNA isolation reagent from Biotecxs Laboratories Inc (Houston, TX) and used as a template for reverse transcription primed by oligo(dT). RNA expression was analyzed by semi-quantitative PCR.
The primer sequences of the sense and antisense were; 5'-GCTGAAGCTA GAGGCCACG-3' and 5'-GCATCACTCCGATTTGCAGC-3' for mouse PLTP; 5'- CCTGCTGAGCCCAGCAGTG-3' and 5'-CTGGACCTCAGGCTGGT CTG-3' for human PLTP; 5'- CCATCAAGGCCATGCGGATC-3' and 5'-CGAGACTGAAGAAACATGTGC-3' for mouse LCAT; 5'-CGAAGTGTTGGATACAGGCC-3' and 5'-GGCAACATCAACAGGACTCC-3' for mouse hypoxanthine guanine phosphoribosyltransferase (mHPRT). PCR products were run on agarose gels, visualized with ethidium bromide staining and quantified.

Quantification of plasma lipids

Cholesterol was determined enzymatically with the F-chol kit of WAKO (Neuss, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer, Mannheim, Germany). Triglycerides were measured with the Sigma GPO-Trinder kit no.337-B (Sigma, St Louis, MO, USA).

Separation of plasma lipoproteins by density gradient ultracentrifugation

Lipoprotein fractions were collected following ultracentrifugation in a Beckman 42.2 Ti rotor (42000 rpm, 3 h, 12 °C) at $d = 1.063$ g/ml. Two fractions were collected for cholesterol determination by tube slicing: a top fraction containing very low density lipoproteins (VLDL) + low density lipoproteins (LDL) and a bottom fraction containing HDL.

Statistical analyses

Data are expressed as means ± SD. Differences between mice fed control chow and fenofibrate-containing chow were analyzed by two sample Wilcoxon rank-sum tests. Differences between the various mouse lines were analyzed by ANOVA, followed by Bonferoni correction. The tests were performed using Intercooled Stata 6.0 software (Stata Corporation, College Station, TX, USA).

Results

Effect of fibrate treatment on PLTP expression in primary human hepatocytes

Human hepatocytes were treated with 200 μM gemfibrozil or 200 μM fenofibric acid in the medium for 48 h, RNA was isolated and the expression of PLTP was measured by real time PCR. There was no difference in PLTP expression of the treated and non-treated cells (Figure 1). Expression of the CPT1 gene, which was measured as a positive control, was increased by 1.55 ± 0.22 and 1.95 ± 0.27 fold respectively in the gemfibrozil and fenofibric acid treated cells.

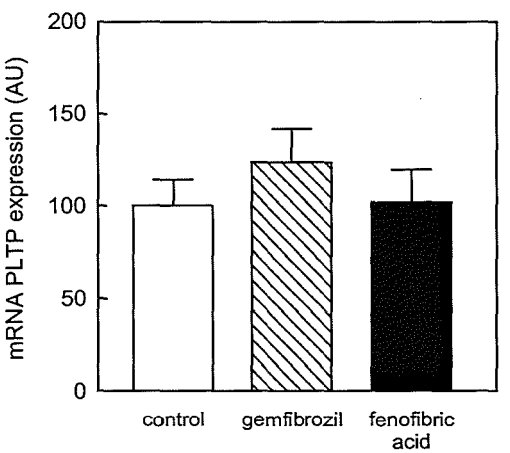

Figure 1. Fibrates do not affect PLTP mRNA levels in human primary hepatocytes. Human primary hepatocytes were treated with gemfibrozil (200 μM; hatched bar) or fenofibric acid (200 μM; black bar). Data were standardized with 28S and the expression in control cells were set to 100%. Values represent means of 5 independent donor preparations ± SD.

Effect of fenofibrate feeding on hepatic PLTP and LCAT mRNA levels in human PLTP transgenic mice

Next, it was tested whether fenofibrate regulates human PLTP *in vivo*. Therefore two lines of transgenic mice that overexpress human PLTP were treated with fenofibrate. Mice from line P4 expresses human PLTP under the control of its native promoter, while mice of line A3 mice express human PLTP under the control of the albumin promoter and enhancer (14). Therefore, with the use of these two lines it would be possible to discriminate between transcriptional and post-transcriptional effects of fenofibrate. Hepatic mRNA levels of PLTP and LCAT were determined by semi-quantitative PCR (Figure 2). The level of hepatic mouse PLTP mRNA was clearly increased by fenofibrate in all groups. By contrast, human PLTP and mouse LCAT mRNA levels were not regulated by fenofibrate treatment.

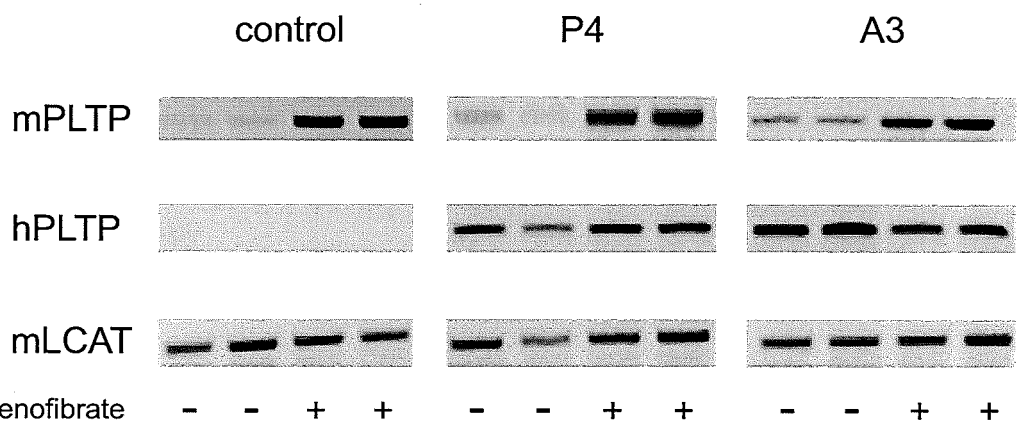

Figure 2. Fenofibrate increases hepatic murine, but not human PLTP expression. Hepatic mRNA levels were determined in wild-type and PLTP overexpressing mice fed control diet (-) or fenofibrate diet (+) for mPLTP, hPLTP and mLCAT. Control: wild-type mice; P4: transgenic mice expressing human PLTP under the control of its native promoter; A3: transgenic mice expressing human PLTP under the control of the albumin promoter and enhancer (14).

Effect of fenofibrate treatment on plasma PLTP and LCAT activities in human PLTP transgenic mice

The effect of fenofibrate feeding on plasma PLTP activity was measured in wild-type mice and two lines of PLTP transgenic mice (Table 1). In addition, the activity of plasma LCAT was measured (Table 1). As expected, plasma PLTP activities were higher in mice overexpressing human PLTP compared with wild-type mice. PLTP activities were clearly elevated in plasma from wild-type mice after fenofibrate treatment (4.4-fold, $P < 0.05$). By contrast, PLTP activity was only marginally increased by fenofibrate in mice overexpressing human PLTP. In line P4, a modest increase of 1.2-fold was found, which was

not statistically significant. The A3 mice showed a 1.3-fold increase. Plasma LCAT activities were increased (about 1.5-fold, $P < 0.05$) in mice overexpressing human PLTP fed normal chow versus wild-type control mice. This effect of the transgene on LCAT activity was absent after feeding fenofibrate. However, fenofibrate feeding caused an increase in LCAT activity in all mouse lines tested ($P < 0.05$).

Table 1. Plasma activity levels of PLTP and LCAT in wild-type and human PLTP transgenic mice.

	Control diet		Fenofibrate diet	
	PLTP	LCAT	PLTP	LCAT
wild-type	209 ± 12	38 ± 3	924 ± 182*	70 ± 14*
P4	1594 ± 319†	54 ± 3†	1856 ± 161†	73 ± 11*
A3	1253 ± 105†	63 ± 6†	1600 ± 238*†	80 ± 12*

Mice were fed control or fenofibrate diet for two weeks ($n = 5$ per group). Plasma activities are expressed as percentage of human reference pool plasma values (%). P4: transgenic mice expressing human PLTP under the control of its native promoter; A3: transgenic mice expressing human PLTP under the control of the albumin promoter and enhancer (14). Differences between the two diets were analyzed by two sample Wilcoxon rank-sum tests. Values are means ± SD. *$P < 0.05$, *versus* control diet; †$P < 0.05$, *versus* control.

Fenofibrate feeding decreases plasma triglycerides

In order to check whether there is an equal response to fenofibrate in the three lines of mice studied, plasma levels of triglycerides were measured. Treatment with fenofibrate caused a decrease in plasma triglyceride levels, both in wild-type mice and in PLTP transgenic mice (Figure 3). The decrease in the wild-type mice was 67%, compared with 48 % and 52% in the transgenic lines P4 and A3, respectively (all $P < 0.05$). There were no statistically significant differences in triglyceride levels between the various mouse lines.

Fenofibrate feeding increases plasma HDL-cholesterol in PLTP transgenic mice, but not in wild-type mice

Plasma HDL levels are known to be reduced in human PLTP transgenic mice (14, 30). Fenofibrate feeding had no effect on HDL-cholesterol in wild-type mice. By contrast, fenofibrate had a substantial HDL-cholesterol raising effect in both PLTP transgenic mouse lines (Figure 4). The increase was 72% in line P4 and 167% in line A3. As the bulk of plasma cholesterol is present in HDL in these mice, we found similar effects of fenofibrate treatment on total plasma cholesterol (increases of 177% and 118% in lines P4 and A3, respectively; results

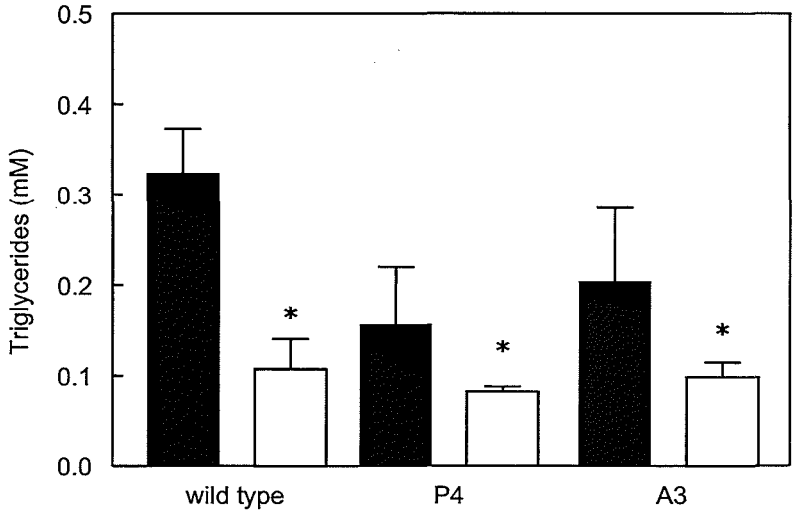

Figure 3. Fenofibrate reduces plasma triglycerides in wild-type and human PLTP transgenic mice. Plasma triglycerides were measured in wild-type mice and PLTP transgenic mice fed control (black bars) or fenofibrate-containing diets (white bars) ($n = 5$ per group). P4: transgenic mice expressing human PLTP under the control of its native promoter; A3: transgenic mice expressing human PLTP under the control of the albumin promoter and enhancer (14). Values represent means ± SD. *$P < 0.05$, control *versus* fenofibrate diet.

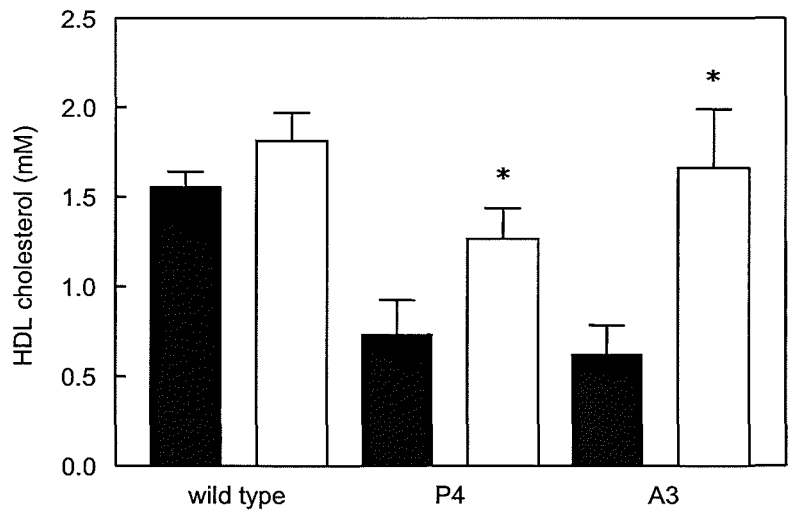

Figure 4. Fenofibrate counteracts the decrease in HDL cholesterol in human PLTP transgenic mice. Plasma HDL-cholesterol was determined in wild-type and PLTP transgenic mice fed control (black bars) or fenofibrate-containing diets (white bars) ($n = 5$ per group). P4: transgenic mice expressing human PLTP under the control of its native promoter; A3: transgenic mice expressing human PLTP under the control of the albumin promoter and enhancer (14). Values represent means ± SD. *$P < 0.05$, control *versus* fenofibrate diet.

not shown). The differences in HDL-cholesterol that normally exist between the wild-type mice and the two PLTP transgenic lines when fed control chow were almost completely counterbalanced by two weeks of fenofibrate feeding.

Discussion

Fibrates increase HDL cholesterol levels in man (31) which has largely been attributed to their action on PPARα, resulting in the transcriptional induction of apoAI and apo AII, the main apolipoproteins of HDL (18) and in the induction of ABCA1 in macrophages (32). In addition to changes in plasma HDL levels, fibrates also increase HDL size in mice via PPARα by inducing PLTP gene expression and decreasing SR-BI expression in the liver (25, 33, 34). Overexpression of human PLTP in mice results in decreased plasma HDL levels (14, 16), but it has not been studied previously how fibrates affect this pro-atherogenic property of PLTP. Actually, the effect of fenofibrate on human PLTP mRNA levels has not been studied before. Therefore, we first investigated the regulation of PLTP expression by fibrates in human hepatocytes. Subsequently, we evaluated the influence of a fenofibrate-containing diet on PLTP activity and expression, as well as on plasma HDL-cholesterol in transgenic mice overexpressing human PLTP.

It was reported earlier that fenofibrate feeding leads to increased plasma PLTP activity in wild-type mice (25). We were able to confirm these findings and observed a 4.4-fold increase in plasma PLTP activity in wild-type mice consistent with the induction of hepatic mouse PLTP mRNA by fenofibrate, as previously reported (25). In contrast, such a strong increase in PLTP activity by fenofibrate was not found in human PLTP transgenic mice. Expression of human PLTP did not respond to fenofibrate treatment, neither in human hepatocytes nor in transgenic mice. Data from *in vitro* promoter analysis already showed that fenofibrate increases the transcriptional activity of the mouse PLTP promoter (35), but not of the human PLTP promoter (36). Thus, PLTP is regulated in a species-specific manner by fibrates. As in both PLTP overexpressing mouse lines an effect of fenofibrate on PLTP expression and activity is absent, a post-transcriptional effect is also excluded.

Elevation of PLTP activity results in decreased plasma HDL levels in mice (9, 14, 16, 37, 38), which is also found in the present study. Treatment with fenofibrate resulted in dramatically increased HDL levels in mouse lines overexpressing human PLTP (2.7-fold and 1.7-fold higher in line A3 and P4 respectively). It has been reported that administration of PPARα agonists leads to elevation of expression of ABCA1, a crucial factor for the generation of HDL, in mice (33) and in human macrophages (32). This may have contributed to the HDL raising

effect of fenofibrate. In the present study, fenofibrate caused an elevation of LCAT activity (Table 1), which also may have contributed to the increase in HDL cholesterol. The increment in HDL levels of PLTP transgenic mice by fenofibrate treatment was to such an extent that it virtually abolished all HDL differences between the wild-type mice and the PLTP transgenic mice. Thus, it can be concluded that fenofibrate is able to counteract the HDL lowering induced by PLTP overexpression and elevated plasma PLTP activities. The HDL raising effect of fenofibrate was accompanied with an increase in total plasma cholesterol, which is in agreement with results from other fenofibrate studies using mouse models (24, 39). This increase in total plasma cholesterol is as expected because HDL is quantitatively the most important plasma lipoprotein in mice.

It is known that fenofibrate treatment increases HDL size in mice by an upregulation of PLTP expression through a PPARα-dependent mechanism (25). LXRs, another group of transcription factors, govern the expression of many of the proteins that are crucial to cholesterol homeostasis, including ABCA1 and the lipid transfer proteins CETP and PLTP (40). Studies in mice showed that LXR-regulated transcriptional activity may lead to the induction of hepatic PLTP expression and elevated plasma PLTP activity, resulting in HDL enlargement (41). In addition there is a possibility of cross talk between LXRs and PPARα (42) and it has recently been demonstrated that co-administration of LXR and PPARα agonists leads to elevation of hepatic PLTP mRNA expression and plasma PLTP activity resulting in enlarged HDL (33). Increased LCAT activity may also result in increased HDL size, mediated via the esterification of plasma cholesterol on HDL (43, 44). ABCA1 is crucial for the synthesis of HDL, and it has been demonstrated that both PLTP and LCAT activities were dramatically reduced in ABCA1-deficient mice (45), indicating their importance in HDL maturation. In the present study, we measured elevated LCAT activities after feeding the fenofibrate-containing diet, both in wild-type mice as well as in both lines of PLTP transgenic mice. However, in an earlier study, the HDL enlargement induced by fenofibrate feeding was accompanied by a slightly decreased plasma LCAT activity (25). This discrepancy might be due to different methodologies used to measure LCAT activity. Together with the increased LCAT activity, we found unchanged hepatic LCAT mRNA levels (25).

PLTP is reported to be a potential risk factor for atherosclerosis in mice (9, 11) and man (12). Its pro-atherogenic action has been related, at least in part, to HDL lowering (14, 16). In this study we showed that fenofibrate is able to counteract the HDL lowering effect of elevated plasma PLTP activity. Therefore we conclude that fenofibrate treatment may prevent lowering of plasma HDL-cholesterol induced by elevated plasma PLTP activity. Hence, fenofibrate appears to be a suitable drug to treat the dyslipidemia observed in

insulin-resistant states such as obesity and type 2 diabetes mellitus, which are characterized by high plasma triglycerides, low HDL cholesterol and elevated PLTP activity (46).

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CHAPTER VII

Discussion

There is a large body of evidence indicating that high plasma levels of HDL cholesterol reduce the risk for development of atherosclerosis. As PLTP has been shown to affect HDL metabolism, it is a potential factor in the development of atherosclerosis. Until recently however, there was no evidence showing whether PLTP was anti-atherogenic or pro-atherogenic. Therefore the aim of this thesis was to clarify the role of PLTP in lipoprotein metabolism and its involvement in the development of atherosclerosis. To this end we used genetically modified mouse models with elevated expression of PLTP, which in addition have a human-like lipoprotein profile. Wild-type mice are resistant to atherosclerosis as they have low plasma levels of cholesterol and the major class of circulating lipoproteins in the fasted state is HDL. This is in contrast to humans, in which the predominant lipoprotein classes in plasma are VLDL and LDL. This discrepancy between the species may be due to several differences in lipoprotein metabolism, including the absence of CETP in mouse plasma and the higher efficiency of murine hepatic LDL receptors, leading to faster clearance of LDL. Therefore we used models that express human CETP and have one inactivated LDL receptor allele (LDL-$R^{+/-}$).

Anti-atherogenic properties of PLTP

It has been well documented that PLTP has multiple effects in lipoprotein metabolism (1-3). Some of its effects are believed to be anti-atherogenic while others are considered pro-atherogenic. A few years ago, PLTP was considered to have anti-atherogenic potential because of its role in preβ-HDL formation (4). Preβ-HDL is the initial acceptor of cellular cholesterol following efflux, which subsequently ends up in larger HDL particles, where cholesterol accumulates before being transferred along the reverse cholesterol transport pathway (5, 6). Thus preβ-HDL may be considered as an anti-atherogenic factor. Still, some clinical data show that relatively high preβ-HDL concentrations are found in conditions of increased risk of cardiovascular disease (7, 8). This could be explained by an inefficient esterification of preβ-HDL by the enzyme LCAT (9). PLTP increases the *formation*, but not necessarily the *concentration* of preβ-HDL particles *in vivo* (6, 10, 11). In addition, plasma from huPLTPtg mice is more efficient in preventing cholesterol accumulation in cultured macrophages compared with plasma from control mice, indicating that the expressed PLTP is mediating an increase in cholesterol efflux (6). Thus the rate of preβ-HDL formation is probably more important than its concentration and may contribute to the anti-atherogenic potential of PLTP.

CETP, the other lipid transfer protein, has also been reported to play a role in preβ-HDL formation (12). In **chapter 2** we established that PLTP rather than CETP is responsible for the generation of preβ-HDL particles using various mouse models expressing either human PLTP, human CETP, or both lipid transfer proteins (11).

In vivo evidence demonstrating that elevated PLTP expression actually enhances reverse cholesterol transport originates from a study showing a 92% decrease in plasma HDL levels in huPLTPtg mice (13). The reduced HDL cholesterol level was caused by a rapid clearance via the liver leading to increased fecal bile acid excretion, indicating increased reverse cholesterol transport. However, these mice have a 15-fold overexpression of PLTP. It remains to be demonstrated whether these findings also apply to mouse models with a more modest increase in PLTP expression.
It has been shown that PLTP is present in human atherosclerotic lesions and is expressed in macrophages (14-16). As PLTP has been demonstrated to enhance cholesterol and phospholipids efflux from cholesterol loaded cells (17, 18), PLTP present in atherosclerotic lesions could play a pivotal role in the removal of excess cholesterol from foam cells, and thereby prevent the progress of atherosclerosis. On the other hand, PLTP associated with the extracellular matrix could mediate lipoprotein retention and lipid accumulation. Whether PLTP is anti-or pro-atherogenic in atherosclerotic lesions requires further investigation.
Finally, another potential anti-atherogenic property of PLTP has been suggested which is not related to the reverse cholesterol transport pathway. PLTP promotes a net mass transfer of vitamin E toward oxLDL or endothelial cells, thereby preventing endothelial dysfunction (19).

Pro-atherogenic properties of PLTP

Most PLTP overexpressing mouse models show decreased HDL levels (10, 11, 20-22). Therefore the question was raised whether PLTP could be pro-atherogenic. The role of PLTP in atherosclerosis has been studied in various mouse models (2) (see also **chapter 1** Table 4). PLTP overexpressing mice are more susceptible to diet induced atherosclerosis than control mice in a PLTP dose-dependent manner (20, 23). Conversely, PLTP knockout mouse models are less susceptible to diet induced atherosclerosis than control mice (24). However, a modest increase in PLTP activity of 1.3-fold does not result in a statistically significant increase in atherosclerosis (22). Likewise, 40-50% decrease of PLTP activity in mice heterozygous for PLTP deficiency did not affect the plasma lipid and lipoprotein levels (25). On the other hand, moderate variations in PLTP activity might affect the development of atherosclerosis in humans, because this is a long term process. Indeed, it was reported recently that patients with coronary heart disease have a statistically signicant 1.1-fold elevation of PLTP activity levels compared with controls independent of other risk factors (26), indicating that PLTP is a potential pro-atherogenic factor.

Possible pro-atherogenic mechanisms of PLTP

The pro-atherogenic effect of PLTP can be explained by several mechanisms: 1) PLTP affects hepatic VLDL secretion. This was demonstrated *in vitro* by using hepatocytes from PLTP deficient mice. The defect could be corrected by reintroduction of PLTP through adenoviral vectors (24). As a follow up, in **chapter 3**, we measured VLDL secretion *in vivo* in mice overexpressing human PLTP as well as human CETP, and found that modest overexpression of PLTP caused a 48% increase in VLDL secretion (27). 2) PLTP increases the susceptibility to atherosclerosis by lowering HDL concentrations as was shown in various PLTP overexpressing mouse models constitutively overexpressing PLTP (20, 23) and in mice expressing PLTP by using an adenovirus associated virus system (22). 3) PLTP may decrease the protection of LDL from oxidation, which is presumably due to accumulation of vitamin E in LDL which was shown in both PLTP deficient and in PLTP overexpressing mice (22, 28), and may therefore accelerate the development in atherosclerosis.

However, a decrease in hepatic VLDL secretion only partially explains the decrease in atherosclerosis in PLTP deficient mice because it could not be demonstrated in all of the tested PLTP deficient mouse models (24). In addition, in PLTP overexpressing mice it was found that hepatic VLDL secretion is not dose dependently related to PLTP activity levels (20). From the results of studies in PLTP overexpressing mice it was concluded that high plasma activity levels of PLTP are pro-atherogenic via a lowering effect on HDL cholesterol (20, 23). In **chapter 4** it is shown that the reduction in HDL levels in PLTP overexpressing mice which are also transgenic for human CETP, coincides with a reduction of the HDL-associated PON and PAF-AH activities. This may contribute to the pro-atherogenicity of PLTP (23), because these enzymes protect LDL from oxidation and hence retard the development of atherosclerosis. Surprisingly, atherosclerosis was increased in these mice in spite of reduced VLDL+ LDL cholesterol. Apparently the adverse effect of the reduction in plasma HDL is more significant than the beneficial effect of the reduction in VLDL+LDL cholesterol.

In **chapter 5** we concluded that the increased atherosclerosis could be attributed to a decrease in plasma HDL, rather than through an increased hepatic secretion of VLDL.

Although decreasing the protection of LDL from oxidation due to accumulation of vitamin E in LDL is another possible pro-atherogenic feature of PLTP, the relationship between vitamin E and atherosclerosis presents a confusing picture in humans (29). PLTP might account for this discordance but this has never been evaluated. However, the conflicting results with vitamin E are inconsistent with the epidemiologically established relation between decreased HDL concentration and increased atherosclerosis.

In view of these considerations, it can be concluded that the most likely pro-

atherogenic mechanism of PLTP is its HDL lowering ability. In addition, it has been demonstrated that a decrease in HDL outweighs the potentially beneficial anti-ahterogenic effect of decreased apoB-containing lipoprotein concentrations.

PLTP and plasma HDL cholesterol

It is remarkable that both PLTP knockout and PLTP transgenic mice show reduced plasma HDL levels. Different mechanisms are involved. In the case of PLTP knockout mice, the transfer of phospholipids and cholesterol from triglyceride-rich lipoproteins into HDL is arrested. This probably prevents the formation of mature HDL by decreasing their lipidation (30). In PLTP transgenic mice the redistribution of phospholipids among lipoproteins facilitated by PLTP causes instability of HDL particles and leads to increased HDL catabolism (10).

Because PLTP activity affects plasma HDL concentration, PLTP possibly interacts with other key factors involved in reverse cholesterol transport like ABCA1 and SRBI.

PLTP activity plays a role in the maturation of HDL which also involves ABCA1 (31-33). ABCA1 facilitates the efflux of phospholipids toward apoAI which may be provided with phospholipids from surface fragments by the action of PLTP. ABCA1 deficiency in mice results in a more than 80% decrease in PLTP activity and changes in phospholipid composition of HDL which affects their maturation (34), and thus plasma HDL concentration. Reports on the relation between ABCA1 expression and the development of atherosclerosis are conflicting (35-38). Both PLTP and ABCA1 are key factors in reverse cholesterol transport, but it is not clear whether they function in concert and how this would affect atherosclerosis. SRBI is another factor that may play a role in the relation between PLTP and plasma HDL concentration. HDL particles are a direct source of cholesteryl esters by selective HDL uptake via SRBI (39, 40). A series of genetically modified mouse models have established that HDL levels are inversely correlated with the level of SRBI expression (41, 42). We observed that the expression of hepatic SRBI mRNA is elevated in huCETPtg/huPLTPtg mice compared with huCETPtg mice, suggesting that overexpression of human PLTP may enhance the uptake of HDL via SRBI (unpublished observation). In the circulation of SRBI deficient mice, an accumulation of cholesterol-rich HDL was observed indicating a decreased delivery to the liver. In addition an increased susceptibility to atherosclerosis has been found in these mice, which is attributed to disrupted cholesterol metabolism in the vascular wall (43).

The PLTP gene is a target of the transcription factors FXR, LXR and PPARα. Therefore, these transcription factors might play a role in the relation between PLTP on the one hand, and plasma HDL composition, plasma HDL concentration and the susceptibility to atherosclerosis on the other hand. Bile

acid is an important component of the high fat high cholesterol diet used to induce atherosclerosis in mice and is a potent activator of FXR. In livers from mice fed a normal chow diet supplemented with bile acid both FXR and PLTP transcription were stimulated (44). These results suggest that FXR might affect HDL metabolism through PLTP.
The impact of LXR was evaluated by treating mice with the LXR agonist, T0901317 (45). This resulted in an elevation of hepatic PLTP mRNA and in increased HDL size and plasma concentration. However, similar effects on HDL were found in PLTP deficient mice, indicating that these were independent from PLTP activity (45).
Fibrates are drugs that activate PPARα, increase HDL cholesterol levels in humans (46, 47) and increase HDL size in mice by inducing PLTP gene expression in liver (48-50). In addition, it has been reported that fenofibrate treatment reduces atherosclerosis in mice (51). In **chapter 6** we demonstrated that fenofibrate restores the decline in HDL caused by PLTP in transgenic mouse models.

Influence of CETP and LDL-R in PLTP mouse models

Although CETP and PLTP function independently in at least some of the metabolic pathways in which the transfer proteins are involved (11, 52), CETP clearly affects the metabolism of both HDL and apoB-containing lipoproteins (53). Therefore, it cannot be excluded that it does play a role in other PLTP functions, like VLDL secretion. In **chapter 3** we investigated whether PLTP-stimulated VLDL secretion is affected by CETP by comparing huCETPtg mice with huCETPtg/huPLTPtg mice. It is concluded that elevation of PLTP increases the rate of VLDL secretion. The presence of CETP did neither affect the VLDL secretion rate nor the composition of the secreted VLDL. These results are in line with findings in other PLTP overexpressing mice which do not express CETP (20). Apparently CETP does not interfere with the hepatic VLDL secretion caused by PLTP overexpression. On the other hand, the LDL-R does have an impact on the VLDL secretion. It has been demonstrated in hepatocytes from PLTP deficient mice that the total absence of the LDL-R prevents the decrease in VLDL secretion which was found in PLTP deficient mice with either apoB transgenic or apoE knockout backgrounds (24). It has been suggested that LDL-Rs might interact with nascent apoB-containing lipoproteins within the secretory pathway (54), suggesting that functional LDL-Rs are necessary for apoB secretion, explaining the unchanged secretion in PLTP deficient mice without LDL-Rs. Therefore the presence of one allele of LDL-R in PLTP overexpressing mice may be sufficient for PLTP to increase hepatic VLDL secretion (20).
In **chapter 4** we performed atherosclerosis studies in huCETPtg/LDL-R$^{+/-}$ mice with various PLTP activities. Increased PLTP activity leads to larger

atherosclerotic lesion areas which were accompanied with a reduction of plasma HDL concentrations. The presence of CETP does not seem to affect the pro-atherogenicity of PLTP when comparing the results of **chapter 4** with earlier published results from PLTP overexpressing mice bred into an LDL-$R^{+/-}$ background without human CETP overexpression (20). CETP is atherogenic in various animal models. In **chapter 4** we demonstrated that CETP activity is decreased in PLTP overexpressing mice and thus cannot be the reason for the increase in atherosclerosis found in these mice. Surprisingly, the increased atherosclerosis occurred in spite of reduced VLDL+ LDL cholesterol. The effect on HDL apparently outweighs the effect on apoB-containing lipoproteins (23). PLTP overexpression in mice with an LDL-$R^{+/-}$ background in the absence of CETP does not affect apoB-containing lipoprotein levels (20). The underlying mechanism is not clear at present. In mice without mutations in the LDL-R the plasma levels of apoB-containing lipoproteins are similar between huCETPtg and huCETPtg/huPLTPtg mice (11). These results suggest that the combination of CETPtg and LDL-$R^{+/-}$ is crucial for the observed decrease in apoB-containing lipoproteins in PLTP overexpressing mice.

Concluding remarks and future directions

The results presented in this thesis indicate that PLTP is pro-atherogenic in genetically modified mouse models. The pro-atherogenic effect of elevated PLTP activity is attributed to its HDL-lowering capacity with a concomitant lowering in anti-oxidant enzyme activities. This was found despite a reduction of atherogenic apoB-containing lipoproteins. The results described in this thesis demonstrate that PLTP affects the plasma lipoprotein distribution and suggest that elevated PLTP activity may be a novel risk factor for atherosclerosis.

Although the atherogenic potential of PLTP is revealed in various mouse models (20, 22-24), it is not yet known whether PLTP is a causal factor for the development of atherosclerosis in humans. High plasma PLTP activity is found in disorders with increased risk for atherosclerosis and coronary heart disease such as diabetes mellitus, obesity and hypertriglyceridemia. In these patients, high PLTP often coincides with low HDL, although not in all cases, notably diabetes mellitus type 1. In addition, it was found that PLTP is highly expressed by macrophages within human atherosclerotic lesions. Although this may indicate that PLTP plays a role in the development of atherosclerosis in humans, further research is needed to clarify this putative role. Obviously a more detailed understanding of its pro-atherogenic mechanism is needed to assess the role of PLTP in atherosclerotic disease.

The relationship between PLTP and atherosclerosis appears to be complex involving other HDL modulating factors such as CETP. PLTP has also been shown to affect lipoprotein metabolism in various ways. As a consequence the mechanism of the atherogenic potential of PLTP has not been fully elucidated.

This can be accomplished by studying the impact of other factors on PLTP. However, it is indisputable that PLTP affects HDL metabolism. Moreover, PLTP is expressed in various tissues where it may serve local functions. In order to determine the mechanism by which PLTP is atherogenic, it may be useful to analyze its local actions. This can be executed by inducing the expression, or exclude the expression of PLTP in certain cell types of mice of which hepatocytes and macrophages are of most interest.
If PLTP turns out to be a causal factor for atherosclerosis, pharmacological modulators of PLTP or gene therapy targeting PLTP may be useful tools to prevent the development of atherosclerosis.

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Summary

Complications of atherosclerosis are the principal cause of mortality in Western societies. Epidemiological studies have shown that a high HDL cholesterol level in plasma is inversely correlated with the risk for atherosclerosis. The exact biological mechanism behind this finding is not known. There are many factors that affect HDL metabolism. *In vitro* and *in vivo* studies demonstrated that plasma phospholipid transfer protein (PLTP) plays a major role in phospholipid transfer processes between lipoproteins, and also in modulating size and composition of HDL particles. The aim of this thesis was to clarify the role of PLTP in HDL metabolism and in the development of atherosclerosis. Normal mice lack cholesteryl ester transfer protein (CETP) and have very low LDL plasma levels compared with humans. Therefore we used a genetically modified mouse model with a humanlike lipoprotein profile. These animals are transgenic for CETP and carry one mutated allele of the LDL receptor (huCETPtg/LDL-$R^{+/-}$).

PLTP could have anti-atherogenic potential due to its ability to generate preβ-HDL. *In vitro* studies indicated that both lipid transfer proteins, *i.e.* CETP and PLTP, are implicated in the formation of preβ-HDL. In **chapter 2** the relative contribution of each of these proteins was investigated in mouse models. We compared human CETP transgenic mice (huCETPtg), human PLTP transgenic mice (huPLTPtg) and mice expressing both lipid transfer proteins (huCETPtg/huPLTPtg). It was shown by using two different techniques that PLTP was much more important than CETP for the formation of preβ-HDL. In both lines of PLTP transgenic mice the formation of preβ-HDL was 3-fold higher than in huCETPtg mice. Thus, PLTP rather than CETP is responsible for the generation of preβ-HDL.

PLTP may also have pro-atherogenic potential. This was demonstrated in PLTP knockout mice, which showed to be less prone to diet-induced atherosclerosis compared with control mice which was attributed to a reduction of hepatic VLDL secretion. We evaluated the influence of PLTP on VLDL secretion in **chapter 3** by using our PLTP transgenic model with appreciable CETP activity. In huCETPtg/huPLTPtg mice, the VLDL secretion was 1.5-fold higher compared with huCETPtg mice. The composition of nascent VLDL was similar in both strains. Thus, PLTP is involved in VLDL secretion *in vivo*.

In **chapter 4** we studied the susceptibility to diet-induced atherosclerosis in huCETPtg/LDL-$R^{+/-}$ mice with moderately and highly elevated plasma PLTP activity. Elevation of PLTP activity gives rise to increased susceptibility to diet-induced atherosclerosis, which was accompanied by a PLTP dose-dependent reduction of HDL as well as by a decrease of apolipoprotein (apo) B-containing lipoproteins. These results indicate that the adverse effect of the reduction in

plasma HDL outweighs the beneficial effect of the reduction in apoB-containing lipoproteins. In addition, we measured the activity of the two HDL-associated anti-atherogenic enzymes, paraoxonase (PON) and platelet-activating factor acetyl hydrolase (PAF-AH). It was found that the PLTP-dependent reduction of HDL coincides with a decrease of these HDL-associated anti-oxidant enzyme activities. Thus, PLTP overexpression is atherogenic. The mechanism may comprise various atheroprotective properties of HDL, including the association with anti-oxidant enzymes.

We described two different mechanisms which could explain the atherogenic potential of PLTP. First, PLTP stimulates hepatic VLDL secretion. Second, PLTP lowers plasma levels of HDL. In **chapter 5** we studied which of these two pro-atherogenic effects is the most important factor in the development of atherosclerosis. To this end, we compared male and female huCETPtg/LDL-$R^{+/-}$ mice with moderate PLTP overexpression. It is well known that male mice are less susceptible to diet-induced atherosclerosis than female mice. This was also found in the present study, as atherosclerosis was approximately 7-fold higher in female mice than in male mice. The reason for this difference is not clear. We used this difference as a starting point to perform a comparative study. We found that PLTP activities were similar in both sexes. No differences were observed in the rate of hepatic VLDL secretion. However, HDL levels were significantly lower in the female mice. From these data we conclude that the atherogenic potential of PLTP is caused by a decrease in HDL and not by an increase of hepatic VLDL secretion.

Fenofibrate lowers triglycerides and increases HDL plasma levels in humans. In **chapter 6** we investigated whether fenofibrate affects PLTP activity levels and plasma HDL in mice transgenic for human PLTP. Hepatic human PLTP mRNA was unchanged by fenofibrate in transgenic PLTP mice whereas hepatic murine PLTP mRNA was increased in both wild-type and PLTP transgenic mice. PLTP activity was elevated by fenofibrate in wild-type mice, but not in mice transgenic for human PLTP. HDL-cholesterol was substantially increased by fenofibrate in PLTP overexpressing mice while it was much less affected in wild-type mice. As a result the decline in plasma HDL-cholesterol in PLTP transgenic mice is almost completely restored by fenofibrate treatment. The data suggest that species differences in PLTP regulation contribute to the differences in HDL response upon fenofibrate treatment.

Finally in **chapter 7**, the results presented in this thesis were discussed in relation to the progress in the field of PLTP-associated atherosclerosis research. The results presented in this thesis indicate that PLTP is pro-atherogenic in genetically modified mouse models. The pro-atherogenic effect of elevated

PLTP activity is attributed to its HDL-lowering capacity. It is not yet known whether PLTP is a causal factor for the development of atherosclerosis in humans. High plasma PLTP activity is found in disorders with increased risk for atherosclerosis and coronary heart disease such as diabetes mellitus, obesity and hypertriglyceridemia. In these patients, high PLTP often coincides with low HDL, although not in all cases, notably diabetes mellitus type 1. In addition, it was found that PLTP is highly expressed by macrophages within human atherosclerotic lesions. Although this may indicate that PLTP plays a role in the development of atherosclerosis in humans, further research is needed to clarify this putative role.
The findings suggest that PLTP may be a novel risk factor for atherosclerosis. A more detailed understanding of its pro-atherogenic mechanism is important in order to conclude that PLTP is causal in atherosclerotic disease. If so, pharmacological modulators of PLTP or gene therapy targeting PLTP may be useful tools to prevent the development of atherosclerosis.

Samenvatting

Hart- en vaatziekten vormen sinds vele tientallen jaren de belangrijkste doodsoorzaak in de Westerse wereld. Er zijn verschillende oorzaken voor deze aandoeningen, maar in veel gevallen gaat het om de gevolgen van atherosclerose ofwel aderverkalking. Atherosclerose is een langdurig proces waarbij vetophopingen in de bloedvatwanden plaatsvinden waardoor de vaten op den duur vernauwd kunnen worden. Er zijn verschillende risico factoren bekend die een belangrijke rol spelen bij het ontstaan van atherosclerose zoals hoge bloeddruk, roken, overgewicht, diabetes mellitus en een verhoogd cholesterol gehalte in het bloed. Cholesterol is essentieel voor het functioneren van het lichaam. Het komt voor in celmembranen en dient als bouwsteen voor o.a. hormonen en componenten van de gal. Cholesterol wordt via het bloed door het lichaam vervoerd. Omdat cholesterol een vetachtige niet in water oplosbare stof is, kan het niet in vrije vorm in het bloed voorkomen. Om toch door het bloed getransporteerd te kunnen worden, wordt cholesterol verpakt in zogenaamde lipoproteïnen. Deze lipoproteïnen bestaan in verschillende klassen die naar dichtheid zijn ingedeeld. De belangrijkste lipoproteïnen zijn de "low density lipoproteins" (LDL) en de "high density lipoproteins" (HDL). LDL vervoert cholesterol naar de vaatwand waardoor cholesterolophoping kan plaatsvinden. Daarom wordt LDL het slechte cholesterol genoemd. In bevolkingsonderzoek wordt dan ook een relatie gevonden tussen een hoog LDL gehalte in het bloed en een verhoogde kans op atherosclerose. Voor HDL is een omgekeerde relatie gevonden. HDL zorgt er juist voor dat het overtollige cholesterol uit de vaatwand kan worden afgevoerd, eerst naar het bloed en daarna naar de lever. In de lever wordt het cholesterol deels omgezet in galzouten en kan het lichaam verlaten via de ontlasting.
Toen met het onderzoek beschreven in dit proefschrift werd begonnen, was het bekend dat het eiwit "phospholipid transfer protein" (PLTP) een rol speelt in de stofwisseling van HDL. Hoe PLTP betrokken is bij de ontwikkeling van atherosclerose was echter nog onduidelijk. De studies beschreven in dit proefschrift zijn uitgevoerd om meer inzicht te krijgen in de werking van PLTP en om de relatie met atherosclerose te onderzoeken.
Om een goed inzicht te verkrijgen in het ontstaan en de ontwikkeling van atherosclerose is het noodzakelijk om gebruik te maken van proefdieren. We hebben gebruik gemaakt van muizen, waarin eiwitten selectief kunnen worden aangemaakt of uitgeschakeld door middel van genetische modificatie. We hebben een muismodel gekozen dat wat betreft de cholesterol stofwisseling eigenschappen vertoont zoals die bij mensen worden gevonden. De dieren bevatten een extra gen coderend voor het menselijke "cholesteryl ester transfer protein" (huCETPtg). Men noemt dit CETP transgene muizen. PLTP en CETP zijn beide lipide transfer eiwitten die circuleren in het bloed. Muizen hebben van nature geen CETP. CETP heeft echter een belangrijke invloed op lipoproteïnen en heeft zeer waarschijnlijk een stimulerend effect op de

ontwikkeling van atherosclerose. Daarnaast hebben muizen van nature zeer lage LDL niveaus in het bloed. Daarom is een extra mutatie ingebracht om het LDL te verhogen. Tenslotte is het menselijke gen voor PLTP ingebracht. Deze muizen zijn vergeleken met dieren die dit transgen niet bevatten om het effect van PLTP op atherosclerose, HDL en lipoproteïnen stofwisseling te kunnen onderzoeken. Atherosclerose is opgewekt door grote hoeveelheden vet en cholesterol aan het muizenvoer toe te voegen.

PLTP zou anti-atherosclerotisch kunnen zijn omdat het in staat is om een specifiek type HDL te genereren nl. preβ-HDL, dat zeer efficiënt is in de opname van cholesterol uit de cel. Uit literatuur was al bekend dat CETP ook in staat is om dit preβ-HDL te kunnen genereren. Het doel van **hoofdstuk 2** was om een vergelijking te maken tussen deze twee lipide transfer eiwitten in dit opzicht. We hebben groepen muizen bestudeerd die transgeen zijn voor : 1) CETP 2) PLTP en 3) zowel CETP als PLTP. Door twee verschillende technieken te gebruiken hebben we overtuigend kunnen aantonen dat PLTP veel belangrijker is voor de vorming van preβ-HDL dan CETP. In de PLTP transgene muizen was de vorming van preβ-HDL drie maal hoger dan in de CETP transgene muizen.

Er zijn ook aanwijzingen dat PLTP pro-atherosclerotisch zou kunnen zijn. Uit experimenten met gekweekte levercellen is gebleken dat PLTP mogelijk betrokken is bij de productie van VLDL deeltjes. Dit zijn grote lipoproteïnen waaruit LDL ontstaat. In **hoofdstuk 3** hebben we onderzocht wat de invloed is van PLTP op de afgifte van VLDL uit de lever naar het bloed. We hebben aangetoond dat in CETP/PLTP transgene muizen de VLDL afgifte 1,5 maal hoger is dan in CETP transgene muizen. PLTP stimuleert dus inderdaad de productie van VLDL in een zoogdier.

Het effect van PLTP op dieet geïnduceerde atherosclerose hebben we in **hoofdstuk 4** getest. Hiervoor hebben we drie muizenlijnen gebruikt die verschillen in PLTP activiteit. De mate van atherosclerose in muizen wordt bepaald door vetafzettingen te meten die zich het eerst ontwikkelen op de aortakleppen. Toenemende PLTP activiteit ging gepaard met meer atherosclerose. Dit ging samen met een eveneens PLTP dosis gerelateerde afname in HDL en LDL. Daaruit kan worden geconcludeerd dat voor de ontwikkeling van atherosclerose vermindering van het goede HDL cholesterol zwaarder weegt dan vermindering van het slechte LDL cholesterol. Ook hebben we de activiteiten van twee enzymen gemeten, die anti-atherosclerotisch zijn door anti-oxidatieve werking. Wij hebben gevonden dat deze beide enzymactiviteiten afnemen bij een oplopende PLTP activiteit. Deze resultaten tonen aan dat PLTP pro-atherosclerotisch is.

Er zijn hierboven twee verschillende mechanismen aangetoond die het gevonden atherosclerose stimulerende effect van PLTP zouden kunnen verklaren. Op de eerste plaats verhoogt PLTP de afgifte van VLDL door de lever aan het bloed en op de tweede plaats verlaagt PLTP het HDL niveau in het bloed. Het doel van **hoofdstuk 5** was vast te stellen welke van de twee genoemde werkingen van PLTP de belangrijkste factor is in de ontwikkeling van atherosclerose. Daartoe hebben we een vergelijking gemaakt tussen mannetjes en vrouwtjes muizen met een matig verhoogde PLTP activiteit. Het is een bekend gegeven dat mannetjes muizen veel minder gevoelig zijn voor de ontwikkeling van dieet-geïnduceerde atherosclerose dan vrouwtjes muizen. Dat blijkt ook uit onze studie, want de gevonden atherosclerose in de vrouwtjes muizen was zeven maal hoger dan in de mannetjes muizen. De oorzaak hiervan is nog onduidelijk. Wij hebben het geslachtsverschil echter als uitgangspunt gebruikt om een vergelijkende studie te kunnen uitvoeren. Het bleek dat de PLTP activiteit vergelijkbaar was tussen mannetjes en vrouwtjes muizen. Er werden geen verschillen waargenomen in de VLDL afgifte door de lever tussen mannetjes en vrouwtjes muizen. De HDL cholesterol waarden in de vrouwtjes waren echter duidelijk lager dan die in de mannetjes. Deze gegevens tonen aan dat atherosclerose in deze muizen niet veroorzaakt wordt door een toegenomen VLDL afgifte maar door een verlaagd HDL niveau.

Fenofibraat is een geneesmiddel dat bij mensen het vetgehalte (triglyceriden) in het bloed verlaagt en het HDL cholesterol niveau verhoogt. Het doel van **hoofdstuk 6** was om het effect van fenofibraat te onderzoeken op PLTP en HDL cholesterol. Hiervoor hebben we controle muizen en PLTP transgene muizen behandeld met fenofibraat. Deze behandeling veroorzaakt een sterke verhoging van het HDL cholesterol in de PLTP transgene muizen waardoor dit gelijk wordt aan de niveaus in de controle muizen. Fenofibraat behandeling resulteert dus in een volledige compensatie van het HDL verlagende effect van PLTP in PLTP transgene muizen.

Als laatste worden in **hoofdstuk 7** de resultaten uit dit proefschrift bediscussieerd. Deze tonen aan dat PLTP pro-atherosclerotisch is in het door ons gebruikte muismodel. Dit wordt waarschijnlijk veroorzaakt doordat verhoogde PLTP activiteit een verlaging geeft van HDL. Of PLTP ook een risicofactor is voor atherosclerose in de mens valt nog niet met zekerheid te zeggen. Er zijn echter wel aanwijzingen voor. Een verhoogde PLTP activiteit is gevonden in mensen met diabetes mellitus, overgewicht en verhoogde triglyceriden in het bloed. In deze groepen is er meestal ook een associatie tussen hoog PLTP en laag HDL, hoewel er ook uitzonderingen bestaan (met name patiënten met diabetes mellitus type 1). In sectie materiaal van overleden patiënten is PLTP aangetoond in de cellen van atherosclerotische bloedvaten.

Hoewel de functie hiervan nog niet is opgehelderd, doet deze bevinding wel vermoeden dat PLTP een rol speelt bij atherosclerose bij mensen.
Om definitieve uitspraken te kunnen doen over de betekenis van PLTP in de ontwikkeling van atherosclerose bij de mens is meer onderzoek noodzakelijk. Op grond hiervan zal kunnen worden vastgesteld in hoeverre PLTP een risicofactor is voor de ontwikkeling van atherosclerose in mensen. Indien PLTP een belangrijke oorzakelijke factor blijkt te zijn kan gedacht worden aan de ontwikkeling van PLTP-verlagende of remmende medicijnen.

Curriculum Vitae

Persoonlijke gegevens

Naam	Jessica Theresa Lie
Geboren	12 april 1970 te Heemstede

Opleidingen

1989 – 1993	Hogeschool van Amsterdam, Hogere Laboratorium Opleiding, richting Biochemie Stage: "Productie en zuivering van oplosbaar runder α1,3-galactosyltransferase in insektencellen" (o.l.v. Dr. D.H. Joziasse, Vrije Universiteit, Amsterdam, Afdeling Medische Chemie)
1993 – 1996	Vrije Universiteit Amsterdam, Medische Biologie Stages: 1. "A strategy for combating Gram-negative bacterial sepsis: Neutralization of endotoxins" (o.l.v. Dr. B.J. Appelmelk en Dr. P.R. Abraham, Vrije Universiteit, Amsterdam Afdeling Medische Microbiologie & Academisch Medisch Centrum, Amsterdam) 2. "Search for low density lipoprotein receptor-related proteins in the fresh water crayfish *Pacifastacus leniusculus*" (o.l.v. Dr. M. Hall, Uppsala University, Sweden, Dept. of Physiological Botany)

Werkervaring

1996 – 1997	Microbiology Research Centre Holland, Amsterdam Functie: biochemicus; taak: Optimaliseren van productie restrictie enzymen en controle DNA ladders
1997 – 1998	Gist-Brocades B.V. (DSM), Bakery Ingredients Division, Delft Functie: Junior Scientist; project "Total Mash Yeast": Verbeteren van bakkersgist d.m.v. genetische modificatie met als doel een toename in respiratoire capaciteit en een toename in de omzetting van suikers naar biomassa productie. Cursus: VMT/GLP
1998 – 1999	Academisch Ziekenhuis Rotterdam, afdeling Cardiologie Functie: administratief medewerkster
1999 – 2003	Erasmus Universiteit Rotterdam, afdelingen Biochemie en Celbiologie & Genetica Functie: Assistent in Opleiding, zie dit proefschrift (o.l.v. Dr. M.P.G. de Crom en Dr. A. van Tol) Cursussen: Transgenesis, Gene transfer and gene therapy, Safe laboratory techniques, Proefdierkunde: artikel 9 functionaris, Engels: Oxford examination

List of Publications

Full papers:

1. J. Lie, R. de Crom, M. Jauhiainen, T. van Gent, R. van Haperen, L. Scheek, H. Jansen, C. Ehnholm and A. van Tol. *Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of preβ-high-density lipoproteins.* Biochem J. 2001; 360(Pt 2):379-385.
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