THE INTERACTION OF PHOSPHOLIPID TRANSFER PROTEIN WITH HIGH DENSITY LIPOPROTEINS

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TABLE OF CONTENTS

SUMMARY

This thesis is concerned with the interaction of the plasma factor, phospholipid transfer protein (PLTP) with HDL. Recent studies have shown that PLTP is of major importance in HDL metabolism. It regulates plasma HDL levels by transferring phospholipids between triglyceride-rich lipoproteins to HDL during lipolysis by lipoprotein lipase (LPL). PLTP also remodels HDL into large and small particles and the dissociation of apoA-I. The remodeling of HDL by PLTP is also enhanced in triglyceride-enriched HDL.

The studies described in this thesis define the mechanism of the remodeling of reconstituted HDL (rHDL) by PLTP and the reasons why triglyceride enrichment of rHDL enhances the remodeling process. Other studies describe why triglyceride enrichment of rHDL enhances PLTP-mediated phospholipid transfers.

The influences of rHDL phospholipid composition on PLTP-mediated phospholipid transfers and remodeling were also determined. In general, the results show that phospholipid transfers from small unilamellar vesicles to rHDL are enhanced as the length and unsaturation of their sn-2 acyl chains increases. The results also show that, while the phospholipid composition of rHDL has no effect on the mechanism by which PLTP remodels the particles, it does have a major influence on the rate at which the processes occur.

The studies with triglyceride-enriched rHDL and rHDL with different phospholipids also show that the apoA-I that dissociates from the particles is complexed with small amounts of lipids. This apoA-I has pre-β migration and may be comparable to the pre-β migrating

HDL that act as the initial cellular cholesterol acceptors in the first step of reverse cholesterol transport (RCT). Thus, this finding supports a potentially anti-atherogenic role for PLTP.

In conclusion, this thesis describes the mechanism of the PLTP-mediated remodeling of rHDL and presents reasons why the remodeling is enhanced in particles that are enriched with triglyceride. The results also show that triglyceride enrichment enhances the PLTP-mediated transfers of phospholipids between rHDL and small unilamellar vesicles. Evidence that the remodeling and phospholipid transfers that are mediated by PLTP are regulated by HDL phospholipid composition is also presented.

DECLARATION

I certify that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Nongnuch Settasatian

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Publications

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ABBREVIATIONS

ABC1	ATP-binding casette transport 1
apo	apolipoprotein
BSA	bovine serum albumin
CAD	coronary artery disease
CE	cholesteryl esters
CE-rHDL	cholesteryl ester containing rHDL
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
d	density
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	1,2-di[1-^{14}C] palmitoyl-L-3-phosphatidylcholine
EL	endothelial lipase
FH	familial hypercholesterolemia
FPLC	fast performance liquid chromatography
GdnHCl	guanidine hydrochloride
HDL	high density lipoproteins
HL	hepatic lipase
ICAM-1	intracellular adhesion molecule-1
IDL	intermediate density lipoproteins
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoproteins
Lp A-I	lipoprotein containing apoA-I
Lp A-II	lipoprotein containing apoA-II
Lp A-I/A-II	lipoprotein containing both apoA-I and apoA-II

LPL	lipoprotein lipase
LRP	LDL receptor-related protein
NF-κB	Nuclear factor κB
PC	phosphatidylcholine
PL	phospholipids
PLTP	phospholipid transfer protein
PON	paraoxonase
PPARα	peroxisome-proliferating agent receptor α
RCT	reverse cholesterol transport
rHDL	reconstituted HDL
SAA	serum amyloid A
SR-BI	scavenger receptor class B type I
TG	triglycerides
TG-rHDL	triglyceride-enriched rHDL
TMA-DPH	1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate
TNF-α	tumour necrosis factor α
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoproteins

CHAPTER 1 INTRODUCTION

1.1 INTRODUCTION

1.1.1 LIPOPROTEINS

1.1.2 APOLIPOPROTEINS

1.1.3 LIPOPROTEIN METABOLISM

Chylomicrons

Very low density lipoproteins

Low density lipoproteins

High density lipoproteins

1.1.4 HIGH DENSITY LIPOPROTEINS AND REVERSE CHOLESTEROL TRANSPORT (RCT)

1.1.5 HIGH DENSITY LIPOPROTEINS AND ATHEROSCLEROSIS

1.1.6 HIGH DENSITY LIPOPROTEINS AND PLASMA FACTORS

Lecithin:cholesterol acyltransferase (LCAT)

Lipoprotein lipase (LPL)

Hepatic lipase (HL)

Endothelial lipase (EL)

Cholesteryl ester transfer protein (CETP)

Phospholipid transfer protein (PLTP)

1.2 SCOPE OF THIS THESIS

1.1 INTRODUCTION

1.1.1 LIPOPROTEINS

Introduction

Lipids synthesized in the liver and small intestine have to be transported to various tissues to accomplish their metabolic functions. Because lipids are not water soluble, they are transported in the plasma as macromolecular complexes called lipoproteins.

Lipoproteins are spherical particles that consist of a core of nonpolar lipids or neutral lipids (cholesteryl esters and triglycerides) surrounded by a surface monolayer of phospholipids, unesterified cholesterol, and apolipoproteins (Fig. 1.1). The polar phospholipid head groups are oriented towards the outside of the particles, while their acyl chains face towards the centre of the particles. Apolipoproteins are amphipathic, with their hydrophobic face embedded in the lipid domain of the lipoproteins and their hydrophilic face exposed to the plasma (Shen et al., 1977; Segrest et al., 1992). Apolipoproteins confer water solubility on lipoproteins.

Classification of lipoproteins

Lipoproteins have been categorized into five major classes according to their physical and chemical properties (Table 1.1). Each of the lipoprotein classes contains different proportions of lipid and protein (Table 1.2).

Lipoproteins can be separated by ultracentrifugation on the basis of their hydrated density into chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (Havel et al., 1955). Lipoproteins can also be separated on the basis of surface charge by

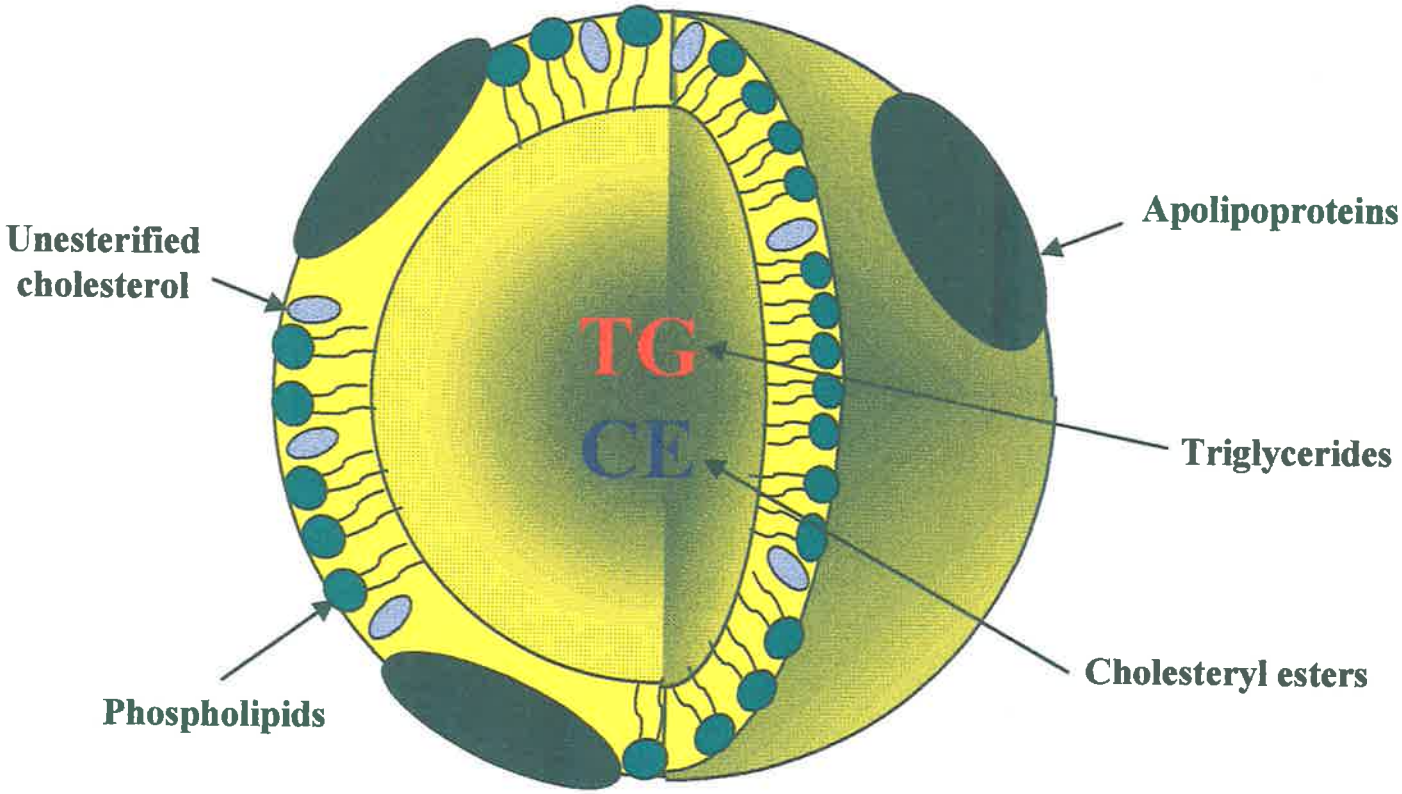


Figure 1.1 Structure of lipoprotein particle (adapted from Davis, 1991).

Table 1.1 Characteristics of human plasma lipoproteins[a]

Variable	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/mL)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Electrophoretic mobility	Origin	Pre-beta	Between beta and pre-beta	Beta	Alpha
Molecular weight (daltons)	$0.4\text{-}30 \times 10^9$	$5\text{-}10 \times 10^6$	$3.9\text{-}4.8 \times 10^6$	2.75×10^6	$1.8\text{-}3.6 \times 10^5$
Diameter (nm)	>70	25-70	22-24	19-23	8-12
Major lipids	Exogenous triglycerides	Endogenous triglycerides	Endogenous triglycerides, cholesteryl esters	Cholesteryl esters	Phospholipids, cholesteryl esters
Major proteins	A-I, B-48, C-I, C-II, C-III	B-100, C-I, C-II, C-III, E	B-100, E	B-100	A-I, A-II

VLDL, Very low density lipoproteins; IDL, Intermediate density lipoproteins; LDL, Low density lipoproteins; HDL, High density lipoproteins. [a]Source from Gotto et al., 1986: Ginsberg, 1990; Barter, 1994.

Table 1.2 Chemical composition (%) of normal human plasma lipoproteins[a]

	Surface components			Core lipids	
	Cholesterol	Phospholipids	Apolipoproteins	Triglycerides	Cholesteryl esters
Chylomicrons	2	7	2	86	3
VLDL	7	18	8	55	12
IDL	9	19	19	23	29
LDL	8	22	22	6	42
HDL_2	5	33	40	5	17
HDL_3	4	25	55	3	13

VLDL, Very low density lipoproteins; IDL, Intermediate density lipoproteins; LDL, Low density lipoproteins; HDL, High density lipoproteins. [a]Source from Eisenberg and Levy, 1975: Shen et al., 1977; Eisenberg, 1984.

agarose gel electrophoresis (Noble, 1968; Sparks and Phillips, 1992; Davidson et al., 1994a), and on the basis of size by non-denaturing polyacrylamide gradient gel electrophoresis (Blanche et al., 1981). When subjected to agarose gel electrophoresis, HDL exhibit α-migration, LDL have β-migration, and VLDL migrate to a pre-β position. IDL migrate to a position intermediate between pre-β and β.

1.1.2 APOLIPOPROTEINS

Apolipoproteins are the protein components of lipoproteins. The characteristics and main known functions of major apolipoproteins are summarized in Table 1.3. Each class of lipoprotein has a variety of apolipoproteins, with the exception of LDL, which contain predominantly apoB-100.

Apolipoprotein A

ApoA-I and apoA-II constitute about 90% of the total HDL protein (Cheung and Albers, 1982; Eisenberg, 1984). ApoA-I and apoA-II appear to have different influences on atherosclerosis. Available evidence suggests that apoA-I protects against atherosclerosis (Kottke et al., 1986). For example, mice transgenic for human apoA-I have high HDL levels and do not develop fatty streak lesions when fed an atherogenic diet (Rubin et al., 1991). Furthermore, rabbits transgenic for human apoA-I have a reduced susceptibility to diet induced atherosclerosis (Duverger et al., 1996). However, a controversial result reported by Li et al. (1993) has shown that apoA-I knockout mice, that have a complete absence of apoA-I, do not develop atherosclerosis, despite their very low plasma HDL levels.

Table 1.3 Classification and properties of major human plasma apolipoproteins[a]

Apolipoprotein	Molecular weight (daltons)	Function[a,b]	Lipoprotein carrier(s)
ApoA-I	28,300	Activator of LCAT; structural protein for HDL; ligand for HDL receptors	Chylomicrons, HDL
ApoA-II	17,414	Ligand for HDL receptors; modulates HL activity	HDL
ApoA-IV	44,500	Activator of LCAT	Chylomicrons, HDL
ApoB-100	550,000	Ligand for LDL receptor; essential for the assembly and secretion of triglyceride-rich lipoproteins	VLDL, IDL, LDL
ApoB-48	264,000	Obligatory structural protein for chylomicrons	Chylomicrons
ApoC-I	6,600	Inhibition of hepatic TGRL uptake; inhibitor of CETP	Chylomicrons, VLDL, HDL
ApoC-II	8,900	Activator of LPL	Chylomicrons, VLDL, HDL
ApoC-III	8,800	Modulates hepatic uptake of apoE-containing lipoproteins	Chylomicrons, VLDL, HDL
ApoE	34,200	Ligand for LDL receptor and apoE receptor	Chylomicrons, VLDL, HDL

VLDL, Very low density lipoproteins; IDL, Intermediate density lipoproteins; LDL, Low density lipoproteins; HDL, High density lipoproteins; TGRL, Triglyceride-rich lipoproteins; LCAT, Lecithin:cholesterol acyltransferase; LPL, Lipoprotein lipase; HL, Hepatic lipase; CETP, Cholesteryl ester transfer protein. [a]Source from Chan and Dresel, 1990; Li and Chan, 1999. [b]Source from Gautier et al., 2000; von Eckardstein et al., 2001.

Atherosclerosis has been demonstrated in a transgenic mouse model expressing human apoA-II (Warden et al., 1993). There is also evidence that the cardioprotection afforded by human apoA-I in transgenic mice is abrogated by co-expressing apoA-II (Schultz et al., 1993). Conversely, apoA-II is also potentially anti-atherogenic. Mice transgenic for human apoA-II are protected against the development of atherosclerosis when fed with a chow diet, despite a marked decrease in HDL cholesterol and apoA-I concentrations. This protection may be related to the reduction in circulating LDL, VLDL, and IDL levels in these mice (Tailleux et al., 2000).

Apolipoprotein A-I

ApoA-I is a 243-residue protein that contains a globular amino-terminal domain and a lipid-binding carboxyl-terminal domain (Segrest et al., 1992; Jonas, 1998; Brouillette et al., 2001). ApoA-I contains eight 22mer and two 11mer tandem amino acid sequence repeats, punctuated by proline turns (Karathanasis et al., 1983; Segrest et al., 1992). Each of these repeats has the periodicity of an amphipathic α-helix (Nolte and Atkinson, 1992). The amphipathic helices of apoA-I are oriented parallel to the lipoprotein lipid surface and penetrate no deeper than the ester linkages of the phospholipids (Clayton and Sawyer, 1999).

Most of the apoA-I in human plasma is secreted from either the liver or intestine and exists as a component of HDL (Ertel Miller et al., 1983; Zannis et al., 1984; Nicolosi and Zannis, 1989). About 20% of the apoA-I in HDL is acquired as a result of transfers from intestinal chylomicrons (Schaefer et al., 1978; Eisenberg. 1984).

ApoA-I can (i) bind lipids (Segrest et al., 1974; Nolte and Atkinson, 1992), (ii) activate lecithin:cholesterol acyltransferase (LCAT) (Jonas et al., 1985; Sparks et al., 1995a; Sorci-Thomas et al., 1998), (iii) promote cellular cholesterol efflux (Stein and Stein, 1973; Atger et al., 1995; Fournier et al., 1996), and (iv) act as a ligand for specific receptors (Francis et al., 1995; Xu et al., 1997).

A detailed model of the secondary structure of apoA-I proposed by Nolte and Atkinson (1992) suggested that the region between residues 44 and 243 of apoA-I is largely α-helical and is involved in lipid binding. Residues 100-143 and the ∝-helix containing residues 100-121 are important for binding to phospholipids, both in vitro (Frank et al., 1997) and in vivo (McManus et al., 2000).

The amino-terminal domain (residues 1-43) may be important for the stability of the protein in the lipid-free state and indirectly modulate its interaction with LCAT (Frank and Marcel, 2000). Studies in a variety of laboratories have shown that both the central (residues 100-122) and carboxyl-terminal (residues 148-186) domains of apoA-I may be critical to the activation of LCAT (Rall et al., 1986; Banka et al., 1991; Minnich et al., 1992; Sorci-Thomas et al., 1993; Sparks et al., 1998). However, the critical importance of the central domain is now in doubt, as more recent studies have shown that a complete deletion of this region in apoA-I has little effect on LCAT activation (Frank et al., 1998). This is not the case for the carboxyl-terminal domain of apoA-I. Sorci-Thomas et al. (1998) have demonstrated that rotation of the hydrophobic face of helix 143-164 of apoA-I by about 80° markedly reduces LCAT activation. The 143-164 region of apoA-I contains a cluster of three strictly conserved arginine residues (R149, R153, and R160). Mutation of these conserved residues drastically decreased LCAT activity (Roosbeck et

al., 2001). However, efficient LCAT activation also requires a certain affinity for phospholipids that is contributed by the carboxyl-terminal domain.

In addition, the lipid binding affinity of the carboxyl-terminal domain appears crucial for the ability of apoA-I to promote cholesterol efflux from cholesterol-loaded macrophages (Frank et al., 1998; Burgess et al., 1999). Analysis of the ability of deletion mutations of apoA-I to promote cholesterol efflux indicates that deletion of helices 8-10 affects the ability of the mutant protein to bind to macrophages and to promote efflux of cholesterol, whereas deletion of any one of helices 4-10 affects passive cholesterol efflux from human skin fibroblasts (Frank et al., 1998; Burgess et al., 1999).

ApoA-I is a ligand for scavenger receptor class B type I (SR-BI), a receptor that mediates selective uptake of HDL cholesteryl esters (Xu et al., 1997). Preliminary studies (Liadaki et al., 1999) suggest that both the amino-terminal and the carboxyl-terminal domains of apoA-I may contribute independently to binding to SR-BI.

Apolipoprotein A-II

ApoA-II, is the second most abundant apolipoprotein in HDL. It is a 154 residue protein and is synthesized in the liver (Eisenberg, 1984). It has a higher affinity for lipids than apoA-I and readily displaces apoA-I from HDL (van Tornout et al., 1980; Edelstein et al., 1982; Rosseneu et al., 1982; Lopez et al., 1994; Mowri et al., 1996). Displacement of apoA-I from the HDL surface by apoA-II reduces the LCAT activation and generates HDL particles that exhibit pre-β migration when subjected to agarose gel electrophoresis (Labeur et al., 1998; Durbin and Jonas, 1999). ApoA-II has been reported to inhibit (Thuren et al., 1991; Weng et al., 1999) and activate (Jahn et al., 1983) hepatic lipase

(HL). Recent studies have resolved this discrepancy by showing that HL has a higher affinity for HDL that contain apoA-II, but hydrolyzes phospholipids and triglycerides more rapidly in HDL that contain apoA-I (Hime et al., 1998).

Apolipoprotein A-IV

ApoA-IV is a glycoprotein found principally in HDL, chylomicrons, and the lipoprotein-free fraction of plasma. It dissociates readily from lipoproteins during ultracentrifugation (Weinberg and Spector, 1985) and associates with HDL that are increasing in size as a result of the action of LCAT (Glomset, 1968; Francone et al., 1989). In humans, the apoA-IV gene is expressed mainly in the intestine (Green et al., 1980). ApoA-IV is found in interstitial fluid at much higher concentration than in plasma (Roheim et al., 1976; Sloop et al., 1983). A number of physiological functions of apoA-IV have been postulated, including fat-soluble vitamin absorption (Weinberg et al., 1990) and activation of LCAT (Chen and Albers, 1985; Emmanuel et al., 1994; Jonas, 2000). ApoA-IV also promotes cholesterol efflux from adipose cells (Steinmetz et al., 1990), macrophages, and smooth muscle cells (Stein et al., 1995).

Apolipoprotein B

ApoB is associated with chylomicrons, VLDL, and LDL. It is essential for the assembly and secretion of chylomicrons and VLDL. ApoB exist in two main forms; apoB-100 and apoB-48 (Mahley et al., 1984). The two proteins are translation products of a single gene (Chen et al., 1987; Hospattankar et al., 1987; Powell et al., 1987; Scott et al., 1988).

Apolipoprotein B-100

ApoB-100 is a single polypeptide of over 4,536 amino acids (Young, 1990). It is the full-length translation product of the apoB gene. ApoB-100 contains a proline-rich 25-amino-acid residue repeat unit and a hydrophobic 52-amino-acid residue repeat unit (Yang et al., 1986; de Loof et al., 1987). The physiological role of these repeats remains to be determined (Yang et al., 1986). ApoB-100 also contains several lipid-binding domains and 25 cysteines, some of which are located in a disulfide cluster within the first 500 amino acid residues of the primary structure (Gotto and Pownall, 1999).

In humans, apoB-100 is synthesized in the liver and secreted into plasma as a constituent of VLDL (Kane, 1983). ApoB-100 is the major apolipoprotein of LDL, the end product of VLDL catabolism. Each VLDL particle contains one molecule of apoB-100. In the fasting state, most of the apoB in plasma is apoB-100. Unlike the other apolipoproteins, apoB-100 cannot move from one lipoprotein particle to another, and the apoB-100 in VLDL remains with the lipoproteins as they are catabolized to LDL (Kane, 1983; Musliner et al., 1987; Demant et al., 1988). ApoB-100 is essential for the removal of LDL from plasma, via the LDL receptor (Brown and Goldstein, 1986).

Apolipoprotein B-48

ApoB-48 contains 2,152 amino acids and is identical to the amino-terminal portion (48%) of apoB-100 (Young, 1990). Although a single gene codes for both proteins, the synthesis of apoB-48 results from the editing of a single codon of apoB mRNA, which converts codon 2,153 (CAA, specifying glutamine) into a premature stop codon (UAA) (Chen et al., 1987; Levy-Wilson, 1995).

ApoB-48 is produced by the small intestine (Powell et al., 1987; Hodges and Scott, 1992). While apoB-48 contains some of the glycosylation and heparin-binding sites as well as the disulfide cluster of apoB-100, it does not contain the receptor-binding domain of apo B-100 that targets LDL to cell surface receptors (Gotto and Pownall, 1999).

Apolipoprotein C

ApoC-I, apoC-II, and apoC-III consist of 57, 78, and 79 amino acids, respectively. They are associated with all lipoproteins except LDL. ApoC-I, the smallest of the C apolipoproteins, has been reported to activate LCAT in vitro (Soutar et al., 1975), inhibit the binding of lipoproteins to several lipoprotein receptors, such as LDL receptor, LDL receptor-related protein (LRP), and the VLDL receptor (Jong et al., 1999), and inhibit the activity of cholesteryl ester transfer protein (CETP) (Gautier et al., 2000). ApoC-II plays an important role in the metabolism of triglyceride-rich lipoproteins (VLDL and chylomicrons) by activating lipoprotein lipase (LPL), the enzyme that hydrolyzes triglycerides in triglyceride-rich lipoproteins (Olivecrona and Bentgsson-Olivecrona, 1993). ApoC-III exist in at least three polymorphic forms (Brunzell, 1995). The precise metabolic function of apoC-III is unknown, but it may inhibit LPL (Bentgsson and Olivecrona, 1980) and therefore regulate the activity of this enzyme.

Apolipoprotein E

ApoE is a 34,200 kDa glycoprotein that is found in chylomicrons, VLDL, HDL, and chylomicron and VLDL remnants. There are three common apoE variants; designed E_2, E_3, and E_4 (Utermann et al., 1980, 1982). These isoforms were initially distinguished by isoelectric focusing (Weisgraber et al., 1982) and have cysteine-arginine interchange at residues 112 and 158 (Rall et al., 1982). ApoE_2 has cysteine residues in both positions

and $apoE_4$ has arginine residues in both positions, whereas $apoE_3$ has cysteine and arginine at position 112 and 158, respectively (Mahley and Rall, 1995). These isoforms are coded for by the three alleles of the apoE gene; ε2, ε3, and ε4. This allelic variation contributes to susceptibility to atherosclerotic cardiovascular disease (Sing and Davignon, 1985; Davignon et al., 1988; de Knijff and Haveks, 1995). The ε3 allele is most common allele, although the relative proportions of the three alleles vary among populations (Dallongeville et al., 1992; Couderc et al., 1993).

ApoE plays a key protective role in atherosclerosis. Its capacity to protect against this disease can be attributed to at least three distinct functions; (i) apoE is a ligand for the LDL receptor, LRP, and scavenger receptors (Cooper et al., 1980; Goldstein et al., 1985; Beisiegel et al., 1989; Choi and Cooper, 1993; Linton et al., 1998; Arai et al., 1999), (ii) apoE facilitates cholesterol efflux from macrophage foam cells within the intima of the lesion (Hara and Yokoyama, 1991; Zhu et al., 1998), and (iii) apoE directly modifies both macrophage- and T-lymphocyte-mediated immune responses that contribute to chronic inflammatory disease (Pepe and Curtiss, 1986; Kelly et al., 1994; Zhou et al., 1996).

1.1.3 LIPOPROTEIN METABOLISM

Chylomicrons

Nascent chylomicrons are assembled from dietary fatty acids and monoglycerides and secreted into the circulation via the thoracic duct. Chylomicrons consist mainly of triglycerides (90% by mass) and apolipoproteins (2% by mass). The main apolipoprotein in chylomicrons is apoB-48 but apoA-I, A-II, and A-IV are also present (Gotto et al., 1986). Shortly after entering the circulation, chylomicrons acquire apoC-I, C-II, C-III, and apoE from circulating HDL (Mahley et al., 1984).

The apoC-II, on the surface of chylomicrons, activates LPL which rapidly hydrolyzes the triglycerides in the particles to free fatty acids and monoglycerides (Nilsson-Ehle et al., 1980; Goldberg, 1996; Zechner, 1997). Simultaneously, some of the phospholipids as well as apoA-I, and apoA-II, are transferred from the chylomicron surface to HDL either by spontaneous transfer to existing HDL or by dissociation from the chylomicrons as surface remnants which are then recycled into new HDL particles (Redgrave and Small, 1979; Tall et al., 1982). ApoB-48 remains associated with the chylomicrons and is a marker for its catabolism. Although the apoE transfers from chylomicron remnants to HDL, a sufficient amount remains with the remnants to mediate its binding to remnant receptors in the liver (Mahley et al., 1984; Davignon et al., 1988; Gregg and Brewer, 1988).

Prolonged plasma residence time of chylomicrons, or elevated levels in the postprandial state, may contribute to the onset of atherosclerosis. Abnormalities in chylomicrons or chylomicron remnant metabolism have been associated with coronary heart diseases (CHD) (Zilversmit, 1979).

Very low density lipoproteins

VLDL are assembled in the liver and secreted into the circulation via the Golgi apparatus (Bamberger and Lane, 1988; Davis, 1988, 1990; Boren et al., 1992). VLDL are triglyceride-rich lipoproteins (55% by mass) that contain apoB-100, apoE, and small amounts of C apolipoproteins. In the plasma additional C apolipoproteins are transferred to VLDL from circulating HDL (Mahley et al., 1984). The initiating event in the catabolism of VLDL is lipolysis by LPL. ApoC-II present on the surface of VLDL

activates LPL on endothelial cells, which leads to the hydrolysis of VLDL triglycerides and the release of fatty acids (Wang et al., 1992a). It is important to note that the rate of hydrolysis of VLDL triglycerides is much lower than that of chylomicron triglycerides (Xiang et al., 1999). As a result, the average residence time of VLDL triglycerides is 15 to 60 min, compared with the 5 to 10 min of chylomicron triglycerides (Havel and Kane, 1995). This difference may be attributed to the fact that VLDL are smaller particles and bind to fewer LPL molecules than chylomicrons (Goldberg, 1996).

The hydrolysis of VLDL triglycerides by LPL reduces the size of the particles and is accompanied by the transfer of the C apolipoproteins back to HDL (Redgrave and Small, 1979; Tall et al., 1982). Furthermore, CETP promotes the exchange of HDL cholesteryl esters for triglycerides from triglyceride-rich lipoproteins (Barter et al., 1982). The net result of the LPL-mediated hydrolysis and the CETP-mediated cholesteryl ester exchange reaction is the replacement of much of triglyceride core of the original VLDL with cholesteryl esters. These processes generate VLDL remnants, some of which are taken up by the liver. The remaining remnants are converted to smaller, more dense IDL (Demant et al., 1988) and, ultimately, LDL (Nicoll and Lewis, 1980; Fan et al., 1994).

Low density lipoproteins

Compared with VLDL and chylomicrons, LDL have a relatively long residence time in the circulation of about 3 days (Havel and Kane, 1995). The mechanism by which LDL are removed from circulation is well understood. LDL receptors recognize and bind apoB-100 of LDL (Brown et al., 1981; Goldstein et al., 1985). The LDL are internalized and delivered to lysosomes where the apoB-100 is degraded by proteases to small peptides and amino acids. Cholesteryl esters are also hydrolyzed by esterases, with the

resulting cholesterol becoming available for the synthesis of cell membranes, steroid hormones in steroidogenic tissues, and bile acids in hepatocytes (Brown and Goldstein, 1984).

LDL can also be taken up by extrahepatic tissues through scavenger receptor type A (SR-A) or non-receptor-mediated pinocytosis (Kodama et al., 1990). The non-receptor-mediated uptake becomes significant as plasma LDL concentrations increase, as in familial hypercholesterolemia (FH). Non-receptor-mediated uptake of LDL is not saturable and not regulated. Scavenger receptors are unregulated as well, and recognize LDL that have been modified in various ways. These receptors are found in macrophages and other cells. Macrophages that become engorged with cholesteryl esters from LDL are called "foam cells" (Brown et al., 1980; Henriksen, 1981, 1983; Brown and Goldstein, 1983), and are precursors of more complex atherosclerotic lesions. Two-thirds of LDL is normally removed by LDL receptors, and the remainder by the scavenger cell system.

High density lipoproteins

Origins of HDL

HDL are secreted from liver and intestine as nascent discoidal particles (Marsh, 1971; Green et al., 1978; Castle et al., 1991; Danielsen et al., 1993) that consist primarily of phospholipids and apoA-I or apoA-II (Hamilton et al., 1976). All nascent HDL have pre-β mobility when subjected to agarose gel electrophoresis. Nascent HDL are also assembled in the plasma from phospholipids that dissociate from triglyceride-rich lipoproteins that are being hydrolyzed by LPL (Redgrave and Small, 1979; Tall et al., 1982; Musliner et al., 1991). ApoA-I that has dissociated from mature HDL can also be recycled into nascent HDL (Barrans et al., 1994; Liang et al., 1994; Francone et al.,

1996b; Jiang et al., 1996; von Eckardstein et al., 1996), which are converted rapidly to spherical particles by the action of LCAT. LCAT is responsible for generating all of the cholesteryl esters in plasma. HDL are the main substrate for LCAT. As cholesteryl esters are formed by LCAT, they partition into the core of HDL and convert the discs into small spherical HDL and then into the large spherical HDL that are found in normal human plasma (Barter et al., 1985; Castro and Fielding, 1988; Francone et al., 1989). The activity of LCAT maintains the movement of cholesterol from cell membranes to the surface of HDL by maintaining a cholesterol gradient between cell membranes and HDL (Jonas, 1991).

Compositions and subclasses of HDL

HDL are the smallest of the lipoproteins, having masses that range from 180-360 kDa. They consist of approximately 50% protein, 25% phospholipids, 20% cholesteryl esters, and 5% triglycerides. ApoA-I and apoA-II represent approximately 90% of the total apolipoprotein content of HDL (Cheung and Albers, 1982). In addition to phosphatidylcholine, the surface of HDL contains other phospholipids such as, sphingomyelin, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine (Davidson et al., 1994a; Bagdade et al., 1995). There is also considerable variation in the length and unsaturation of HDL phosphatidylcholine acyl chains (Subbaiah and Monshizadegan, 1988). Enzymes and transfer proteins are also present on the surface of HDL. These include LCAT (Cheung et al., 1986; Francone et al., 1989), CETP (Pattnaik and Zilversmit, 1979; Cheung et al., 1986; Francone et al., 1989; Marcel et al., 1990), and phospholipid transfer protein (PLTP) (Tall et al., 1983a; Tall, 1993; Day et al., 1994). Together with HL (Marques-Vidal et al., 1997), these plasma factors remodel HDL and

are responsible for their heterogeneous size, composition, density, shape, and electrophoretic mobility.

Non-denaturing polyacrylamide gradient gel electrophoresis has been used to identify five distinct HDL subfractions in human plasma; HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, and HDL_{3c} (Blanche et al., 1981). HDL can also be subdivided on the basis of apolipoprotein composition into particles that contain both apoA-I and apoA-II (LpA-I/A-II), and those that contain apoA-I but not apoA-II (LpA-I) (Cheung and Albers, 1982; McVicar et al., 1984). A minor population of particles containing only apoA-II (LpA-II) has also been identified (Bekaert et al., 1992). The apoA-I in HDL is distributed approximately equally between LpA-I and LpA-I/A-II in most human subjects. More than 90% of the apoA-II is in LpA-I/A-II (Cheung and Albers, 1982). In normolipidemic male subjects, plasma levels of LpA-I/A-II are 2.5 times higher than that of LpA-I. Most LpA-I/A-II are present in the HDL_3 subfraction (James et al., 1989; Mowri et al., 1994), while approximately 60% of the LpA-I are in the HDL_2 density range (James and Pometta, 1990).

In addition to the HDL subpopulations described above, human plasma also contains several minor populations of particles that consist of apoA-I, unesterified cholesterol, and phospholipids. These subpopulations have pre-β electrophoretic mobility (Castro and Fielding, 1988) and are designated; pre-β_1 HDL, pre-β_2 HDL, and pre-β_3 HDL (Fielding and Fielding, 1995). Pre-β_1 HDL were first identified in 1985 (Kunitake et al., 1985). They are approximately 67 kDa and contain a single molecule of apoA-I complexed with a small amount of phospholipids and unesterified cholesterol (Castro and Fielding, 1988). The phospholipid component of pre-β_1-migrating HDL is characterized by a high content

of sphingomyelin. Pre-β_1 HDL has been observed in both plasma (Asztalos et al., 1993a) and peripheral lymph (Asztalos et al., 1993b).

Pre-β migrating HDL can be produced by several distinct metabolic pathways. The first pathway involves the interaction of lipid-free or lipid-poor apoA-I with cell membranes (Huang et al., 1995). Pre-β migrating HDL are also formed when apoA-I dissociates from spherical HDL that are being remodeled by lipolytic enzymes and lipid transfer proteins. For example, the conversion of triglyceride-rich HDL_2 into HDL_3 by HL generates pre-β migrating HDL (Clay et al., 1990, 1991, 1992; Barrans et al., 1994; Marques-Vidal et al., 1997). As mentioned above, CETP promotes transfers of core lipids between HDL and triglyceride-rich lipoproteins. Under some circumstances this can lead to an overall reduction in HDL core lipid content (Barter et al., 1982, 1990; Rye et al., 1995), a reduction in HDL size (Barter et al., 1990; Rye et al., 1995), and the dissociation of lipid poor apoA-I from the particles (Kunitake et al., 1992; Hennessy et al., 1993; Liang et al., 1994; Rye et al., 1995). In addition, the incubation of the HDL_3 with PLTP generates large, HDL_2-like particles, as well as small, pre-β migrating HDL (Jauhiainen et al., 1993; Day et al., 1994; Pussinen et al., 1995; Jiang et al., 1996; Lusa et al., 1996; von Eckardstein et al., 1996).

Pre-β migrating HDL are the initial acceptors of cellular cholesterol (Castro and Fielding, 1988; von Eckardstein et al., 1994; Sviridov and Fidge, 1995; Barrans et al., 1996). Pre-β migrating HDL remove cholesterol from cell membranes by two different pathways; (i) passive diffusion down a chemical gradient (Phillips et al., 1987; Johnson et al., 1991), and (ii) an active transport pathway via HDL or apoA-I receptors such as SR-BI and

ATP-binding cassette transport 1 (ABC1) (Luciani et al., 1994; Krieger et al., 1999; Lawn et al., 1999; Rust et al., 1999).

Small, protein-rich particles containing apoA-IV or apoE, that are present in plasma and lymph, also contribute to cholesterol efflux in vitro (Asztalos and Roheim, 1995), and possibly in vivo (Sloop et al., 1987; Roheim et al., 1990). However, it should be noted that these HDL subclasses take up phospholipids and cholesterol from different cellular pools by different kinetics (Fielding and Fielding, 1995).

Catabolism of HDL

Unlike apoB-containing lipoproteins, all components of HDL are exchangeable through spontaneous or protein-mediated transfer mechanisms. Therefore, the lipids and proteins of HDL can be catabolized independently of one another and of the HDL particle. For example, the kidney is the major catabolic site of apoA-I (Glass et al., 1985) via co-expression of cubilin and megalin receptors (Moestrup et al., 1998; Kozyraki et al., 1999). Other studies have shown that HDL cholesteryl esters are selectively removed by the adrenal glands and liver via SR-BI (Acton et al., 1996; Wang et al., 1996; Rigotti et al., 1997; Krieger, 1999). SR-BI also promotes the hepatic uptake of HDL cholesterol and its transport into bile (Ji et al., 1999). The liver accounts for 60-80% of total HDL cholesteryl ester clearance in vivo (Pittnan and Steinberg, 1984; Glass et al., 1985; Woollett and Spady, 1997).

Staels (2000) has identified the nuclear receptors, peroxisome-proliferating agent receptor α (PPARα) as transcriptional regulators of HDL metabolism. These receptors act by influencing the transcription of the genes that determine HDL levels such as, apoA-I and

apoA-II. In addition, absence of their expression profoundly perturbs HDL metabolism. Moreover, PPARα activators also induce SR-BI and ABC1 mediated efflux in human macrophages (Chinetti et al., 2000).

1.1.4 HIGH DENSITY LIPOPROTEINS AND REVERSE CHOLESTEROL TRANS-PORT (RCT)

Reverse cholesterol transport (RCT) is the term used to describe a pathway by which (i) the excess cholesterol in peripheral tissues, including the arterial wall, is incorporated into HDL, (ii) esterified by LCAT, (iii) transferred to LDL as well as to triglyceride-rich lipoproteins by CETP, and (iv) transported to the liver where it is taken up by receptor-mediated processes and either recycled or excreted into the intestine as a component of bile (Fig. 1.2) (Glomset, 1973; Pieters et al., 1994). RCT may protect against the development of atherosclerosis (Glomset, 1968).

RCT pathway

The initial step of RCT is the efflux of cholesterol from the peripheral tissues and its incorporation into pre-β_1-migrating HDL (Castro and Fielding, 1988; Kawano et al., 1993; Fielding and Fielding, 1995). Experiments with reconstituted HDL have shown that several factors such as, apolipoprotein composition (Mahlberg et al., 1991; Mahlberg and Rothblat, 1992), phospholipid composition (Davidson et al., 1995a), and particle size (Agnani and Marcel, 1993; Davidson et al., 1995b) affect the capacity of the acceptor particle to acquire cellular cholesterol. Cholesterol and phospholipid efflux can occur by passive diffusion (Phillips et al., 1987; Johnson et al., 1991), or it may involve HDL or apoA-I receptors such as, SR-BI and ABC1 (Luciani et al., 1994; Krieger et al., 1999; Lawn et al., 1999; Rust et al., 1999).

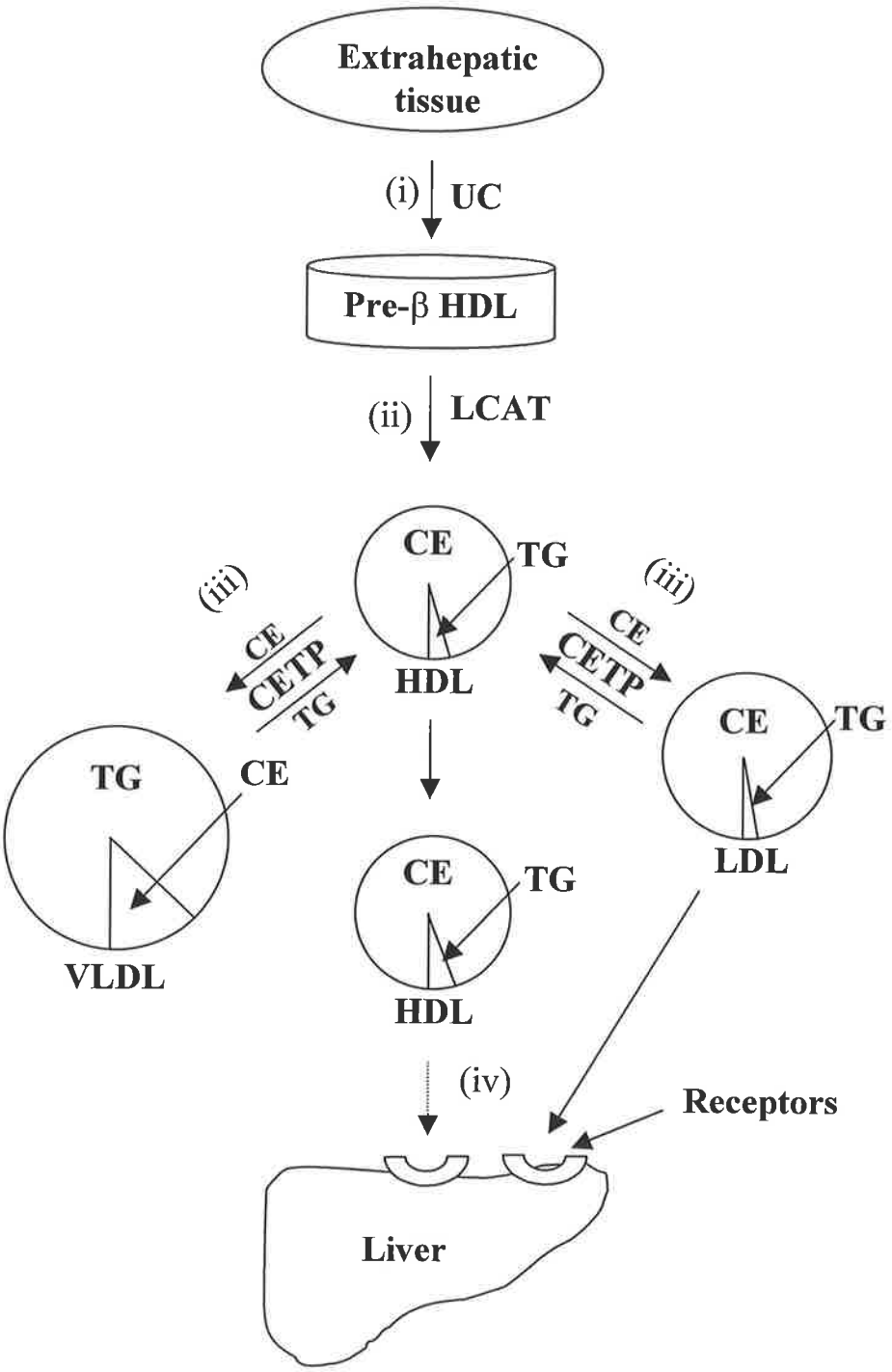


Figure 1.2 Reverse cholesterol transport
UC, Unesterified cholesterol; CE, Cholesteryl esters; TG, Triglycerides; LCAT, Lecithin:cholesterol acyltransferase; CETP, Cholesteryl ester transfer protein; HDL, High density lipoproteins; LDL, Low density lipoproteins; VLDL, Very low density lipoproteins (adapted from Barter and Rye, 1996).

The next step is esterification of HDL cholesterol by LCAT. This produces cholesteryl esters that are sequestered into the HDL core, and convert discoidal HDL into small spherical HDL (Barter et al., 1985; Castro and Fielding, 1988; Francone et al., 1989). As LCAT esterifies cholesterol in HDL, a concentration gradient is established that enables the HDL to attract more free cholesterol from cell membranes or from the excess surface constituents of chylomicrons and VLDL that are being lipolyzed by LPL. As cholesterol esterification proceeds, the size of the HDL increases.

The cholesteryl esters are then transferred from HDL to LDL or to triglyceride-rich lipoproteins in exchange for triglycerides in a process mediated by CETP (Barter et al., 1982; Drayna et al., 1987; Hesler et al., 1987; Tall, 1995).

HDL cholesteryl esters can be returned to the liver by; (i) the selective uptake without concomitant degradation of HDL protein. This pathway was first elucidated by Pittman and coworkers (Glass et al., 1983) and has recently been shown by Acton et al. (1996) to be mediated by SR-BI, and (ii) uptake of holo-HDL particles via a pathway that may involve proteoglycans and apoE as well as the degradation of HDL apolipoproteins such as apoA-I (Stow et al., 1985; Gwynne, 1989; Hamilton et al., 1990; Ji et al., 1993).

Recent studies have indicated that interactions of HDL with PLTP may also influence RCT by (i) transferring phospholipids and cholesterol from triglyceride-rich lipoproteins, which are ungergoing lipolysis to HDL (Tall et al., 1985; Jiang et al., 1999), (ii) remodeling HDL and regenerating pre-β migrating HDL or lipid-free apoA-I that can participate in the first step of RCT (Day et al., 1994; Pussinen et al., 1995; Jiang et al.,

1996; Lusa et al., 1996; von Eckardstein et al., 1996; van Haperen et al., 2000), and (iii) accelerating the hepatic uptake of HDL surface and core lipids (Föger et al., 1997).

There are, however, studies that do not support the role of HDL in RCT. In a steady-state, the rate of cholesterol acquisition by extrahepatic tissues equals the rate of cholesterol return to the liver for excretion. Under these conditions, the rate of cholesterol acquisition in extrahepatic tissues is a measure of RCT (Spady, 1999). This approach has been used to show that plasma HDL cholesterol concentrations can be varied over a wide range with no change in the rate of RCT (Woollett et al., 1997). Spady et al. (1999) observed that while polyunsaturated fatty acids in the diet lowers the plasma HDL cholesteryl ester concentration in the hamster, the rate of RCT is not affected. Recently, Alam et al. (2001) have shown that unlike nascent HDL, overexpression of 7α-hydroxylase, SR-BI, LCAT, or apoA-I in the liver do not stimulate cholesterol efflux from any extrahepatic tissues. Thus, although HDL enhance cholesterol efflux from extrahepatic tissues and increase net cholesterol movement to the liver, cholesterol flux through the entire RCT pathway is not increased.

1.1.5 HIGH DENSITY LIPOPROTEINS AND ATHEROSCLEROSIS

The evidence that HDL protect against atherosclerosis is now irrefutable. A strong negative correlation has been established between plasma HDL and the risk of CHD (Miller and Miller, 1975; Castelli et al., 1986; Genest et al., 1999; Stein and Stein, 1999), although the mechanisms underlying this protective effect are incompletely understood. HDL may exert protective effects by (i) stimulating RCT from extrahepatic tissues to the liver for excretion (Glomset, 1968; Fielding and Fielding, 1995), (ii) protecting LDL from oxidation (Watson et al., 1995; Banka, 1996), and (iii) inhibiting endothelial

adhesion molecule expression and inflammatory responses to endotoxins (Cockerill et al., 1995; van Lenten et al., 1995).

Reverse cholesterol transport (RCT)

HDL may be anti-atherogenic by virtue of their ability to promote the efflux of cholesterol from cells in the artery wall in the first step in RCT (Fielding and Fielding, 1995; Oram and Yokoyama, 1996). The details of RCT are described above in Section 1.1.4. In support of this mechanism, it has been shown that HDL promote the efflux of cholesterol from cholesterol loaded cells and that they reduce the cholesterol content of foam cells (Hara and Yokoyama, 1991; Asztalos et al., 1997). Lagrost et al. (1995) have reported that LpA-I are better acceptors for cellular cholesterol than LpA-I/A-II. LpA-I may also be more protective against atherosclerosis than LpA-I/A-II (Parra et al., 1992; Duverger et al., 1996). Transgenic animal studies are also consistent with these observations (Schultz et al., 1992, 1993). Disruption of RCT may favor the deposition of cholesterol within the arterial wall and thereby contribute to the development of atherosclerosis. Recently new insights into the mechanisms involved in RCT have been obtained following the identification of ABC1 as the major apoA-I mediated pathway for the efflux of cellular cholesterol (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Lawn et al., 1999; Rust et al., 1999). Defects in ABC1 are present in patients with Tangier disease and in selected patients with familial hypoalphalipoproteinemia. As a result of these defects, apoA-I is poorly lipidated. This leads to enhanced catabolism of apoA-I and low plasma HDL levels (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). Furthermore, apoA-I-mediated cholesterol efflux is severely decreased by the inhibition of ABC1 with either antisense oligonucleotides or pharmacological

compounds and is increased by the overexpression of ABC1 (Lawn et al., 1999; Hamon et al., 2000).

Antioxidant properties

The anti-atherogenic capacity of HDL may also be related to its antioxidant properties (Parthasarathy et al., 1990; Mackness and Durrington, 1995). It is now generally accepted that oxidation of LDL plays a significant pathogenic role in atherosclerosis (Berliner et al., 1995; Holvoet and Collen, 1998; Steinberg, 1999). Products generated from peroxidized lipids modify LDL to a more electronegative form that is capable of interacting with the macrophage scavenger receptors (Steinbrecher et al., 1989). Under conditions of mild oxidative modification, HDL can take up, transport, and neutralize highly reactive oxidized lipid species from LDL (Hessler et al., 1979; Christison et al., 1995; Garner et al., 1998). Parthasarathy et al. (1990) demonstrated that HDL inhibit the oxidation of LDL by metal ions in endothelial cells in vitro by reducing both the net negative charge of LDL and macrophage degradation. Recently, Panzenboeck et al. (2000) have found that selective oxidation of apoA-I methionine residues in HDL to methionine sulfoxides does not decrease the potential anti-atherogenic properties of apoA-I. This is consistent with the overall hypothesis that detoxication of lipid hydroperoxides by HDL is potentially anti-atherogenic.

Paraoxonase (PON) is a Ca^{2+}-dependent esterase bound to HDL (Watson et al., 1995). It hydrolyzes many synthetic substrates including organophosphates. In vitro and in vivo studies suggest that PON is the enzyme primarily responsible for the antioxidant activity of HDL (Mackness et al., 1993; Mackness and Durrington, 1995; Watson et al., 1995; Shih et al., 1998). Purified human PON decreases the generation and accumulation of

lipoperoxides in LDL (Mackness et al., 1991). In addition, PON, by destroying oxidized phospholipids, reduces the ability of oxidized LDL to induce monocyte binding and transmigration and thus, inflammation in the vessel wall. (Watson et al., 1995). Furthermore, targeted deletion of the PON gene in the mouse led to an increase sensitivity of HDL and LDL to oxidation and enhanced susceptibility of the animals to atherosclerosis when placed on an atherogenic diet (Shih et al., 1998).

Anti-inflammatory properties

It is generally recognized that atherosclerosis is among the group of chronic disease in which over-recruitment of leukocytes, in this case monocytes and to a lesser extent T-cells, is the cause of the pathology (Ross, 1999). Another mechanism by which HDL may protect against atherosclerosis is by inhibiting the expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin in endothelial cells (Cockerill et al., 1995; Xia et al., 1999). This prevents recruitment of monocytes by endothelial cells in what is recognized as one of the initiating events in atherosclerosis. It has been shown that both the apolipoproteins and lipids of HDL must be present to produce the inhibitory effect (Ashby et al., 1998). However, the apolipoprotein composition of HDL does not appear to be important in inhibiting adhesion molecule expression (Calabresi et al., 1997; Ashby et al., 1998; Baker et al., 1999).

One mechanism by which HDL inhibit adhesion molecule expression involves the signaling pathway which begins with tumour necrosis factor α (TNF-α) and ends with nuclear factor κB (NF-κB) (Barter et al., 1998). NF-κB is an inducible transcription factor essential for the expression of VCAM-1, ICAM-1, and E-selectin (Collins, 1993).

The involvement of this pathway in the inhibitory effect of HDL is supported by the study of Xia et al. (1999) which showed that HDL interrupt the sphingosine kinase signaling pathway. However, a further study challenges the view that HDL have a general inhibitory effect on the NF-κB pathway (Cockerill et al., 1999).

1.1.6 HIGH DENSITY LIPOPROTEINS AND PLASMA FACTORS

It is important to understand the factors that regulate the remodeling of HDL. Remodeling is the conversion of one HDL subpopulation into another by various plasma factors. Remodeling accounts for much of the heterogeneity of HDL in human plasma. Remodeling also has important implications in terms of the anti-atherogenic and other functional properties of HDL. Several plasma proteins are involved in the remodeling of HDL. These include LCAT, LPL, HL, endothelial lipase (EL), CETP, and PLTP.

Lecithin:cholesterol acyltransferase (LCAT)

Physical characteristics of LCAT

LCAT is a hydrophobic glycoprotein of molecular weight 68 kDa (Albers et al., 1976). LCAT is synthesized in the liver and secreted into plasma, where it circulates bound predominantly to HDL (Cheung et al., 1986; Francone et al., 1989; Navab et al., 1998). Jonas (1987) and Fielding et al. (1991) have shown that about equal amounts of plasma LCAT is associated with the LpA-I and LpA-I/A-II fractions. However, other investigators have reported that 60-80% of the LCAT in plasma is associated with LpA-I, while 10-20% is associated with LpA-I/A-II (Cheung et al., 1986; Cheung, 1993; Duverger et al., 1993). LCAT is present in normal human plasma at a concentration of about 6 mg/L.

Enzymatic functions of LCAT

LCAT is responsible for all of the esterification of cholesterol in plasma lipoproteins (Castro and Fielding, 1988; Francone et al., 1989). This enzyme displays two activities; a phospholipase A_2 activity, which hydrolyzes sn-2 acyl ester bonds in phosphatidylcholine, and an acyltransferase activity, which transfers the resulting fatty acids to the 3-β hydroxy group of free cholesterol. These processes generate cholesteryl esters and lysophosphatidyl-choline (Glomset, 1968). The LCAT reaction takes place mainly on the surface of HDL and accounts for the formation of most of the cholesteryl esters in human plasma (Glomset, 1968). The lysophosphatidylcholine that is generated either binds to albumin or acts as an acyl group acceptor and is converted back to phosphatidylcholine by LCAT (Subbaiah et al., 1985; Czarnecka and Yokoyama, 1993). The conformation and charge of apoA-I plays a central role in LCAT activation and these parameters are influenced by the amount of cholesterol in the surface of HDL particles (Jonas et al., 1985; Sparks et al., 1995a).

In studies aimed at identifying the residues essential for LCAT activity, the loss of activity that accompanied deletion of residues 394-398 suggested a structural role for this part of LCAT (Francone et al., 1996a). In other studies amino acid residue 149 was shown to determine the phospholipase A_2 and transacylase fatty acyl specificity of LCAT (Wang et al., 1997).

It is note worthy that there are two different LCAT activities in normal human plasma. One is α-LCAT, which esterifies the HDL cholesterol and the other is β-LCAT which acts on VLDL and LDL (Carlson and Holmquist, 1985). The preferred substrates for LCAT are HDL, which also contain the major activator for LCAT, apoA-I. HDL_3 has

higher reactivity with LCAT than HDL_2 (Jahani and Lacko, 1982; Barter et al., 1984), and discoidal HDL are superior as substrates to small spherical HDL (Barter et al., 1985; Jonas, 1991). There is also evidence that LpA-I are better LCAT substrates than LpA-I/A-II (Ohta et al., 1993; Francone et al., 1995).

LCAT-mediated HDL remodeling

LCAT is responsible for the maturation of nascent, discoidal HDL into the large spherical particles that are present in normal human plasma. The cholesteryl esters generated by LCAT partition into the HDL core and increase their size. Coincident with the LCAT-mediated increase in core lipids, the HDL surface acquires additional apoA-I, either in the lipid-poor form (Liang et al., 1995) or in a process which involves particle fusion (Liang et al., 1996). The LCAT-mediated fusion of small, spherical LpA-I generates large, unstable particles with four molecules of apoA-I. These particles either shed a molecule of apoA-I, forming HDL with three molecules of apoA-I, or may persist as particles containing four molecules of apoA-I. The importance of LCAT in the generation of spherical HDL particles is evident from mutations of the LCAT gene, which result in familial LCAT deficiency and Fish-eye disease (Wiebusch et al., 1995; Contacos et al., 1996; Kuivenhoven et al., 1997). Both of these conditions show an absence of normal, spherical HDL (von Eckardstein et al., 1995; Kuivenhoven et al., 1997). Comparable observations have also been made in mice with targeted disruption of the LCAT gene (Ng et al., 1997; Sakai et al., 1997).

LCAT and atherosclerosis

As LCAT facilitates one of the intermediate steps of RCT, it can be regarded as potentially anti-atherogenic. However, it must also be recognized that the cholesteryl

esters generated by LCAT can be transferred by CETP to potentially atherogenic apoB-containing lipoproteins.

LCAT may also prevent the development of atherosclerosis by hydrolyzing oxidized phosphatidylcholine on LDL and inhibiting their accumulation by macrophage scavenger receptors (Itabe et al., 1999; Vohl et al., 1999). However, other studies have shown that polar lipids isolated from minimally oxidized LDL reduce LCAT activity, impair RCT, and thus increase atherogenesis (Bielicki and Forte, 1999; Davit-Spraul et al., 1999).

Overexpression of human LCAT in rabbits results in a two-fold increase in plasma HDL and a three-fold increase in plasma cholesterol esterification rate (Hoeg et al., 1996b). The predominant mechanism by which LCAT overexpression increases plasma HDL concentration in rabbits is by lowering the apoA-I fractional catabolic rate (Brousseau et al., 1996). When rabbits transgenic for LCAT were fed an atherogenic diet, non-HDL cholesterol was reduced and HDL cholesterol increased relative to control animals. These changes were associated with a marked reduction in atherosclerosis (Hoeg et al., 1996a). However, when these experiments were repeated in mice transgenic for human LCAT, atherosclerosis was enhanced in spite of high plasma HDL concentrations (Berard et al., 1997). In these mice, the selective uptake of ^{3}H-cholesteryl ester by the liver was reduced, apparently due to the compositional changes of the particle. It is important to emphasize that human LCAT transgenic rabbits, unlike human LCAT transgenic mice, had significant reductions in LDL cholesterol levels when fed an atherogenic diet due to enhanced catabolism of LDL apoB-100 (Brousseau et al., 1997). This, together with the absence of CETP in the mouse, is probably the major reason for the differences observed in the atherosclerosis susceptibility of these two transgenic models.

Lipoprotein lipase (LPL)

Physical characteristics of LPL

LPL is a homodimeric glycoprotein found on the luminal surface of extrahepatic vascular endothelial cells (Garfinkel et al., 1983; Eckel, 1989). The active enzyme is a dimer of two identical 55-60 kDa glycoprotein subunits that loses hydrolytic activity upon dissociation (Osborne et al., 1985; Wion et al., 1987).

LPL is synthesized in adipocytes, myocytes, milk-producing cells, and macrophages (Olivecrona and Olivecrona, 1994). The major sites of LPL secretion are adipose tissue, cardiac and skeletal muscle, breast tissues, kidney, and lung. LPL is translocated from sites of synthesis to sites of utilization (O'Brien et al., 1994). Saxena et al. (1991) and Lutz et al. (2001) have found that heparan sulphate proteoglycans, which are present throughout the vascular tree, are required for LPL binding and activity. Some LPL is present in the circulation bound to lipoproteins (Peterson et al., 1985; Vilella et al., 1993), but its concentration is low due to effective uptake in liver whereby the LPL is degraded (Wallinder et al., 1984).

Enzymatic functions of LPL

LPL hydrolyzes triglycerides in chylomicrons and VLDL (Olivecrona and Bengtsson-Olivecrona, 1993). ApoC-II plays an important role in activating LPL. However, there is growing evidence that LPL is a multifunctional protein. In addition to mediating lipolysis of triglycerides, LPL is also involved in the uptake and degradation of lipoproteins by cells (Beisiegel et al., 1991; Williams et al., 1992; Mulder et al., 1993; Obunike et al., 1994). Initially, Felts et al. (1975) suggested that lipoprotein-associated LPL directs these

particles for catabolism to the liver. Beisiegel et al. (1991) later showed an LPL-induced increase in the binding of apoE-rich lipoproteins to cells in vitro. LPL also promotes the binding of apoE-containing liposomes to the LRP (Herz et al., 1988; Beisiegel et al., 1991). Direct binding of LPL to LRP has been demonstrated as well, and this interaction apparently mediates lipoprotein degradation (Beisiegel et al., 1991; Chappell et al., 1992; Nykjaer et al., 1993). Based on these observations it was suggested that LPL stimulates the cellular catabolism of triglyceride-rich lipoproteins and that the specific mechanism involves an interaction between LPL, lipoproteins, and LRP.

The potential role of LPL in HDL metabolism has not been well explored. In human postheparin plasma, LPL mass and activity elute on gel filtration at positions similar to HDL and LDL (Goldberg et al., 1986; Vilella et al., 1993). Rinninger et al. (1998) have reported that LPL increases the selective uptake of HDL cholesteryl esters by hepatic cells in culture. This effect is dependent on cell surface heparan sulfate proteoglycans but independent of lipolysis and of endocytosis mediated by LRP or LDL receptors (Goldberg, 1996).

In LPL knockout mice surviving up to 18 h after birth, massive hypertriglyceridemia was accompanied by very low plasma HDL (Weinstock et al., 1995). In LPL-deficient mice rescued by crossbreeding with mice expressing LPL in cardiac muscle, plasma triglycerides and HDL were normalized in adult animals (Levak-Frank et al., 1999).

Conversely, the overexpression of LPL drastically lowered triglycerides, VLDL, and increased HDL (Shimada et al., 1993; Clee et al., 1997). Transgenic overexpression of LPL is associated with reduced atherosclerosis in LDL receptor-deficient (Shimada et al.,

1996) and apoE-deficient mice (Yagyu et al., 1999). Pharmacological upregulation of LPL in rats raises HDL levels and reduces atherosclerosis (Tsutsumi et al., 1993).

Hepatic lipase (HL)

Physical characteristics of HL

HL is a 476 amino acids lipolytic glycoprotein (Datta et al., 1988) with molecular weight of 53-60 kDa. HL is synthesized in hepatocytes (Doolittle et al., 1987). It is anchored by heparan sulfate proteoglycans to the luminal surface of endothelial cells, which line the sinusoids of the liver (Kuusi et al., 1979; Stanhke et al., 1987; Datta et al., 1988; Sanan et al., 1997). Experimental data is consistent with lipolytically active HL existing as a dimer (Hill et al., 1996).

Functions of HL

The substrate specificity of HL is broad. It hydrolyzes monoglycerides, diglycerides, triglycerides, and phospholipids (Waite, 1987; Waite et al., 1991). Although HDL triglycerides and phospholipids are the preferred substrates of HL, this enzyme can hydrolyze triglycerides and phospholipids in all lipoprotein fractions (Ehnholm et al., 1975; Kuusi et al., 1979; Musliner et al., 1979; Shirai et al., 1981; Groot et al., 1983; Deckelbaum et al., 1992).

Within the HDL fraction, HL has the greatest affinity for HDL which contain apoA-II (Hime et al., 1998). Several studies have shown that the main apolipoproteins in HDL, apoA-I and apoA-II, affect the HL-mediated hydrolysis of HDL triglycerides and phospholipids, but the results have been inconsistent. While some studies have found that apoA-II enhances the HL-mediated hydrolysis of triglycerides and phospholipids in HDL,

others have shown that the hydrolysis is inhibited by apoA-II. The reason for this discrepancy has recently been resolved. Homogenous preparations of spherical reconstituted HDL (rHDL) containing either apoA-I only (A-I rHDL) or apoA-II only (A-II rHDL) were incubated with HL (Hime et al., 1998). The results showed that HL has a great affinity (K_m) for A-II rHDL than for A-I rHDL, but that the maximal rate (V_{max}) of triglyceride and phospholipid hydrolysis is greater in A-I rHDL than in A-II rHDL. In addition, the K_m and V_{max} of the phospholipid hydrolysis in rHDL containing both apoA-I and apoA-II (A-I/A-II rHDL) is intermediate between that of A-I rHDL and A-II rHDL. These results show that apoA-I and apoA-II are major determinants of the interaction of HDL with HL (Hime et al., 1998). Tansey et al. (1997) have also shown that spherical HDL particles are better substrates for HL-catalyzed phospholipid hydrolysis than discoidal HDL particles.

HL is also involved in the SR-BI-mediated selective uptake of cholesteryl esters from HDL (Lambert et al., 1999). The involvement of HL in selective cholesteryl ester uptake is inhibited by anti SR-BI antibodies (Wang et al., 1996; Thuren et al., 1998; Vieira-Van Bruggen, 1998). HL is also involved in chylomicron remnant uptake. This process is independent of its lipolytic activity (de Faria et al., 1996). HL also acts as a ligand for the removal of apoB-containing lipoproteins from plasma (Choi et al., 1998; Dichek et al., 1998).

In vitro, HL converts larger LDL into small LDL (Musliner et al., 1979). When HL is deficient, conversion of IDL to LDL is almost totally inhibited (Demant et al., 1988) and the LDL become enlarged and enriched in triglycerides (Connelly et al., 1990).

HL-mediated HDL remodeling

HL mediates the hydrolysis of the triglycerides in HDL in a process that decreases the core volume and size of the particles and is accompanied by the dissociation of lipid poor apoA-I (Clay et al., 1990, 1991, 1992; Barrans et al., 1994; Marques-Vidal et al., 1997).

The importance of HL in HDL remodeling is highlighted by studies of human subjects with HL deficiency, whose plasma contains enlarged, triglyceride-rich HDL (Knudsen et al., 1996, 1997; Connelly and Hegele, 1998). Similar particles are also found in the rabbit, a species which is naturally deficient in HL (Clay et al., 1989), as well as in mice in which the HL gene has been inactivated (Homanics et al., 1995; Applebaum-Bowden et al., 1996). HL also remodels HDL which have not been enriched with triglycerides. Under these conditions, HDL phospholipids are the substrate for HL (van Tol et al., 1980; Parret et al., 1987). Although the resulting phospholipid-depleted HDL are reduced in size, it is not known whether lipid-poor apoA-I is generated under these circumstances.

HL and atherosclerosis

Despite the evidence showing that HL plays a prominent role in lipoprotein metabolism, it is not known whether it decreases or increases the risk of developing atherosclerosis.

Subjects with HL deficiency, as well as HL knockout mice, have increased levels of plasma HDL (Homanics et al., 1995; Santamarina-Fojo et al., 1998), and premature manifestations of atherosclerosis (Cohen et al., 1999; Thuren, 2000). Dugi et al. (2000) also found that, despite the inverse correlation between HL activity and HDL levels, low HL activity is associated with the presence of atherosclerosis. However, in apoE-deficient mice targeted mutation of HL decreases atherosclerosis and increases cholesterol efflux

from cells (Mezdour et al., 1997). Similar effects were seen in mice transgenic for human LCAT that are also deficient in HL (Amar et al., 1997). In middle-aged men with established coronary artery disease (CAD) and dyslipidemia, the -514C→T polymorphism of the HL gene significantly predicts changes in coronary stenosis with lipid-lowering treatment (Zambon et al., 2001). Intensive lipid-lowering therapy lowers HL activity, increases LDL and HDL buoyancy, and promotes CAD regression (Zambon et al., 2001).

Overexpression of human HL in transgenic rabbits resulted in an 80% reduction in plasma HDL levels (Fan et al., 1994). Dichek et al. (1998) found that this reduction in the level of HDL was due to the HL-mediated hydrolysis of HDL phospholipids. HL also enhances the selective uptake of HDL cholesteryl esters in transfected hepatoma (Ji et al., 1997) and Chinese hamster ovary (CHO) cells (Komaromy et al., 1996). When these results are considered together, it is uncertain whether inhibition or stimulation of HL will exert beneficial effects in atherosclerosis.

Endothelial lipase (EL)

Physical characteristics of EL

The triglyceride lipase gene family plays a central role in intestinal lipid absorption, energy homeostasis, plasma lipoprotein metabolism, and atherosclerosis. A new member of this gene family, termed endothelial lipase (EL), was recently discovered (Hirata et al., 1999; Jaye et al., 1999). The apparent molecular weight of EL is 68 kDa. Though cloned from a human placental cDNA library, the new lipase is expressed in human umbilical vein and coronary artery endothelial cells. EL mRNA was detected in the lung, liver, and kidney as well as in the placenta, where expression was the greatest. EL, like LPL and

HL, may be anchored to the luminal endothelial surface via heparan sulfate proteoglycans (Rader and Jaye, 2000). The primary sequence of EL has homology with other members of the triglyceride lipase family, including LPL (45%), HL (40%), and pancreatic lipase (27%) (Hirata et al., 1999).

Functions of EL

While HL has both triglyceride lipase and phospholipase activities and LPL has triglyceride lipase activity but very low phospholipase activity, EL has high phospholipase activity and minimal triglyceride lipase activity. EL is not dependent on apoC-II or any other serum factor for activity (Hirata et al., 1999; Jaye et al., 1999).

EL and atherosclerosis

Overexpression of EL in mice results in a striking reduction of plasma HDL concentrations (Jaye et al., 1999). It is possible that endothelial EL expression could influence atherosclerosis directly (through expression at the site of atherosclerotic lesions) or indirectly (through modulation of lipoprotein metabolism and function) (Rader and Jaye, 2000). Further studies will help define the physiological role of the newly discovered lipase in HDL metabolism.

Cholesteryl ester transfer protein (CETP)

Physical characteristics of CETP

CETP is an extremely hydrophobic, heat stable glycoprotein (Barter et al., 1982; Hesler et al., 1987; Morton and Steinbrunner, 1993), containing 476 amino acids (Jarnagin et al., 1987) with a high proportion of hydrophobic residues and six potential N-glycosylation sites (Tall, 1995). The molecular weight of mature CETP is approximately 74 kDa. Many

cell types secrete CETP but the major source of the protein in humans is the liver (Quinit et al., 1991; Agellon et al., 1992). Significant amounts of CETP are also produced by adipose tissue and muscle cells (Jiang et al., 1991). CETP and PLTP together with lipopolysaccharide-binding protein (LBP), and bactericidal/permeability-increasing protein (BPI) belong to the lipopolysaccharide binding/lipid transfer protein family. These proteins have all evolved from a common ancestral gene (Tu et al., 1995).

Most of the CETP in human plasma is bound to HDL (Pattnaik and Zilversmit, 1979; Cheung et al., 1986; Francone et al., 1989; Marcel et al., 1990). There is evidence that the hydrophobic face of a putative carboxyl-terminal amphipathic helix (residues 465-476) of CETP is necessary for cholesteryl ester and triglyceride transfers (Swenson et al., 1989; Wang et al., 1992b). However, HDL binding also involves other regions of the protein. Evidence of this comes from a study where deletion of residues 470-475 resulted in impaired cholesteryl ester and triglyceride transfers without affecting HDL binding activity (Wang et al., 1992b). The binding of CETP to lipoproteins involves hydrophobic and ionic interactions (Nishida et al., 1993). Binding is increased as the lipoprotein surface charge becomes increasing negative. For example, lipolysis increases the binding of CETP to VLDL as a result of enrichment of VLDL with negatively charged fatty acids (Sammett and Tall, 1985). Increasing the negative charge of LDL by chemical modification, or by addition of anionic detergents, also enhances the binding of CETP to these particles and improves their ability to serve as substrates (Nishida et al., 1993). Removal of phospholipid head groups from the surface of HDL by phospholipase C treatment abolishes binding of CETP (Pattnaik and Zilversmit, 1979). These and other findings suggest that the phospholipid head groups are the primary sites for the

interaction of CETP with lipoproteins and that the stability of the complexes increases with the negative charge of the lipoproteins.

Functions of CETP

CETP mediates the transfer and exchange of neutral lipids and phospholipids between plasma lipoproteins (Barter et al., 1982; Hesler et al., 1987; Tall, 1995). It also exchanges the cholesteryl esters of HDL for triglycerides from triglyceride-rich lipoproteins. These transfers are not always equimolar (Barter et al., 1982, 1990; Liu and Bagdade, 1995), as the magnitude of cholesteryl ester transfers out of HDL may be greater than the transfers of triglyceride into the HDL. This can deplete the HDL of cholesteryl esters and enrich them with triglycerides (Deckelbaum et al., 1986; Barter et al., 1990; Rye et al., 1995). Triglyceride enrichment of HDL enhances their interaction with PLTP (Rye et al., 1998), and also generates HDL which are the preferred substrates for HL (Perret et al., 1987).

CETP-mediated HDL remodeling

CETP decreases HDL size (Barter et al., 1990; Rye et al., 1995; Lagrost et al., 1996), and promotes the dissociation of lipid-poor apoA-I from the particles (Liang et al., 1994; Rye et al., 1995). The combined activities of CETP and HL, in the presence of triglyceride-rich lipoproteins are particularly effective in reducing HDL size and mediating the dissociation of apoA-I (Hopkins and Barter, 1986; Liang et al., 1994). In the absence of other lipoproteins, CETP promotes HDL fusion and remodels the resulting fusion product into a larger number of smaller particles without generating lipid-poor apoA-I (Rye et al., 1997).

CETP and atherosclerosis

There have been conflicting opinions regarding the role of CETP in the development of atherosclerosis. CETP may be anti-atherogenic by virtue of its ability to mediate the transfer of cholesteryl esters from HDL to VLDL/LDL, delivery of the cholesteryl esters to the liver and their elimination from the body as a component of bile. However, the fact that CETP redistributes cholesteryl esters from non-atherogenic HDL to the potentially atherogenic VLDL/LDL implies that it may also be proatherogenic.

Several studies have shown that CETP deficiency increases HDL levels (Brown et al., 1989; Inazu et al., 1990; Takahashi et al., 1993) and particle size (Brown et al., 1989). CETP deficiency also changes HDL composition (Chiba et al., 1997). Although HDL levels are increased in CETP deficient subjects, CHD is not attenuated (Haraki et al., 1997). In hypertriglyceridemic subjects, low CETP (due to a 405V gene mutation), is also associated with high HDL and possibly with increased CHD (Bruce et al., 1998). Contrary, to expectations, an increase in CHD, rather than decrease, has also been found in subjects with various mutations of the CETP gene (Zhong et al., 1996). These findings were true only for subjects with moderate elevation of HDL. Those with HDL cholesterol levels of >60 mg/dL had lower rates of CHD (Zhong et al., 1996).

Human apoC-III transgenic mice have high levels of triglyceride-rich lipoprotein remnants and develop atherosclerosis. Introduction and expression of the CETP gene into these animals reduce the extent of atherosclerosis (Hayek et al., 1995). The development of atherosclerosis in apoC-III-transgenic mice indicated that the proatherogenic potential of the remnants exceeds the anti-atherogenic potential of HDL. When CETP is expressed

in the animals, the resulting increase in RCT may provide an anti-atherogenic balance to the remnant particles and hence, reduce athersclerosis.

In studies of cholesterol-fed, atherosclerosis-prone mice, the introduction and overexpression of the Simian CETP gene increases atherosclerosis compared to non-expressing controls (Marotti et al., 1993). Similar results are also observed in human CETP overexpression in apoE-knockout mice and LDL receptor-knockout mice (Plump et al., 1999). It was concluded in those studies that the enhancement of atherosclerosis by CETP was secondary to a redistribution of cholesterol from HDL to the VLDL/LDL pool.

Phospholipid transfer protein (PLTP)

Physical characteristics of PLTP

PLTP is a temperature-sensitive hydrophobic glycoprotein consisting of 476 amino acids. The human PLTP gene contains 16 exons, spanning approximately 13.3 kbp (Tu et al., 1995). The highest mRNA expression levels in human tissues were observed in ovary, thymus, and placenta (Albers et al., 1995). Taking into account the organ size, liver and adipose tissue also appear to be important sites of PLTP biosynthesis (Jiang and Bruce, 1995). PLTP has six potential N-glycosylation sites and numerous potential O-glycosylation sites, which may account for the discrepancy between 54 kDa calculated by protein molecular mass and the 81 kDa estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Day et al., 1994; Huuskonen et al., 1998). The protein also contains four cysteine residues at positions 5, 129, 168, and 318 and has the potential to form two intra-chain disulfide bonds (Day et al., 1994). Secondary structure predictions suggest that PLTP has two potential transmembrane regions spanning

residues 169 through 181, and residues 288 through 304 (Albers et al., 1996). PLTP has a high content of hydrophobic residues (>40%).

PLTP shares 20, 26, and 24% amino acid identity to CETP, LBP, and BPI, respectively (Day et al., 1994). The recently presented molecular model of PLTP structure (Huuskonen et al., 1999) is based on the sequence alignment of the entire protein family and the crystallographic structure of BPI (Beamer et al., 1997). The model predicts a boomerang-shaped, two-domain structure. Each end of the protein has barrel-type structural units, with a central β-sheet forming an interface between these units. Two lipid-binding pockets, one in each domain, consist of nonpolar residues, and are found in all the members of this protein family (Beamer et al., 1997; Huuskonen et al., 1999). Several single amino acid substitutions in the lipid-binding pockets decreased the specific phospholipid transfer activity of PLTP, demonstrating the functional importance of the conserved lipid-binding pockets (Huuskonen et al., 1999). It has been suggested that arginine 218 and 245 may mediate the electrostatic interaction of PLTP with lipoproteins (Lagrost et al., 1998). In support of this view, recent data demonstrated that the phospholipid transfer activity of PLTP is markedly influenced by the electrostatic charge of lipoproteins. Striking similarities of the effect of lipoprotein electronegativity on lipid transfer activities of CETP and PLTP were also observed (Desrumaux et al., 1998). The phospholipid transfer and conversion activities reside in the same structural motifs of the PLTP molecule and phospholipid transfers are required for HDL conversion process (Huuskonen et al., 2000).

Pulse-chase analysis revealed that the major secreted forms of PLTP carry complex N-glycans, which are crucial for efficient secretion (Huuskonen et al., 1998). Furthermore,

DTT treatment of the transfected cells caused a reversible arrest of PLTP secretion, suggesting a role of disulfide bonds in the correct folding of the polypeptide. Analysis of carboxyl-terminally truncated PLTP variants demonstrated the importance of the carboxyl-terminus for secretion as well as phospholipid transfer activity (Huuskonen et al., 1998).

Oka et al. (2000) have reported the differences in the distribution of PLTP mass and activity in human plasma. These results suggested that human plasma contains two types of PLTP, one that is in an active form and the other in an inactive form.

Functions of PLTP

PLTP-mediated PL transfer

PLTP transfers a wide range of phospholipids between HDL and other plasma lipoproteins (Tall et al., 1983b, 1985; Albers et al., 1984; Tollefson et al., 1988b; Rao et al., 1997). It transfers diacylglyceride > phosphatidic acid > sphingomyelin > phosphatidylcholine > phosphatidylglycerol > cerebroside > phosphatidylethanolamine. Unsaturation of one phospholipid acyl chain greatly increases the transfer rate, whereas increasing chain length and exchanging sn-1/sn-2 position have only small effects (Rao et al., 1997). However, Huuskonen et al. (1996) found that when the length of the phospholipid acyl chains increased from 6 to 14 carbons, there was a uniform decrease in the transfer rate. These investigators also found no difference in transfer rate was observed when the sn-1/sn-2 positions were exchanged. PLTP mediated equally the transfer of the various head group derivatives except phosphatidylethanolamine , which was transferred 2-3 fold slower. The rate of phospholipid transfer mediated by PLTP

decreases as HDL unesterified cholesterol content increases and HDL size decreases (Rao et al., 1997).

Recent in vivo data from PLTP knockout mice showed that transfers of phospholipids from triglyceride-rich lipoproteins to HDL during lipolysis is facilitated by PLTP and this plays a major role in the regulating plasma HDL levels (Jiang et al., 1999). Moreover, it has been reported recently that PLTP-mediated transfers of phospholipids between HDL and other lipoprotein classes are not interchangeable with the phospholipid transfers that are mediated by CETP (Kawano et al., 2000).

PLTP-mediated HDL remodeling

PLTP remodels HDL in a process that generates large and small particles and promotes the dissociation of apoA-I (Rye and Barter, 1986; Jauhiainen et al., 1993; Tu et al., 1993; Lusa et al., 1996). There is evidence that particle fusion, rather than net lipid transfer or particle aggregation, is responsible for the large HDL particles that are formed during incubation with PLTP (Lusa et al., 1996; Korhonen et al., 1998). The PLTP-mediated dissociation of apoA-I from the HDL surface constitutes a key step in the HDL conversion process (Lusa et al., 1996). The remodeling of HDL by PLTP is enhanced in HDL which contain triglycerides in their core (Rye et al., 1998). Both human PLTP and mouse recombinant PLTP are able to convert human or mouse HDL into larger and smaller particles (Albers et al., 1995). The PLTP in pig plasma also has the ability to convert either human or pig HDL into larger and smaller particles (Pussinen et al., 1995).

PLTP binds to both apoA-I and apoA-II and can promote the conversion of both LpA-I and LpA-I/A-II to populations of larger and smaller particles. Both the formation of large

particles and the release of apoA-I are inhibited by increasing concentrations of apoA-II in the HDL particle (Pussinen et al., 1997).

ApoA-I is required for PLTP-mediated HDL fusion (Pussinen et al., 1998). The polyclonal antibody, R33, and the monoclonal antibody A-I-1 (epitope between amino acids 27-48) reduced PLTP binding by 70%. These results indicate that the PLTP binding domain of apoA-I resides in the amino-terminal region of the protein, between amino acids 27-48 (Pussinen et al., 1998). However, the addition of purified serum amyloid A (SAA) to HDL_3 facilitates the ability of these particles to undergo HDL conversion, despite the lowered content of apoA-I in SAA-HDL (Pussinen et al., 2001).

The in vivo relevance of PLTP-mediated HDL conversion is evident in PLTP knockout mice, and also in transgenic and recombinant adenovirus-infected mouse models of PLTP. On a chow diet, homozygous PLTP knockout mice show a marked decrease in HDL phospholipids, cholesterol and apoA-I (Jiang et al., 1999). Jiang et al. (1996) also found increased plasma levels of pre-β migrating apoA-I in transgenic mice that express human PLTP. These results suggest that PLTP increases the efflux of phospholipids and cholesterol from cell membranes into HDL, leading to an increase in potentially anti-atherogenic pre-β HDL particles. Plasma from mice overexpressing PLTP by 2.5-4.5 fold showed a 2-3 fold increase in the formation of pre-β HDL in the presence of an LCAT inhibitor, compared with plasma from wild-type mice (van Haperen et al., 2000). It has also been reported that the adenovirus-mediated overexpression of PLTP in mice increases the size and decreases the density of HDL (Ehnholm et al., 1998). Jiang et al. (1996) and Föger et al. (1997), by contrast, have both reported that overexpression of PLTP in mice does not increase HDL size. The absence of size changes in those studies

may be due to either increased selective uptake of HDL cholesteryl esters by SR-BI (Wang et al., 1996; Vieira-Van Bruggen et al., 1998), or to the remodeling of the large conversion products by circulating mouse HL (Shirai et al., 1981; Groot et al., 1983; Hopkins and Barter, 1986; Clay et al., 1991; Marques-Vidal et al., 1997). Overall, it appears that PLTP might exert a dual beneficial effect on RCT through the concomitant generation of pre-β HDL, which constitute the initial acceptors of cellular cholesterol, and the formation of large cholesteryl ester-riched α-HDL, which can bring large amounts of cholesterol back to the liver (Lagrost et al., 1998).

Other functions of PLTP

PLTP facilitates the transfer of free cholesterol from single bilayer vesicles containing phosphatidylcholine and cholesterol to HDL_3 (Nishida and Nishida, 1997). PLTP can also mediate the exchange/transfer of α-tocopherol between lipoproteins and cells (Kostner et al., 1995). PLTP, by supplying oxidized LDL and endothelial cells with α-tocopherol through a net mass transfer reaction may play at least two distinct beneficial roles in preventing oxidative damage to endothelial cells by; (i) protecting LDL against oxidation, and (ii) preserving vascular endothelial cell relaxation (Desrumaux et al., 1999). Furthermore, PLTP and LBP both bind and transfer lipopolysaccharide (Vesy et al., 2000) but PLTP is unable to transfer lipopolysaccharide to CD14 and thus does not mediate responses of cells to lipopolysaccharide (Hailman et al., 1996).

PLTP also mediates the proteolytic cleavage of intact apoA-I to a 23 kDa fragment (Jauhiainen et al., 1999). This proteolysis is evident in both native HDL and lipid-free apoA-I. The cleavage site resides in the carboxyl-terminal portion of apoA-I between amino acid residue 196 (alanine) and 197 (threonine).

PLTP and atherosclerosis

Recent reports suggest an anti-atherogenic role for PLTP (von Eckardstein et al., 1996; Jiang, et al., 1999; van Haperen et al., 2000). As described previously, PLTP remodels HDL, generates pre-β migrating HDL, the initial acceptors of cellular cholesterol in the first step of RCT, and also facilitate the preβ-LpA-I-mediated uptake of cellular cholesterol and phospholipids from cholesterol-loaded human skin fibroblasts (Wolfbauer et al., 1999). PLTP may protect against atherosclerosis by facilitating the hepatic uptake of phospholipids and cholesteryl esters from HDL (Wolfbauer et al., 1999). In addition, PLTP transfers α-tocopherol between lipoproteins and between lipoproteins and cells, suggesting that PLTP might play an important role in maintaining the antioxidant status of cells (Kostner et al., 1995).

1.2 SCOPE OF THE THESIS

The work in this thesis has arisen from the observation that triglyceride enrichment of HDL enhances PLTP-mediated remodeling of HDL into large and small particles (Rye et al., 1998). The mechanism of action of PLTP is also presented. These studies have focused on the remodeling of (i) rHDL that contain cholesteryl esters on the sole core lipid, (ii) rHDL that are enriched with triglyceride and (iii) rHDL that vary in their phospholipid composition.

As PLTP also facilitates the transfer of phospholipids between HDL and the other plasma lipoproteins, the influence of triglyceride enrichment of HDL on PLTP-mediated phospholipid transfers are also presented. While a great deal is known about the ability of apolipoproteins to regulate the structure, function, and remodeling of HDL, little is

known about the influence of phospholipids on these events. This thesis also describes the effects of HDL phospholipid composition on PLTP-mediated phospholipid transfers and remodeling of HDL.

CHAPTER 2 MATERIALS AND METHODS

2.1 ISOLATION OF APOA-I

2.2 ISOLATION OF LIPOPROTEINS

2.3 ISOLATION OF PLASMA FACTORS

Isolation of LCAT

Isolation of CETP

Isolation of PLTP

2.4 PREPARATION OF RECONSTITUTED HDL (rHDL)

Discoidal rHDL

Spherical rHDL

Triglyceride-enriched spherical rHDL (TG-rHDL)

2.5 PREPARATION OF SPHERICAL rHDL CONTAINING DIFFERENT PHOSPHOLIPIDS

Preparation of discoidal rHDL containing either POPC, PLPC, PAPC or PDPC

Preparation of spherical rHDL containing either POPC, PLPC, PAPC or PDPC

2.6 COMPOSITIONAL ANALYSES

2.7 ELECTROPHORESIS

Non-denaturing polyacrylamide gradient gel electrophoresis

Agarose gel electrophoresis

SDS-polyacrylamide gel electrophoresis

Immunoblotting

2.8 GEL PERMEATION CHROMATOGRAPHY

2.9 CROSS-LINKING OF APOA-I

2.10 SPECTROSCOPIC STUDIES

2.11 CHEMICALS AND REAGENTS

2.1 ISOLATION OF APOA-I

HDL were isolated from pooled autologously donated human plasma (Gribbles Pathology, Adelaide, South Australia) by ultracentrifugation in the $1.07 < d < 1.21$ g/mL density range, with a single 16 h spin at d 1.07 g/mL and two 26 h spins at d 1.21 g/mL (Rye et al., 1992, 1993). These procedures were carried out using a 55.2 Ti rotor at a speed of 55,000 rpm in a Beckman L8-M ultracentrifuge maintained at 4 °C. Density adjustments were made by the addition of solid KBr (Hatch and Lees, 1963). The isolated HDL were dialyzed against 3 x 5 L of 5 mM ammonium bicarbonate solution, then delipidated as described by Osborne (1986). The resulting apoHDL was dissolved in 20 mM Tris, pH 8.2, lyophilised, and stored at -20 °C.

ApoA-I and apoA-II were isolated from the apoHDL by anion exchange chromatography on an XK 26/40 column of Q Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) attached to an Fast Performance Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech) (Weisweiler, 1987; Rye, 1990). This procedure was carried out at room temperature. The apoA-I appeared as a single band after electrophoresis on a 20% sodium dodecyl sulphate (SDS) polyacrylamide gel (Phast System, Amersham Pharmacia Biotech) and staining with Coomassie Blue. The purified apolipoproteins were dialyzed against 3 x 5 L of 20 mM ammonium bicarbonate, lyophilized, and stored at -20 °C. Prior to use, they were reconstituted in 10 mM Tris/3 M guanidine-HCl/0.01% (w/v) EDTA-Na_2, pH 8.2 for 1 h, then dialyzed against 5 x 1 L of Tris-buffered saline (10 mM Tris/150 mM NaCl, pH 7.4) containing 1 mM EDTA-Na_2 and 0.01% (w/v) NaN_3 (TBS).

2.2 ISOLATION OF LIPOPROTEINS

Isolation of HDL and HDL_3

Fresh blood samples were collected from subjects after a 12 h fast by standard venipuncture into tubes containing EDTA-Na_2 (final concentration 1 mg/mL). The samples were placed immediately on ice. Plasma was separated by centrifugation at 5,000 rpm, 4 °C for 10 min. HDL and HDL_3 were isolated by ultracentrifugation in the density range of $1.07 < d < 1.21$ and $1.13 < d < 1.21$ g/mL, respectively, with a single spin at the lower density and two spins at the upper density (Rye et al., 1993). The spins were conducted at 100,000 rpm for 16 h using a TLA 100.4 rotor in a Beckman TL-100 Tabletop ultracentrifuge maintained at 4 °C. The HDL were recovered by tube slicing and dialyzed against 3 x 1 L of TBS before use.

Isolation of LDL

LDL were isolated from pooled human plasma in a 55.2 Ti rotor maintained at a speed of 55,000 rpm in a Beckman L8-M ultracentrifuge with a single 16 h spin at d 1.019 g/mL and two 16 h spins at d 1.055 g/mL (Rye et al., 1992). These procedures were carried out at 4 °C. The LDL were dialyzed against 3 x 1 L of TBS before use.

2.3 ISOLATION OF PLASMA FACTORS

Isolation of LCAT

LCAT was isolated from samples of pooled human plasma as described, with some modifications (Mahadevan and Soloff, 1983; Rajaram and Barter, 1985). Two litres of pooled human plasma were subjected to precipitation at 35% saturation of ammonium sulphate. The precipitated proteins were removed by centrifugation at a speed of 12,000

rpm then 125 mL of 1 M citric acid was added dropwise to the supernatant. The precipitated proteins were suspended in 200 mL of Milli Q water and the pH of the sample was raised to 7.4 by the addition of a saturated solution of Na_2CO_3. The solution was dialyzed against 2 x 5 L of Milli Q water then ultracentrifuged at a density of 1.25 g/mL. This procedure was carried out at 55,000 rpm for 26 h using a 55.2 Ti rotor in a Beckman L8-M ultracentrifuge maintained at 4 °C. After the initial spin, the d < 1.25 g/mL fraction was recovered by tube slicing and subjected to a further 26 h of ultracentrifugation at a density of 1.25 g/mL. The d > 1.25 g/mL fractions from both of the spins were pooled and applied to an XK 50/60 containing Phenyl Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) pre-equilibrated with 1 L of 3 M NaCl. After washing with 0.15M NaCl, LCAT was eluted with Milli Q water/0.02% (w/v) NaN_3/0.01% (w/v) EDTA-Na_2 at a flow rate of 10 mL/min. The active fractions were pooled and dialyzed against 2 x 5 L of 20 mM Tris, pH 7.4, containing 1 mL β-mercaptoethanol/L. The same buffer was used to pre-equilibrate an XK 26/40 column packed with DEAE Sepharose Fast Flow (Amersham Pharmacia Biotech). The LCAT was loaded onto this column and eluted with 20 mM Tris/500 mM NaCl, pH 7.4, containing 1 mL β-mercaptoethanol/L. Ten mL fractions were collected at a flow rate of 10 mL/min. Fatty acid-free bovine serum albumin (BSA, final concentration 1 mg/mL) was added to the fractions that contained LCAT. The fractions were stored in 10 mL aliquots at -70 °C until use.

Assay for LCAT activity

Activity of LCAT was determined as described by Piran and Morin (1979) using discoidal rHDL containing β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (POPC):un-esterified cho-lesterol (UC):apoA-I) labelled with [1α,2α(n)-^{3}H] cholesterol ([^{3}H]UC)

(Amersham Pharmacia Biotech) as a substrate. The final concentration of UC in the substrate was 0.13 nmol/μL. Twenty-five μL of substrate, together with 50 μL of 10 mg/mL fatty acid-free BSA, 5 μL of β-mercaptoethanol (final concentration 48.5 mM), and 47 μL of TBS were pre-incubated at 37 °C under nitrogen for 30 min. LCAT (20 μL), TBS or the d > 1.25 g/mL fraction of pooled, human plasma (positive control) were added to the mixtures and the incubations were continued at 37 °C under nitrogen for a further 1 h. The final volume of the incubation mixtures was 147 μL. The reaction was stopped by addition of 0.5 mL of 1% digitonin in 95% ethanol. The mixtures were vortexed for 15 sec to denature the proteins and extract lipids. Excess UC (25 μL of 5 mg/mL UC in ethanol) was then added and the mixtures were vortexed again then centrifuged at 1,500 x g for 10 min. A 400 μL aliquot, which contained radiolabelled CE, was taken from the supernatant, added to 10 mL of aqueous scintillation cocktail (Ready safe, Beckman, USA) and counted in a Beckman LS 6000 TA liquid scintillation system (Beckman Instruments, Inc., Fullerton, CA, USA) for 5 min. The assay was linear when less than 30% of the [^{3}H]UC was esterified. LCAT activity was expressed as μmol of CE formed/mL LCAT/h.

Isolation of CETP

CETP was isolated from 2 L of pooled human plasma as described by Rye et al. (1995). Plasma proteins were precipitated between 35 to 55% saturation of ammonium sulphate and suspended in 250 mL Milli Q water. The proteins were dialyzed against 5 L of Milli Q water, then subjected to ultracentrifugation at a density of 1.25 g/mL. The details of the spins were the same as described above for the preparation of LCAT. The pooled d > 1.25 g/mL fractions were subjected to hydrophobic interaction chromatography on an XK 50/30 column containing Macroprep Hydrophobic Interaction gel (Bio-Rad Laboratories,

Hercules, CA, USA), which had been pre-equilibrated with 500 mL of 3 M NaCl. Unbound proteins were eluted with 3 M NaCl. The bound, hydrophobic proteins were eluted at a flow rate of 10 ml/min with Milli Q water/0.02% (w/v) NaN_3/0.01% (w/v) EDTA-Na_2. The active fractions were pooled and adjusted to 50 mM NaOAc by addition of 0.5 M NaOAc, pH 4.5, then subjected to cation exchange chromatography on an XK 26/40 column containing CM (Carboxyl-Methyl)-Sepharose Fast Flow (Amersham Pharmacia Biotech) pre-equilibrated with 500 mL of 50 mM NaOAc, pH 4.5. CETP was eluted from the column at a flow rate of 10 mL/min with a linear gradient of 0-0.4 M NaCl in 50 mM NaOAc, pH 4.5. The fractions were dialyzed against 3 x 5 L of 20 mM Tris, pH 7.4 and assayed for CETP activity. The active fractions were pooled and subjected to chromatography on a Mono Q HR 5/5 anion exchange column (Amersham Pharmacia Biotech). The column was pre-equilibrated with 10 mL of 20 mM Tris, pH 7.4. CETP was eluted from the column with a 0-0.5 M NaCl gradient at a flow rate of 1 mL/min (Barter et al., 1988). The active fractions were pooled and stored at -70 °C in 1 mL aliqouts. No LCAT and PLTP activities were detected in these preparations.

Assay for CETP activity

Activity of CETP was assessed as the transfer of [^{3}H]CE from [^{3}H]CE-HDL_3 to LDL (Burstein et al., 1970; Tollefson et al., 1988a). [^{3}H]CE-HDL_3 (final total cholesterol (TC) concentration 80 nmol/mL), LDL (final TC concentration 240 nmol/mL), and 50 µL of purified CETP, or the d > 1.25 g/mL fraction of pooled, human plasma (positive control), were incubated in the presence of 10 µL of 14.2 mg/mL 5,5'-dithio-bis(nitrobenzoic acid), DTNB (to inhibit LCAT activity). TBS was added to a total volume to 175 µL. The incubations were carried out at 37 °C for 3 h. At the end of incubation, the LDL were precipitated with 25 µL of heparin and $MnCl_2$ solution (2500 IU/mL heparin/1 M $MnCl_2$)

(Burstein et al., 1970) then centrifuged for 5 min at 1,500 x g. A 200 µL aliquot of the supernatant was placed into 10 mL of aqueous scintillation cocktail and counted in a Beckman LS 6000 TA liquid scintillation system for 5 min. The assay was linear when less than 30% of [^{3}H]CE transferred from HDL_3 to LDL. Activity is expressed in units/mL, with 1 unit being the transfer activity of 1 mL of a preparation of pooled, human lipoprotein-deficient plasma.

Isolation of PLTP

PLTP was isolated from 2 L of pooled human plasma as described (Rye et al., 1998) with some modifications. The proteins that precipitated between 35 to 45% saturation with ammonium sulphate were suspended in ~100 mL water and adjusted to a density of 1.25 g/mL. The d > 1.25 g/mL fraction was isolated by ultracentrifugation as described above for the preparation of LCAT. The pooled d > 1.25 g/mL fractions were applied at a flow rate of 10 mL/min to an XK 50/30 column containing a Macroprep Hydrophobic Interaction gel which was pre-equilibrated with 1 L of 3 M NaCl. Hydrophobic proteins were eluted from the column with Milli Q water/0.02% (w/v) NaN_3/0.01% (w/v) EDTA-Na_2. The eluted proteins were dialyzed against 3 x 5 L of 10 mM Tris, pH 7.4, and applied at a flow rate of 10 mL/min to a pre-equilibrated XK 26/40 column packed with DEAE Sepharose Fast Flow. PLTP was eluted from the DEAE column with a 0-250 mM NaCl gradient. The fractions containing PLTP were pooled, dialyzed against 3 x 5 L of 10 mM Tris/50 mM NaCl/1 mM EDTA-Na_2, pH 7.4, and applied at 10 mL/min to a pre-equilibrated HR 10/30 column containing Heparin Sepharose Fast Flow (Amersham Pharmacia Biotech). PLTP was eluted from this column with 10 mM Tris/500 mM NaCl/1 mM EDTA-Na_2, pH 7.4. The active fractions were pooled, dialyzed against 3 x 5

L of 20 mM Tris, pH 7.4, mixed with BSA (final concentration 1 mg/mL), and stored in 1 mL aliquots at -70 °C. The PLTP preparations were free of CETP and LCAT activities.

Assay for PLTP activity

PLTP activity was determined as the transfer of 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine (DPPC) (Amersham Pharmacia Biotech) from [^{14}C]-DPPC-labelled egg phosphatidylcholine small unilamellar vesicles to HDL (Damen et al., 1982). The incubations contained HDL (final protein concentration 0.625 mmol/mL), [^{14}C]-DPPC-labelled egg PC vesicles (final phospholipid (PL) concentration 0.375 μmol/mL), and 50 μL of PLTP. TBS was added to make up the total volume to 400 μL and the incubations were conducted at 37 °C for 2 h. When the incubations were complete, the vesicles were precipitated with 300 μL of heparin and $MnCl_2$ solution (0.47 units/μL of heparin/215 mM $MnCl_2$/500 mM NaCl) (Burstein and Scholnick, 1973; Warnick and Albers, 1978). A 500 μL aliquot of the supernatant was placed into 10 mL of aqueous scintillation cocktail and counted in a Beckman LS 6000 TA liquid scintillation system for 2 min. PLTP activity was expressed in μmol of PL transferred/mL PLTP/h.

2.4 PREPARATION OF RECONSTITUTED HDL (rHDL)

Discoidal rHDL

ApoA-I was reconstituted with 10 mM Tris/3 M guanidine-HCl/0.01% (w/v) EDTA-Na_2, pH 8.2 and dialyzed against 5 x 1 L of TBS as described in Section 2.1. POPC-discoidal rHDL were prepared by the cholate dialysis method using POPC, UC, and apoA-I (Matz and Jonas, 1982), at a molar ratio of 100:10:1. The POPC and UC were both dissolved in chloroform:methanol solution 2:1 (v/v) at a concentration of 100 and 10 mg/mL, respectively. The POPC and UC were placed in clean dry test tubes and dried as a thin

film onto the wall of the tube using nitrogen. The test tubes were then dried at ~40 °C under nitrogen for 2 h. A solution of sodium cholate (30 mg/mL in TBS) was added to the dried lipids to give a molar ratio of POPC:UC:cholate of 100:10:100. TBS was added to each tube to bring the volume to 500 µL. The test tubes were kept in ice and vortexed every 15 min until the mixtures became clear. A 6-8 mg/mL solution of apoA-I was then added into each tube (2 mg apoA-I/tube). After another 2 h on ice, the contents of the tubes were pooled and dialyzed against 5 x 1 L of TBS to remove the sodium cholate.

Spherical rHDL

Spherical rHDL containing CE as the sole core lipid were prepared as described (Rye et al., 1993; Rye and Barter, 1994). Discoidal rHDL (final apoA-I concentration 355.9 µg/mL), fatty acid-free BSA (final concentration 40 mg/mL), and β-mercaptoethanol (final concentration 4 mM) were incubated for 24 h at 37 °C with LDL (final protein concentration 1.4 mg/mL) and LCAT. The final volume of the incubation was 78.7 mL. The spherical rHDL were isolated by ultracentrifugation in the $1.07 < d < 1.21$ g/mL density range with two 24 h spins at the lower density and a single 16 h spin at the upper density. The spins at d 1.07 g/mL were carried out at 55,000 rpm using a Beckman Ti 55.2 rotor in a Beckman L8-M ultracentrifuge. The d 1.21 g/mL spin was carried out at a speed of 100,000 rpm using a Beckman TLA 100.4 rotor in a Beckman TL-100 Tabletop ultracentrifuge. These procedures were all conducted at 4 °C. The spherical rHDL were dialyzed against 3 x 1 L of TBS before use.

Triglyceride-enriched spherical rHDL (TG-rHDL)

Spherical rHDL (final CE concentration 0.1 µmol/mL) were enriched with triglyceride (TG) (TG-rHDL) by incubation at 37 °C for 20 min with the phospholipid/TG emulsion,

Intralipid (Kabi Vitrum AB, Stockholm, Sweden) (final TG concentration 4.0 μmol/mL), and CETP (final activity 2.6 units/mL) (Rye et al., 1998). The final volume of the incubation was 68.0 mL. When the incubations were complete, the TG-rHDL were isolated by ultracentrifugation in the $1.063 < d < 1.21$ g/mL density range, at 4 °C, with two 16 h spins at the lower density and a single 16 h spin at the upper density. The spins were carried out at a speed of 100,000 rpm using a Beckman TLA 100.4 rotor in a Beckman TL-100 Tabletop ultracentrifuge. The TG-rHDL were dialyzed against 3 x 1 L of TBS before use.

2.5 PREPARATION OF SPHERICAL rHDL CONTAINING DIFFERENT PHOSPHOLIPIDS (POPC-rHDL, PLPC-rHDL, PAPC-rHDL AND PDPC-rHDL)

The preparation of rHDL which contain a single type of phospholipid has been recently developed in this laboratory. The rHDL are prepared by using unesterified cholesterol dissolves in ethanol instead of LDL as the source of cholesterol for the LCAT reaction. This avoids the transfer of phospholipids from LDL to rHDL during the incubation with LCAT (Rye et al., 1996) and ensures that the phospholipids in the spherical rHDL are identical to the phospholipids in the discoidal rHDL from which they were derived.

Preparation of discoidal rHDL containing either POPC, PLPC, PAPC or PDPC as the sole phospholipid

Discoidal rHDL containing a single type of phospholipid were prepared by the cholate dialysis method as described above (Matz and Jonas, 1982). The phospholipids used in these preparations were POPC; γ-palmitoyl-β-linoleoyl-L-α-phosphatidylcholine, PLPC; γ-palmi-toyl-β-arachidonoyl-L-α-phosphatidylcholine, PAPC; and γ-palmitoyl-β-docosa-hexaenoyl-L-α-phosphatidylcholine, PDPC. The initial molar ratio of PC:UC:A-I was

110:5:1. The discoidal rHDL were dialyzed against 5 x 1 L of TBS containing 50 μM diethylenetriamine pentaacetic acid (DETAPAC) and 10 μM BHT/0.006% (w/v) NaN_3, pH 7.4 before use. Chelex resin (2.0 mg/mL)was also added to the dialysis to protect against oxidation of unsaturated phospholipid acyl chains. After dialysis, a small amount of Chelex resin was added directly into the discoidal rHDL.

Preparation of spherical rHDL containing either POPC, PLPC, PAPC or PDPC

Spherical rHDL containing a single type of phospholipid were prepared from discoidal rHDL containing either POPC, PLPC, PAPC or PDPC. In a typical experiment, discoidal POPC-rHDL (final UC concentration 189 nmol/mL) were mixed with 2.14 mL of LCAT, fatty acid-free BSA (final concentration 40 mg/mL), and β-mercaptoethanol (final concentration 4 mM). The LCAT used for this preparation generated 1,765 nmol CE/mL LCAT/h. TBS containing 50 μM DETAPAC and 10 μM BHT/0.006% (w/v) NaN_3, pH 7.4 was also added to bring the total volume of the mixture to 10.6 mL. After the mixture was incubated at 37 °C for 15 min, 43 μL of 25 mM UC in ethanol, and 133 μL of the same preparation of LCAT was added to the incubation. Fatty acid-free BSA and β-mercaptoethanol were also added to maintain their concentrations of 40 mg/mL and 4 mM, respectively. These additions were repeated at 30 min intervals until the total incubation time was 7 h. The final incubation volume was 36.6 mL. The incubation was then continued without further additions until the total incubation time was 24 h. The resulting spherical rHDL were isolated by ultracentrifugation in the $1.07 < d < 1.21$ g/mL density range with a single spin at the upper and lower densities. The 24 h spin was conducted at 55,000 rpm using a Beckman Ti 55.2 rotor in a Beckman L8-M ultracentrifuge. The 16 h spin was carried out at 100,000 rpm using a Beckman TLA 100.4 rotor maintained at 4 °C in a Beckman TL-100 Tabletop ultracentrifuge. The

resulting spherical rHDL were dialysed against 3 x 1 L TBS containing 50 μM DETAPAC and 10 μM BHT/0.006% (w/v) NaN_3, pH 7.4 and Chelex resin.

As the reactivity of discoidal rHDL with LCAT varies according to their phospholipid content, there was wide variation in the amount of LCAT required to convert the discs into spheres. While a total of 133 μL of LCAT was used to convert the discoidal POPC-rHDL into spherical POPC-rHDL, the discoidal PLPC-rHDL required 551 μL of LCAT. The discoidal PAPC-rHDL and PDPC-rHDL required 1.12 and 2.85 mL of LCAT, respectively, for conversion into spheres.

2.6 COMPOSITONAL ANALYSES

Compositional analyses were carried out using a Cobas Fara Centrifugal Analyzer (Roche Diagnostics, Zurich, Switzerland). ApoA-I concentrations were determined either by the method of Lowry et al. (1951), using BSA as a standard, or by an immunoturbidometric assay (Reipponen et al., 1987; Hopkins and Barter, 1989). Enzymatic kits (Boehringer Mannheim GmbH, Germany) were used to determine PL, UC and TC concentrations. CE concentrations were calculated as the different between TC and UC concentrations. TG was quantitated enzymatically as described (Bergmeyer, 1985).

2.7 ELECTROPHORESIS

Non-denaturing polyacrylamide gradient gel electrophoresis

The hydrated diameter of the rHDL was determined by electrophoresis on 3-40% non-denaturing polyacrylamide gradient gels, using a GE-2/4 LS Gel Electrophoresis Apparatus (Amersham Pharmacia Biotech). The gels were prepared according to the method of Rainwater et al. (1992). Samples were pre-mixed with 40% (w/v)

sucrose/0.01% (w/v) bromophenol blue solution then applied to the gel. Electrophoresis was carried out in 0.09 M Tris/0.08 M boric acid/0.003 M EDTA-Na_2 buffer, pH 8.4, at 150-180 volts for a total of 3,000 volt-hours. The gel was fixed with 10% (w/v) 5-sulphosalicylic acid for 30-60 min, stained with 0.04% (w/v) Coomassie Brilliant Blue G in 3.5% (v/v) perchloric acid for 1-2 h, and destained with 5% (v/v) acetic acid until the background was clear. A Sharp JX-610 scanner (Sharp, Japan) was used to scan the gels. Image Master software (Sharp, Japan) was used to quantitate particle size by reference to a series of high molecular weight standards of known hydrated diameter (Amersham Pharmacia Biotech).

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate lipoproteins on the basis of surface charge (Sparks and Phillips, 1992; Rye and Barter, 1994). A Bio-Rad Mini Sub gel electrophoresis system (Hercules, CA, USA) was used for this procedure. The running buffer, contained 10 mM barbitone/50 mM sodium barbitone, pH 8.7. The 0.6% agarose gel was prepared by dissolving 0.18 g agarose in 30 mL running buffer. The mixture was poured into a gel tray and left at room temperature for about 25 min or until the gel set. The samples (10-15 μL containing about 20 μg of protein) were pre-mixed with small amount of tracking dye (40% w/v sucrose and 0.01% w/v bromophenol blue) then applied to the gel. The gel was electrophoresed at 100 volts at room temperature for 1 h, fixed with ethanol:water:acetic acid 6:3:1 (v/v/v) for 10 min, stained with Coomassie Brilliant Blue G for 30 min, destained with 45% ethanol for overnight, and scanned as for non-denaturing polyacrylamide gradient gel. Electrophoretic mobility was calculated as described by Sparks and Phillips, 1992.

$$\text{Mobility} = \frac{\text{Migration distance (}\mu\text{m)/time (sec)}}{\text{Voltage (v)/length of the gel (cm)}}$$

SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was carried out on 20% homogenous polyacrylamide gels in the Phast System (Amersham Pharmacia Biotech). The samples (protein concentration ~ 1mg/mL) were boiled with 10 µL of SDS sample buffer (0.01 M Tris/0.001 M EDTA-Na_2/2.5% (w/v) SDS/0.025% (w/v) bromophenol blue) for 5 min, and then applied to the gel. The electrophoresis was carried out for 95 volt-hours. The gels were stained with Coomassie Brilliant Blue R, destained with methanol:acetic acid:water 3:1:6 (v/v/v), and preserved with glycerol:acetic acid:water 1:1:8 (v/v/v).

For silver staining, the gels were incubated with 20% $AgNO_3$:water 1:200 (v/v) then destained with 3% (w/v) Na_2CO_3, followed by 5% acetic acid until the gel lightened in colour. Glycerol:acetic acid:water 1:1:8 (v/v/v) was used for preserving the gel.

Immunoblotting

Aliquots of incubation mixtures which had not been ultracentrifuged were separated on the basis of size by non-denaturing 3/40% polyacrylamide gradient gel electrophoresis as described above. The samples were transferred electrophoretically from the gel to a nitrocellulose membrane (MFS Membrane filters mixed cellulose ester, Advantec MFS, Inc., CA, USA) using a Bio-Rad Trans-blot electrophoresis unit (Bio-Rad Laboratories). The buffer used for this procedure was 0.025 M Tris/0.2 M glycine/20% (v/v) methanol, pH 8.3. The transfer was carried out at 200 mA, at 4 °C for 24 h. The membrane was then immunoblotted with polyclonal sheep antihuman apoA-I antiserum 1:5,000 (Boehringer

Mannheim GmbH, Germany) in Blotto (50 mg/mL of skim milk powder, containing a small amount of thimerosal and anti-foam). This procedure was carried out at room temperature for 1 h. The membrane was washed with 0.05% Tween 20 in TBS (3 x 5 min) then Blotto (3 x 5 min) and incubated at room temperature for 1 h with 1:10,000 anti-sheep/goat antiserum conjugated to horseradish peroxidase (Silenus Laboratories Pty. Ltd., Hawthorn, Australia). The membrane was then washed with 0.05% Tween 20 in TBS (3 x 5 min) and TBS (3 x 5 min). The transferred bands were detected by ECL (Amersham Pharmacia Biotech) (Liang et al., 1994).

2.8 GEL PERMEATION CHROMATOGRAPHY

This procedure was used for resolving the conversion products that were formed when the rHDL were incubated with PLTP. The ultracentrifugally isolated rHDL were concentrated by ultrafiltration using a CF25 membrane cone (Amicon®, MA, USA), then applied to a pre-equilibrated HR 10/30 Superose 6 column (Amersham Pharmacia Biotech). The rHDL were eluted from the column with TBS using a flow rate of 0.3 mL/min. Fractions were collected at 1 min intervals.

2.9 CROSS-LINKING OF APOA-I

The number of apoA-I molecules/rHDL particle was determined by covalent cross-linking with the bifunctional cross-linking reagent, bis(sulfosuccinimidyl) suberate (BS) (Staros, 1982). The samples were adjusted to a protein concentration of 0.5 mg/mL and dialyzed against 3 x 1 L of 50 mM sodium phosphate buffer, pH 7.4. After the dialysis, 200 μL of each sample was mixed with 50 μL of 10 mM BS in phosphate buffer and left at room temperature for 30 min. The mixtures were then dialyzed against 1 L of SDS sample buffer, containing 0.01 M Tris/0.001 M EDTA-Na_2/1% (w/v) SDS, pH 8.0. The

samples were incubated at 37 °C for 45 min, then subjected to SDS polyacrylamide gel electrophoresis. The electrophoresis was carried out on 3-40% polyacrylamide gradient gel, using a SDS buffer containing 0.04 M Tris/0.02 M sodium acetate/0.002 M EDTA-Na_2/0.2 % (w/v) SDS, pH 7.4. The samples were loaded onto a gel that had been pre-equilibrated at 70 volts for 1 h. The electrophoresis was carried out at 300 volts for 10 min, then at 150 volts until the tracking dye had migrated off the bottom of the gel. The electrophoresis was continued at 150 volts for further 1 h. The gel was fixed in isopropanol:acetic acid:water 25:10:65 (v/v/v) for 1 h and stained with 0.1% (w/v) Coomassie blue, containing 25% (v/v) methanol and 10% (v/v) acetic acid. A solution of methanol:acetic acid:water 25:10:65 (v/v/v) was used for destaining. The gel was scanned and the number of apoA-I molecules/particle was determined by reference to a sample of cross linked, lipid-free apoA-I.

2.10 SPECTROSCOPIC STUDIES

These studies were carried out with a Perkin-Elmer LS-50 luminescence spectrometer fitted with a thermostatted cell holder and polarizers. Sample temperatures were controlled by a Lauda RM6T recirculating water bath (Lauda Königshofen, Germany) and monitored with digital temperature probe (Baker Medical Research Institute, Melbourne, Australia).

Phospholipid acyl chain and head group packing order

Phospholipid acyl chain and head group packing order was determined by labelling the rHDL with 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) (Prendergast et al., 1981; Lentz et al., 1976). The rHDL (final PL concentration 0.5 mM) were mixed with 7 μL of 0.1

mM DPH in tetrahydrofuran (THF) or TMA-DPH in dimethylformamide (DMF), respectively. In both cases, the molar ratio of rHDL phospholipid:probe was 500:1. The final volume of the mixtures was 707 μL. The mixtures were maintained in the dark for 30 min to allow for labelling of the rHDL to proceed. Steady state fluorescence polarization of the DPH- and TMA-DPH-labelled rHDL was measured at 5 °C intervals from 5 to 50 °C using an excitation wavelength of 366 nm and an emission wavelength of 400 nm. The excitation and emission slit widths were 5 and 6 nm, respectively (Rye and Barter, 1994).

Unfolding of rHDL apoA-I

Unfolding of apoA-I in rHDL was assessed by incubation with increasing concentrations of guanidine hydrochloride (GdnHCl) (Pace, 1986; Rye et al., 1995). The rHDL (final apoA-I concentration 20 μg/mL) were added to aliquots of 50 mM Tris-HCl, pH 8.0 containing 0-8 M GdnHCl. The final volume of mixtures was 625 μL. Wavelengths of maximum fluorescence were determined from 300 to 380 nm emission scans using an excitation wavelength of 295 nm. The respective excitation and emission band passes were 10 and 5 nm. Initial readings (t = 0 h) were made at 25 °C, immediately after the rHDL had been added to the GdnHCl solutions. Measurements at t = 2, 5, 8, and 24 h were made after the samples had been incubated at 25 °C for the appropriate time.

The wavelengths of maximum fluorescence obtained when rHDL were mixed with varying concentrations (0-8 M) of GdnHCl and incubated at 25 °C for 5 h were used to calculate the concentration of GdnHCl required to achieve 50% unfolding of apoA-I ($[GdnHCl]_{1/2}$) as described below. All calculations are based on the assumption that the unfolding of apoA-I is represented by a two-state process, such that at a given time, the

only species present at significant concentrations are either completely folded or completely unfolded (Pace, 1986). The central, linear regions of the unfolding curves were used for the calculations. For a given concentration of GdnHCl, the fraction of unfolded apoA-I was calculated as

$$f_u = \frac{y_f - y}{y_f - y_u}$$

where y_f, y_u, and y represent the respective wavelengths of maximum fluorescence in the folded, unfolded, and transition states.

The equilibrium constant (K) for unfolding was calculated as

$$K = \frac{f_u}{1 - f_u}$$

and the free energy of unfolding was calculated as

$$\Delta G = - RT \ln K$$

where R is the gas constant (1.987 cal/degree/mol) and T is the absolute temperature (293.15 °K). The concentration of GdnHCl at the midpoint of the denaturation curve was calculated from plots of ΔG versus the concentration of GdnHCl using values of ΔG between -1.5 and +1.5 kcal/mol.

2.11 CHEMICALS AND REAGENTS

Adenosine 5' triphosphate, disodium salt (ATP)	Sigma Chemicals A-5394
Agarose	Sigma Chemicals A-6013

4-Aminoantipyrine	Sigma Chemicals A-4382
Ammonium bicarbonate	BDH chemicals 103025 E
Ammonium sulphate	BDH Chemicals 10033.6E
Anti-apolipoprotein A-I	Boehringer Mannheim 726478
Antifoam A	Sigma Chemicals A-5758
Anti-sheep/goat Ig, HRP conjugated	Silenus Lab. UAH
β-Arachidonoyl-γ-palmitoyl-L-α-phosphatidylcholine (PAPC)	Sigma Chemicals P-4203
Barbitone	BDH Chemicals 10415 3 P
Barbitone, sodium	BDH Chemicals 103654 E
Boric acid	BDH Chemicals 10058.3 R
Bovine serum albumin (BSA)	Sigma Chemicals A-8022
Bovine serum albumin, fatty acid-free	Sigma Chemicals A-3803
Bromophenol blue	BDH Chemicals 44305
Butylated hydroxy toluene (BHT)	Sigma Chemicals B-1378
Chelex resin	Bio-Rad 142-2832
Chloroform	BDH Chemicals 10077.6 B
Cholesterol, unesterified	Sigma Chemicals C-8667
[1α, 2α (n)-^{3}H] cholesterol	Amersham Pharmacia Biotech TRK 330 B 80
Cholic acid, sodium salt	Sigma Chemicals C-1254
Citric acid	BDH Chemicals 27781
Coomassie Brilliant Blue G-250	Bio-Rad 161-0406
Coomassie Brilliant Blue R-350 (Phast Gel)	Amersham Pharmacia Biotech 17-0518-01

Diethylenetriamine pentaacetic acid	Sigma Chemicals D-1133
Diethyl ether	BDH Chemicals 10094.6 B
Digitonin	Sigma Chemicals D-5628
5, 5'-Dithio-bis(nitrobenzoic acid) (DTNB)	Sigma Chemicals D-8130
β-Docosahexaenoyl-γ-palmitoyl-L-α-phosphatidylcholine (PDPC)	Sigma Chemicals P-5204
ECL reagent	Amersham Pharmacia Biotech RPN 2106
Ethanol	BDH Chemicals 10107.7 Y
Ethylenediaminetetraacetic acid, disodium salt (EDTA-Na_2)	BDH Chemicals 10093.5 V
Folin & Ciocalteu's phenol reagent	Sigma Chemicals F-9252
Formaldehyde	BDH Chemicals 10113
Free cholesterol reagent	Boehringer Mannheim 310328
Glacial acetic acid	BDH Chemicals 100015 N
Glutaraldehyde	Sigma Chemicals G-6403
Glycerokinase	Boehringer Mannheim 127159
Glycerol	BDH Chemicals 010118.2500
L-Glycerol-3-phosphate oxidase (GPO)	Boehringer Mannheim 775797
Glycine	BDH Chemicals 10119. CU
Guanidine (aminomethanamidine) hydrochloride	Sigma Chemicals G-3272
Heparin, sodium salt	Sigma Chemicals H-9399
High molecular weight electrophoresis calibration kit	Amersham Pharmacia Biotech 17-0445-01
20% Homogenous polyacrylamide gel	Amersham Pharmacia Biotech 17-0624-01

Hydrochloric acid	BDH Chemicals 10307 8 R
Isopropanol	BDH Chemicals 10224
β-Linoleoyl-γ-palmitoyl-L-α-phosphatidylcholine (PLPC)	Sigma Chemicals P-9648
Lipase	Sigma Chemicals L-9518
Low molecular weight standard electrophoresis calibration kit	Amersham Pharmacia Biotech 17-0446-01
Magnesium sulphate	Merck Chemicals 10151
Manganous chloride	Ajax Chemicals D 3247
β-Mercaptoethanol	Merck Chemicals 805740
Methanol	BDH Chemicals 10158. BG
Nitrocellulose membrane	Advantec MFS, Inc., A 020 A304 C
β-Oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (POPC)	Sigma Chemicals P-3017
1,2-di[1-^{14}C]Palmitoyl-L-3-phosphatidylcholine (DPPC)	Amersham Pharmacia Biotech CFA 604 B 38
Perchloric acid	BDH Chemicals 101754 W
Peroxidase (POD)	Boehringer Mannheim 413470
L-α-Phosphatidylcholine (egg PC)	Sigma Chemicals P-5388
Phospholipid reagent	Roche Diagnostics 691844
Polyethylene glycol	Sigma Chemicals P-2139
Potassium bromide	BDH Chemicals 101954 F
Potassium ferrocyanide	Sigma Chemicals P-9387
SDS buffer strips	Amersham Pharmacia Biotech 17-0516-01

Silver nitatre	Ajax Chemicals 449
Skim milk powder	Diploma, Bonlac Foods Ltd., Australia
Sodium acetate	BDH Chemicals 10236.4 Q
Sodium azide	Sigma Chemicals S-2002
Sodium bromide	BDH Chemicals 301164 S
Sodium carbonate	BDH Chemicals 10240.4 H
Sodium chloride	BDH Chemicals 10241.3000
Sodium dihydrogen orthophosphate	BDH Chemicals 10245
Sodium dodecyl sulphate (SDS)	BDH Chemicals 442444 H
di-Sodium hydrogen orthophosphate	BDH Chemicals 10249
Sodium hydroxide	BDH Chemicals 10252.4 X
Sucrose	BDH Chemicals 010274.0500
5-Sulphosalicylic acid	BDH Chemicals 103464 A
Thimerosal	Sigma Chemicals T-5125
Total cholesterol reagent	Roche Diagnostics 2016630
Tris (hydroxymethyl) aminomethane	Sigma Chemicals T-1378
Triton X-100	Merck Chemicals 30632
Tween 20	BDH chemicals 66368
Urea	BDH Chemicals 10290. BG

CHAPTER 3

PHYSICAL CHARACTERIZATION OF rHDL CONTAINING ONLY CHOLESTERYL ESTERS IN THEIR CORE (CE-rHDL) AND rHDL ENRICHED WITH VARIOUS AMOUNTS OF TRIGLYCERIDE (TG-rHDL)

3.1 INTRODUCTION

3.2 METHODS

Preparation of cholesteryl ester containing rHDL (CE-rHDL) and triglyceride-enriched rHDL (TG-rHDL)

Physical properties of CE-rHDL and TG-rHDL

3.3 RESULTS

3.4 DISCUSSION

3.1 INTRODUCTION

A great deal of information about the remodeling of HDL by PLTP has been obtained by using native HDL isolated from human and animal plasma. While these studies have generated much information of value, the intrinsic heterogeneity of native HDL can make the results difficult to interpret. While it is theoretically possible to circumvent this problem by isolating specific HDL subpopulations, this is not feasible because the concentrations of many of the HDL subpopulations in plasma are extremely low and the techniques which are currently available for resolving HDL subpopulations are not sufficiently sensitive.

In this thesis an alternate approach, which eliminates the problems caused by the heterogeneity and low concentrations of native HDL subpopulations, was used. For several years our laboratory has been assembling reconstituted HDL (rHDL) from the individual protein and lipid constituents. These techniques have enabled us to generate large quantities of spherical rHDL (Rye et al., 1992, 1995, 1998). These preparations are indistinguishable from their plasma counterparts in terms of their interactions with CETP (Rye et al., 1995), LCAT (Liang et al., 1996), and HL (Hime et al., 1998). Because the size as well as the lipid and apolipoprotein composition of rHDL can be strictly controlled, these preparations are ideal models to study HDL-PLTP interactions.

Recently, Rye et al. (1998) showed that the PLTP-mediated remodeling of HDL is markedly enhanced when the particles are enriched with triglyceride. The reasons for this enhancement are investigated in the first part of this thesis

The preparation of CE-rHDL and their enrichment with triglyceride are described in the present chapter. A complete physical characterization of the CE-rHDL and TG-rHDL is also presented.

3.2 METHODS

Preparation of cholesteryl ester containing rHDL (CE-rHDL) and triglyceride-enriched rHDL (TG-rHDL)

Spherical rHDL, containing cholesteryl esters (CE) as the sole core lipid were prepared as described in Chapter 2. TG-rHDL containing increasing amounts of triglyceride in their core were prepared. These samples were used to determine how triglyceride enrichment affects phospholipid acyl chain and head group packing order as well as unfolding of apoA-I. The TG-rHDL were prepared by incubating spherical CE-rHDL (final CE concentration 0.1 μmol/mL) with Intralipid (final triglyceride (TG) concentration 4.0 μmol/mL) and CETP (final activity 2.6 units/mL) at 37 °C for 2 and 20 min. Control samples containing spherical CE-rHDL and Intralipid, but no CETP, were either maintained at 4°C or incubated at 37 °C for 20 min. The final volume of the incubation mixtures was 5.0 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation in the $1.063 < d < 1.21$ g/mL density range and dialyzed against 3 x 1 L of TBS. Triglyceride comprised either 12.2 or 32.4% of the total TG-rHDL core lipids.

rHDL particle size was determined by non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. The stoichiometry of CE-rHDL and TG-rHDL was determined using a Cobas Fara Centrifugal Analyzer. The number of apoA-I molecules/particle was determined by covalent cross-linking and the surface charge of the rHDL was determined by agarose gel electrophoresis. The details are described in Chapter 2.

The phospholipid acyl chain and head group packing order of the CE-rHDL and TG-rHDL (12.2 and 32.4% TG) was studied by labelling the samples with either 1,6-diphenyl-1,3,5-hexatriene (DPH) or 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluene-sulfonate (TMA-DPH) (Prendergast et al., 1981; Lentz et al., 1976).

The rHDL (final PL concentration 0.5 mM) were mixed with 7 μL of either 0.1 mM DPH or 0.1 mM TMA-DPH. The molar ratio of rHDL phospholipids:DPH or TMA-DPH was 500:1. The final volume of the mixtures was 707 μL. The samples were placed in the dark for 30 min to allow for uptake of label. Steady state fluorescence polarization of the DPH- and TMA-DPH-labelled CE-rHDL and TG-rHDL was measured as described in Chapter 2.

3.3 RESULTS

Physical properties of CE-rHDL and TG-rHDL (Figures 3.1-3.2, Table 3.1)

Figure 3.1 shows scans of the Coomassie Blue-stained non-denaturing gradient gels to which CE-rHDL and TG-rHDL were applied. Particle diameters were calculated by reference to known high molecular weight standards. The CE-rHDL and TG-rHDL were homogeneous in size. The diameter of CE-rHDL was 9.2 nm while those of TG-rHDL with 12.2 and 32.4% TG were 9.3 and 9.5 nm, respectively.

The composition of the rHDL was determined as the molar ratio of PL/UC/CE/TG/apoA-I (Table 3.1). Means of triplicate determinations which varied by less than 10% are shown. The TG-rHDL were prepared by incubating spherical CE-rHDL with Intralipid and CETP for 2 and 20 min. Control samples, CE-rHDL, were prepared by mixing

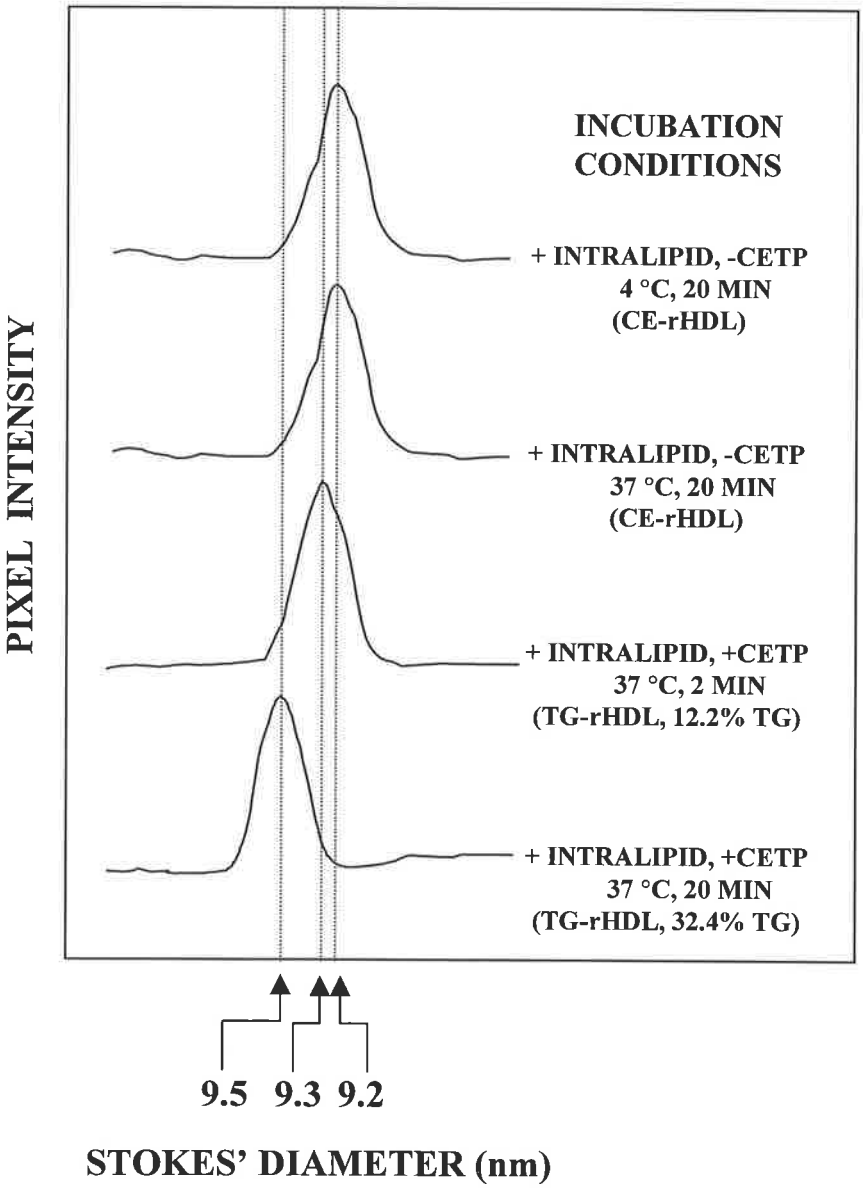


Figure 3.1 Size distribution of CE-rHDL and TG-rHDL
CE-rHDL and TG-rHDL were subjected to non-denaturing 3-40% poly-acrylamide gradient gel electrophoresis and stained with Coomassie Blue as described in Chapter 2. Laser densitometric scans of the gels are shown.

Table 3.1 Physical properties of CE-rHDL and TG-rHDL containing increasing amounts of triglyceride

Incubation Conditions	Stoichiometry PL/UC/CE/TG/apoA-I[a]	$\frac{\text{moleTG}}{\text{mole (TG+CE)}}$	Stokes' Diameter[b]	Number of apoA-I molecules/particle[c]	Electrophoretic mobility[d]
	(mol/mol)	*(%)*	*(nm)*		*($\mu m.s^{-1}/V.cm^{-1}$)*
+Intralipid-CETP, 4 °C, 20 min	84.6/5.7/59.7/0.6/3.0	1.0	9.2	3	-0.49
+Intralipid-CETP, 37 °C, 20 min	88.5/5.1/61.8/1.8/3.0	2.8	9.2	3	-0.49
+Intralipid+CETP, 37 °C, 2 min	91.8/6.0/58.5/8.1/3.0	12.2	9.3	3	-0.49
+Intralipid+CETP, 37 °C, 20 min	103.2/6.7/43.8/21.0/3.0	32.4	9.5	3	-0.49

Spherical CE-rHDL (final CE concentration 0.1 μmol/mL) were mixed with Intralipid (final TG concentration 4.0 μmol/mL) and either maintained at 4 °C, incubated at 37 °C for 20 min in the absence of CETP or incubated at 37 °C for 2 and 20 min in the presence of CETP (final activity 2.6 units/mL). The final volume of the incubation mixtures was 5.0 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation. The stoichiometries represent the mean of triplicate determinations which varied by less than 10%.

[a]PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride.

[b]Determined by non-denaturing 3/40% polyacrylamide gradient gel electrophoresis.

[c]Determined by cross-linking.

[d]Determined by agarose gel electrophoresis.

spherical CE-rHDL with Intralipid only and either maintained at 4°C or incubated at 37 °C for 20 min.

After incubation with Intralipid and CETP, the molar ratio of CE/apoA-I in the rHDL had decreased from 61.8/3.0 to 58.5/3.0 (incubation time 2 min) and 43.8/3.0 (incubation time 20 min). While the molar ratio of TG/apoA-I increased from 1.8/3.0 to 8.1/3.0 and 21.0/3.0, respectively. This indicates that the rHDL gained triglyceride and lost cholesteryl esters and is in agreement with previous studies (Rye et al., 1995, 1996, 1998).

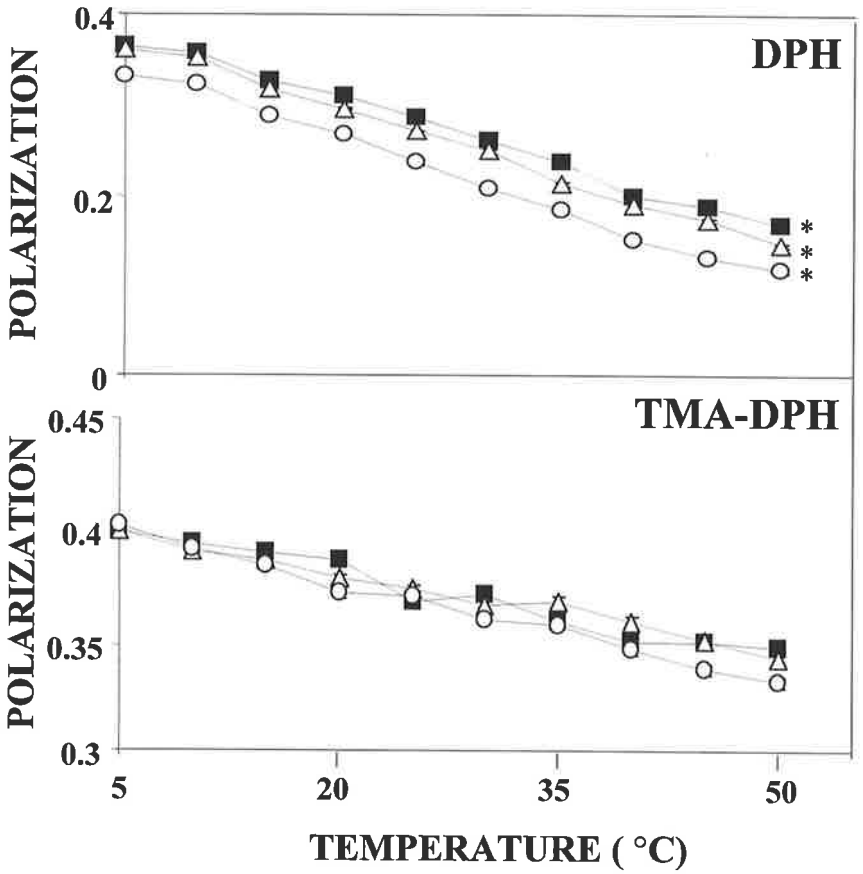
Cross-linking with bis(sulfosuccinimidyl) suberate (BS) showed that the CE-rHDL and TG-rHDL all contained 3 molecules of apoA-I/particle. Consequently, the stoichiometries in Table 3.1 are expressed relative to 3 molecules of apoA-I.

As judged by agarose gel electrophoresis, triglyceride enrichment did not affect rHDL surface charge, with the CE-rHDL and TG-rHDL all displaying electrophoretic mobilities of -0.49 $\mu m.s^{-1}/V.cm^{-1}$.

The acyl chain and head group packing order of the rHDL phospholipids was determined from the steady state fluorescence polarization of CE-rHDL and TG-rHDL (12.2 and 32.4% TG) labelled with DPH and TMA-DPH (Figure 3.2). The DPH-labelled CE-rHDL that were maintained at 4 °C (data not shown) or incubated at 37 °C in the absence of CETP (closed squares) had comparable polarization values. As the triglyceride content of the rHDL increased, phospholipid acyl chain packing order decreased as evidenced by the progressive reduction in the polarization values for TG-rHDL containing 12.2% TG

Figure 3.2 Phospholipid acyl chain and head group packing order of CE-rHDL and TG-rHDL

Spherical CE-rHDL (final CE concentration 0.1 μmol/mL) were mixed with Intralipid (final TG concentration 4.0 μmol/mL) and either maintained at 4 °C (data not shown), incubated at 37 °C for 20 min in the absence of CETP (■) or incubated at 37 °C for 2 (Δ) and 20 min (O) in the presence of CETP (final activity 2.6 units/mL). The final volume of the incubation mixtures was 5.0 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation and labelled with DPH and TMA-DPH. Steady state fluorescence polarization values for the DPH- and TMA-DPH-labelled rHDL are shown. Values represent the mean±sd of at least three determinations. *Significant different from all other samples by ANOVA, $p < 0.001$


DPH
TMA-DPH
POLARIZATION
POLARIZATION
0.4
0.2
0
0.45
0.4
0.35
0.3
5
20
35
50
TEMPERATURE (°C)
*
*
*

(open triangles) ($p < 0.001$ compared to all other samples by ANOVA) and 32.4% TG (open circles) ($p < 0.001$ compared to all other samples by ANOVA).

The polarization values for the TMA-DPH-labelled CE-rHDL that were maintained at 4 °C (data not shown) or incubated at 37 °C in the absence of CETP (closed squares) and the TMA-DPH-labelled TG-rHDL with 12.2% TG (open triangles) and 32.4% TG (open circles) were comparable. This indicates that triglyceride enrichment does not affect rHDL phospholipid head group packing order.

3.4 DISCUSSION

As judged by non-denaturing 3-40% polyacrylamide gradient gel elctrophoresis, the CE-rHDL and TG-rHDL were homogeneous in size (Figure 3.1). Triglyceride enrichment increased the diameter of the particles slightly from 9.2 to 9.5 nm (Table 3.1). This increase is not surprising given that the volume of a triglyceride molecule is approximately 1.4 times greater than that of a cholesteryl ester molecule (Sata et al., 1972).

The small amount of triglyceride in CE-rHDL (Table 3.1) probably represents trace amounts of Intralipid co-isolating with the rHDL rather than spontaneous transfer of triglyceride from Intralipid to the rHDL (Rye et al., 1995). The triglyceride enrichment was accompanied by a concomitant decrease in the cholesteryl ester content of the particles. This is consistent with what has been reported earlier by Rye et al. (1995, 1996, 1998).

Even though triglyceride comprised 12.2 and 32.4% of the total core lipids in the TG-rHDL, their surface charge was indistinguishable from that of the CE-rHDL (Table 3.1). This indicates that rHDL can contain substantial amounts of triglyceride without affecting the conformation of apoA-I on the particle surface. This is not the case when all of the cholesteryl esters are replaced by triglyceride (Curtiss et al., 2000).

Previous studies have shown that when spherical rHDL are incubated with Intralipid and CETP for 24 h, the rHDL become depleted of core lipids, their size decreases and apoA-I dissociates from the particles (Rye et al., 1995). These changes did not occur in the present study because the incubation was only 20 min in duration. Under these circumstances, the rHDL were enriched with triglyceride without dramatically changing their physical properties. As a result, these CE-rHDL and TG-rHDL are the ideal models determining how triglyceride enrichment influences HDL-PLTP interactions.

The results of the steady state fluorescence polarization studies showed that increasing the triglyceride content of rHDL decreases phospholipid acyl chain packing order. This is probably caused by the relatively bulky triglyceride molecules partitioning from the core into the rHDL surface (Hamilton and Small, 1981; Miller and Small, 1983).

CHAPTER 4

EFFECTS OF TRIGLYCERIDE ENRICHMENT ON PLTP-MEDIATED PHOSPHOLIPID TRANSFERS BETWEEN rHDL AND PHOSPHOLIPID VESICLES

4.1 INTRODUCTION

4.2 METHODS

Preparation of CE-rHDL and TG-rHDL

Preparation of small unilamellar POPC vesicles

Preparation of [^{14}C]-POPC-labelled CE-rHDL and [^{14}C]-POPC-labelled TG-rHDL

PLTP-mediated phospholipid transfers between [^{14}C]-POPC-labelled small unilamellar vesicles and unlabelled rHDL

PLTP-mediated phospholipid transfers between unlabelled small unilamellar POPC vesicles and [^{14}C]-POPC-labelled rHDL

4.3 RESULTS

4.4 DISCUSSION

4.1 INTRODUCTION

PLTP transfers a wide range of phospholipids between HDL and other plasma lipoproteins (Tollefson et al., 1988b; Rao et al., 1997). In these systems PLTP facilitates the first step of the reaction: the desorption of phospholipid molecules from the surface of the donor particles (Lalanne and Ponsin, 2000). Studies in PLTP knockout mice have also shown that the transfer of phospholipids from triglyceride-rich lipoproteins to HDL during lipolysis by LPL is facilitated by PLTP. These transfers play a major role in regulating plasma HDL levels (Tall et al., 1985; Jiang, et al., 1999).

Triglyceride-enriched HDL are the preferred substrates for HL (Perret et al., 1987; Clay et al., 1992). This is consistent with the concentration of triglycerides in HDL correlating positively with the rate of PLTP-mediated phospholipid transfers in human plasma (Cheung, et al., 1996). The latter study suggests that triglycerides are significant modulators of intravascular phospholipid transport. However, the influence of triglyceride enrichment of rHDL on PLTP-mediated phospholipid transfers has not been investigated directly.

This issue is addressed in the present chapter by quantitating PLTP-mediated phospholipid transfers between [^{14}C]-POPC-labelled small unilamellar vesicles and unlabelled rHDL as well as between unlabelled small unilamellar POPC vesicles and [^{14}C]-POPC-labelled rHDL. The reasons for the enhanced phospholipid transfers in triglyceride-enriched rHDL are also addressed.

4.2 METHODS

Preparation of CE-rHDL and TG-rHDL

The spherical rHDL (final CE concentration 0.1 μmol/mL) were enriched with Intralipid (Kabi Vitrum AB, Stockholm, Sweden) (final TG concentration 4.0 μmol/mL) by incubation in the presence of CETP (final activity 2.6 units/mL) at 37 °C for 20 min (Rye et al., 1998). Control samples of CE-rHDL were incubated with Intralipid alone. The final volume of the incubation mixtures was 68.0 mL. When the incubations were complete, the CE-rHDL and TG-rHDL were isolated from the incubation mixtures by ultracentrifugation in the $1.063 < d < 1.21$ g/mL density range and dialyzed against 3 x 1 L of TBS. Triglyceride comprised 27.4% of the total TG-rHDL core lipids.

Preparation of small unilamellar POPC vesicles

[^{14}C]-POPC small unilamellar vesicles were prepared as described (Damen et al., 1982) by adding to a clean, dry test tube 39 μL of POPC (100 mg/mL) in chloroform/methanol 2:1 (v/v), 20 μL of 10 μCi of 1-palmitoyl-2-[1-^{14}C]oleoyl-L-3-phosphatidylcholine ([^{14}C]-POPC) (Amersham Pharmacia Biotech), and 20 μL of 1mM butylated hydroxytoluene (BHT) in ethanol. The lipids were dispersed as a thin film on the walls of the tube and dried under N_2 at 40 °C for 2 h. The phospholipids were resuspended in 0.5 mL of TBS and sonicated for 3 x 5 min, using a Sonifier B-12 (Branson Sonic Power Company, Danbury, CT, USA) equipped with a microtip. The mixture was then centrifuged at 15,000 rpm for 10 min and the supernatant, containing the [^{14}C]-POPC-labelled small unilamellar vesicles, was collected.

Unlabelled small unilamellar POPC vesicles were prepared as described in the preceding paragraph, but without adding the radiolabelled phospholipid.

Preparation of [^{14}C]-POPC-labelled CE-rHDL and [^{14}C]-POPC-labelled TG-rHDL

[^{14}C]-POPC-labelled CE-rHDL were prepared by mixing unlabelled spherical CE-rHDL (6.6 μmol PL) with [^{14}C]-POPC small unilamellar vesicles (0.66 μmol PL), and fatty acid free BSA (final concentration 20 mg/mL). The mixture was incubated with PLTP (final activity 247 nmol PL transferred/mL PLTP/h) at 37 °C for 3 h. The final volume of the incubation mixture was 5.5 mL. When the incubation was complete, the [^{14}C]-POPC-labelled CE-rHDL were isolated by ultracentrifugation at 100,000 rpm in the density range $1.063 < d < 1.21$ g/mL using a TLA-100.4 rotor with one 6 h spin at the lower density and a second 16 h spin at the higher density. The ultracentrifugations were carried out at 4 °C. The [^{14}C]-POPC-labelled rHDL were dialyzed against 3 x 1 L of TBS before use.

The [^{14}C]-POPC-labelled CE-rHDL were then incubated at 37 °C for 20 min with Intralipid (final TG concentration 4.0 μmol/mL), or enriched with triglyceride by incubation at 37 °C for 20 min with Intralipid (final TG concentration 4.0 μmol/mL) and CETP (final activity 2.6 units/mL). The final volume of the incubation mixture was 20.5 mL. The resulting [^{14}C]-POPC-labelled CE-rHDL and [^{14}C]-POPC-labelled TG-rHDL were isolated by ultracentrifugation as described for unlabelled CE-rHDL and TG-rHDL.

PLTP-mediated phospholipid transfers between [^{14}C]-POPC labelled small unilamellar vesicles and unlabelled rHDL

PLTP-mediated phospholipid transfers were determined as described by Damen et al. (1982). [^{14}C]-POPC small unilamellar vesicles (final PL concentration 0.375 μmol/mL) were mixed with either unlabelled CE-rHDL (final apoA-I concentration 0.625 mg/mL)

or unlabelled TG-rHDL (27.4% TG) (final apoA-I concentration 0.625 mg/mL) and PLTP (final activity 234 nmol PL transferred/mL PLTP/h). TBS was added to make the total volume 100 μL and the incubations were conducted at 37 °C for 1, 3, 5, 10, and 20 min. At the end of the incubation, the vesicles were precipitated with 600 μL of $MnCl_2$/heparin solution (0.47 units/μL heparin/215 mM $MnCl_2$/500 mM NaCl). The [^{14}C]-POPC content of the rHDL in the supernatant was determined by liquid scintillation counting as described in Chapter 2. Precipitation of the vesicles with $MnCl_2$/heparin solution was quantitative, while more than 95% of the rHDL remained in solution. Transfer rates were calculated as the slope of the initial, linear section of plots as the % phospholipids transferred between the rHDL and the vesicles as a function of time.

PLTP-mediated phospholipid transfers between unlabelled small unilamellar POPC vesicles and [^{14}C]-POPC-labelled rHDL

The study was carried out as described in the preceding paragraph but with unlebelled small unilamellar POPC vesicles and either [^{14}C]-POPC-labelled CE-rHDL or [^{14}C]-POPC-labelled TG-rHDL.

4.3 RESULTS

Physical properties of CE-rHDL and TG-rHDL (Figure 4.1, Table 4.1)

Figure 4.1 shows scans of the Coomassie Blue-stained non-denaturing gradient gels. The CE-rHDL and TG-rHDL were homogeneous in size, with diameters of 9.3 and 9.5 nm, respectively. The composition of the rHDL was shown in Table 4.1. Triglyceride comprised 27.4% of total TG-rHDL core lipids.

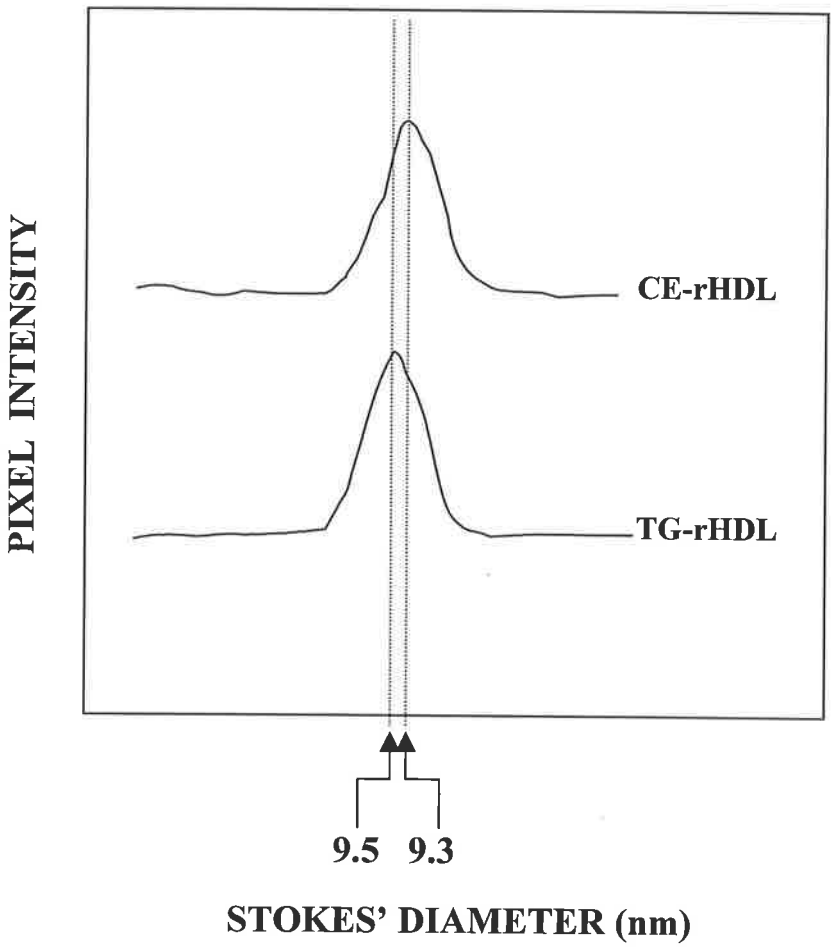


Figure 4.1 Size distribution of CE-rHDL and TG-rHDL
CE-rHDL and TG-rHDL were subjected to non-denaturing 3-40% poly-acrylamide gradient gel electrophoresis and stained with Coomassie Blue as described in Chapter 2. Laser densitometric scans of the gels are shown.

Table 4.1 Physical properties of CE-rHDL and TG-rHDL

rHDL	Stoichiometry PL/UC/CE/TG/apoA-I[a]	Stokes' Diameter[b]
	(*mol/mol*)	(*nm*)
CE-rHDL	95.3/6.8/68.1/1.8/3.0	9.3
TG-rHDL	103.2/6.2/54.2/22.9/3.0	9.5

Spherical rHDL (final CE concentration 0.1 μmol/mL) were incubated with Intralipid (final TG concentration 4.0 μmol/mL) in the absence and presence of CETP (final activity 2.6 units/mL) at 37 °C for 20 min. The final volume of the incubation mixtures was 68.0 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation. Stoichiometries were determined as the mean of triplicate determinations which varied by less than 10%

[a] PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride.

[b]Determined by non-denaturing 3/40% polyacrylamide gradient gel electrophoresis.

PLTP-mediated phospholipid transfers between [^{14}C]-POPC small unilamellar vesicles and unlabelled rHDL (Figure 4.2)

To determine whether transfers of phospholipids from small unilamellar POPC vesicles to TG-rHDL were enhanced relative to CE-rHDL, the rHDL were incubated with [^{14}C]-POPC small unilamellar vesicles and PLTP. Transfers of [^{14}C]-POPC from the vesicles to CE-rHDL (open squares) and TG-rHDL (closed squares) are shown in Figure 4.2. The initial rate of transfer from the vesicles to TG-rHDL was 7.1 μmol POPC transferred/mL PLTP/h, compared to 3.9 μmol POPC transferred/mL PLTP/h for CE-rHDL.

PLTP-mediated phospholipid transfers between unlabelled small unilamellar POPC vesicles and [^{14}C]-POPC-labelled rHDL (Figure 4.2)

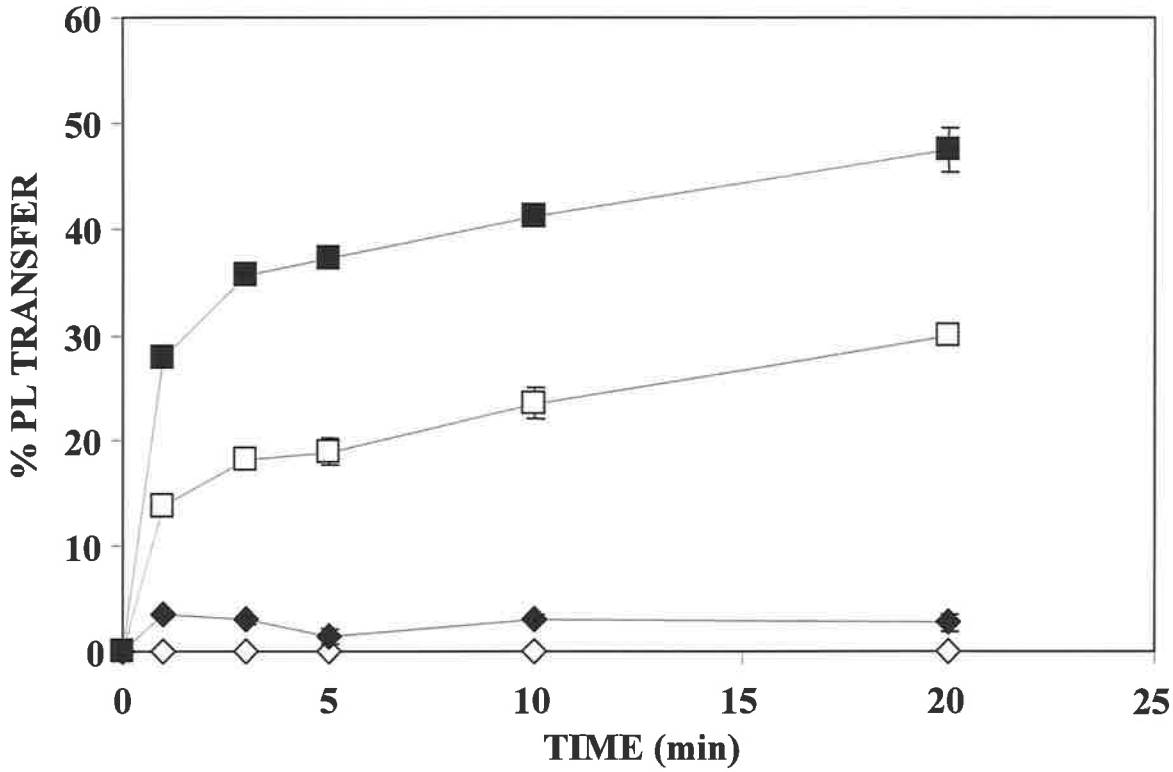
To determine whether the different rates of transfers could be explained by modification of the vesicles as a consequence of transfers surface constituents from the rHDL to the vesicles, transfers of phospholipids from rHDL to the vesicles were also examined. In these experiments [^{14}C]-POPC-labelled CE-rHDL and [^{14}C]-POPC-labelled TG-rHDL were incubated with PLTP and unlabelled small unilamellar POPC vesicles. There was no detectable transfer of POPC from the [^{14}C]-POPC-labelled CE-rHDL to the vesicles (open diamonds). Less than 5% of the [^{14}C]-POPC transferred from the TG-rHDL to the vesicles (closed diamonds) (Figure 4.2). This indicates that the different rates of transfer of phospholipids from the vesicles to the rHDL cannot be explained by rHDL surface constituents altering the vesicle structure.

4.4 DISCUSSION

The results of these experiments show that PLTP-mediated phospholipid transfers from small unilamellar POPC vesicles to TG-rHDL are enhanced relative to CE-rHDL. These

Figure 4.2 PLTP-mediated phospholipid transfers between the vesicles and rHDL

CE-rHDL (□) and TG-rHDL (■) (final apoA-I concentration 0.625 mg/mL) were incubated with PLTP (final activity 234 nmol PL transferred/mL PLTP/h) and [^{14}C]-POPC small unilamellar vesicles (final PL concentration 0.375 μmol/mL) at 37 °C for 1, 3, 5, 10, and 20 min. The final incubation volume was 100 μL. Identical conditions were used for incubations [^{14}C]-POPC-labelled CE-rHDL (◇) and [^{14}C]-POPC-labelled TG-rHDL (◆) with unlabelled small unilamellar POPC vesicles and PLTP. When the incubations were complete, the vesicles were precipitated with a $MnCl_2$/heparin solution and the [^{14}C]-POPC content of the rHDL was determined by liquid scintillation counting. Data points represent the mean±sd of triplicate determinations.


60
50
40
30
20
10
0
% PL TRANSFER
0
5
10
15
20
25
TIME (min)

findings are consistent with the observation of Cheung et al. (1996) who found that the triglyceride content of HDL correlates positively with the rate of PLTP-mediated phospholipid transfers in human plasma.

The results of the steady state fluorescence polarization studies in Chapter 3 show that increasing the triglyceride content of rHDL core decreases their phospholipid acyl chain packing order. This may be a result of triglyceride in TG-rHDL partitioning from the core into the rHDL surface (Hamilton and Small, 1981; Miller and Small, 1983) and generating packing defects in the particle surface. This may enhance the ability of the TG-rHDL to accommodate additional phospholipids that are transferred from the vesicles.

The additional finding that there is minimal transfer of phospholipids in the reverse direction from the rHDL to the vesicles shows unequivocally that the different rates of phospholipid transfers from the vesicles to the CE-rHDL and TG-rHDL cannot be explained by rHDL surface constituents altering the properties of the vesicles.

CHAPTER 5

THE REMODELING OF CE-rHDL AND TG-rHDL BY PLTP

5.1 INTRODUCTION

5.2 METHODS

PLTP-mediated remodeling of CE-rHDL and TG-rHDL

Resolution of the CE-rHDL and TG-rHDL conversion products by gel permeation chromatography

Physical properties of the pooled rHDL fractions

Time course of the remodeling of CE-rHDL and TG-rHDL by PLTP

5.3 RESULTS

5.4 DISCUSSION

5.1 INTRODUCTION

As described in Chapter 1, PLTP promotes the remodeling of HDL. The interaction of PLTP with HDL in vitro results in the formation of large and small particles, and the dissociation of lipid-poor or lipid-free apoA-I in a process involving particle fusion. (Jauhiainen et al., 1993; Tu et al., 1993; Lusa et al., 1996; Korhonen et al., 1998). This is also the case in vivo where adenovirus mediated overexpression of PLTP in mice promotes the remodeling of HDL into large HDL particles as well as pre-β HDL (Ehnholm et al., 1998). There is a clear correlation between serum PLTP activity in mice that overexpress PLTP and the formation of pre-β HDL (Ehnholm et al., 1998). This suggests that one function of PLTP in vivo is to generate pre-β HDL during HDL remodeling (Ehnholm et al., 1998). The physiological significance of the remodeling of HDL by PLTP is highlighted by a study in which the incubation of plasma with purified PLTP generated pre-β HDL and an enhanced efflux of cellular cholesterol into these particles (von Eckardstein et al., 1996).

The PLTP-mediated remodeling of HDL is controlled by several factors; (i) HDL apolipoprotein composition, (ii) the phospholipid transfer activity of PLTP, and (iii) the core lipid composition of HDL. Both the formation of large particles and the dissociation of apoA-I are inhibited by increasing the concentration of apoA-II in HDL (Pussinen et al., 1997). Employing both chemically modified PLTP and PLTP mutants with reduced phospholipid transfer activity, Huuskonen et al. (2000) demonstrated that phospholipid transfers are a prerequisite for efficient PLTP-mediated HDL remodeling. It has also been shown that remodeling by PLTP is enhanced in spherical rHDL or pig HDL that are enriched with triglycerides (Rye et al., 1998).

Triglyceride enrichment also enhances the PLTP-mediated remodeling of HDL in vivo. Murakami et al. (1995) have reported that hypertriglyceridemic patients have HDL that are enriched with triglycerides and remodeled faster than the HDL of normal subjects. The levels of small HDL in these subjects are also increased.

The mechanism of the remodeling of HDL by PLTP is poorly understood. Although, there is evidence that particle fusion and the dissociation of lipid-poor or lipid-free apoA-I are involved (Lusa et al., 1996; Korhonen et al., 1998), nothing is known about how the interaction of PLTP with HDL is regulated, or the events that occur when HDL are remodeled into large and small particles.

In order to define the mechanism of PLTP-mediated HDL remodeling, the CE-rHDL and TG-rHDL were incubated with PLTP, and the incubation products were isolated and characterised. The time course of the remodeling of HDL by PLTP was also studied.

5.2 METHODS

PLTP-mediated remodeling of CE-rHDL and TG-rHDL

CE-rHDL and TG-rHDL, prepared in Chapter 4, (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C or incubated at 37 °C for 24 h in the absence or presence of PLTP (final activity 7.8 μmol PL transferred/mL PLTP/h). The final volume of the incubation mixtures was 4.79 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation in the $1.063 < d < 1.25$ g/mL density range with a single spin at the upper and lower densities. The spins were conducted at 100,000 rpm for 16 h using a TLA 100.4 rotor in a Beckman TL-100 Tabletop ultracentrifuge maintained at 4 °C.

The ultracentrifugally isolated rHDL were concentrated by ultrafiltration using a CF 25 membrane cone (Amicon®, MA, USA), then applied to a pre-equilibrated HR 10/30 Superose 6 column as described in Chapter 2. The rHDL were eluted from the column with TBS at a flow rate of 0.3 mL/min. Fractions were collected at 1 min intervals. The concentrations of PL, UC, CE, TG, and apoA-I were determined for each fraction as described in Chapter 2.

Selected fractions were pooled and subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. The composition and the number of apoA-I molecules/particle in the pooled samples were determined as described in Chapter 2.

Time course of the remodeling of CE-rHDL and TG-rHDL by PLTP

CE-rHDL and TG-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (final activity 5.9 μmol PL transferred/mL PLTP/h). The final volume of the incubation mixtures was 270 μL. Aliquots of the unprocessed incubation mixtures (0.25 μg of apoA-I) were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis, transferred electrophoretically to nitrocellulose membranes and immunoblotted with polyclonal sheep antihuman apoA-I antibody. The transferred bands were detected by ECL.

5.3 RESULTS

PLTP-mediated remodeling of CE-rHDL and TG-rHDL (Figure 5.1)

CE-rHDL and TG-rHDL size was not affected by incubation for 24 h in the absence of PLTP (Figure 5.1). After 24 h of incubation in the presence of PLTP, approximately 76% of the original CE-rHDL were converted into large (11.3 nm) and small (7.7 nm) particles. The remaining CE-rHDL were unchanged in size. When the TG-rHDL were incubated for 24 h with PLTP, they were completely converted into large (11.3 nm) and small (7.7 nm) particles. This confirms the earlier study of Rye et al. (1998) which showed that triglyceride enrichment enhances the remodeling of HDL by PLTP.

Resolution of the CE-rHDL and TG-rHDL conversion products by gel permeation chromatography (Figures 5.2-5.3)

The ultracentrifugally isolated rHDL were concentrated and resolved by gel permeation chromatography. Elution profiles from the size-exclusion chromatographic procedure are shown in Figure 5.2. The rHDL either maintained at 4 °C or incubated at 37 °C for 24 h in the absence of PLTP eluted as a single peak. This reflects the monodispersity of the rHDL preparations. The rHDL that were incubated at 37 °C for 24 h with PLTP eluted as two peaks.

The concentrations of the individual rHDL constituents in each fraction are shown in Figure 5.3. The large particles generated by incubation in the presence of PLTP were enriched in cholesteryl esters, while the small particles were enriched in apoA-I. The scales on the ordinates for Figure 5.3 are smaller for rHDL incubated with PLTP because the constituents are spread over a large number of fractions. Selected fractions were pooled as indicated (↔).

Figure 5.1 PLTP-mediated remodeling of CE-rHDL and TG-rHDL

CE-rHDL and TG-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C or incubated at 37 °C for 24 h in the absence and presence of PLTP (final activity 7.8 μmol PL transferred/mL PLTP/h). The final volume of the incubation mixtures was 4.79 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation and subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. The profiles represent scans of Coomassie Blue-stained gels. Particle diameters were calculated by reference to known high molecular weight standards.

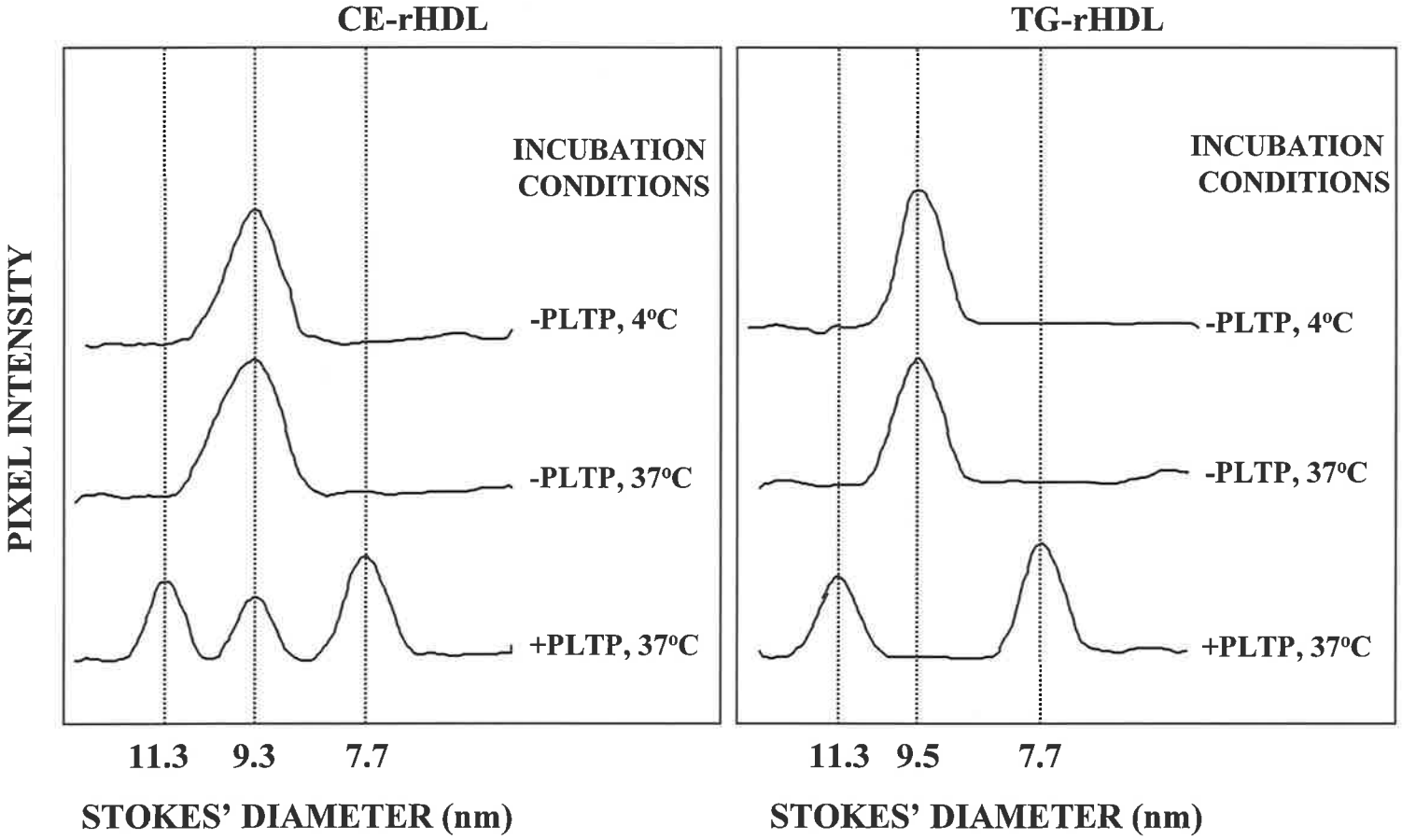

CE-rHDL
TG-rHDL
INCUBATION CONDITIONS
-PLTP, 4°C
-PLTP, 37°C
+PLTP, 37°C
PIXEL INTENSITY
11.3
9.3
7.7
11.3
9.5
7.7
STOKES' DIAMETER (nm)
STOKES' DIAMETER (nm)

Figure 5.2 Elution profiles of rHDL after gel permeation chromatography

The ultracentrifugally isolated rHDL, obtained from the mixtures of rHDL either maintained at 4 °C or incubated at 37 °C for 24 h in the absence and presence of PLTP, were concentrated and resolved by gel permeation chromatography. The rHDL were eluted from the column with TBS at a flow rate of 0.3 mL/min. Fractions were collected at 1 min intervals.

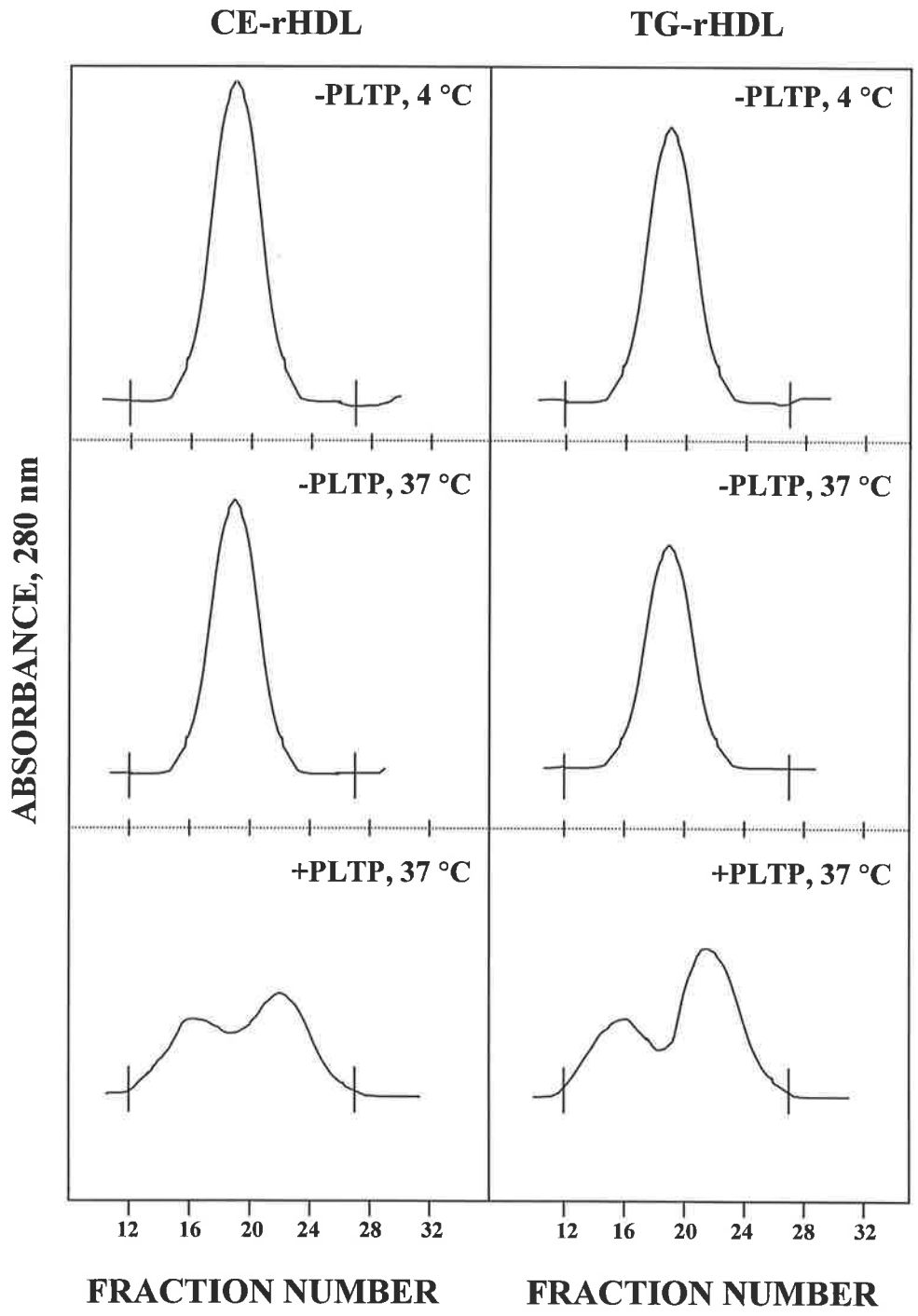
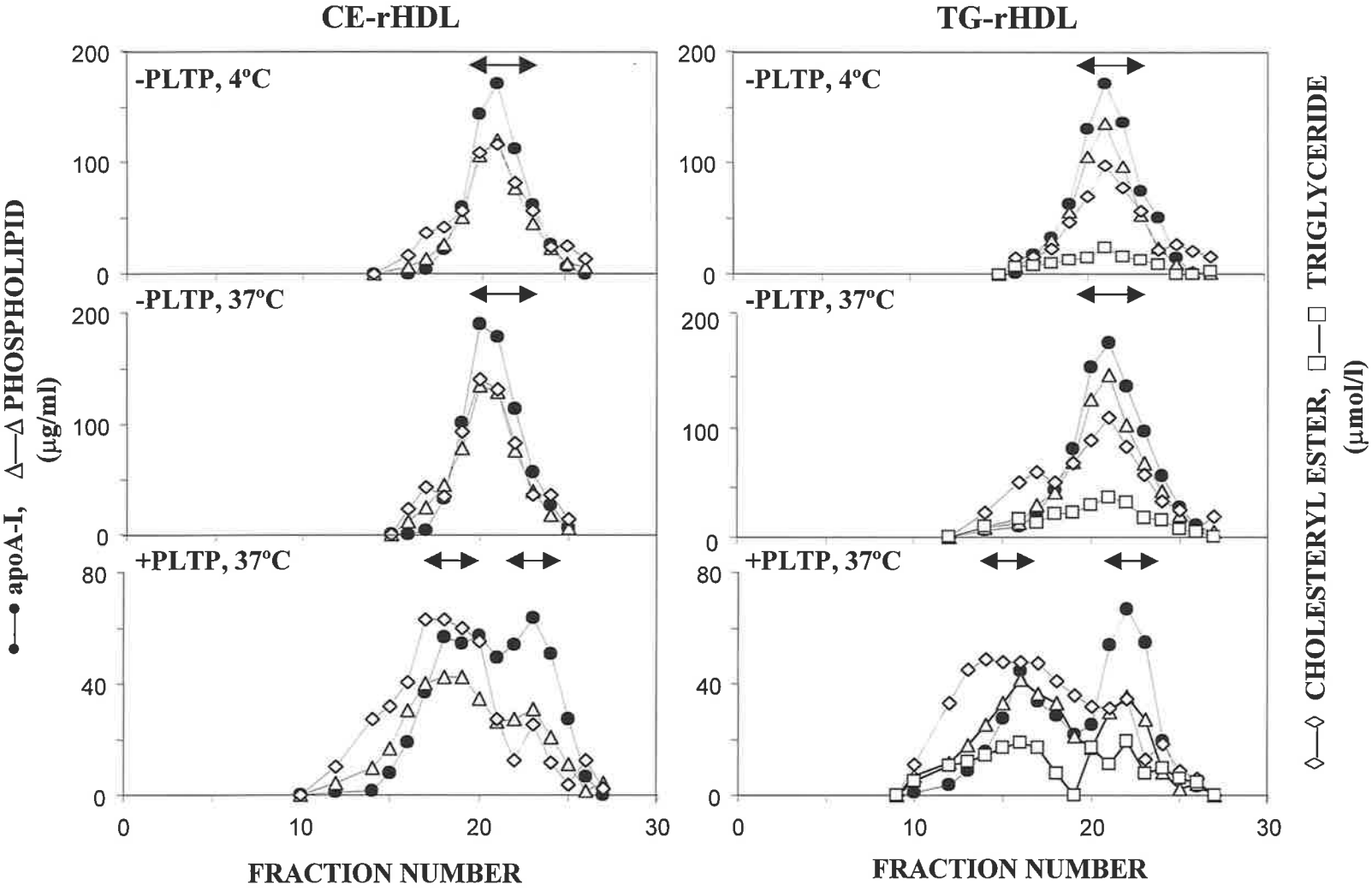

CE-rHDL
TG-rHDL
-PLTP, 4 °C
-PLTP, 4 °C
-PLTP, 37 °C
-PLTP, 37 °C
+PLTP, 37 °C
+PLTP, 37 °C
ABSORBANCE, 280 nm
12
16
20
24
28
32
12
16
20
24
28
32
FRACTION NUMBER
FRACTION NUMBER

Figure 5.3 The composition of elution fractions of CE-rHDL and TG-rHDL

The concentrations of the individual rHDL constituents in each of the gel permeation chromatographic fractions are shown. The values represent the mean of triplicate determinations which varied by less than 10%. Selected fractions were pooled as indicated (↔).


CE-rHDL
TG-rHDL
-PLTP, 4°C
-PLTP, 37°C
+PLTP, 37°C
200
100
0
80
40
0
10
20
30
FRACTION NUMBER
●—● apoA-I, Δ—Δ PHOSPHOLIPID (μg/ml)
◇—◇ CHOLESTERYL ESTER, □—□ TRIGLYCERIDE (μmol/l)

Physical properties of the pooled rHDL fractions (Figure 5.4, Table 5.1)

The pooled fractions were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. Figure 5.4 shows laser densitometric scans of the Coomassie Blue-stained gels. Profiles A and B, respectively, show the CE-rHDL and TG-rHDL that were either maintained at 4 °C or incubated at 37 °C for 24 h in the absence of PLTP. Profiles C and D represent the large (11.3 nm) and small (7.7 nm) conversion products generated by incubation of CE-rHDL and TG-rHDL with PLTP at 37 °C for 24 h.

The composition of the pooled samples and the recovery of the individual rHDL constituents are shown in Table 5.1. The large particles contained approximately twice as many phospholipids and core lipid molecules as the original rHDL, indicating that they must be derived from a fusion product. Following incubation with PLTP, the recovery of rHDL core lipids exceeded that of the surface constituents. This indicates that apoA-I and phospholipids as well as unesterifed cholesterol dissociated from the CE-rHDL and TG-rHDL during the incubation.

The number of apoA-I molecules/particle in the pooled samples was determined by cross-linking (Table 5.1). Whereas the original CE-rHDL and TG-rHDL contained three molecules of apoA-I/particle, the large and small conversion products contained four and two molecules of apoA-I/particle, respectively.

Time course of the remodeling of CE-rHDL and TG-rHDL by PLTP (Figure 5.5)

The time dependence of the remodeling of CE-rHDL and TG-rHDL by PLTP is shown in Figure 5.5. Tracks A and B, show rHDL that were either maintained at 4 °C or incubated

Figure 5.4 Size distribution of the pooled rHDL fractions

Pooled samples from Figure 5.3 were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. Scans of the Coomassie Blue-stained gels are shown. Profiles A and B, respectively show the pooled CE-rHDL and TG-rHDL fractions that were either maintained at 4 °C or incubated at 37 °C for 24 h in the absence of PLTP. Profiles C and D are the large and small conversion products generated by incubation of CE-rHDL and TG-rHDL with PLTP at 37 °C for 24 h.

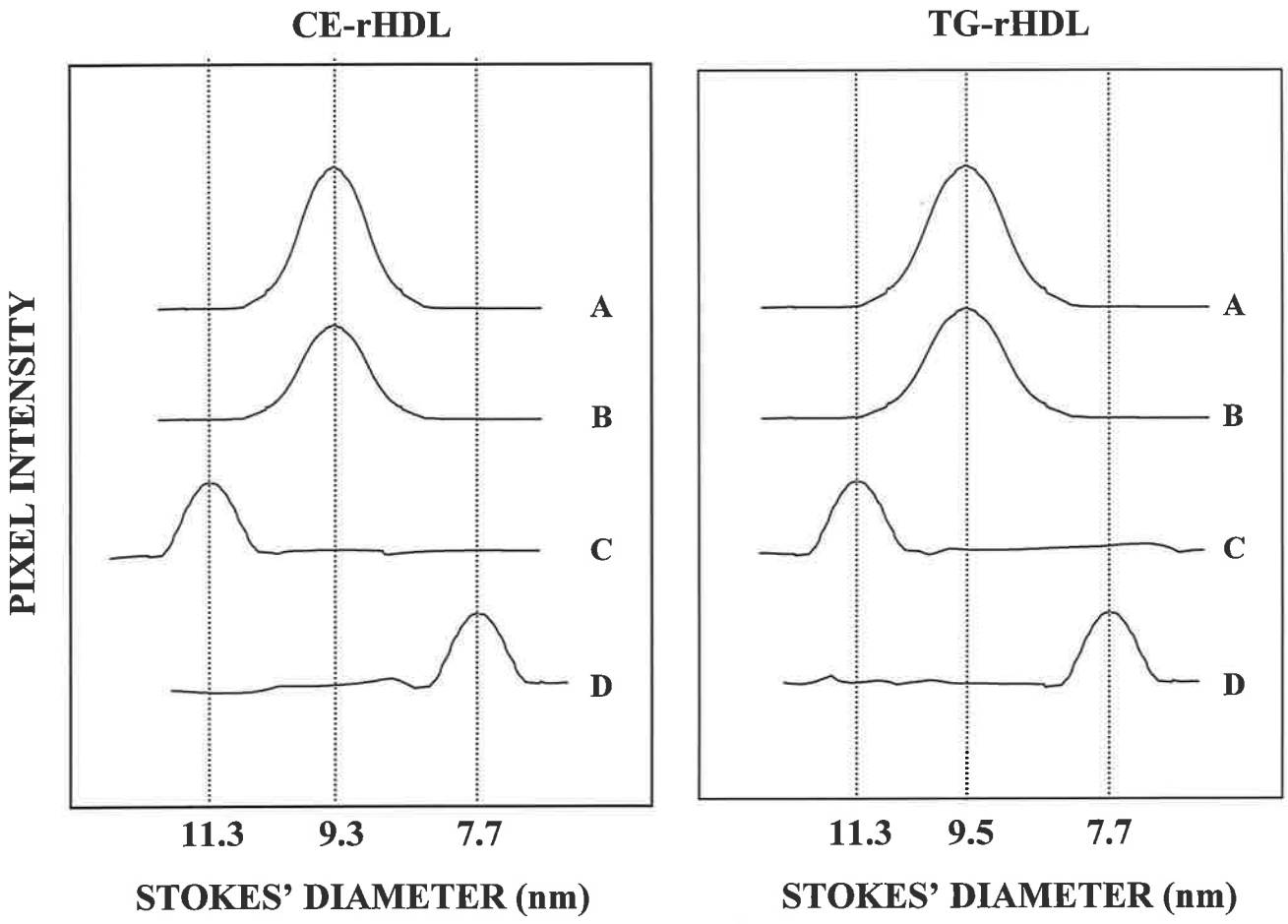

CE-rHDL
TG-rHDL
PIXEL INTENSITY
A
B
C
D
11.3
9.3
7.7
11.3
9.5
7.7
STOKES' DIAMETER (nm)
STOKES' DIAMETER (nm)

Table 5.1 Physical properties of the pooled rHDL fractions

Incubation Conditions	Stoichiometry PL UC CE TG apoA-I[a]	Recovery PL UC CE TG apoA-I[b]	Stokes' Diameter[c]	Number of apoA-I molecules/particle[d]
	(mol/mol)	*(%)*	*(nm)*	
CE-rHDL				
- PLTP, 4°C	73.5 7.0 71.7 2.0 3.0	100 100 100 100 100	9.3	3
- PLTP, 37°C	78.3 6.9 72.6 2.0 3.0	99 105 98 103 101	9.3	3
+PLTP, 37°C				
Large conversion products	148.3 10.8 157.7 4.2 4.0	36 23 48 42 29	11.3	4
Small conversion products	48.8 3.0 44.8 1.5 2.0	29 22 34 39 35	7.7	2
		(65) (45) (82) (81) (64)		
TG-rHDL				
- PLTP, 4°C	76.8 6.0 72.9 21.3 3.0	100 100 100 100 100	9.5	3
- PLTP, 37°C	71.1 6.9 75.0 22.5 3.0	99 109 95 101 94	9.5	3
+PLTP, 37°C				
Large conversion products	143.6 12.0 153.5 50.8 4.0	27 11 44 35 20	11.3	4
Small conversion products	54.6 3.6 52.6 16.0 2.0	23 11 28 35 33	7.7	2
		(50) (22) (72) (70) (53)		

CE-rHDL and TG-rHDL were incubated with PLTP as described in Chapter 5. Selected fractions were pooled as indicated (↔) in Figure 5.3. The composition, size and number of apoA-I molecules/particle were determined as described in Chapter 2. The stoichiometries represent the mean of triplicate determinations which varied by less than 10%.

[a]PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride.

[b]Recovery of individual constituents is expressed relative to the recovery of the constituents in the non-incubated controls. Values in parenthesis represent the total recovery of constituents in the large and small conversion products.

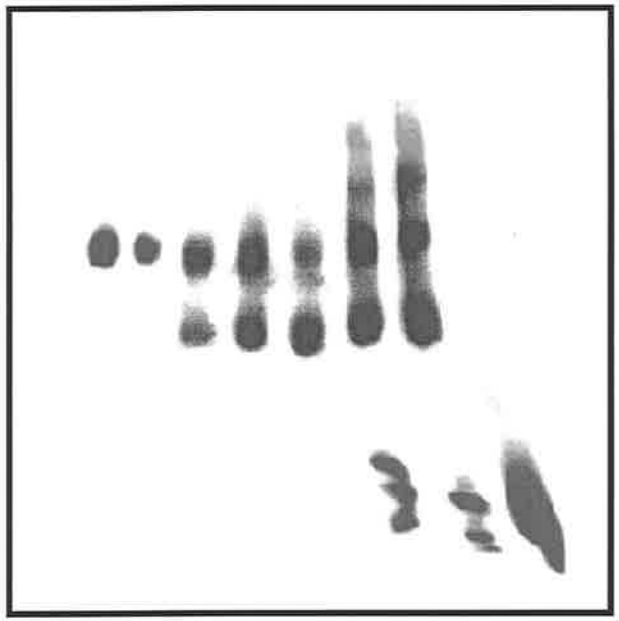
[c]Determined by non-denaturing 3-40% polyacrylamide gradient gel electrophoresis.

[d]Determined by cross-linking.

Figure 5.5 Time course of the remodeling of CE-rHDL and TG-rHDL by PLTP

CE-rHDL and TG-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP (Tracks A and B, respectively) or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (final activity 5.9 μmol PL transferred/mL PLTP/h) (Tracks C, D, E, F, and G, respectively). The final volume of the incubation mixtures was 270 μL. An aliquot of each incubation mixture (0.25 μg apoA-I) was subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. Lipid-free apoA-I was also applied to the gels (Track H). The samples were transferred to nitrocellulose membranes, immunoblotted with sheep antihuman apoA-I antiserum and detected by ECL.

CE-rHDL



A B C D E F G H

TG-rHDL

A B C D E F G H

at 37 °C for 24 h in the absence of PLTP. Tracks C, D, E, F, and G represent rHDL that were incubated at 37 °C in the presence of PLTP for 1, 3, 6, 12, and 24 h. Lipid-free apoA-I is shown in Track H. A proportion of the CE-rHDL was converted into small particles during the first 6 h of incubation with PLTP. Large CE-rHDL conversion products and dissociated apoA-I were apparent after 12 h of incubation. In the case of TG-rHDL, small particles were apparent at 1 h, while the large conversion products and dissociated apoA-I appeared after 3 h of incubation with PLTP.

5.4 DISCUSSION

The ability of PLTP to remodel HDL into large and small particles in processes involving particle fusion and the dissociation of apoA-I from HDL is well documented (Jauhiainen et al., 1993; Tu et al., 1993; Lusa et al., 1996; Korhonen et al., 1998). The experiments in this chapter gave an insight into the events that mediate these changes. The data in Table 5.1 show that the large CE-rHDL and TG-rHDL conversion products contain more surface and core lipid constituents/particle than the original rHDL. This indicates that the large conversion products must have been formed by particle fusion. This is consistent with what has been reported by Lusa et al. (1996) and Korhonen et al. (1998). Furthermore, the recoveries of all of the rHDL surface constituents were decreased compared to the recovery of core lipids (Table 5.1). This demonstrates that apoA-I and phospholipids as well as unesterified cholesterol must have dissociated from the CE-rHDL and TG-rHDL during the incubation with PLTP.

The relationship between the time sequence of the remodeling of rHDL into large and small particles and the dissociation of apoA-I was also studied by incubating CE-rHDL and TG-rHDL with PLTP and monitoring rHDL size changes as well as the dissociation

of apoA-I (Figure 5.5). These results showed that in both cases the small conversion products were formed before the large conversion products. In addition, the appearance of the large conversion products coincided with the dissociation of apoA-I. Both the large and small TG-rHDL conversion products were generated more rapidly than the CE-rHDL conversion products. This indicates that triglyceride enrichment does not affect the mechanism in by which the rHDL are remodeled.

In the case of CE-rHDL, small particles were formed during the first 6 h of incubation with PLTP, while the large conversion products and dissociated apoA-I appeared after 12 h of incubation. These time differences indicate that the large and small conversion products are formed by independent processes. Furthermore, the finding that the formation of the large particles coincided with the dissociation of apoA-I suggest that they are generated via a common pathway.

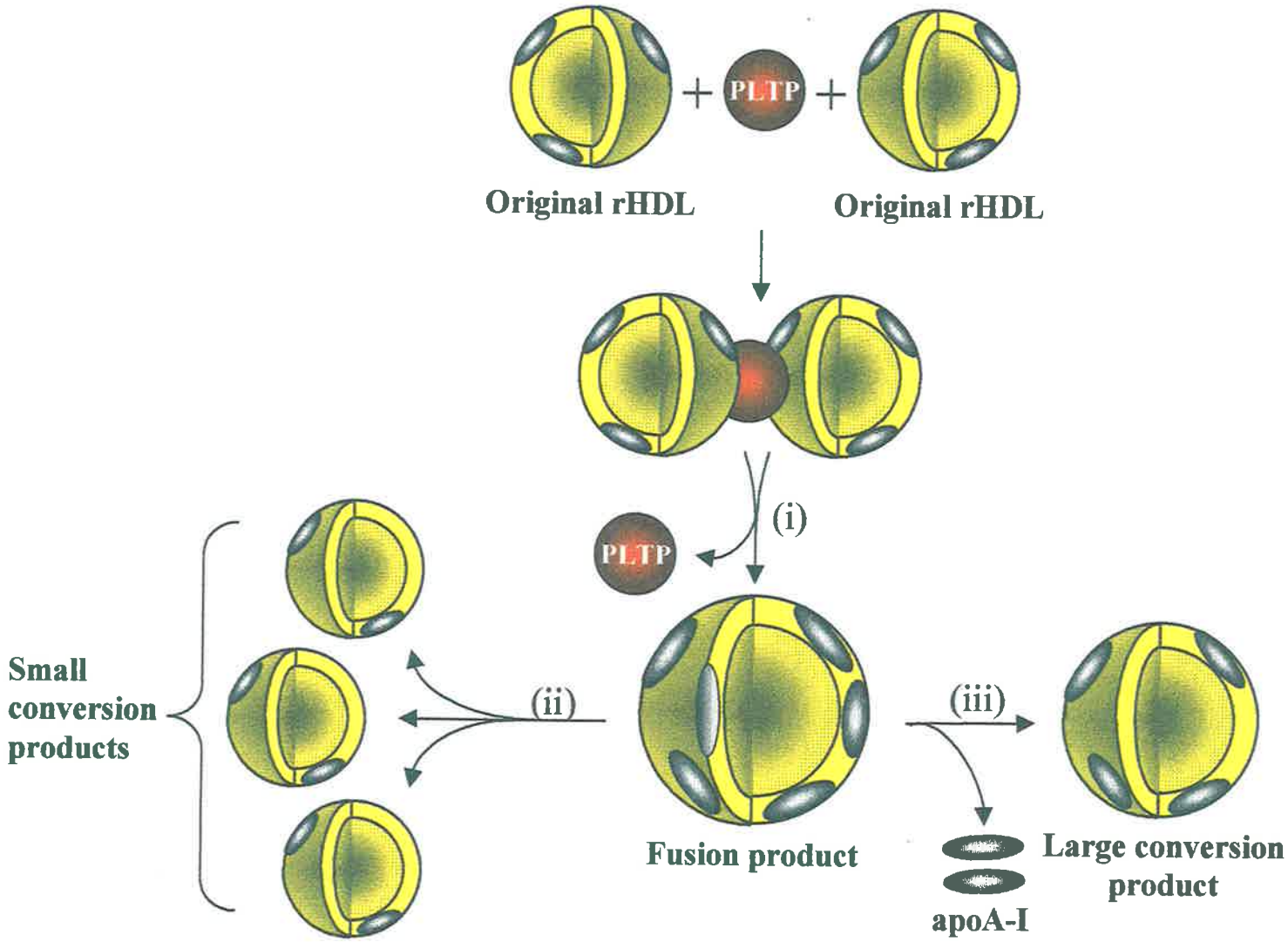
The apoA-I that dissociated from the CE-rHDL and TG-rHDL during the incubations with PLTP appeared as three bands (Figure 5.5). One of the bands is comparable in size to lipid-free apoA-I. The smallest band is possibly the 23 kDa fragment of apoA-I that is generated when either lipid-free or lipid-associated apoA-I are incubated with PLTP (Jauhiainen et al., 1999). The largest of the three bands may represent apoA-I complexed with small amounts of rHDL lipids. This is consistent with the reduced recovery of surface constituents relative to core lipids in the large and small conversion products (Table 5.1). It is possible that the apoA-I that is associated with small amounts of lipid may be comparable to the pre-β_1 migrating HDL in human plasma that have been identified as the initial acceptors of cellular cholesterol from peripheral tissues (Castro and Fielding, 1988; von Eckardstein et al., 1996).

The results in Table 5.1 and Figure 5.5 indicate that the mechanism of PLTP-mediated remodeling of rHDL involves the following events (Figure 5.6); (i) interaction of PLTP with two rHDL particles to give a large, unstable fusion product containing six molecules of apoA-I. The fusion product has two fates; (ii) it either rearranges into three small particles, each of which contains two molecules of apoA-I/particle, in a process that is not accompanied by the dissociation of apoA-I, or (iii) forms a more stable, large conversion product with four molecules of apoA-I with the dissociation of two molecules of apoA-I from the particle.

The possibility that the small conversion products are further remodeled into large conversion products was also considered. The data in Table 5.1 indicate that this pathway requires interactions between three small conversion products and the concomitant dissociation of two molecules of apoA-I. Although the results in Figure 5.5 indicate that formation of large conversion products by this pathway is feasible, it is likely that trimolecular collisions of this type are not energetically favourable. As such, this pathway is unlikely to be a major source of the large conversion products.

Figure 5.6 Proposed mechanism for the remodeling of rHDL by PLTP

(i) PLTP mediates the fusion of two rHDL particles, each of which contains three molecules of apoA-I, to give an unstable particle with six apoA-I molecules. The fusion product either (ii) rearranges into three small particles with two molecules of apoA-I/particle or (iii) is converted into a large particle with four molecules of apoA-I in a process that is accompanied by the dissociation of two molecules of apoA-I.


PLTP
Original rHDL
Original rHDL
PLTP
(i)
Small
conversion
products
(ii)
Fusion product
(iii)
Large conversion
product
apoA-I

CHAPTER 6

EFFECTS OF TRIGLYCERIDE ENRICHMENT ON THE STABILITY OF APOA-I IN SPHERICAL rHDL

6.1 INTRODUCTION

6.2 METHODS

Unfolding of apoA-I in CE-rHDL and TG-rHDL

6.3 RESULTS

6.4 DISCUSSION

6.1 INTRODUCTION

Cabana et al. (1996) have reported that the HDL in hypertriglyceridemic animals are enriched with triglycerides and that apoA-I dissociates from these particles. This is in agreement with what has been reported by Lewis et al. (1998) and Lamarche et al. (1999b). These investigators have found that hypertriglyceridemic states in humans leads to triglyceride enrichment of HDL and enhanced clearance of HDL apoA-I and cholesterol from the circulation. Furthermore, studies in hypertriglyceridemic patients have shown that their HDL are remodeled rapidly compared to the HDL in control subjects (Murakami et al., 1995). Hypertriglyceridemic subjects also have increased levels of small HDL.

Lamarche et al. (1999a) have reported that HDL apoA-I and cholesterol levels, as well as HDL particle numbers are also reduced in hypertriglyceridemic states. This may be due to any one, or a combination, of the following; (i) rapid clearance of the small HDL particles which are formed by the intravascular lipolysis of triglyceride-enriched HDL (Lewis et al., 1998), (ii) decreased binding of apoA-I to HDL (Cabana et al., 1996), and (iii) the lipolysis process of triglyceride-enriched HDL leading to a reduction in HDL particle numbers and enhanced dissociation and clearance of apoA-I (Lewis et al., 1998; Lamarche et al., 1999b).

The results in Chapter 5 have shown that triglyceride enrichment of rHDL enhances PLTP-mediated remodeling of rHDL into large and small particles and the dissociation of apoA-I. However, the reasons why the remodeling is faster in TG-rHDL than in CE-rHDL are unknown. In order to address this issue, the stability of apoA-I in CE-rHDL

and TG-rHDL was determined by incubating the samples with increasing concentrations of guanidine hydrochloride.

6.2 METHODS

Unfolding of apoA-I in CE-rHDL and TG-rHDL

The preparations and physical properties of the CE-rHDL and TG-rHDL used in these studies are described in Chapter 3.

The unfolding of apoA-I in CE-rHDL and TG-rHDL (12.2 and 32.4% TG in their core) was determined by incubating the samples with 0-8 M of guanidine hydrochloride (GdnHCl) at 25 °C for 0, 2, 5, 8, and 24 h (Pace, 1986; Rye et al., 1995). The rHDL (final apoA-I concentration 20 μg/mL) were added to aliquots of 50 mM Tris-HCl, pH 8.0 containing 0-8 M GdnHCl. The final volume of the incubation mixtures was 625 μL. Wavelengths of maximum fluorescence were determined from 300 to 380 nm emission scans using an excitation wavelength of 295 nm. The respective excitation and emission band passes were 10 and 5 nm. Initial readings (t = 0 h) were made at 25 °C, immediately after the rHDL had been added to the GdnHCl solutions. Measurements at t = 2, 5, 8, and 24 h were made after the samples had been incubated at 25 °C for the appropriate time. The details are described in Chapter 2.

The wavelengths of maximum fluorescence obtained when rHDL were mixed with varying concentrations (0-8 M) of GdnHCl and incubated at 25 °C for 5 h were used to calculate of concentration of GdnHCl required to achieve 50% unfolding of apoA-I ($[GdnHCl]_{1/2}$) as described in Chapter 2.

6.3 RESULTS

Unfolding of apoA-I in CE-rHDL and TG-rHDL (Figures 6.1-6.2, Table 6.1)

The unfolding of apoA-I in CE-rHDL and TG-rHDL is shown in Figure 6.1. The rHDL were mixed with 0-8 M GdnHCl for 0 (closed diamonds), 5 (open squares), and 24 h (closed triangles). The results for the CE-rHDL are shown in Panel A while Panels B and C represent TG-rHDL containing 12.2 and 32.4% TG, respectively. Table 6.1 shows the concentrations of GdnHCl required to achieve 50% unfolding of apoA-I. These values were either calculated directly from Figure 6.1, or from a plot of the concentration of GdnHCl versus the free energy of unfolding of apoA-I as described in Chapter 2. The results show that the concentration of GdnHCl required to unfold apoA-I decreased with increasing triglyceride content in the rHDL core lipids.

Figure 6.2 shows the kinetics of the unfolding of apoA-I in CE-rHDL (closed diamonds) and TG-rHDL containing 12.2% (open squares) and 32.4% TG (closed triangles) following incubation with 4.0 M GdnHCl at 25 °C for 0-24 h. The results show that the rate at which the apoA-I unfolds increases as the triglyceride content of rHDL increases.

6.4 DISCUSSION

When the results from Figures 6.1 and 6.2 and Table 6.1 are considered together, it follows that triglyceride enrichment of rHDL decreases the stability of apoA-I in rHDL. This destabilisation of apoA-I may be caused by triglyceride molecules partitioning from the core into particle surface and preventing apoA-I α helices from intercalating between the rHDL phospholipid acyl chains (Frank et al., 1997). Given that the mechanism of the remodeling of rHDL by PLTP that is shown in Figure 5.6 involves the dissociation of apoA-I, this observation could explain why the PLTP-mediated remodeling of rHDL is

Figure 6.1 Unfolding of apoA-I in CE-rHDL and TG-rHDL

Spherical CE-rHDL were mixed with Intralipid and either incubated at 37 °C for 20 min in the absence of CETP (Panel A) or for 2 (Panel B) and 20 min (Panel C) in the presence of CETP as described in the legend to Table 6.1. When the incubations were complete, the rHDL were isolated by ultracentrifugation then incubated with 0-8 M GdnHCl at 25 °C for 0, 2, 5, 8, and 24 h. The data for 0 (◆), 5 (□), and 24 (▲) h are shown. Values represent the mean of at least three determinations. Experimental errors for the wavelength of maximum fluorescence are ± 1.0 nm.

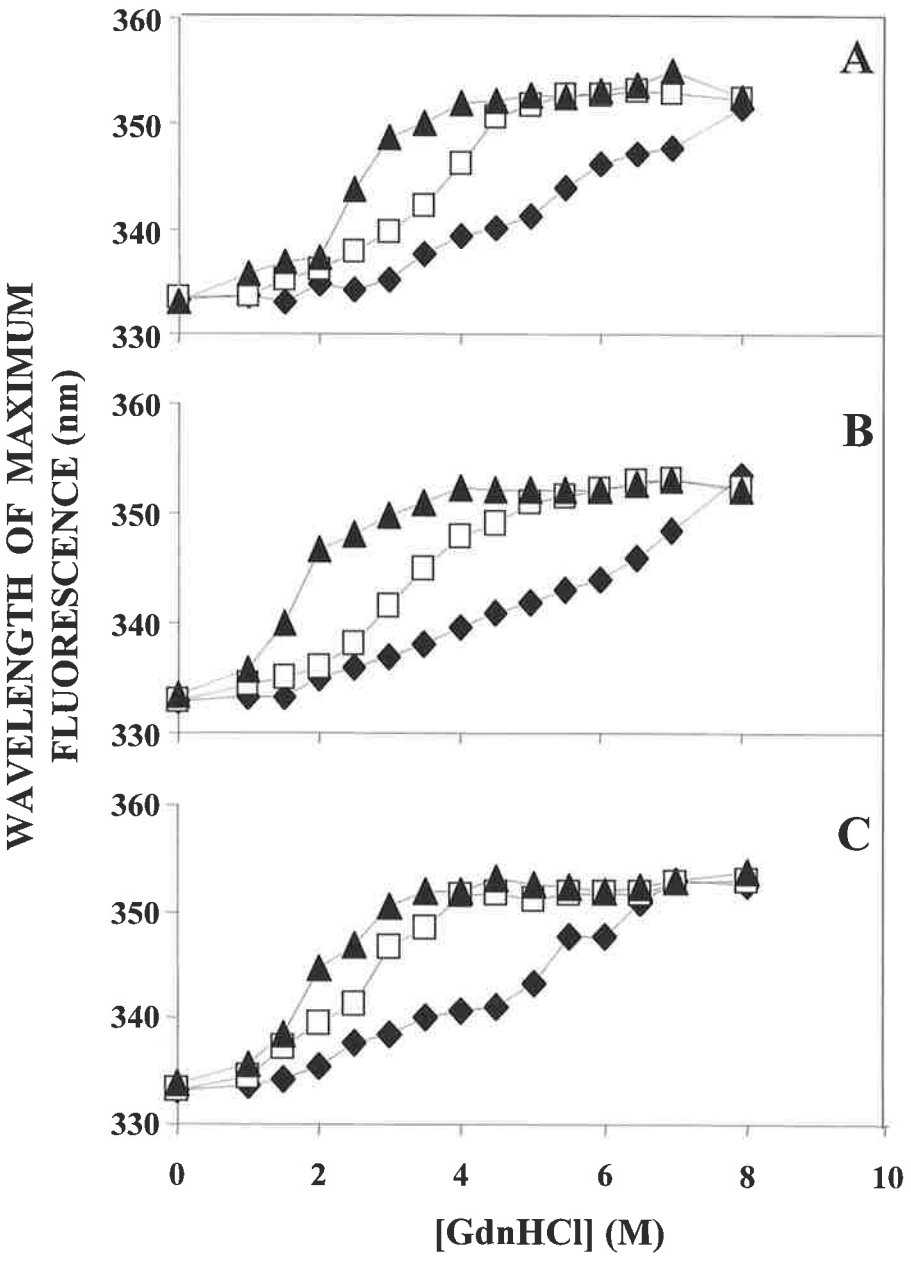

WAVELENGTH OF MAXIMUM FLUORESCENCE (nm)
360
350
340
330
A
B
C
0
2
4
6
8
10
[GdnHCl] (M)

Table 6.1 Unfolding of apoA-I in CE-rHDL and TG-rHDL

Incubation Conditions	Stoichiometry PL/UC/CE/TG/apoA-I[a]	$\frac{\text{mole TG}}{\text{mole (TG+CE)}}$	Stokes' Diameter[b]	$[GdnHCL]_{1/2}$[c]	$[GdnHCl]_{1/2}$[d]
	(mol/mol)	*(%)*	*(nm)*	*(M)*	*(M)*
Intralipid-CETP, 37 °C, 20 min	88.5/5.1/61.8/1.8/3.0	2.8	9.2	3.5±0.13	3.2±0.02
Intralipid+CETP, 37 °C, 2 min	91.8/6.0/58.5/8.1/3.0	12.2	9.3	3.2±0.08	3.0±0.04
Intralipid+CETP, 37 °C, 20 min	103.2/6.7/43.8/21.0/3.0	32.4	9.5	2.5±0.09	2.3±0.03

Spherical CE-rHDL (final CE concentration 0.1 μmol/mL) were mixed with Intralipid (final TG concentration 4.0 μmol/mL) and either incubated at 37 °C for 20 min in the absence of CETP or incubated at 37 °C for 2 and 20 min in the presence of CETP (final activity 2.6 units/mL). The final volume of the incubation mixtures was 5.0 mL. When the incubations were complete, the rHDL were isolated by ultracentrifugation. The stoichiometries represent the mean of triplicate determinations which varied by less than 10%.

[a]PL, phospholipid; UC, unesterfied cholesterol; CE, cholesteryl ester; TG, triglyceride.

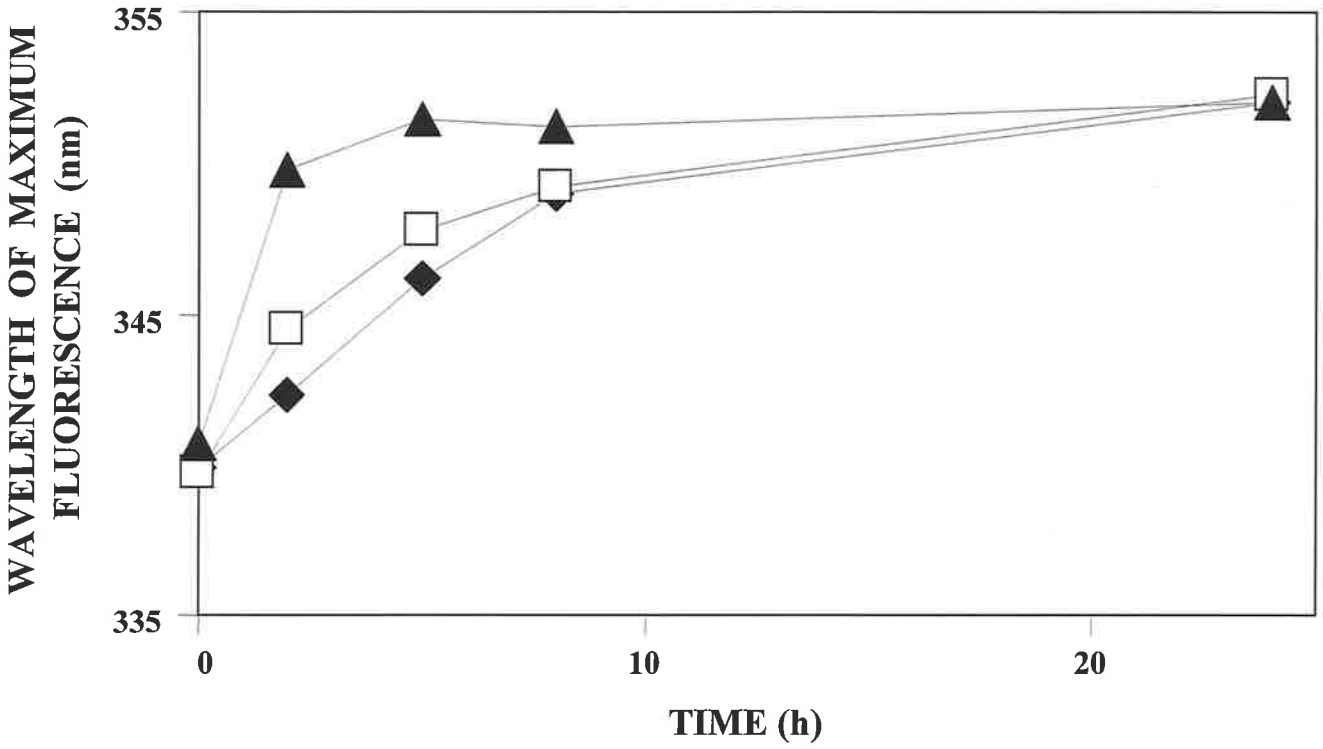
[b]Determined by non-denaturing 3/40% polyacrylamide gradient gel electrophoresis.

[c]Concentration of GdnHCl required to achieve 50% unfolding of apoA-I. Determined directly from Figure 6.1.

[d]Concentration of GdnHCl required to achieve 50% unfolding of apoA-I. Calculated as described in Chapter 2.

Figure 6.2 The kinetics of unfolding of apoA-I in CE-rHDL and TG-rHDL

Spherical CE-rHDL were mixed with Intralipid and either incubated at 37 °C for 20 min in the absence of CETP (◆) or for 2 (□) and 20 (▲) min in the presence of CETP as described in the legend to Table 6.1. When the incubations were complete, the rHDL were isolated by ultracentrifugation then incubated with 4.0 M GdnHCl for 0-24 h. Values for the wavelength of maximum fluorescence represent the mean of triplicate determinations. Experimental errors are ±1.0 nm.


WAVELENGTH OF MAXIMUM FLUORESCENCE (nm)
355
345
335
0
10
20
TIME (h)

enhanced by triglyceride enrichment. The exclusion of apoA-I α-helices from the surface monolayer of TG-rHDL could enhance their ability to dissociate from the particles.

The result is consistent with what has been reported by Sparks et al. (1995b), who found that the apoA-I in triglyceride containing HDL is less stable than cholesteryl ester containing HDL. This is also in agreement with the studies in hypertriglyceridemic subjects which show that triglyceride enrichment of HDL enhances the dissociation of apoA-I from the particles and the clearance of apoA-I from the circulation (Lewis et al., 1998; Lamarche et al., 1999a, 1999b).

CHAPTER 7

PLTP-MEDIATED PHOSPHOLIPID TRANSFERS AND REMODELING OF SPHERICAL rHDL OF VARYING PHOSPHOLIPID COMPOSITION

7.1 INTRODUCTION

7.2 METHODS

Preparation of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

Physical properties of POPC-rHDL, PLPC-rHDL PAPC-rHDL and PDPC-rHDL

Preparation of [^{14}C]-POPC small unilamellar vesicles

PLTP-mediated phospholipid transfers between [^{14}C]-POPC small unilamellar vesicles and unlabelled POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

PLTP-mediated remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

Time course of the remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL by PLTP

7.3 RESULTS

7.4 DISCUSSION

7.1 INTRODUCTION

Phospholipids consist of a glycerol backbone with fatty acyl chains at sn-1 and sn-2 positions and a head group at the sn-3 position. The phospholipid acyl chains in HDL vary widely in length and degree of unsaturation. The sn-1 acyl chain is usually saturated, whereas the sn-2 acyl chain is unsaturated. While most HDL phospholipids have a choline head group and are termed phosphatidylcholine (PC) (Subbaiah and Monshizadegan, 1988; Subbaiah and Pritchard, 1989), other phospholipids such as, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin are also present in these lipoproteins (Winkler and Marsh, 1989; Kawano et al., 1993).

HDL phospholipids influence several of the potentially anti-atherogenic functions of HDL, such as the selective efflux of cellular cholesterol (Sola et al., 1993; Davidson et al., 1995a), the activation of LCAT and the stability of apoA-I in HDL (Parks and Gebre, 1997) and the ability of HDL to inhibit the cytokine-induced expression of VCAM-1 in human umbilical vein endothelial cells (Baker et al., 2000).

Several studies have shown that the ability of HDL to accept cellular cholesterol is related to the amount of phospholipids in the particle (Agnani and Marcel, 1993; Davidson et al., 1994b). There is also evidence that the types of phospholipids present in HDL may be important for cellular cholesterol efflux. Sola et al. (1993) have demonstrated that diet-induced changes in the phospholipid acyl chain composition of HDL_3 influences their ability to remove cholesterol from cultured fibroblasts. It has also been reported that the ability of HDL_3 to accept cellular cholesterol decreases as the their phospholipid acyl chain saturation increases. This effect has been attributed to a reduction in the fluidity of the HDL_3 surface (Sola et al., 1990). Davidson et al. (1995a) have also

reported that the ability of discoidal rHDL to accept cellular cholesterol increases as phospholipid acyl chain length and unsaturation increases. These results suggest that rHDL containing highly fluid surfaces sequester cholesterol molecules that have diffused from the cell membrane at a significantly faster rate than those containing a more organised lipid surface with restricted phospholipid acyl chain mobility.

Phospholipid acyl chain length also influences the ability of rHDL to act as a substrate for LCAT (Jonas et al., 1987; Parks and Gebre, 1997). The catalytic efficiency of LCAT, the activation energy of the LCAT reaction, and the stability of apoA-I decreases as sn-2 acyl chain length increases (Jonas et al., 1987; Parks and Gebre, 1997). It is well documented that the α-helical content and stability of apoA-I increases following interaction with phospholipids (Zorich et al., 1987; Sparks et al., 1992). Huggins et al. (1998) have used discoidal rHDL to show that these stabilising interactions decrease as phospholipid acyl chain length and unsaturation increases. Parks and Thuren (1993) have shown that the molecular surface areas of phospholipids increase as phospholipid acyl chain length and unsaturation increases. When taken together these results suggest that the binding of apoA-I to HDL decreases as phospholipid acyl chain length and unsaturation increases. One of the physiological consequences of this reduced binding may be the decreased conversion of nascent, discoidal HDL to mature spherical HDL by LCAT. This may account for the lower HDL and apoA-I concentrations and the smaller HDL that are found in the plasma of animals fed a diet rich in polyunsaturated fatty acids (Wolfe et al., 1993; Thornburg et al., 1995).

The phospholipid composition of HDL also regulates their ability to inhibit the cytokine-induced expression of VCAM-1 in human umbilical vein endothelial cells. rHDL

containing PLPC or PAPC have a much greater inhibitory activity than those containing POPC (Baker et al., 2000).

At present, little is known about the influence of HDL phospholipid composition on PLTP-mediated phospholipid transfers and remodeling of HDL. Huuskonen et al. (1996) have found that as phospholipid acyl chain length increased from 6 to 14 carbons, PLTP-mediated phospholipid transfers from the vesicles to HDL_3 decreased. No difference in phospholipid transfer rate was observed for exchanging the sn-1/sn-2 positions. Rao et al. (1997) have also shown that unsaturation of one phospholipid acyl chain greatly increases transfer rate, whereas increasing acyl chain length and exchanging sn-1/sn-2 position have only small effect.

The effect of phospholipid composition on the remodeling of HDL by PLTP has not been studied. This is because it is very difficult to obtain subpopulations of HDL that vary systematically in their phospholipid composition, but are comparable in all other aspects.

In order to examine the effects of phospholipid composition of rHDL on PLTP-mediated phospholipid transfers and remodeling of rHDL, a technique for preparing monodisperse populations of spherical rHDL which contain a single type of phospholipid has been developed and used in this study.

7.2 METHODS

Preparation of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

Spherical rHDL containing apoA-I, unesterified cholesterol and either γ-palmitoyl-β-oleoyl-L-α-phosphatidylcholine (POPC), γ-palmitoyl-β-linoleoyl-L-α-phosphatidyl-

choline (PLPC), γ-palmitoyl-β-arachidonoyl-L-α-phosphatidylcholine (PAPC) or γ-palmitoyl-β-docosahexa-enoyl-L-α-phosphatidylcholine (PDPC) were prepared as described in Chapter 2. The rHDL were isolated by ultracentrifugation. The size of the particles was determined by non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. A Cobas Fara Centrifugal Analyzer was used to determine the composition of the rHDL as described in Chapter 2.

Preparation of [^{14}C]-POPC small unilamellar vesicles

[^{14}C]-POPC small unilamellar vesicles were prepared by adding to a clean, dry test tube 39 μL of POPC (100 mg/mL) in chloroform/methanol 2:1 (v/v), 20 μL of 10 μCi of [^{14}C]-POPC (Amersham Pharmacia Biotech), and 20 μL of 1mM of BHT in ethanol as described in Chapter 2 (Damen et al., 1982).

PLTP-mediated phospholipid transfers between [^{14}C]-POPC small unilamellar vesicles and unlabelled POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

PLTP-mediated phospholipid transfers were carried out as described by Damen et al. (1982). [^{14}C]-POPC small unilamellar vesicles (final PL concentration 0.375 μmol/mL) were mixed with either POPC-rHDL, PLPC-rHDL, PAPC-rHDL or PDPC-rHDL (final apoA-I concentration 0.625 mg/mL), and PLTP (final activity 234 nmol PL transferred/mL PLTP/h). TBS was added to make the total volume 100 μL and the incubation was conducted at 37 °C for 1, 3, 5, 10, 20, and 60 min. When the incubation was complete, the vesicles were precipitated with 600 μL of a $MnCl_2$/heparin solution. The [^{14}C]-POPC content of the rHDL in the supernatant was determined by liquid scintillation counting as described in Chapter 2. Transfer rates were calculated from the

slope of the initial, linear section of plots as the % phospholipids transferred between the rHDL and the vesicles as a function of time.

PLTP-mediated remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (final activity 4.0 μmol PL transferred/mL PLTP/h), respectively. The final volume of the incubation mixtures was 270 μL. When the incubations were complete, the rHDL were isolated by ultracentrifugation at a density of 1.25 g/mL. A single spin was conducted at 100,000 rpm for 16 h using a TLA 100.1 rotor maintained at 4 °C in a Beckman TL-100 Tabletop ultracentrifuge. Non-denaturing 3-40% polyacrylamide gradient gel elctrophoresis was used to quantitate rHDL size.

Time course of the remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL by PLTP

POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (final activity 5.9 μmol PL transferred/mL PLTP/h). The final volume of the incubation mixture was 270 μL. Aliquots of the unprocessed incubation mixtures (0.25 μg of apoA-I) were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis, transferred electrophoretically to nitrocellulose membrane and immunoblotted with

polyclonal sheep antihuman apoA-I antibody. The transferred bands were detected by ECL. The details are described in Chapter 2.

7.3 RESULTS

Physical properties of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (Figure 7.1, Table 7.1)

The size of the rHDL preparations was determined by non-denaturing 3/40% polyacrylamide gradient gel electrophoresis. Figure 7.1 shows scans of the Coomassie Blue-stained gels. Particle diameters were calculated by reference to known high molecular weight standards. The POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL were stable and monodisperse, with diameters of 9.5, 9.9, 9.1, and 9.8 nm, respectively. The stoichiometries of the rHDL preparations is shown in Table 7.1. The smaller size of the PAPC-rHDL compared to the other HDL preparations is reflected in their lower PL/apoA-I molar ratio.

PLTP-mediated phospholipid transfers between [^{14}C]-POPC small unilamellar vesicles and unlabelled POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (Figure 7.2)

When the rHDL preparations were incubated with [^{14}C]-POPC small unilamellar vesicles and PLTP, the rate of [^{14}C]-POPC transfer from the vesicles to PAPC-rHDL (closed diamonds) was enhanced relative to the transfer to POPC-rHDL (open circles), PLPC-rHDL (closed triangles), and PDPC-rHDL (open squares) rHDL (Figure 7.2). The initial rate of transfer from the vesicles to PAPC-rHDL was 7.2 μmol POPC transferred/mL PLTP/h compared to 1.7, 0.9, and 1.2 μmol POPC transferred/mL PLTP/h for POPC-rHDL, PLPC-rHDL, and PDPC-rHDL, respectively (See inset).

Figure 7.1 Size distribution of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis and stained with Coomassie Blue as described in Chapter 2. Laser densitometric scans of the gels are shown.

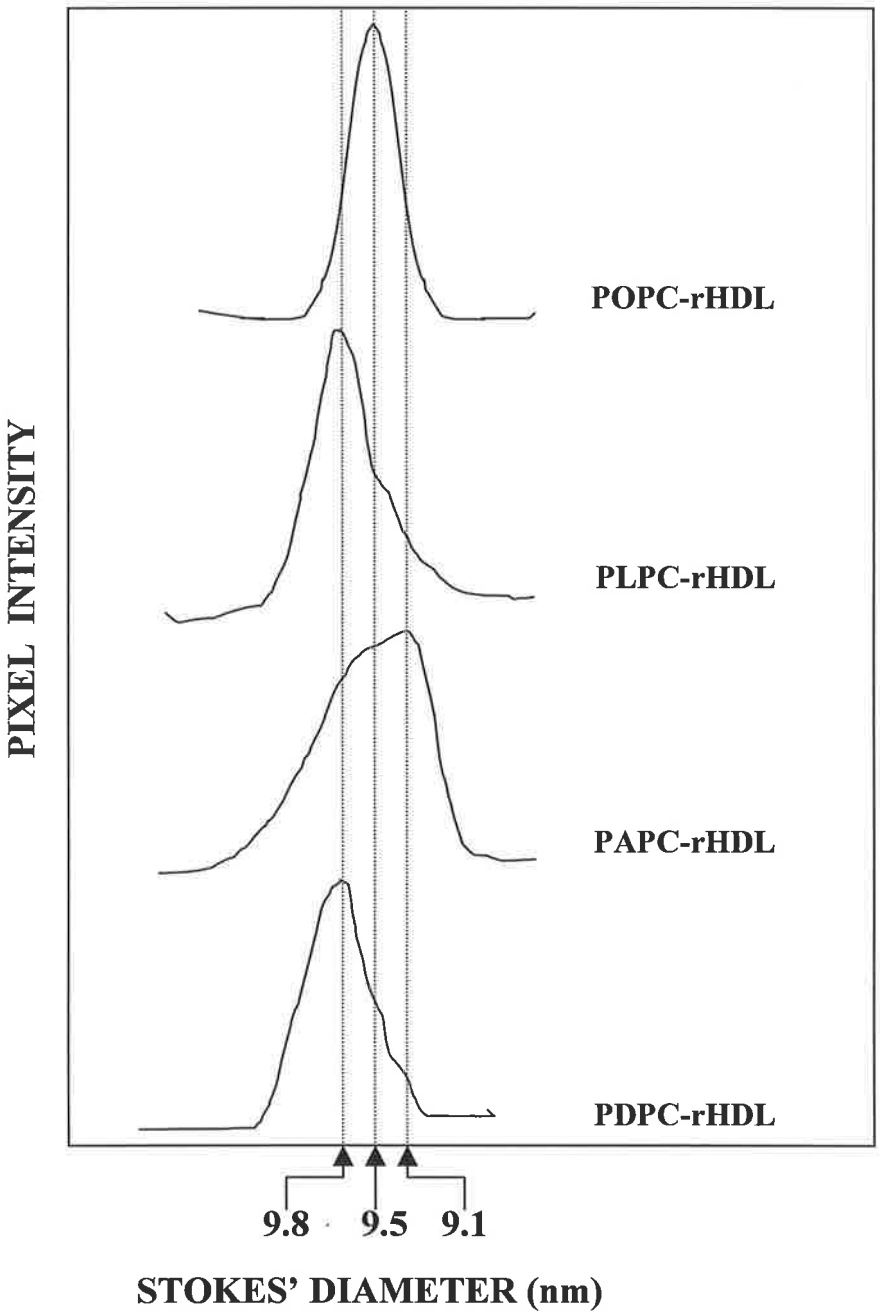

PIXEL INTENSITY
POPC-rHDL
PLPC-rHDL
PAPC-rHDL
PDPC-rHDL
9.8
9.5
9.1
STOKES' DIAMETER (nm)

Table 7.1 Physical properties of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

rHDL	Acyl chain composition sn-1-sn-2	Stoichiometry PL/UC/CE/apoA-I[a]	Stokes' Diameter[b]
		(mol/mol)	*(nm)*
POPC-rHDL	16:0-18:1	61.3/2.0/31.3/1.0	9.5
PLPC-rHDL	16:0-18:2	60.8/1.8/32.7/1.0	9.9
PAPC-rHDL	16:0-20:4	41.5/1.0/44.6/1.0	9.1
PDPC-rHDL	16:0-22:6	60.8/2.6/55.2/1.0	9.8

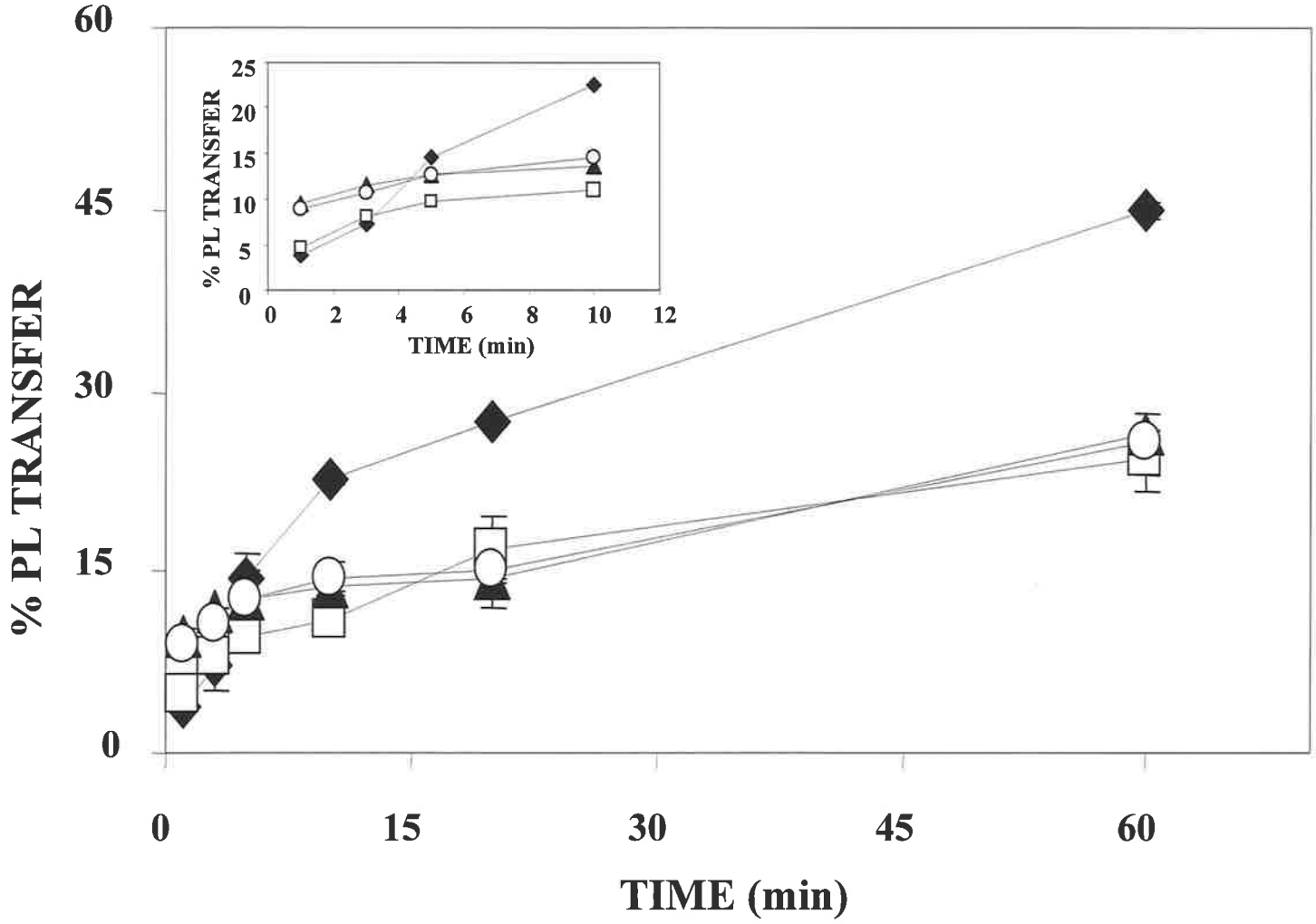
POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL were prepared as described in Chapter 2. Stoichiometries were determined as the mean of triplicate determinations which varied by less than 10%.

[a]PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester.

[b]Determined by non-denaturing 3-40% polyacrylamide gradient gel electrophoresis.

Figure 7.2 PLTP-mediated phospholipid transfers between [^{14}C]-POPC small unilamellar vesicles and unlabelled POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

[^{14}C]-POPC small unilamellar vesicles (final PL concentration 0.375 μmol/mL) were mixed with POPC-rHDL (O), PLPC-rHDL (▲), PAPC-rHDL (◆) and PDPC-rHDL (□) (final apoA-I concentration 0.625 mg/mL), and PLTP (final activity 234 nmol PL transferred/mL PLTP/h). The final volume of the incubation mixtures was 100 μL. The mixtures were incubated at 37 °C for 0-60 min. After incubation, the vesicles were precipitated with a $MnCl_2$/heparin solution. The [^{14}C]-POPC content of the rHDL remaining in the supernatant was determined by liquid scintillation counting. Data points represent the mean±sd of triplicate determinations.


% PL TRANSFER
60
45
30
15
0
0
15
30
45
60
TIME (min)
% PL TRANSFER
25
20
15
10
5
0
0
2
4
6
8
10
12
TIME (min)

PLTP-mediated remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (Figure 7.3, Table 7.2)

Figure 7.3 shows scans of the Coomassie Blue-stained gels of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL that were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP (Profiles A and B, respectively) or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (Profiles C, D, E, F, and G, respectively). The size of the rHDL was not affected by incubation for 24 h in the absence of PLTP. When the rHDL were incubated with PLTP, the rHDL were converted into large (10.3-11.5 nm) and small (7.7-7.8 nm) particles.

The proportion of the original rHDL that was converted into large and small particles varied according to the phospholipid composition of the particles (Table 7.2). The remodeling of rHDL into large and small particles by PLTP increased as the length and unsaturation of the phospholipid sn-2 acyl chains increased. In the case of the POPC-rHDL, 32.8% of the particles were converted into large particles, 11.1 nm in diameter, 26.2% were 7.8 nm in diameter and 41.0% were unchanged. In the case of PLPC-rHDL, 33.9% of the particles were unchanged, 38.7% were converted into large particles (diameter 11.4 nm), and 27.4% were converted into small particles, 7.8 nm in diameter. While 23.8% of PAPC-rHDL were unchanged, the diameter of 59.5% of the particles increased to 10.3 nm, while the diameter of 16.7% of the particles decreased to 7.7 nm. In the case of PDPC-rHDL, 16.9% of the particles were unchanged, 44.1% were converted into large particles (11.5 nm), and 39.0% were converted into small particles (7.8 nm).

Figure 7.3 PLTP-mediated remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL

POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP (Profiles A and B, respectively) or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (final activity 4.0 μmol PL transferred/mL PLTP/h) (Profiles C, D, E, F, and G, respectively). When the incubations were complete, the rHDL were isolated by ultracentrifugation. The rHDL were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis and stained with Coomassie Blue as described in Chapter 2. Laser densitometric scans of the gels are shown.

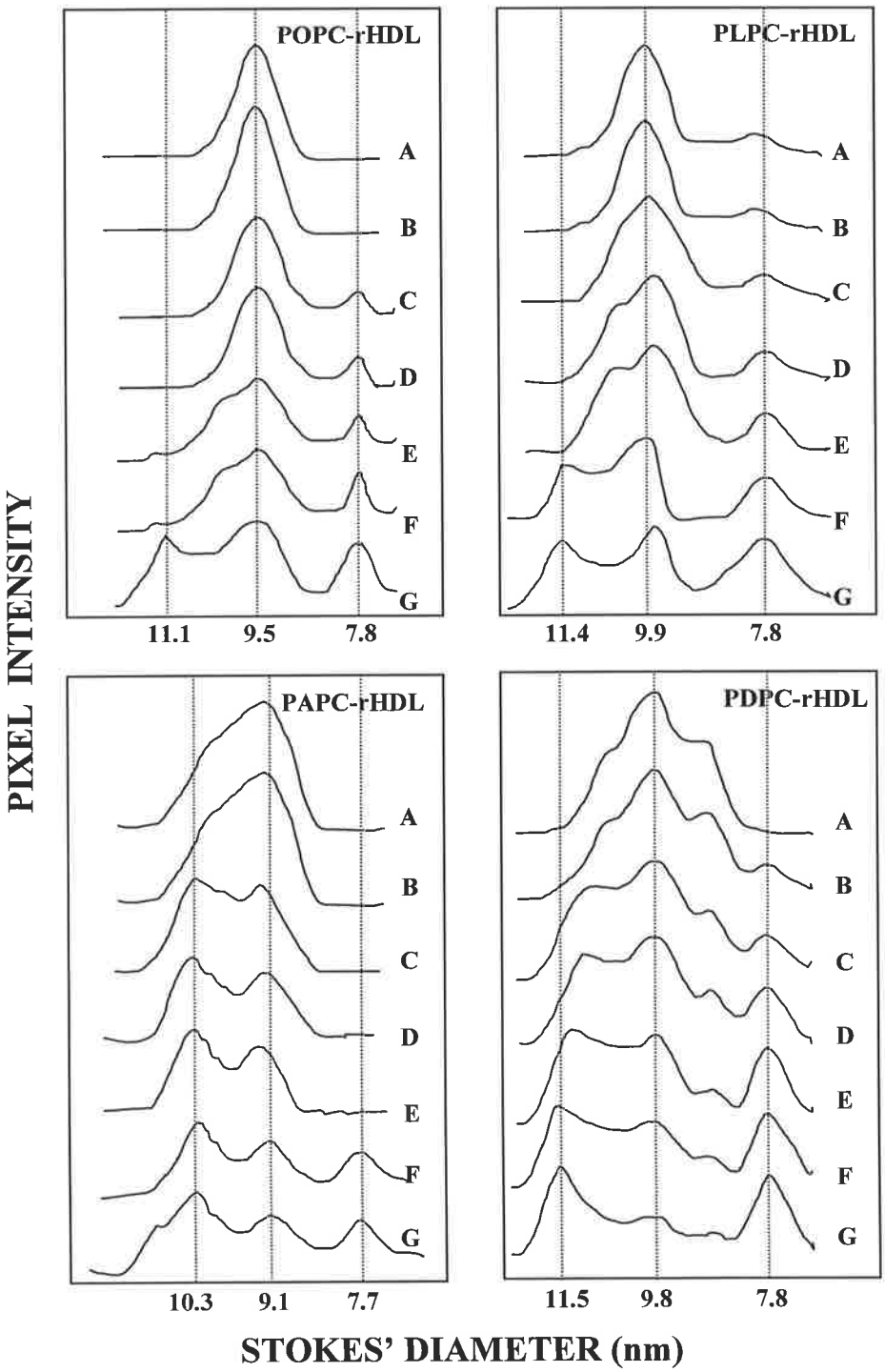

POPC-rHDL
A
B
C
D
E
F
G
11.1
9.5
7.8
PLPC-rHDL
A
B
C
D
E
F
G
11.4
9.9
7.8
PAPC-rHDL
A
B
C
D
E
F
G
10.3
9.1
7.7
PDPC-rHDL
A
B
C
D
E
F
G
11.5
9.8
7.8
PIXEL INTENSITY
STOKES' DIAMETER (nm)

Table 7.2 The amounts of large, original, and small particles of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL after incubation at 37 °C for 24 h with PLTP

rHDL	Large particles	Original particles	Small particles
	(%)	*(%)*	*(%)*
POPC-rHDL	32.8	41.0	26.2
PLPC-rHDL	38.7	33.9	27.4
PAPC-rHDL	59.5	23.8	16.7
PDPC-rHDL	44.1	16.9	39.0

rHDL (final apoA-I concentration 92.5 μg/mL) were incubated at 37 °C for 1-24 h with PLTP (final activity 4.0 μmol PL transferred/mL PLTP/h). The final volume of the incubation mixtures was 270 μL. The ultracentrifugally isolated rHDL were subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. The Coomassie blue-stained gels were scanned with a Sharp JX-610 scanner. The values in the Table 7.2 represent the relative concentrations of the original rHDL as well as the large and small conversion products that were present after 24 h of incubation with PLTP. This was determined by multiplying the height of the peak by the width at half height.

Time course of the remodeling of POPC-rHDL, PLCP-rHDL, PAPC-rHDL and PDPC-rHDL by PLTP (Figure 7.4)

Figure 7.4 shows the time dependence of the remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL by PLTP. Tracks A and B, show rHDL that either maintained at 4 °C or incubated at 37 °C for 24 h in the absence of PLTP. Tracks C, D, E, F, and G represent rHDL that were incubated at 37 °C in the presence of PLTP for 1, 3, 6, 12, and 24 h. Lipid-free apoA-I is shown in Track H.

The results show that the small conversion products were formed before the large conversion products, and the appearance of the large conversion products coincided with the dissociation of apoA-I. This sequence of events is identical to what was described in Chapter 5. As shown in Figure 7.4, dissociated apoA-I was apparent at 3 h in the incubation that contained PAPC-rHDL and at 12 h in the PDPC-rHDL incubation. Dissociation of apoA-I from the POPC-rHDL and PLPC-rHDL was, by contrast, minimal.

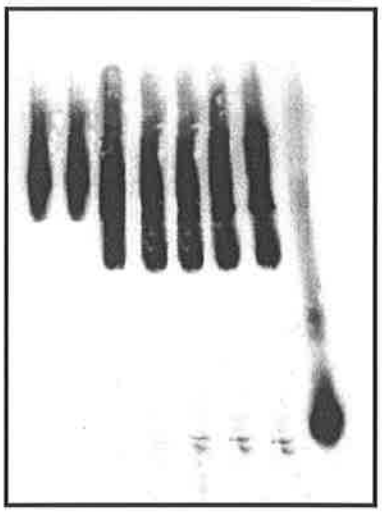
7.4 DISCUSSION

There is a considerable amount of evidence showing that phospholipid acyl chain length and unsaturation directly affects the physical state of phospholipids and influences the structure and function of discoidal rHDL. Structural studies of discoidal rHDL have shown that increases in the acyl chain length and unsaturation of rHDL phosphatidylcholine increases phosphatidylcholine packing defects and surface hydration (Jonas et al., 1987; Davidson et al., 1995a; Huggins et al., 1998).

Figure 7.4 Time course of the remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL by PLTP

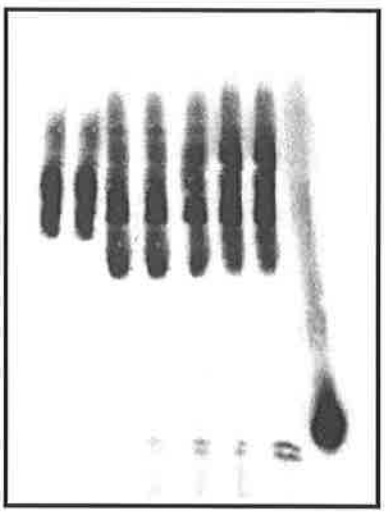
POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL (final apoA-I concentration 92.5 μg/mL) were either maintained at 4 °C, incubated at 37 °C for 24 h in the absence of PLTP (Tracks A and B, respectively) or incubated at 37 °C for 1, 3, 6, 12, and 24 h in the presence of PLTP (final activity 5.9 μmol PL transferred/mL PLTP/h) (Tracks C, D, E, F, and G, respectively). The final volume of the incubation mixtures was 270 μL. An aliquot of each incubation mixture (0.25 μg apoA-I) was subjected to non-denaturing 3-40% polyacrylamide gradient gel electrophoresis. Lipid-free apoA-I was also applied to the gels (Track H). The samples were transferred to nitrocellulose membranes, immunoblotted with sheep antihuman apoA-I antiserum and detected by ECL.

POPC-rHDL



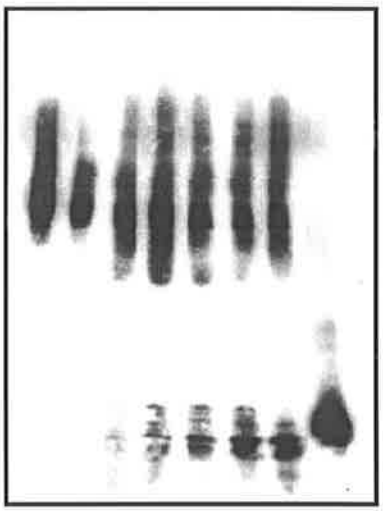
A B C D E F G H

PLPC-rHDL



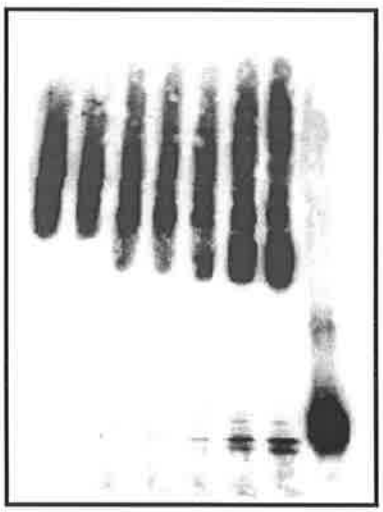
A B C D E F G H

PAPC-rHDL



A B C D E F G H

PDPC-rHDL



A B C D E F G H

The present study shows that phospholipid acyl chain length and unsaturation also affects the structure and properties of spherical rHDL. In the first study described in this chapter, transfers of phospholipids between [^{14}C]-POPC small unilamellar vesicles and either POPC-rHDL, PLPC-rHDL, PAPC-rHDL or PDPC-rHDL were examined. These results showed that an increase of one double bond in the sn-2 phospholipid acyl chain from 18:1 (POPC-rHDL) to 18:2 (PLPC-rHDL) does not affect the rate of [^{14}C]-POPC transfer from the vesicles to the rHDL. However, when the length of the sn-2 phospholipid acyl chain is increased from 18 to 20 carbons and the number of double bonds is increased from 2 to 4 (PAPC-rHDL), the transfer rate increases significantly. This may be due to increased phospholipid packing decfects and surface hydration in PAPC-rHDL (Jonas et al., 1987; Davidson et al., 1995a; Huggins et al., 1998). These structural changes may accelerate PLTP-mediated phospholipid transfer from the vesicles to accommodate on the surface of PAPC-rHDL. However, this was not the case for PDPC-rHDL where the phospholipid transfer rate was comparable to that of POPC-rHDL and PLPC-rHDL. The reasons for this observation are unclear.

The results in Figures 7.3 and 7.4 and Table 7.2 show that the phospholipid composition of rHDL has a profound effect on their PLTP-mediated remodeling into large and small particles and the dissociation of apoA-I. In general, the results indicate that remodeling is enhanced as the length and unsaturation of the rHDL phospholipid sn-2 acyl chains increase. This may be due to the larger molecular surface areas of long chain, polyunsaturated phospholipids decreasing the binding, and hence the stability, of apoA-I.

The processes involved in the remodeling of POPC-rHDL, PLPC-rHDL, PAPC-rHDL and PDPC-rHDL by PLTP are identical to what was observed for the CE-rHDL and TG-

rHDL in Chapter 5, with small particles being formed before large particles and the appearance of the large particles being accompanied by the dissociation of apoA-I.

The apoA-I that dissociated from the PAPC-rHDL and PDPC-rHDL during the incubations with PLTP appeared as three bands in Figure 7.4. This also is in agreement with what was found in Chapter 5. Those bands correspond to the 23 kDa fragment of apoA-I, lipid-free apoA-I, and apoA-I complexed with small amounts of rHDL lipids. It is likely that this apoA-I complexed with small amounts of rHDL lipids may be comparable to pre-β migrating HDL (Castro and Fielding, 1988; von Eckardstein et al., 1996).

When taken together these results show that the phospholipid composition of rHDL is of considerable importance for the PLTP-mediated remodeling of rHDL and the generation of pre-β migrating HDL, the initial cellular cholesterol acceptors in reverse cholesterol transport (RCT).

In conclusion, the results of these experiments show that, while the phospholipid composition of rHDL has no effect on the mechanism by which PLTP remodels the particles, it does have a major influence on the rate at which the processes occur particularly the formation of pre-β migrating HDL. Given that the phospholipid composition of HDL varies according to dietary fat intake (Hodge et al., 1993; Sola et al., 1993), these findings suggest that diet may be directly involved in the regulation of RCT and that it may be possible to regulate the remodeling of HDL by PLTP in ways that upregulate RCT.

CHAPTER 8

CONCLUDING COMMENTS

An inverse relationship between HDL levels and the development of atherosclerosis has been observed in many epidemiological studies (Miller and Miller, 1975; Castelli et al., 1986; Genest et al., 1999; Stein and Stein, 1999). However, the exact mechanisms behind the atheroprotective role of HDL are still not fully understood. HDL may exert protective effects by; stimulating reverse cholesterol transport (RCT) (Glomset, 1968; Fielding and Fielding, 1995), preventing the oxidation of LDL (Watson et al., 1995; Banka, 1996), and inhibiting endothelial adhesion molecule expression and inflammatory responses to endotoxins (Cockerill et al., 1995; van Lenten et al., 1995).

During recent years, many studies have focused on the properties and functions of PLTP as its importance in the regulation of HDL metabolism. The transfer of phospholipids from triglyceride-rich lipoproteins to HDL during lipolysis by LPL is facilitated by PLTP. These transfers play a major role in regulating plasma HDL levels (Tall et al., 1985; Jiang, et al., 1999). PLTP also remodels HDL into large and small particles in a process involving particle fusion and the dissociation of lipid-poor or lipid-free apoA-I (Jauhiainen et al., 1993; Tu et al., 1993; Lusa et al., 1996). The studies in this thesis describe the mechanism of this process. The remodeling of HDL by PLTP is of considerable physiological significance because it generates pre-β migrating HDL (Castro and Fielding, 1988) that may act as acceptors of cellular cholesterol and phospholipids in the first step of RCT.

Recently, Rye et al. (1998) have shown that PLTP-mediated remodeling of HDL is markedly enhanced when the HDL are enriched with triglyceride. The studies described herein also explain why remodeling is enhanced in these particles.

The results in Chapter 4 show that PLTP-mediated phospholipid transfers from small unilamellar POPC vesicles to rHDL are enhanced by triglyceride enrichment of rHDL. While the evidence is not direct, these results suggest that enhanced phospholipid transfers from triglyceride-enriched rHDL may be due triglyceride partitioning from the core into the rHDL surface (Hamilton and Small, 1981; Miller and Small, 1983) and generating packing defects of surface phospholipid acyl chains. These changes can enhance the ability of the triglyceride-enriched rHDL to accommodate additional phospholipids from the vesicles.

The experiments in Chapter 5 elucidate the mechanism of the remodeling of rHDL by PLTP. The results show unequivocally that PLTP is a fusogenic protein. It mediates rHDL fusion and subsequent rearrangement of the fusion product by two independent processes into large and small particles. Evidence that the appearance of the large particles is accompanied by the dissociation of apoA-I is also presented. Some of the dissociated apoA-I is complexed with small amounts of rHDL lipids and may be comparable to pre-β migrating HDL (Castro and Fielding, 1988; von Eckardstein et al., 1996). Thus, this study supports the notion that PLTP plays a potentially anti-atherogenic role in RCT by generating pre-β migrating HDL which are available for participation in the first step of RCT.

The results in Chapter 5 also show that triglyceride enrichment does not affect the mechanism by which the rHDL are remodeled by PLTP but increases the rate at which the large and small conversion products are formed. As shown in Chapter 6, this enhancement is probably due to a reduction in the stability of the apoA-I in triglyceride-enriched rHDL. This is consistent with what has been reported by Sparks et al. (1995b)

and is also in agreement with the studies in hypertriglyceridemic subjects which show that triglyceride enrichment of HDL enhances the dissociation of apoA-I from the particles and the clearance of apoA-I from the circulation (Lewis et al., 1998; Lamarche et al., 1999a, 1999b). The destabilisation of apoA-I from triglyceride-enriched rHDL may be caused by the triglyceride molecules partitioning from the core into the rHDL surface and preventing apoA-I α-helices from intercalating between the rHDL phospholipid acyl chains (Frank et al., 1997).

The final part of this thesis describes the effects of rHDL phospholipid composition on the phospholipid transfers and the remodeling that are mediated by PLTP. This was achieved by preparing rHDL that contained apoA-I as the sole apolipoprotein and either POPC, PLPC, PAPC or PDPC as the sole phospholipid. These results show that phospholipid transfers from small unilamellar vesicles to the rHDL is most rapid for PAPC-rHDL. This may be due to the long polyunsaturated sn-2 acyl chains in the PAPC-rHDL introducing packing defects in the surface of the particles (Jonas et al., 1987; Davidson et al., 1995; Huggins et al., 1998) which enables the rHDL to accommodate additional phospholipids.

The experiments in Chapter 7 also show that the phospholipid composition of rHDL has a profound effect on their PLTP-mediated remodeling into large and small particles and the dissociation of apoA-I. The results indicate that remodeling is enhanced as the length and unsaturation of the rHDL phospholipid acyl chain increases. The reason for this may be due to the larger molecular surface areas of the phospholipids with long, polyunsaturated acyl chains decreasing the binding, and hence the stability, of apoA-I (Parks and Thuren, 1993; Parks and Gebre, 1997).

The mechanism of the remodeling of rHDL containing different type of phospholipids is identical to what has been proposed in Chapter 5; with large and small particles being formed by different pathways and the dissociation of apoA-I being closely associated with the formation of large particles.

The finding that the phospholipid composition of rHDL influences the rate at which they are remodeled by PLTP is of considerable physiological significance. Given that the phospholipid composition of HDL varies according to dietary fat uptake (Hodge et al., 1993; Sola et al., 1993), these findings suggest that it may be possible to regulate the remodeling of HDL by PLTP in ways that upregulate RCT.

In conclusion, the findings in this thesis describe (i) the mechanism of the remodeling of rHDL by PLTP, (ii) the reasons for the enhanced remodeling of triglyceride-enriched rHDL by PLTP, (iii) the enhancement of PLTP-mediated phospholipid transfers in triglyceride-enriched rHDL and finally, (iv) the capacity of rHDL phospholipids to regulate the PLTP-mediated phospholipid transfers and remodeling of the particles.

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THESIS CORRECTION

THESIS TITLE: **THE INTERACTION OF PHOSPHOLIPID TRANSFER PROTEIN WITH HIGH DENSITY LIPOPROTEINS**

p11, line 15	change 'diseases' to 'disease'
p18, line 2	change 'PPARα' to 'PPARα and PPARγ'
p22, line 14	change 'in vitro by reducing' to 'in vitro reducing'
p22/23	'PON, acting as an esterase, is expected to inhibit LDL oxidation' to 'PON is expected to inhibit LDL oxidation'
p30, line 6	change 'amino acids' to 'amino acid'
p31, line 5	change 'great affinity' to 'greater affinity'
p33, line 4	change 'changes in stenosis' to 'reduction in stenosis'
p35, line 17	change 'increasing' to 'increasingly'
p44, line 5	change 'facilitate' to 'facilitates'
p44, line 17	change 'esters on the sole' to 'esters as the sole'
p49, last line	change 'cho-lesterol' to 'cholesterol'
p55, line 13	change 'dissolves' to 'dissolved'
p55, line 2 from bottom	change 'palmi-toyl' to 'palmitoyl'
p56, line 10	In a typical experiment, the volume of discoidal rHDL depended on the concentration of unesterified cholesterol. The final concentration of unesterified cholesterol was 189 nmol/mL.
p58, line 1	Samples (approximately 10 μg protein) were pre-mixed with 10 μL tracking dye (40% (w/v) sucrose/0.01% (w/v) bromophenol blue solution) then applied to the gel.
p59, line 7	Samples (protein concentration ~ 10 μg/mL or 5 μL of samples) were boiled with 10 μL of SDS sample buffer for 5 min.
p78, paragraph 1	PLTP was efficiently removed from the incubation mixture by ultracentrifugation at 100,000 rpm in the density range $1.063 < d < 1.21$ g/mL using a TLA-100.4 rotor with one 6 h spin at the lower density and a second 16 h spin at the higher density.
p79, line 14	change 'unlebelled' to 'unlabelled'
Figure 7.1 and p 102, line 10	omit 'monodisperse'
Table 1.2	The chemical composition of normal human plasma lipoproteins was expressed as percentage by weight.