REMODELLING OF HIGH DENSITY LIPOPROTEINS BY PLASMA FACTORS

by

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SUMMARY

The protective effect of high density lipoprotein (HDL) against coronary heart disease has stimulated a sustained interest in the metabolism and regulation of this lipoprotein fraction. Of particular interest has been the remodelling of HDL that takes place during their circulation in plasma. Several factors are known to operate in plasma to modify the lipid compositions of HDL. These include lecithin:cholesterol acyltransferase (LCAT) which catalyses the esterification of cholesterol in HDL; cholesteryl ester transfer protein (CETP), which transfers cholesteryl esters from HDL to other lipoproteins in exchange for either triglyceride or cholesteryl esters; hepatic lipase (HL), which hydrolyses HDL triglyceride and phospholipid; and phospholipid transfer protein (PLTP) which transfers phospholipid to HDL. These factors all have substantial effects on the concentration and composition of HDL lipids and on HDL particle size. However, there are little research done into the effect of these factors on apolipoprotein A-I (apo A-I), the main protein constituent of HDL, whose concentration in plasma is in a inverse correlation with incidence of coronary heart disease.

This thesis examines the effect of remodelling HDL on the metabolism of apo A-I. The major focus is on the effects of CETP and LCAT in the regulation of apo A-I concentration in HDL. Chapter one reviews the literature with respect to the intraplasma metabolism of lipoproteins and the remodelling of HDL by various plasma factors. Chapter two describes the general methods and materials used in the studies presented in this thesis.

In chapter three the effects of incubation of HDL with CETP in the presence of VLDL and/or LDL on apo A-I concentration in HDL are examined. It is demonstrated that coincident with the reduction in HDL particle size, a proportion of apo A-I dissociated from HDL during the incubation. The dissociation of apo A-I was time-dependent. The percentage of apo A-I that dissociated from HDL correlated positively with the

concentrations of VLDL, LDL, and CETP but negatively with the concentration of HDL in the incubation.

The characterization of the dissociated apo A-I from HDL is presented in Chapter four. The dissociated apo A-I was essentially free of cholesterol and phospholipid and was not associated with other apolipoproteins. When subjected to agarose gel electrophoresis, the dissociated apo A-I migrated to a prebeta position which is identical to that of purified, lipid-free apo A-I. This is distinct from the alpha migration of the bulk HDL in plasma.

Studies in chapter five demonstrate that the dissociation of apo A-I from HDL mediated by CETP is preventable and reversible in a process dependent on LCAT activity. After the first incubation with CETP, HDL particles reduced in size and lost a proportion of apo A-I. The resulting HDL was isolated from the incubation and reincubated with lipid-free apo A-I in the presence of LCAT. Due to the generation of cholesteryl esters by LCAT, HDL particles became bigger in size. Coincided with this increase in HDL size was a proportional rise in HDL apo A-I content, which was comparable to that of native HDL prior to incubation. The reincorporation of apo A-I into HDL implies a way in which apo A-I is preserved in vivo.

Chapter six explores the mechanism by which HDL apo A-I content is increased. The studies were performed by using homogenous reconstituted HDL (rHDL). The increase in cholesteryl ester content of rHDL by LCAT not only resulted in an increase in rHDL particle size, but also resulted in a rise in the numbers of apo A-I molecules in HDL, from two to three per particle. The incubations conducted in the absence of lipid-free apo A-I did not cause changes in the concentration of rHDL-associated apo A-I, indicating that the increase from two to three molecules of apo A-I per particle was achieved at the expense of a one third reduction in the number of rHDL particles, a process that must have involved particle fusion between HDL. In contrast, when the incubation mixture was supplemented by lipid-free apo A-I, there was an increase in the

concentration of HDL-associated apo A-I of approximately 50%, indicating that under these conditions, the increase from two to three in the number of apo A-I molecules per rHDL particles was achieved by a direct incorporation of lipid-free apolipoproteins without fusion of HDL particles.

In conclusion, these observations provide in vitro evidence of a cyclic dissociation and reassociation of apo A-I with HDL. These processes are regulated by plasma factors which modify HDL lipids. This thesis expands our understanding of the HDL remodelling and the regulation of apo A-I concentration in plasma.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university or other tertiary institution; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is 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.

Hui-Qi Liang

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PUBLICATIONS AND ABSTRACTS

Publications

1. Barter PJ, Rajaram OV, Liang H-Q and Rye K-A. (1993) "Relationship between the size and phospholipid content of low-density lipoproteins". Biochim. Biophys. Acta 1166: 135-137.

2. Liang H-Q, Rye K-A and Barter PJ. (1994) "Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins". J. Lipid Res. 35: 1187-1199.

3. Barter PJ, Liang H-Q, Clay MA and Rye K-A. (1994) "Roles of hepatic lipase and cholesterol ester transfer protein in HDL metabolism". Proceedings of the 10th International Symposium on Atherosclerosis, pp 731-735, Editors FP Woodford, J Davignon and A Sniderman. Montreal, October 9-14.

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1. Barter PJ, Liang H-Q and Rye K-A. "Role of cholesteryl ester transfer protein in remodelling high density lipoproteins". Australian Atherosclerosis Society 19th Annual Meeting, Melbourne, 7-9 December, 1993, pp 22.

2. Liang H-Q, Rye K-A and Barter PJ. "Incorporation of lipid-free apolipoprotein A-I into high density lipoproteins". Australian Atherosclerosis Society 20th Annual Meeting, Glenelg, 6-9 September, 1994, pp 16.

3. Liang H-Q, Rye K-A and Barter PJ. "Incorporation of lipid-free apolipoprotein A-I into high density lipoproteins". Atherosclerosis. Vol. 109, Nos 1,2, 15 September 1994, pp 224.

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ABBREVIATIONS

ACAT	Acyl-CoA: cholesterol acyltransferase
apo	apolipoprotein
BSA	Bovine serum albumin
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
d	density
DTNB	5,5-dithio-bio(2-nitrobenzoic acid)
FC	Free cholesterol
HDL	High density lipoproteins
HDL_2	subfraction 2 of high density lipoproteins
HDL_3	subfraction 3 of high density lipoproteins
HDL-C	HDL-cholesterol
HL	Hepatic lipase
IDL	Intermediate density lipoproteins
L	Litre
LCAT	Lecithin: cholesterol acyltransferase
LDL	Low density lipoproteins
Lp (a)	Lipoprotein (a)
Lp A-I	apoA-I-containing lipoprotein
Lp A-I/A-II	lipoproteins containing both apo A-I and apo A-II
Lp A-II	apoA-II-containing lipoprotein
LPDP	Lipoprotein-deficient plasma
LPL	Lipoprotein lipase
LRP	LDL-receptor-related protein
NEFA	Non-esterified fatty acids
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PL	Phospholipid
PLTP	Phospholipid transfer protein
POPC	Palmitoyl-oleoyl phosphatidylcholine
RCT	Reverse Cholesterol Transport

rHDL	recombinant HDL or reconstituted HDL
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TC	Total cholesterol
TG	Triglyceride
TGRL	Triglyceride-rich lipoprotein
UC	Unesterified cholesterol
VLDL	Very low density lipoproteins

CHAPTER 1 INTRODUCTION

INTRODUCTION TO LIPOPROTEINS

1.1 CLASSIFICATION OF LIPOPROTEINS

1.2 PLASMA LIPID TRANSPORT BY LIPOPROTEINS

1.3 LIPOPROTEIN METABOLISM

1.3.1 Metabolism of Apo B-Containing Lipoproteins
1.3.2 High Density Lipoproteins (HDL)
1.3.3 Metabolism of HDL

PLASMA FACTORS THAT REMODEL HDL

1.4 PLASMA FACTORS THAT REMODEL HDL

1.4.1 Cholesteryl Ester Transfer Protein (CETP)
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1.4.4 Lipoprotein Lipase (LPL)
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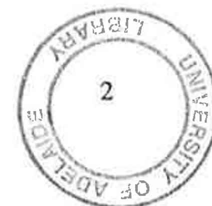
1.5 SCOPE OF THIS THESIS



INTRODUCTION TO LIPOPROTEINS

Cardiovascular disease remains a major cause of death and disability in industrialized countries. Clinical and epidemiologic studies have linked changes in blood lipids to coronary heart disease (CHD) and later have indicated that this is mediated through the changes in the plasma levels of the major lipid carriers: lipoproteins. The atherogenicity of different lipoprotein classes has been studied extensively and we have now learned that increased cholesterol in the low density lipoprotein (LDL) fraction and decreased cholesterol in the high density lipoprotein (HDL) fraction are strongly associated with the risk of CHD (Kannel et al., 1971).

In human plasma, lipids include free (or unesterified) cholesterol (FC or UC), cholesteryl esters (CE), phospholipids (PL), triglycerides (TG) and free (or non-esterified) fatty acids (NEFA). Plasma lipids are combined with a family of proteins, apolipoproteins, to form water-soluble particles which enable the lipid to be transported within the intravascular compartment. These macromolecular complexes of lipid and apolipoproteins are called lipoproteins. Each lipoprotein particle has a nonpolar core containing various amounts of neutral, hydrophobic lipids (triglyceride and cholesteryl esters). The core is surrounded by a monolayer of phospholipids, with free cholesterol and apolipoproteins interdigitated between the polar-head groups of phospholipids. The polar-head groups are oriented towards the aqueous phase of the plasma, which stabilizes the lipoprotein particle so that it can remain in solution in plasma. Due to their amphipathic character, the apolipoproteins are partly embedded in the lipid domain of the lipoprotein and with a portion of the molecule protruding from the surface of the lipoprotein into the aqueous phase of the plasma. This structure allows apolipoproteins serve as an interface between lipid and aqueous environments and thus hold a key function in the regulation of lipid transport and lipoprotein metabolism.

1.1 CLASSIFICATION OF LIPOPROTEINS

Lipoprotein particles are composed of different classes of apolipoproteins and different amounts of lipids, which lead to them vary in composition, size, density, function and metabolism (Table 1.1 and Table 1.2). Major classes of lipoproteins have been well defined by their physical-chemical characteristics. The most widely used classification of lipoproteins is based on their hydrated density (Havel et al. 1955). In this system, there are five major lipoprotein classes: chylomicron (CM, density < 0.095 g/ml), very low density lipoprotein (VLDL, d 0.095 - 1.006 g/ml), intermediate density lipoprotein (IDL, d 1.006 - 1.019 g/ml), low density lipoprotein (LDL, d 1.019 - 1.063 g/ml) and high density lipoprotein (HDL, d 1.063 - 1.21 g/ml). Other classification systems are based on the charge of the lipoprotein particle (Lees and Hatch, 1963) or their apolipoprotein composition (Alaupovic, 1991).

The concentration and distribution of lipids and apolipoproteins among different lipoprotein classes are shown in Table 1.3. The characteristics of major apolipoproteins found in human plasma are listed in Table 1.4. Apolipoproteins are not only the structural protein on lipoprotein particles, they also have several functions (Table 1.5) which are important in the regulation of lipid transport and lipoprotein metabolism.

Numerous epidemiologic studies have shown that different lipoprotein classes may play different roles in the evolution of atherosclerosis. This has led to the extensive studies on the metabolism of different lipoprotein classes. From the moment of secretion from the liver and small intestine, especially after entering the circulation, lipoproteins are under continuing and usually rapid modification by processes such as lipid esterification, lipolysis and transfers of apolipoprotein and lipid components between different particles. These processes are mediated by various enzymes and lipid transfer proteins, including lecithin:cholesterol acyltransferase (LCAT) (Glomset, 1968), hepatic lipase (HL) (Shirai et al., 1981), lipoprotein lipase (LPL) (Eckel, 1989), cholesteryl ester transfer protein

Table 1.1 Classification and Properties of Human Plasma Lipoproteins [a]

	Density range (g/ml)	Diameter (nm)	Molecular weight (Daltons)	Electrophoretic mobility	Flotation rate	Synthesis sites
CM	d<0.95	100 - 500	400×10^6	origin	$S_{f\,(1.063)} > 400$	Intestine
VLDL	0.95 - 1.006	30 - 100	$10 - 80 \times 10^6$	pre-β	$S_{f\,(1.063)}$ 60 - 400	Liver, intestine
IDL	1.006 - 1.019	25 - 30	$5 - 10 \times 10^6$	slow pre-β	$S_{f\,(1.063)}$ 12 - 60	Intravascular
LDL	1.019 - 1.063	20 - 25	2.3×10^6	β	$S_{f\,(1.063)}$ 0 - 12	Intravascular
HDL	1.063 - 1.21	7.4 - 12	$1.7 - 3.6 \times 10^5$	α	$S_{f\,(1.20)}$ 0 - 9	Intestine, liver

[a] Source - Lees and Hatch, 1963; Gotto et al., 1986; Ginsberg, 1990

Table 1.2 Chemical Composition of Normal Human Plasma Lipoproteins [a]

	Main lipids	Main apolipoproteins	Composition (% mass)		Surface components (mol % of surface)			Core lipids (mol % of core)	
			Lipid	Protein	Chol.	PL	Apos	TG	CE
CM	TG	B_{48}, C	98 - 99	1 - 2	35	63	2	95	5
VLDL	TG	B_{100}, C	90	10	43	55	2	76	24
IDL	TG, Chol.	B_{100}, E	85	15	38	60	2	78	22
LDL	Chol.	B_{100}	75	25	42	58	0.2	19	81
HDL_2	Chol., PL	A-I, A-II	60	40	22	75	2	18	82
HDL_3	Chol., PL	A-I, A-II	45	55	23	72	5	16	84

[a] Source - Eisenberg & Levy, 1975; Shen et al., 1977; Eisenberg, 1984.

Table 1.3 Concentration and Distribution of Lipids and Apolipoproteins in Normal Fasting Plasma

	Plasma concentration	VLDL	IDL	LDL	HDL
TG (mmol/L) [a]	0.5 - 2.0	0.3 - 1.0	0.1 - 0.2	0.1 - 0.3	0.1 - 0.2
Chol. (mmol/L) [a]	4.0 - 5.2	0.1 - 0.4	0.1 - 0.2	2.0 - 3.0	1.0 - 1.6

	Concentration [b] (µg/ml)	Distribution in Lipoproteins [c]			
A-I	900 - 1300				++++
A-II	300 - 500				++++
B-100	800 - 1000	+	++	+++	
C-I	40 - 70	+	Tr		+++
C-II	30 - 80	+	+		+++
C-III	80 - 150	+	+	Tr	++
D	80 - 100				++++
E	30 - 60	++	++		++

i) Chylomicron, at various stages of its metabolism, contains samll amounts of all the apolipoproteins except apo B-100.
ii) Apo B-48 is present exclusively in chylomicron and its remnants, its plasma concentration is about 10 µg/ml.
iii) Plasma apo A-IV is mainly unassociated with lipoproteins in the d<1.21 g/ml fraction.
iv) Tr - Trace amount.

[a] Source - Barter, 1994
[b] Source - Herbert et al., 1983
[c] Source - Chan & Dresel, 1990

Table 1.4 Characteristics of the Major Apolipoproteins [a]

	Origin	Molecular weight (Daltons)	Major lipoproteins	Major functions
Apo A-I	Liver, intestine	28,300	HDL	Structural protein for HDL; LCAT activitor; ligand for HDL receptor
Apo A-II	Liver, intestine	17,500	HDL	Structural protein for HDL; HL activitor; ligand for HDL receptor
Apo A-IV	Intestine	46,000	HDL, CM	Activator of LPL and LCAT
Apo B-48	Intestine	~260,000	CM	Structural protein for CM
Apo B-100	Liver	~500,000	VLDL, IDL, LDL	Structural protein for VLDL, IDL, LDL; ligand for LDL receptor
Apo C-I	Liver	7,000	CM, VLDL, HDL	LCAT activitor
Apo C-II	Liver	8,800	CM, VLDL, HDL	LPL activator
Apo C-III	Liver	8,800	CM, VLDL, HDL	LPL inhibitor; inhibit hepatic uptake of CM and VLDL remnants
Apo D	Liver	22,000	HDL	Possibly involved in reverse cholesterol transport
Apo E	Liver	34,000	Remnant, VLDL, LDL, HDL	Ligand for binding to remnant (apo E) receptor
Apo (a)	Liver	500,000	Lp (a)	Structrual protein for Lp (a); inhibitor of plasminogen activitor

[a] Source - Chan & Dresel, 1990; Patsch & Gotto, 1996

(CETP) (Barter et al., 1982) and phospholipid transfer protein (PLTP) (Tall et al., 1983). Lipoproteins also interact with cell surface receptors. The results of these reactions are the acquisition or loss of lipids from lipoprotein, or the uptake of the whole lipoprotein particles by cells.

Table 1.5 Functions of Apolipoproteins in Lipoprotein Metabolism *

Structural, secretion of lipoproteins	apo B48, apo B100, apo A-I, apo A-II
Enzyme activation/inhibition	HL activator - apo A-II LPL activator - apo C-II LPL inhibitor - apo C-III LCAT activator - apo A-I, A-IV, C-I
Interaction of lipoproteins with cellular receptor:	
- ligand for LDL receptor (B/E receptor)	apo B100, apo E
- ligand for Chylomicron remnant receptor (E receptor)	apo E
- ligand for HDL binding	apo A-I, apo A-II
- regulation of cell growth	apo E

* Chan & Dresel, 1990; Patsch & Gotto, 1996

1.2 PLASMA LIPID TRANSPORT BY LIPOPROTEINS

Figure 1.1 summarizes the transport of plasma lipids by different lipoprotein classes. Chylomicrons are responsible for the delivery of dietary lipids (exogenous lipids) from the intestine to the liver. Dietary lipids are absorbed into the cells of small intestine as fatty acids and cholesterol. Inside the intestinal mucosal cells, re-esterification to triglyceride and cholesterol ester occurs. These lipids are then incorporated into chylomicrons which are secreted into lymph in which they are transported to the plasma. Once in the plasma, dietary triglyceride is delivered to tissues as fatty acids following their hydrolysis by LPL. This leaves a chylomicron remnant which delivers dietary cholesterol to the liver, where it can be used for bile acid synthesis, membrane synthesis or resecretion as lipoprotein cholesterol.

Hepatic lipids (endogenous lipids) are transported to peripheral tissues via the VLDL/IDL/LDL cascade. The hepatic lipids, either taken up from plasma or produced in the liver, are packed into VLDL. Once in the plasma, VLDL interacts with LPL and is converted to IDL. A proportion of IDL particles are catabolized by the liver after interacting with LDL receptors on hepatocyte membranes. The IDL remaining in the plasma compartment is converted to LDL following further removal of triglyceride by lipase. One function of LDL is to supply cholesterol to cells. For this purpose, LDL binds to LDL receptors to be internalized into the cells by receptor-mediated endocytosis.

HDL is involved in the process of reverse cholesterol transport (RCT) whereby cholesterol is transported from peripheral tissues back to the liver. HDL receives free cholesterol from peripheral tissues and from chylomicrons and VLDL undergoing lipolysis. The free cholesterol is subsequently esterified by LCAT to form cholesteryl esters which are incorporated into the core of the HDL. The HDL triglycerides are hydrolyzed by hepatic lipase which is located on hepatic endothelial cells. The HDL

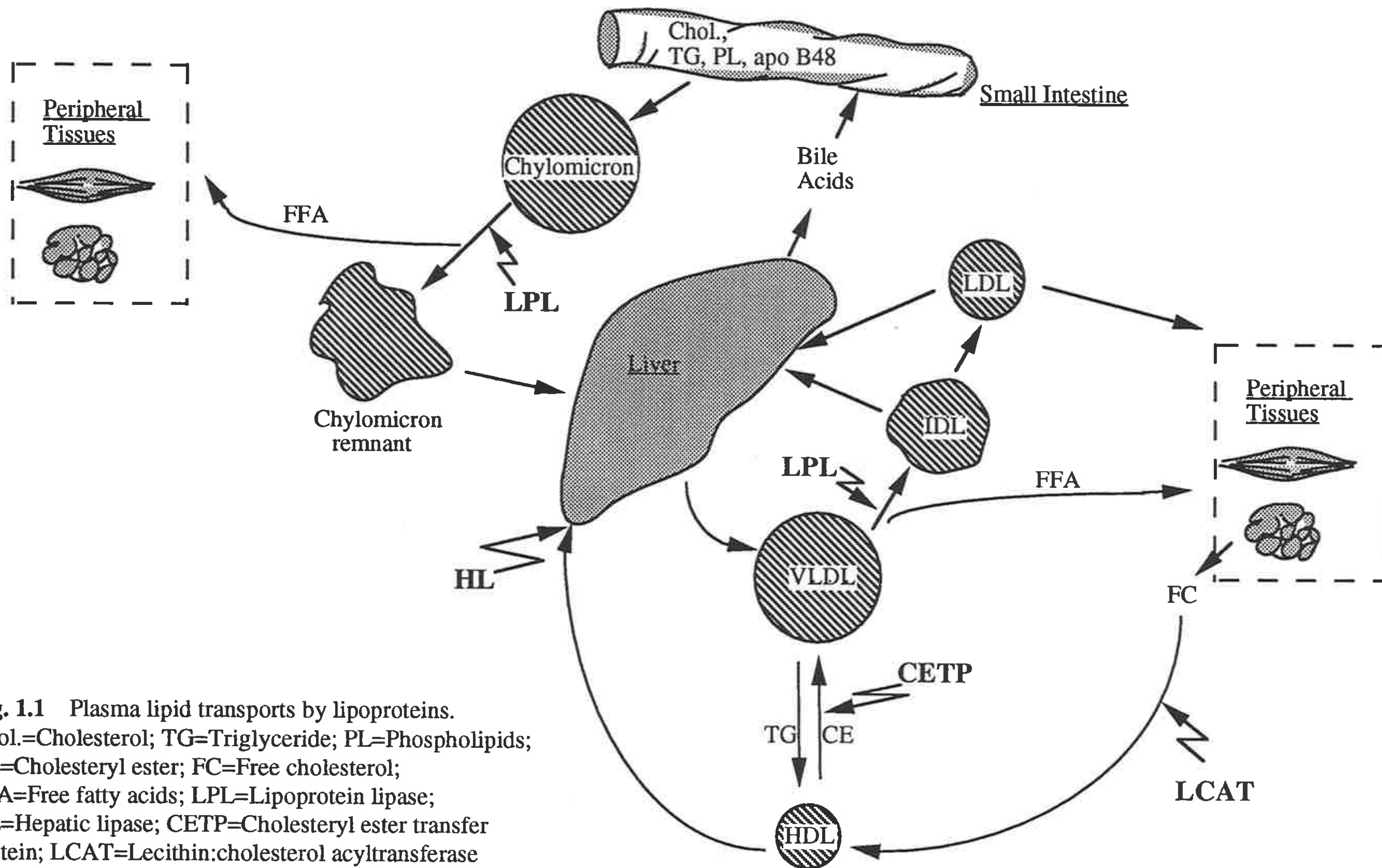


Fig. 1.1 Plasma lipid transports by lipoproteins. Chol.=Cholesterol; TG=Triglyceride; PL=Phospholipids; CE=Cholesteryl ester; FC=Free cholesterol; FFA=Free fatty acids; LPL=Lipoprotein lipase; HL=Hepatic lipase; CETP=Cholesteryl ester transfer protein; LCAT=Lecithin:cholesterol acyltransferase

cholesteryl esters may be taken up by liver following binding to a putative HDL receptor or transferred to the lower density lipoproteins by CETP.

1.3 LIPOPROTEIN METABOLISM

1.3.1 Metabolism of Apo B-Containing Lipoproteins

<u>Chylomicron</u>
<u>VLDL</u>
<u>IDL</u>
<u>LDL</u>
<u>Atherogenicity of apo B-containing lipoproteins</u>

Apolipoprotein B (apo B) is the major protein constituent of chylomicrons, VLDL, IDL and LDL. Apo B is secreted from both the intestine and liver. The intestinal apo B contains only 48% of amino acids of the liver protein: it is referred to as apo B-48 and is found only in chylomicrons (Kane, 1983). The apo B synthesized in liver is referred to be apo B-100. As it is named, apo B-100 is translated from the full-length mRNA. Apo B-100 is the major apolipoprotein of VLDL, IDL and LDL.

<u>Chylomicrons</u>

The largest lipoprotein particles are the chylomicrons. Chylomicrons are synthesized by the intestine in response to a fat-containing meal and transport mainly dietary triglycerides. Chylomicron particles are formed within enterocytes from dietary lipids, with a core of triglycerides and cholesteryl esters and a surface layer consisting of phospholipids, unesterified cholesterol and apolipoproteins (mainly apo B-48 but also apo A-I, A-II and A-IV). After being secreted into the intercellular space, chylomicrons are transported in lymph and eventually enter the general circulation. Once exposed to plasma, chylomicrons gain additional apolipoproteins such as apo C-I, C-II, C-III, and E from HDL. In the capillaries of skeletal muscle and adipose tissue, the chylomicrons adhere to binding sites on the vessel walls, where they are hydrolyzed by LPL (facilitated

by apo C-II) to supply fatty acids for storage or energy needs. The triglyceride hydrolysis reduces the size of chylomicrons and some surface components (PL, apo A-I, apo A-IV and apo C) become redundant and are transferred to HDL. HDL is important in the metabolism of chylomicrons since it acts as a reservoir for apolipoproteins, especially apo C and apo E, which initiate lipolysis and receptor uptake of these lipoproteins particles.

The chylomicron remnant, enriched in cholesteryl esters and containing apo B-48 and apo E, is removed from the circulation by the liver primarily via the hepatic remnant receptor, which recognizes the apo E on the particle (Davignon et al., 1988; Gregg and Brewer, 1988). The LDL-receptor-related-protein (LRP), a receptor described by Herz et al. (1988), also appears to be a potential candidate for the chylomicron remnant receptor (Beisiegel et al., 1991). The LRP has been shown to be a multifunctional receptor (Herz, 1993), but it is not yet certain whether the LRP is actually the remnant receptor.

Following the degradation of chylomicron remnants in hepatocytes, their cholesterol is either excreted into bile or reused for the synthesis of other lipoproteins and membrane. Under normal conditions, chylomicron remnants are rapidly and quantitatively removed from plasma. Since chylomicron remnants can be taken up by cells of artery wall, prolonged plasma residence times of chylomicrons or elevated levels in the postprandial state may contribute to the onset of atherosclerosis (Zilversmit, 1979).

VLDL

VLDLs are assembled in and then secreted by the liver. Triglycerides, cholesterol and phospholipid are packaged together with apo B-100 into VLDL. VLDL acquires apo Cs and apo E from HDL in the plasma compartment. As with chylomicron metabolism, VLDL triglyceride is hydrolyzed by LPL in capillary beds of various tissues. As the VLDLs lose triglyceride, they also shed apolipoproteins other than apo B-100 and are subsequently converted into VLDL remnants, also known as IDL. IDL can either be

returned to the liver cell following binding to hepatic receptors or further catabolised to LDL. The extent to which VLDL in normal individuals is converted to LDL has been estimated to be less than 50% (Packard et al., 1984; Nestel, 1987). It appears that larger VLDL are more likely to be removed directly from plasma without being converted to LDL, whereas smaller, more dense VLDL are more efficiently converted to LDL (Redgrave and Maranhao, 1985).

IDL

IDLs are intermediate with respect to size and density between VLDL and LDL. In fasting human subjects most IDL and LDL arise from VLDL within the plasma compartment (Demant et al., 1988). While some of IDL particles are removed from the circulation by the liver, a large proportion are rapidly hydrolyzed further to LDL by hepatic lipase.

LDL

LDLs are formed mainly as degradation products of VLDL through IDL intermediates (Sigurdsson et al., 1975; Demant et al., 1988). They carry about two-thirds of the plasma cholesterol in normolipidemic individuals. The core of LDL particles contains mainly cholesteryl esters; the predominant protein component of LDL is apoB-100. Overall about 60% to 70% of LDL is taken up by tissues (mainly by the liver) following its binding to LDL receptors. The LDL receptor recognises both apo B-100 and apo E on the surface of LDL and they are present in most tissues but are expressed mainly in the liver (Brown and Goldstein, 1983).

The number and activity of LDL receptors are major determinants of the concentration of LDL in plasma. Genetic deficiency or dysfunctional receptors results in an impaired uptake of LDL by tissues and a consequent increased level of LDL cholesterol in plasma. Other determinants of plasma LDL levels include plasma VLDL level and the efficiency with which VLDL are converted to LDL.

The regulation of cellular cholesterol homeostasis is mediated by several mechanisms as described by Brown and Goldstein (1986). When intracellular cholesterol content increases, the regulatory responses that assure cholesterol homeostasis include (1) suppression of the synthesis of LDL receptors on the cells surface, which prevents further uptake of cholesterol by cells; (2) suppression of the activity of HMG-CoA-reductase, a rate limiting enzyme in cellular cholesterol synthesis, which turns off cholesterol synthesis by the cell; (3) activation of acyl-CoA: cholesterol acyltransferase (ACAT), which re-esterifies excess cholesterol intracellularly for storage as cholesteryl ester droplets.

Lipoprotein (a) (or Lp(a)) is a variant of LDL. It is an important independent risk factor for the development of premature cardiovascular disease (Armstrong et al., 1986; Ütermann, 1989). Lp(a) is a cholesterol-rich lipoprotein that closely resembles LDL in lipid composition but carries an extra apolipoprotein, apo (a), linked to apo B-100 by a single disulfide bridge (Scanu, 1993a). The hydrated density of Lp(a) is 1.055-1.088 g/ml, but its particle size is larger that LDL. Apo (a) is a large glycoprotein, with isoproteins ranging in size from 400 to 700 kDa. There is a highly significant association of individual apo (a) phenotypes with the plasma Lp(a) levels: the apo (a) isoproteins of higher and lower molecular weight are associated with lower and higher plasma concentrations of Lp(a), respectively. The plasma concentration of Lp(a) appears to be independent of diet and is genetically determined, with a normal value range from less that 1 to more than 100 mg/dl. Population studies showed that a level of Lp(a) of 30 mg/ml is associated with a twofold increase in the relative risk of premature cardiovascular disease.

<u>Atherogenicity of apo B-containing lipoproteins</u>

All of the apo B lipoproteins are potentially atherogenic. Abnormalities in chylomicron or chylomicron remnant metabolism have been associated with the presence of CHD. It

appears that many individuals with CHD have increased levels of these lipoproteins in the circulation in the postprandial state (Zilversmit, 1979). The uptake of chylomicron remnants by the liver is reported to involve the LRP, which binds apo E. The clearance of the chylomicron remnant particles seems dependent on the individual's apo E phenotype (Weintraub et al., 1987a). In some clinical conditions such as dysbetalipoproteinemia, the clearance of chylomicron remnants is delayed considerably as a consequence of the presence of a variant of apo E (apo $\varepsilon 2$) with reduced affinity for the lipoprotein receptors (Mahley and Innerarity, 1983; Weintraub et al., 1987b).

Increased plasma levels of LDL (Kannel et al., 1971; Kannel, et al., 1979; Castelli et al., 1986) and Lp (a) (Scanu and Fless, 1990; Scanu, 1993b) are associated with the development of CHD. The pathophysiological mechanisms involved in the atherosclerosis associated with LDL are being elucidated (Van Lenten and Fogelman, 1990; Steinberg, 1991). Oxidative modification of LDL increases the intimal macrophage uptake of these cholesterol-rich lipoproteins by specific macrophage cellular receptors, the scavenger receptors (Steinberg et al., 1989). Unlike the normal LDL receptor, the scavenger receptor recognizes oxidized LDL and is not down-regulated by increasing intracellular cholesterol pool (Goldstein et al., 1979). This results in the generation of arterial lipid-laden foam cells which are characteristic of the early atherosclerotic lesion. Smooth muscle cells in the arterial media may also take up atherogenic lipoproteins and undergo conversion to foam cells.

LDL heterogeneity may also be important in the development of CHD. A classification of plasma LDL patterns has been proposed on the basis of gradient gel electrophoresis profiles (Austin and Krauss, 1986; Williams et al. 1990). Pattern A is defined as an LDL pattern containing mainly large particles (apparent diameter > 25.5 nm) with minor subpopulations of smaller size. In pattern B, there is a predominance of small, dense LDL particles (mean apparent diameter < 25.5 nm), and minor subpopulations of larger LDL. Pattern A is observed in 65-75 % of the population, whereas pattern B is

observed in 20-30% of the population. The LDL subclass pattern B is associated with an increased risk of CHD (Austin et al., 1988; Tornvall et al., 1991; Campos et al., 1992). In fact, the association between LDL size and coronary heart disease is dependent on other established risk factors such as LDL cholesterol and HDL cholesterol (Campos et al., 1992), and plasma triglyceride levels (McNamara et al., 1992).

1.3.2 High Density Lipoproteins (HDL)

HDL subpopulations
Functions of HDL
Variation in plasma HDL concentration
Anti-atherogenicity of HDL

HDL contains about 50% protein and 50% lipid by weight. Their diameters range between 7 and 14 nm and their molecular mass range between 200 K to 400 K Daltons (Eisenberg, 1984). Circulating HDL consist primarily of spherical particles which are composed of a monomolecular surface layer of phospholipids, unesterified cholesterol and apolipoproteins that surrounds a core of neutral lipids (Shen et al., 1977; Edelstein et al., 1979). The major core-lipid is cholesteryl ester, together with variable amounts of triglycerides (Eisenberg, 1984). The major apolipoproteins of HDL are apo A-I and apo A-II, which account for 70% and 20% of the protein content of HDL respectively (Cheung and Albers, 1982). A variety of minor, but possibly metabolically important apolipoproteins are also been found associated with HDL; these include the apo C's (C-I, C-II and C-III), apo E, apo A-IV, apo D and apo J.

HDL subpopulations

HDL consist of particles with heterogeneous composition, density, size and shape (Blanche et al., 1981; Eisenberg, 1984). Nascent HDLs are disc-shaped particles consisting of a phospholipid bilayer, together with apo A-I or apo E and unesterified cholesterol molecules (Hamilton et al., 1976; Bisgaier and Glickman, 1983). Mature HDLs are spherical particles that can be subfractionated into several discrete subclasses

according to their electrophoretic mobility (Ishida et al., 1987), density (Patsch et al., 1980), size (Blanche et al., 1981) or apolipoprotein composition (Cheung and Albers, 1982). At least nine HDL subspecies have been identified in human plasma (Cheung et al., 1987). More recently, Asztalos et al. (1993) have identified twelve distinct apo A-I-containing HDL subpopulations in human plasma by using two-dimensional electrophoresis based on particle size and charge.

Based on their density, HDLs in human plasma exist as two major subfractions: the larger, less dense HDL2 and the smaller, more dense HDL3 (Patsch et al., 1980). On average, HDL2 and HDL3 account for one-third and two-thirds of HDL cholesterol respectively, although this varies widely from subject to subject. The main features of HDL subpopulations are shown in Table 1.6. The diameters of HDL2 particles range from 8.8 to 12.9 nm, as compared to that of HDL3 which range from 7.2 to 8.8 nm. HDL2 is lighter than HDL3 and contains more lipids per particle.

In addition, a minor population, HDL1, has been identified in human plasma. HDL1 is present in large quantities only in non human species such as rats. HDL1 particles are lighter and larger than HDL2 and they have a diameter of 13 - 14 nm. HDL1 particles contain large amount of apo E (50-60% of the total protein), they also contain apo C and small amount of apo A-I and apo A-II (Eisenberg, 1984). Another HDL population which is denser and smaller than HDL3, referred to as HDL4, has been identified in some pathological conditions such as familial LCAT deficiency (Mitchell et al., 1980). These particles are spherical, contain mainly apo A-I and apo A-II, and range in diameter between 6.5 -7.0 nm (Deckelbaum et al., 1982).

As summarised in Table 1.6, separation of HDL on the basis of particle size by non-denaturing polyacrylamide gradient gel electrophoresis has enabled the quantification of HDL2 and HDL3 into five distinct subclasses: HDL2a, 2b and HDL 3a, 3b, 3c (Blanche

Table 1.6 Characterization of Alpha-migrating HDL Subpopulation

	HDL_1	HDL_{2b}	HDL_{2a}	HDL_{3a}	HDL_{3b}	HDL_{3c}
Density (g/ml)[a,c]	1.04-1.09	1.063-1.100	1.100-1.125	1.125-1.147	1.147-1.167	1.167-1.200
Diameter (nm)[a,c]	13-14	9.8-12.9	8.8-9.8	8.2-8.8	7.8-8.2	7.2-7.8
Main Apolipoproteins[b,c]	apo E, C	A-I	A-I, A-II	A-I, A-II	A-I, A-II	A-I

	HDL_2	HDL_3
Molecular Weight (average)[c]	400,000	200,000
Composition (moles/mole lipoprotein)[c]		
A-I	4	3
A-II	1	1
C	1	1
PL	189	93
FC	52	11
CE	109	44
TG	18	9

[a]. Nichols et al., 1986; [b]. Cheung and Albers, 1984; Lagrost, 1992; [c]. Eisenberg, 1984.

et al., 1981; Nichols et al., 1986; Williams et al., 1990). In normal human plasma, over 80% of the HDL are HDL3a and HDL2b (Pritchard and Frohlich, 1994).

HDL2 and HDL3 are interconvertable under certain circumstances. In vitro studies showed that HDL3 was converted into HDL2 following the incorporation of molecules released from the surface of lipolysed VLDL and the formation of cholesteryl esters via the LCAT reaction (Eisenberg, 1984). The hypothesis that HDL3 is the precursor of HDL2 is supported by the report that variability of plasma HDL levels reflect variable HDL2 concentration, while HDL3 levels remain relatively constant (Anderson et al., 1978). HDL2 may also be converted to HDL3 by the depletion of lipids and apolipoproteins of HDL2 via lipolysis (by HL) or via core lipid transfer reaction (by CETP).

Apolipoprotein specific HDL particles can be isolated by use of immunoaffinity columns with specific antibodies against apo A-I or A-II (Cheung and Albers, 1982; Cheung and Albers, 1984). Using this technique, HDLs separate into those containing only apo A-I (LpA-I) and those containing both apo A-I and apo A-II (LpA-I/A-II). A minor population of particles containing only apo A-II (LpA-II) has also been reported (Bekaert et al., 1992). In normolipidemic male subjects, plasma levels of LpA-I/A-II are 2.5 times higher than levels of LpA-I. The LpA-I/A-II family is a polydisperse system of particles present throughout the HDL density range with its major part occurring in the HDL3 subfraction (Cheung and Albers, 1982; James et al., 1989; Mowri et al., 1994). For LpA-I, it was estimated that 60% and 33% of the LpA-I particles are found in HDL2 and HDL3, respectively (Atmeh et al., 1983; James and Pometta, 1990). More than 70% of the particles in HDL2 are LpA-I, while the majority of HDL3 particles are LpA-I/A-II (Atmeh, et al., 1983; Cheung and Albers, 1984). In other words, LpA-I compares closely but is not identical to HDL2 while LpA-I/A-II compares closely but is not identical to HDL3 (Cheung and Albers, 1984).

LpA-I exists in two discrete sizes: particles with a diameter of 10.6 nm contain 4 molecules of apo A-I and particles with a diameter of 8.8 nm contain 3 molecules of apo A-I (Leroy et al., 1993). There are three size classes of LpA-I/A-II particles: 9.6 nm, 8.7 nm and 8.0 nm; the largest of these particles contain 2 molecules of both apo A-I and apo A-II, while the apolipoprotein compositions of the smaller particles are not known (Leroy et al., 1993).

A third aspect of HDL heterogeneity relates to the charge of the HDL particle. While the bulk of HDL have an alpha mobility on an agarose gel, up to 10% of HDL from freshly isolated plasma has a pre-beta mobility (Kunitake et al., 1985; Neary and Gowland, 1987; Duval et al., 1989; O'Kane et al., 1992). Further analysis of HDL using two-dimensional electrophoresis has revealed that pre-beta HDL are also heterogeneous in size. Prebeta-migrating particles include small pre-beta-1 HDL and larger pre-beta-2 HDL and pre-beta-3 HDL (Castro and Fielding, 1988). Pre-beta-1 HDL particles have received a great deal attention in recent years due to their reported role in reverse cholesterol transport. It has been shown that they are the preferred initial acceptor of cell cholesterol (Castro and Fielding, 1988). As described in the following section, pre-beta HDL are converted into mature HDL with alpha mobility by the action of LCAT (Castro and Fielding, 1988; Kunitake et al. 1992a). More recently, Asztalos et al. (1993) have modified and improved the two-dimensional electrophoresis technique and have identified at least 12 subpopulations of apo A-I-containing HDL particles. These twelve separate subpopulations of HDL are $\alpha_{1,2,3}$, pre $\alpha_{1,2,3,4}$, pre $\beta_{1a,\ 1b}$, and pre $\beta_{2a,\ 2b,\ 2c}$.

Functions of HDL

HDL is an essential component of the process of reverse cholesterol transport (RCT) (Glomset, 1968; Glomset, 1970; Fielding & Fielding, 1982) -- a pathway by which cholesterol is transported from peripheral tissues to the liver via plasma. HDL particles act as acceptors of both phospholipids and free cholesterol released from cells. Following the transfer of cholesterol from cell membranes to HDL, the cholesterol is

esterified by LCAT. The cholesteryl esters so formed are then transferred from HDL to other lipoproteins in a process mediated by CETP. The cholesteryl esters are then removed from plasma mainly as a result of an uptake of LDL by the liver.

In vitro studies have shown that HDL particles are not equally effective as acceptors of cell cholesterol. Following incubation of native plasma and a cultured cell monolayer labelled with isotopic cholesterol, a major part of cholesterol radioactivity is recovered in a minor subfraction of HDL (pre-beta-1 HDL) (Castro and Fielding, 1988), indicating pre-beta-1 HDL may be the preferred primary acceptor of cell cholesterol. Pre-beta-1 HDL contains apo A-I, phospholipid and free cholesterol and has a molecular weight of 74 kDa (Castro and Fielding, 1988). Further study of the metabolism of these small pre-beta-1 HDLs have shown that they gradually increase in size (become pre-beta-2 HDL and pre-beta-3 HDL) following esterification of the free cholesterol by LCAT.

HDL also acts as an acceptor of lipolysis by-products (phospholipid, free cholesterol, apo A-I, apo A-II, apo E and apo C) released during lipolysis of triglyceride in chylomicron and VLDL by lipoprotein lipase. As the triglyceride is hydrolysed, chylomicron and VLDL become smaller and a proportion of their surface lipids and apolipoproteins are transferred to HDL. This leads to the conversion of HDL3 to larger HDL2 (Tall and Small, 1978).

Other functions of HDL include a possible role in the inhibition of LDL oxidation (Ohta et al., 1989; Parthasarathy et al., 1990; Kunitake et al., 1992b); inhibition of the expression of adhesion molecules on endothelial cells (Cockerill et al., 1995); inhibition of the cellular uptake and degradation of LDL by competing with LDL for apo B/E receptor (Miller et al., 1977) and stimulation of vascular endothelium (Tauber et al. 1981).

Variation in plasma HDL concentration

Alterations in plasma HDL levels are not only affected by changes in the synthesis or catabolism of the major HDL proteins, but are also influenced by the activities of endothelial lipases, plasma lipid transfer proteins and LCAT. It has been shown that variations in HDL cholesterol (HDL-C) and apo A-I levels between individuals can be explained in part by differences in the fractional catabolic rate of apo A-I (Brinton et al., 1990) and by differences in the production of apo A-I (Rader et al., 1993). Hypertriglyceridemic subjects with low HDL-C and apo A-I levels have an increased fractional catabolic rate of apo A-I (Le and Ginsberg, 1988). Apart from variations in synthesis and catabolism of HDL apolipoproteins, an increase in activity of LPL (such as by physical exercise) or decrease in activity of HL (by estrogens) elevates plasma HDL levels (Tikkanen et al., 1981; Taskinen et al., 1982). On the other hand, increase in HL activity or lipid transfer activity by CETP are associated with a reduction in HDL levels (Tikkanen and Nikkilä, 1987; Chiesa et al., 1993).

Some of the physiopathologic factors which alter HDL levels are listed in Table 1.7. HDL-C levels are 40-50% higher in male endurance athletes than in sedentary controls (Herbert et al., 1984; Thompson et al., 1991). An enhancement of LPL activity produced by exercise training and increased plasma TG clearance may also contribute to elevated HDL-C levels (Thompson, 1990; Podl et al., 1994). Cigarette smokers tend to have lower plasma HDL-C level than non-smokers (Freeman et al., 1993).

The variability of plasma HDL levels usually reflects a difference in the relative proportions of HDL subpopulations. For example, a high or low level of HDL tends to reflect a high or low HDL2 concentration, while HDL3 levels remain relatively constant (Anderson et al., 1978; Patsch et al., 1982). Females tend to have higher levels of total HDL and HDL2 than males. Consistent with the finding that HDL2 are mainly LpA-I, it has been found that LpA-I levels are higher in females than males. In females, about 35-

45% of total plasma apo A-I is associated with LpA-I, compared to 26-42% in males (Fruchart, 1990; Parra, et al., 1990).

Table 1.7 Some Factors That Alter Plasma HDL Levels*

Increase HDL	Female sex Physical exercise Alcohol intake Nicotinic acid, fibrates; phenytoin Oestrogens
Decrease HDL	Low fat diet High polyunsaturated fat diet Obesity Probucol, β-blockers, progestins, and androgens Smoking

* Tall, 1990.

Changes in plasma levels of HDL also correlate to changes in HDL particles size: low levels of HDL in plasma are usually associated with a shift in the average size of HDL to smaller particles, e.g., people at risk for CHD have a greater proportion of the smaller subpopulations (HDL3b, 3c) (Fruchart et al., 1993).

Anti-atherogenicity of HDL

Numerous epidemiological studies have verified a negative correlation between CHD and plasma levels of HDL cholesterol (Miller and Miller, 1975; Gordon and Rifkind, 1989). More specifically, the protective effect of HDL is better reflected in the plasma levels of HDL2 than in total HDL or HDL3 (Patsch and Gotto, 1987). Consistent with the finding that most HDL2 particles are LpA-I (Atmeh, et al., 1983; Cheung and Albers, 1984),

LpA-I has been suggested to be more antiatherogenic than LpA-I/A-II (Pritchard and Frohlich, 1994). In a study comparing patients with angiographically verified CAD with controls, it was found that the presence of CAD was inversely correlated with levels of LpA-I, but not LpA-I/A-II (Puchois et al., 1987). Consistently, a strong inverse correlation is found between plasma levels of apo A-I and CAD (Brunzell et al., 1984; Miller, 1987). The well-established male-female difference in plasma levels of HDL2 was shown to result from increased concentrations of LpA-I in the HDL2 fraction of females (James et al., 1989).

Although the association between low HDL and CHD is proven, the mechanism underlying the association is not known. The most widely accepted explanation for the apparent protective effects of HDL is the involvement of HDL in reverse cholesterol transport. According to this view, the level of HDL cholesterol is a marker of the efficiency of a system of reverse cholesterol transport (Tall, 1990). This suggests that HDL is antiatherogenic by virtue of its potential to promote the efflux of cholesterol from the artery wall and transport it from peripheral tissues to the liver for secretion or reuse. In supporting this proposition, Fielding and Fielding (1995) found that pre-beta HDL particles are present in high concentrations within the aortic intima and in large vessel lymph, indicating its potential role in transporting cholesterol away from the peripheral tissues.

It is clear from animal and human studies that cholesterol is removed from atheroma when plasma LDL-C levels are lowered and HDL-C levels are increased. In tissue culture studies HDL can remove cholesterol from cells, including CE-loaded macrophage foam cells (Brown and Goldstein, 1983). In animal studies, the development of diet-induced atherosclerosis can be prevented (Badimon et al., 1989) and even reversed (Badimon et al., 1992) by infusions of the homologous plasma HDL fraction.

As discussed in the section "Functions of HDL", the esterification of HDL cholesterol by LCAT and the transfer of CE from HDL to other lipoprotein are key steps in the pathway of reverse cholesterol transport. However, the reverse cholesterol transport theory does not explain why in genetic HDL deficient states such LCAT deficiency and Tangier disease, HDL levels are low but atherosclerosis is not particularly prevalent (Schaeffer et al., 1980). Also, in individuals with the apo A-I_{Milano} variant (Franceschini et al., 1980), where HDL2 is virtually absent from plasma and HDL cholesterol is very low, accelerated atherosclerosis has not been reported. The explanation for this is not known. But these studies do suggest an additional or alternative anti-atherogenic mechanism for HDL. In fact, other anti-atherosclerosis mechanisms by HDL have been proposed, these include inhibition of expression of adhesion molecules on endothelial cells (Cockerill et al., 1995); inhibition of the uptake and degradation of LDL by competing with LDL for apo B/E receptor (Miller et al., 1977); inhibition on platelet function (Surya and Akkerman, 1993; Pieters et al., 1994); protection of cells against the cytotoxicity of LDL or prevent the formation of oxidized LDL (Witztum and Steinberg, 1991).

Recent studies of transgenic mice have provided powerful evidence in support of a direct action of HDL in protecting against the development of atherosclerosis. In these studies, the gene for human apo A-I and/or apo A-II have been introduced into mice. The overexpression of apo A-I increases plasma apo A-I and HDL cholesterol levels (Walsh et al., 1989). These apo A-I transgenic mice are protected against the development of atherosclerotic lesions produced by an atherogenic diet (Rubin et al., 1991). When both human apo A-I and apo A-II are expressed in high levels in transgenic mice, protection against atherosclerosis is reduced (Rubin et al., 1992). It seems apo A-II does not confer protection against atherosclerosis (Schultz et al., 1993; Warden et al., 1993). In fact, in some mouse strains increased expression of apo A-II is associated with enhanced fatty streak development despite increased HDL cholesterol (Mehrabian et al., 1993).

Another hypothesis for the inverse correlation between HDL and CAD is that low HDL level is simply a marker for the accumulation of chylomicron or VLDL remnants in plasma (Patsch et al., 1982). Individuals with high levels of HDL cholesterol and HDL2 are less prone to CHD, suggesting their lipid transport system is well-functioning. The effective lipolysis of triglyceride-rich lipoproteins will lead to an increase in HDL (HDL2) levels; the low levels of HDL may result from both inefficient lipolytic transfer of lipids into HDL and the accelerated catabolism of the TG-rich HDL2 (Patsch and Gotto, 1987; Tall, 1990).

1.3.3 Metabolism of HDL

Formation of HDL
Catabolism of HDL
Remodelling of HDL in plasma

Formation of HDL

Plasma HDL originates from three sources: (1) direct secretion of "nascent" HDL particles from intestinal and hepatic cells; (2) lipid-protein complexes released during the lipolysis of chylomicrons and VLDL; and (3) the association of lipid-free apolipoproteins with phospholipids (Eisenberg, 1984; Patsch and Gotto, 1987; Clay and Barter, 1996). Nascent HDL particles are secreted into the circulation as discoidal, precursor particles containing apolipoproteins, PL (mainly phosphatidylcholine) and free cholesterol but no cholesteryl esters (Marsh, 1974; Hamilton et al., 1976). Nascent intestinal HDL contains apo A-I and apo A-IV, while nascent hepatic HDL contains apo A-I, apo A-II and apo E. Once in the plasma, nascent HDL acquire additional apolipoproteins, including apo C's released during the hydrolysis of TG-rich lipoproteins (Havel et al., 1973). The discoidal HDL particles are excellent acceptors of cellular cholesterol and they are also highly reactive with LCAT. This results in the generation of cholesteryl esters which form a core in the particle, rendering it spherical (Glomset, 1968). The transformation from discoidal to spherical HDL is sufficiently rapid that under normal conditions, only

the spherical particles are found in plasma. One exception is in subjects deficient of LCAT in whom discoidal HDL predominates (Mitchell, et al., 1980).

A second source of HDL is constituents released from the surface of lipolyzed triglyceride-rich lipoproteins (Tall and Small, 1978; Eisenberg, 1984). The mechanism for the formation of HDL in this process has been proposed as the release of fragments of redundant surface materials from the "shrinking" chylomicron or VLDL particles. The shed fragments, composed of lipid and apolipoprotein molecules, form the basis of new HDL particles in the plasma (Eisenberg, 1984).

A third pathway by which HDL precursors can be formed is through apolipoprotein-phospholipid associations. According to this mechanism, phospholipids are derived from either cell membranes or intact lipoproteins or the TG-rich lipoproteins undergoing lipolysis; lipid-free apolipoproteins may be newly synthesized and secreted into plasma or released from released from lipolyzed lipoproteins. Apolipoprotein-phospholipid complexes have been shown to be formed in vitro and may have either discoidal or spherical structure (Forte et al., 1993; Forte et al., 1995; Yancey et al., 1995). Direct evidence for the formation of HDL precursors by this pathway is also supported by the work of Clay and Barter (1996), which shows lipid-free apo A-I can form new HDL particles in the presence of NEFA and other lipoprotein fractions. In that study, the new HDL particles are predominantly discoidal in shape and their major constituents are apo A-I, phospholipid and unesterified cholesterol.

Both phosphatidylcholine and unesterified cholesterol in HDL precursor particles serve as substrates for the cholesterol-esterifying enzyme LCAT (Glomset, 1968). LCAT, which requires the amphipathic helical regions of apo A-I as its activator (Pownall et al., 1980), acts on the nascent HDL to generate a core of CE and hence converts the HDL to spherical particles. Further generation of CE by the LCAT reaction leads to enlargement of small HDL3 particles to larger HDL2 particles. During the enlargement of the HDL,

the particles accommodate additional apolipoproteins such as apo C-II, apo C-III and apo E on their surfaces. In LCAT deficiency, HDLs remain as discoidal and vesicular particles (Glomset et al., 1973).

Catabolism of HDL

There are at least three pathways involved in the metabolism of HDL: (1) CE is transferred from HDL to apo B-containing lipoproteins to be ultimately taken up by the liver in a LDL receptor-mediated process (Barter et al., 1987; Brown et al., 1990; Tall, 1993); (2) removal of the entire particle from plasma: as HDLs accumulate CE, they become enriched in apo E and may be cleared by receptors recognizing apo E in the liver and in other tissues (Franceschini et al., 1991; Tall, 1992); and (3) selective uptake of CE - a process in which HDL delivers CE to hepatocytes directly without internalization of the entire particle (Pittman et al., 1987a; Pittman et al., 1987b; Franceschini et al., 1991; Tall, 1992).

Among the above pathways involved in HDL metabolism, the first one predominates in species with a high CETP activity, such as humans. It has been estimated that at least 80% of the LCAT-derived CEs end up in lower density lipoprotein fractions (Eisengerg, 1984). Similarly, kinetic studies in rabbits (which also have a high plasma CETP activity) suggested that about 70% of HDL CE were cleared from plasma after being transferred to other lipoproteins (Goldberg et al., 1991). The CETP-mediated transfer of CE from HDL to other lipoprotein classes is accompanied by a reciprocal transfer of triglyceride in the reverse direction (Deckelbaum et al., 1986; Tall, 1992). A recent study in this reciprocal transfer of neutral lipids showed that this process is not an equimolar heteroexchange, with the number of molecules of CE lost from HDL was almost three times greater than the molecules of TG it acquired (Liu and Bagdade, 1995). The TG accumulates in the HDL particles and are subsequently hydrolyzed by HL and possibly also by LPL (Deckelbaum et al., 1986). The combination of the loss of CE and the

hydrolysis of TG result in a reduction in HDL size. This enables the particle to initiate another cycle of delivery of cholesterol into the liver via CETP or other pathways.

HDL receptors have been proposed to be involved in the endocytosis of HDL and the selective uptake of CE from HDL by hepatic cells. It is an HDL-binding protein on cell surface which mediates the translocation of cholesterol between HDL and cells. In fact, in many cell types, high-affinity binding sites for HDL exist, and binding shows specificity for apo A-I, apo A-II and apo A-IV (Oram, 1990). The number of binding sites increases when cells loaded with cholesterol (Oram et al., 1983). More detail features of the HDL receptors are yet to be investigated.

Studies designed to identify the catabolic sites for HDL have suggested that HDL lipids and apolipoproteins have different ultimate catabolic sites. The liver is the major degradation site for HDL lipids (Stein et al., 1983), while apo A-I is catabolized primarily by the kidney (Glass et al., 1983).

Remodelling of HDL in plasma

HDL undergoes constant modification in the circulation, due to the action of enzymes (such as lipoprotein lipase, hepatic lipase, LCAT) and lipid transfer proteins (CETP, PLTP). These plasma factors act primarily on the lipid composition of the HDL particles. However, alterations in lipid composition may be followed by changes in the whole structure of the particle, which also causes changes in apolipoprotein composition, particle size and density of the HDL. For example, remodelling of the triglyceride-enriched HDL by hepatic lipase leads to a reduction of HDL size and triggers a loss of apo A-I from the HDL particles (Goldberg et al., 1990; Clay et al., 1992).

This thesis is concerned with remodelling of HDL by plasma factors, specifically with the effects of this remodelling on the metabolism of apo A-I. In the next section, various

plasma factors which modify HDL are discussed, with focus on their effects in terms of changes in size and lipid and apolipoprotein compositions of HDL.

1.4 PLASMA FACTORS THAT REMODEL HDL

1.4.1 Cholesteryl Ester Transfer Protein (CETP)

Physical characteristics of CETP
Lipid transfer activities of CETP
Remodelling of HDL by CETP
Relationship between CETP and HDL in vivo

Physical characteristics of CETP

Cholesteryl ester transfer protein (CETP) is a plasma protein that promotes transfers and exchanges of cholesteryl esters (CE), triglycerides (TG) and phospholipids (PL) between plasma lipoproteins (Barter et al., 1982; Quig & Zilversmit, 1990; Rye & Barter, 1992; Tall, 1993; Barter & Rye, 1994; Tall, 1995). It has also been referred to as cholesteryl ester exchange protein, lipid transfer protein (LTP) (Albers et al., 1984), lipid transfer protein-1 (LTP-1) and HDL conversion factor (Barter et al., 1988). CETP is responsible for all of the cholesteryl esters and triglycerides transfers and about half of the phospholipids transfers between lipoproteins in human plasma (Hesler et al., 1988; Yen et al., 1989). The remaining transfer of phospholipids is mediated by a plasma phospholipid transfer protein (PLTP) or LTP-2 (Tollefson et al., 1988a).

CETP has been isolated from human plasma in a highly purified form. It is an extremely hydrophobic, heat stable glycoprotein with an apparent Mr of 66,000 to 74,000 determined by SDS-polyacrylamide gel electrophoresis (Albers et al., 1984; Drayna et al., 1987; Hesler et al., 1987; Jarnagin et al., 1987). However, the cloning of the human CETP cDNA revealed a 476 amino acid protein of Mr 53,000 (Drayna et al., 1987). At high concentrations, purified CETP forms dimers and higher order-multimers due to self-association (Hesler et al., 1987). Antibodies raised against human CETP have been

shown to cross-react with CETP from monkey (Quinet et al., 1991) and rabbit (Ko et al., 1993), suggesting the protein is partly conserved between species. The CETP gene is located on the long arm of chromosome 16 near the LCAT locus (Lusis et al., 1987). Mammalian adipose tissue, muscle and liver have been reported to be the major sources of CETP mRNA (Jiang et al., 1991; Pape et al., 1991).

Plasma CETP levels are about 1.1-1.7 mg/l in normolipidemic males and about 25% higher in females (Marcel et al., 1990; Fukasawa et al., 1992; Ritsch et al., 1993). It has been established that most of the CETP in human plasma are bound to HDL (Moulin et al., 1994). At physiological concentrations, CETP binds to HDL particles as a monomer (Bruce et al., 1995). On agarose gel electrophoresis, a major portion of CETP is associated with pre-beta migrating HDL (Francone et al., 1989; Marcel et al., 1990). Most CETP is found in the LpA-I fraction, although the proportion of CETP in LpA-I/A-II is increased in hyperlipidemic males (Moulin et al., 1994). The distribution of CETP among lipoproteins is estimated to be 74%, 24%, and 1% associated with HDL, LDL, and VLDL, respectively, with only about 1% of CETP in free form in plasma (Nishida et al., 1993).

<u>Lipid transfer activities of CETP</u>

CETP facilitates the exchange and net mass transfer of neutral lipids between lipoproteins. During incubation of plasma, CETP mediates transfers of cholesteryl esters from HDL to VLDL and LDL in exchange for triglyceride (Nichols and Smith, 1965). CETP not only promotes the equilibration of both cholesteryl esters and triglyceride between different lipoprotein fractions, but also facilitates the exchange of these neutral lipids between particles within each lipoprotein class. Even though CETP also mediates phospholipid exchange between lipoproteins, net mass phospholipid transfer is mediated by PLTP (Tollefson et al., 1988a).

In vitro studies have shown that CETP mediates transfer of isotopically labelled cholesteryl esters and triglyceride in either direction between HDL and LDL, between HDL and VLDL and between LDL and VLDL (Barter and Jones, 1979; Barter and Lally, 1979; Nestel et al., 1979; Barter et al., 1982). Recent reports clearly indicate that CETP can promote a net movement of cholesteryl esters into HDL (Dullart et al., 1989; Fielding et al., 1989; Van Tol et al., 1991). Those reports suggested there was substantial net transfer of cholesteryl ester from LDL into HDL in normolipidemic fasting plasma. On the contrary, incubations of normal postprandial plasma (Dullart et al., 1989; Fielding et al., 1989; Castro and Fielding, 1985), or hyperlipidemic plasma of baboons (Fielding et al., 1989) and humans (Dullart et al., 1989; Van Tol et al., 1988) have demonstrated a reduction in HDL-cholesterol resulting from a net transfer of CE from HDL to LDL.

Cholesteryl ester transfer to LDL was thought to be anti-atherogenic, as protein turnover studies suggested that LDL turnover was much faster than that of HDL and therefore lipid transfer to this fraction may promote hepatic clearance of cholesteryl ester (Barter et al., 1982). However, CETP may also be proatherogenic if the net movement of cholesteryl ester into LDL exceeds the normal rate of hepatic LDL clearance, or if there is a defect in the hepatic LDL receptor function. Under both conditions the plasma LDL level would be increased. Some investigators (Tall, 1986) have suggested that low HDL-C levels may be a result of increased CETP activity, which stimulates increased transfer of cholesteryl ester from HDL to LDL.

The CETP-mediated lipid transfers is regulated by several factors, including factors which influence plasma CETP concentration, the plasma CETP inhibitor, the concentrations and compositions of donor and acceptor lipoproteins and the factors which influence the interaction of CETP with lipoproteins (Barter & Rye, 1994). Plasma CETP levels in human can be increased by dietary cholesterol (Martin et al., 1993), hyperlipidemia (Bagdade et al., 1991) and probucol therapy (McPherson et al., 1991a). On the other hand, physical exercise (Seip et al., 1993) and alcohol intake (Savolainen et

al., 1990) reduce CETP concentrations. Although several studies have reported plasma proteins of Mr 29,000 - 35,000 which inhibited CETP-mediated lipid transfers (Nishida et al., 1989; Kushwaha et al., 1993), the role and identity of CETP inhibitor proteins remains to be determined (Barter & Rye, 1994). Non-esterified fatty acids (NEFA), which are released from lipoproteins by the actions of lipases, increase the transfer of CE from HDL to VLDL and LDL and, paradoxically, decrease the transfer of TG from VLDL to HDL (Sammett & Tall, 1985; Barter et al., 1990a, 1990b). This results in reduction in the core lipid content and the particle size of the HDL (Barter et al., 1990c).

Further factors influencing the rate of the CETP-mediated lipid transfers include the concentrations and compositions of donor and acceptor lipoproteins. In vitro studies have shown that the rate of CE transfer from HDL to VLDL or LDL is increased by elevating the concentrations of both LDL and VLDL, but decreased by increasing the concentration of HDL (Barter & Jones, 1980; Barter et al., 1982). The inhibitory effect of high concentration of HDL has been suggested to be due to the high affinity of CETP for HDL; as a consequence, lipid transfers between HDL particles are increased and less CETP is available to transfer lipids between HDL and other lipoproteins (Barter & Rye. 1994). The lipid transfers mediated by CETP in vitro have also shown to be influenced by variations in the unesterified cholesterol and triglyceride contents (Sparks & Pritchard, 1989), the apolipoprotein composition (Sparks & Pritchard, 1989) and the surface charge (Nishida et al., 1993) of lipoprotein.

Under certain experimental conditions, LCAT (Nishida et al., 1990) and PLTP (Albers, 1990) have also been shown to enhance CETP-mediated transfer of CE. Studies on the effect of LCAT (Nishida et al., 1990) indicate that such enhancement is secondary to an interaction between LCAT and CETP at the lipid-water interface of lipoprotein particles; this facilitates the optimal orientation of the CETP-neutral lipid binding site and thus increases the accessibility of the binding site to CE. These studies also suggest that the

presence of apo A-I may be needed for the optimal interaction of LCAT and CETP at the lipid-water interface.

Remodelling of HDL by CETP

As mentioned above, CETP promotes the equilibrium of neutral lipids between lipoproteins. Under physiological conditions, CETP-mediated lipid transfers between the relatively CE-rich HDL and the TG-rich VLDL will result in a net mass transfer of cholesteryl esters from HDL to VLDL and a net mass transfer of triglyceride from VLDL to HDL. However, in vitro studies showed that the lipid exchange between HDL and VLDL is not on a one-mole-to-one-mole basis. Rather, the transfers of cholesteryl ester from HDL to VLDL generally exceed those of triglyceride in the reverse direction (Barter et al., 1990a). As a result, HDL depletes core lipids and in turn, reduces in particle size (Barter et al., 1990c) despite the fact that molecular volume of triglyceride is 1.5 times larger than that of cholesteryl ester (Shen et al., 1977). In fact, recent studies have shown that the number of molecules of CE lost from HDL3 to VLDL is almost three times greater than the molecules of TG it re-acquires (Liu & Bagdade, 1995).

Other studies have shown that anti-CETP monoclonal (Lagrost et al., 1990) and polyclonal (Barter et al., 1990c) antibodies are able to abolish the CETP-mediated changes to the particle size of HDL. Similar conversion in particle size by CETP has been found in studies using reconstituted HDL (rHDL) complexes, despite the fact that discoidal rHDL does not contain core lipids (Rye, 1989).

In the presence of VLDL or LDL, CETP promotes the formation of pre-beta migrating HDL (Kunitake et al., 1992a). Since pre-beta HDL is proposed to be the preferred acceptor of cellular cholesterol (Castro and Fielding, 1988), it is possible to speculate that the conversion activity of CETP may have an important role in the formation of small HDL particles that are initial cholesterol acceptors in the reverse cholesterol transport.

The role of lipolysis on the triglyceride-rich HDL has also been explored (Hopkins et al., 1985; Newnham and Barter, 1990). A synergism has been found between CETP and hepatic lipase in terms of both CE transfer and reduction in HDL particle size. This synergism is probably due to the effect of non-esterified fatty acids (NEFA) released during lipolysis, which has the paradoxical effect of increasing the CE transfer from HDL to VLDL and LDL and decreasing the triglyceride transfer from VLDL to HDL (Sammett & Tall, 1985; Barter et al., 1990a, 1990b).

In summary, CETP has the capacity to modulate the lipid composition and the particle size of HDL. Even though this is concluded from in vitro studies, it is also supported by in vivo studies as discussed in the following section.

Relationship between CETP and HDL in vivo

Cross-sectional population studies (Marcel et al., 1990) do not show any strong relationships between plasma CETP and HDL levels. However, studies on dyslipidemic subjects (Groener et al., 1989; McPherson et al., 1991b) indicate that plasma CETP levels are markedly increased in dysbetalipoproteinemia (Type III hyperlipidemia) and moderately increased in combined hyperlipidemia. In these subjects, the CETP levels can be correlated to both LDL and VLDL cholesterol levels. Elevated concentrations of triglyceride-rich lipoproteins are known to decrease plasma HDL levels and HDL particle size (Saku et al., 1985; Hong et al., 1988), possibly as the result of the action of CETP.

Investigations (Koizumi et al., 1985; Brown et al., 1989; Inazu et al., 1990; Yamashita et al., 1991) into human subjects with a genetic deficiency of CETP have suggested a link between CETP and atherosclerosis, even thought it is yet to be proven. These CETP-deficient subjects have very high concentrations of HDL cholesterol, low concentrations of LDL cholesterol and an apparent resistance to atherosclerosis. Their HDL particles are very large and are markedly enriched in cholesteryl esters. In contrast, their LDL particles are depleted of cholesteryl esters and are smaller than in normal subjects.

The in vivo effect of CETP of reducing the concentration of HDL-C has been illustrated in transgenic mice. Mice, which are naturally deficient in activity of CETP, are engineered to overexpress human (Agellon et al., 1991) or simian (Marotti et al., 1992) CETP. These mice have higher concentrations of LDL cholesterol, lower concentrations of HDL cholesterol and a reduced HDL particle size when compared to controls. They are also have a markedly increased susceptibility to diet-induced atherosclerosis.

The in vitro observation that CETP modulates the HDL lipid contents and particle size is also supported in studies of the rat, an animal which is deficient in CETP and thus have large and CE-rich HDL particles. When rats are injected intravenously with human CETP (Ha et al., 1985), cholesterol is redistributed from HDL to VLDL and LDL, with a concomitant reduction in HDL particle size.

1.4.2 Phospholipid Transfer Protein (PLTP)

Physical characteristics of PLTP

Lipid transfer activity of PLTP

Remodelling of HDL by PLTP

Physical characteristics of PLTP

PLTP (previously called LTP-II) has been isolated from human plasma (Tall et al., 1983; Albers et al., 1984; Tollefson and Albers, 1986). PLTP has a Mr of 75 000 (Jauhiainen et al., 1993) and its cDNA encodes a polypeptide of 476 amino acids (Mr 55,000) (Day et al, 1994). Human PLTP mRNA is found in many organs throughout the body (Tall, 1995). In contrast to CETP, PLTP binds heparin and is heat labile with most of the PL transfer activity lost after one hour incubation at 58 °C (Tollefson et al., 1988a).

Lipid transfer activity of PLTP

PLTP has no neutral lipid transfer activity (Tall et al., 1983; Tollefson et al., 1988a; Brown et al. 1990; Lagrost et al., 1994), but it is responsible for about half of the phospholipid transfer activity between lipoproteins and for all of the net transfer of phospholipids from VLDL to HDL. In the postprandial state, there are significant amounts of PL transferred from TG-rich lipoproteins to HDL following lipolysis of TG-rich lipoproteins. Although PLTP itself has no effect on neutral lipid transfer, in vitro studies have shown that the transfer of PL from VLDL to HDL promoted by PLTP significantly enhances the CETP-mediated transfer of cholesterol (Albers, 1990).

Remodelling of HDL by PLTP

Recently, PLTP has been found to have HDL conversion activity which is similar to that of CETP (Jauhiainen et al., 1993). In the absence of other lipoproteins, incubation of HDL3 (diameter 8.7 nm) with highly purified preparations of PLTP induces a conversion of the homogeneous population of HDL particles into two main populations of particles, one larger (diameter 10.9 nm) and one smaller (diameter 7.8 nm), indicating the phospholipid transfer between HDL particles. However, when LDL is present, the size of reconstituted HDL was reduced upon incubation with partially purified PLTP, due to the transfer of PL mass from HDL-like particles into LDL (Jonas et al., 1988). It has been concluded that the direction of PL transfer medicated by PLTP is a function of the thermodynamic gradient of the PL activity in a particular system. Antibodies to PLTP which inhibited net phospholipid transfer activity, blocked the increase in size of HDL observed in incubated plasma (Tu et al., 1993). Apparantly, PLTP may have an important role in the remodelling of HDL.

1.4.3 Lecithin:cholesterol Acyltransferase (LCAT)

Physical characteristics of LCAT

Enzymatic functions of LCAT

Substrate specificity of LCAT

Role of LCAT in reverse cholesterol transport

Remodelling of HDL by LCAT

Physical characteristics of LCAT

LCAT is a glycoprotein with a Mr of 68,000 (Albers et al., 1976) which is secreted into the plasma from the liver (Jonas, 1991). LCAT has been purified from human plasma to homogeneity (Kostner et al., 1974; Albers et al., 1976; Matz and Jonas, 1982a). LCATs from other species (rat and pig) have been recently purified and characterised (Pownall et al., 1985; Knipping, 1986; Furukawa et al., 1989; Yüksel et al., 1989) and they have similar properties to the human enzyme.

LCAT is present in normal human plasma at a concentration of about 6 mg/L (Albers et al., 1981). Most of the LCAT is associated with HDL and only a minor proportion of LCAT protein is associated with LDL (Chung et al., 1982; Francone et al., 1989; Fielding et al., 1991). Jonas (1987) and Fielding et al. (1991) showed that about equal amounts of plasma LCAT is associated with the LpA-I and LpA-I/A-II fractions. However, other investigators reported that 60-80% of the plasma LCAT is associated with LpA-I and only 10-20% of LCAT is associated with LpA-I/A-II (Chueng et al., 1986; Cheung, 1993; Duverger et al., 1993).

Enzymatic functions of LCAT

The esterification of free cholesterol in human plasma lipoproteins is enzymatically catalysed by LCAT (Glomset, 1968). LCAT catalyzes the hydrolysis of fatty acyl groups from the *sn* -2 position of phosphatidylcholine (PC) and the transesterification of the fatty acyl group to the 3-β hydroxyl group of unesterified cholesterol, generating CE and

lysophosphatidylcholine (lyso-PC). Lyso-PC is soluble and is transferred rapidly away from lipoprotein through the aqueous phase where it binds to albumin. In the absence of cholesterol, LCAT hydrolyzes PC to produce lyso-PC and non-esterified fatty acids. LCAT also has the ability to catalyze acyl transfer to lyso-PC on the surface of LDL (Subbaiah, 1986), suggesting that LCAT may play a role in the repair of oxidatively damaged PCs by re-esterifying the lyso-PC (Jonas, 1991).

Substrate specificity of LCAT

The transesterification catalysed by LCAT occurs preferentially on the surface of the HDL particle (Fielding et al., 1972). About 1% of HDL particles in plasma contain bound enzyme. The reaction of LCAT with PC is activated by apo A-I. Studies on the interaction of rHDL and LCAT have suggested that apo A-I is required for substrate orientation in discoidal HDL, functioning at the interface between phospholipid and water; this facilitates the formation of an acyl-LCAT common interface (Jonas et al., 1987). There is an important site for LCAT activation near the middle of the 243 amino acid apo A-I molecule. Apo A-I may also stabilize the CE in mature spherical HDL particles. Other apolipoproteins, such as apo A-IV, apo C-I and apo E, have significant but limited cofactor activity for LCAT compared to apo A-I (Steinmetz & Utermann, 1985).

HDL is preferred to other lipoprotein classes as the substrate for LCAT (Glomset, 1968; Marcel, 1982; Rajaram and Barter, 1985; Yen et al., 1989). Triglyceride-rich lipoproteins is almost nonreactive with LCAT in vitro (Fielding and Fielding, 1971; Rajaram and Barter, 1985). Among HDL particles, HDL3 has higher reactivity with LCAT when compared to HDL2 (Jahani and Lacko, 1982; Barter et al., 1984). Larger HDL2 appear to either be non-reactive or to inhibit the LCAT reaction probably by competing with HDL3 for the enzyme (Barter et al., 1984).

Reactivity with LCAT also varies within the HDL3 subfractions: smaller particles are more reactive than larger species (Barter et al., 1985) and the LpA-I particles are more reactive than Lpa-I/A-II (Ohta et al., 1993). Ohta et al. (1992) reported that the function of LpA-I is closely linked to the LCAT activity while that of LpA-I/A-II is not. Experiments with rHDL suggest that the difference in reactivity with LCAT among HDL subpopulations may relate to the conformation of apo A-I at the protein-lipid interface (Jonas and McHugh, 1984).

Role of LCAT in reverse cholesterol transport

It is postulated that the LCAT reaction plays a central role in reverse cholesterol transport by continuously depleting the HDL surface of free cholesterol, thereby maintaining a concentration gradient which promotes cholesterol efflux from peripheral cells to HDL (Pieters et al., 1994). Studies have been conducted to follow the transfer of isotopically-labelled cholesterol from cell membranes to plasma (Fielding, 1990). The cholesterol was first detected in plasma in very small prebeta-migrating HDL. It then appeared in larger prebeta HDL where esterification was directly demonstrated.

Through its function of producing cholesteryl esters, LCAT is involved in the transformation of nascent HDL to mature spherical HDL. In liver perfusion studies, when LCAT is inhibited, lipoproteins with this discoidal structure accumulate in the perfusate (Hamilton et al., 1976). The transformation of nascent HDL into mature HDL by LCAT has been clearly demonstrated by adding LCAT to the plasma derived from LCAT-deficient patients (Glomset et al., 1980; Glomset et al., 1983; Chen et al., 1984). HDL is present in LCAT-deficient plasma as discoidal and small spherical particles, which may represent nascent HDL because they are very similar to those secreted by human hepatoblastoma-derived Hep G2 cells (McCall et al., 1989). In the presence of exogenous LCAT, these HDL species are converted into spherical species which have comparable chemical composition to normal mature HDL (Nichols et al., 1987).

Remodelling of HDL by LCAT

LCAT has been implicated in modifying the particle size of HDL by esterifying unesterified cholesterol in the particles. In vitro incubations of HDL3 with LCAT (Rajaram and Barter, 1986) have shown that when 70% of the HDL unesterified cholesterol is esterified, the HDL cholesteryl ester content is only increased by about 10% and there are minimal changes to the particle size of HDL and no formation of particles in the size range of HDL2. However, if LDL is also present in the incubation (Rajaram and Barter, 1986; Cheung and Wolf, 1989) as an additional source of unesterified cholesterol, both the molar rate of cholesteryl ester formation and the cholesteryl ester content of HDL are markedly increased. As a result, LCAT converts HDL3 into particles of the size of HDL2 in a step-wise manner. These newly formed HDL2 are markedly enriched with cholesteryl esters and depleted of phospholipids and unesterified cholesterol.

In addition to change the lipid contents and particle size of HDL, LCAT also changes the apolipoprotein content of HDL. In particular, it has been shown that in association to the increase in HDL size, the number of molecules of apo A-I per HDL particle also increases (Nichols et al., 1985; Nichols et al., 1987). Nichols and co-worker have shown that in the presence of LDL, incubation of LCAT with rHDL causes a change in the number of apo A-I molecules in rHDL from 2 to 3 per particle. Coincidently, they found one-fourth of apo A-I have been lost and no longer associated with the rHDL. It has been presumed that the LCAT-mediated increase in the number of apo A-I molecules per particle is due to the fusion of the HDL-like particles, which is followed by the release of excess apo A-I from the fusion product.

1.4.4 Lipoprotein Lipase (LPL)

Physical characteristics of LPL
Enzymatic functions of LPL
Remodelling of HDL by LPL
Relationship between LPL and HDL in vivo

Physical characteristics of LPL

Lipoprotein lipase (LPL) and hepatic lipase (HL) are the two lipases which are found on the capillary endothelial cells and are involved in the lipoprotein metabolism in the circulation. They are part of the family of lipases which have strongly related exon structure and coding sequences. While LPL and HL hydrolyses TG in extrahepatic and hepatic vascular site respectively, pancreatic lipase hydrolyses dietary lipid in the intestine, hormone-sensitive triglyceride lipase hydrolyses the ester linkages of TG within adipocytes.

LPL (EC 3.1.1.34) is a homodimeric glycoprotein found on the luminal surface of extrahepatic vascular endothelial cells (Eckel, 1989). The binding to the vessel wall is mediated through heparan sulphate proteoglycans on the endothelial cells. 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). Heparin and lipid seem to stabilize the dimeric form; heparin displaces LPL from its binding site on the endothelium and releases it into the circulation as heparin-LPL complexes (Eckel, 1989), resulting in lipolysis of plasma lipoproteins.

LPL is not synthesized in vascular endothelial cell, but in other cells such as adipocytes, myocytes, milk-producing cells and macrophages (Olivecrona and Olivecrona, 1994). The major sites of LPL secretion are the adipose tissue, cardiac and skeletal muscle, breast tissue, kidney and lung. LPL is translocated from sites of synthesis to sites of utilization (O'Brien et al., 1994). Studies of the transport of LPL from sites of synthesis

to endothelial cells (Saxena et al., 1991) suggested that heparan sulfate proteoglycans are required. Since heparan sulfate proteoglycans are present throughout the vascular tree, the distribution of the enzyme to sites where there is no local synthesis results from the transport of LPL in the blood and the attachment of the enzyme to binding sites (Olivecrona and Olivecrona, 1994). Some LPL in the circulation is 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 is needed for effective hydrolysis of circulating chylomicrons and VLDL (Eckel, 1989; Olivecrona and Bengrsson-Olivecrona, 1990). It cleaves the 1- and 3-ester bonds of the TG molecule, producing 2-monoglycerides which are further broken down into glycerol and free fatty acids for utilisation. The fatty acids are taken up by tissues for various reasons: adipose tissue for storage, muscles for oxidation and breast tissue for secretion in milk. LPL also hydrolyzes PL and di- and mono-glycerides (McLean, 1986). Thus, LPL is responsible for the removal of both endogenous and exogenous TG from the circulation. In addition, LPL also effects the metabolism of CE-rich lipoproteins. It has been reported that it enhances the cellular uptake and degradation of LDL (Rumsey et al., 1992).

Apo C-II is required as a cofactor for LPL to function (Breckenridge et al., 1978). LPL has functional domains which include binding sites for apo C-II, membrane-bound heparin sulphate and interfacial lipids, and the catalytic site (Eckel, 1989). Many lipid-binding proteins can inhibit LPL, including apo C-III and apo E. Deficiency in LPL or its activator, apo C-II, causes massive hypertriglyceridemia (Fojo, 1992).

LPL hydrolyzes TG in plasma lipoproteins at the endothelial "binding-lipolysis" sites where the enzyme is noncovalently attached to heparan sulfate chains (Eckel, 1989). The active enzyme is a noncovalent homodimer which, when converted to the monomeric

form, loses its catalytic activity (Iverius and Östlund Lindqvist, 1976; Osborne, 1985). The degradation of LPL is in the liver (Wallinder et al, 1984; Vilaro and Llobera, 1988). A minor amount of LPL has been noted in human plasma (Olivecrona et al., 1986; Ikeda et al., 1990; Kern et al., 1990; Viella and Joven, 1991; Peterson et al., 1992); however this circulating LPL is mainly inactive and associated with remnants of chylomicrons and VLDL (Goldberg et al., 1986) or cholesterol-rich lipoproteins (Vilella et al., 1993).

Remodelling of HDL by LPL

During LPL-mediated hydrolysis of TG-rich lipoproteins, the surface lipids and apolipoproteins released, such as phospholipids and apolipoprotein C, are transferred to HDL (Patsch et al., 1978). This results in (1) the formation of discoidal complexes which under the action of LCAT may develop into mature HDL (Eisenberg and Olivecrona, 1979; Tall et al, 1979), and (2) the enlargement of pre-existing HDL with formation of HDL2 from HDL3 (Patsch et al., 1978; Taskinen and Nikkilä, 1981).

When triglyceride in TG-rich lipoproteins is hydrolysed by LPL, the availability of triglyceride for transfer into HDL in exchange for CE is reduced. Hence, the CETP-mediated lipid transfers between HDL and other lipoproteins is limited. As a consequence, the reduction in HDL particle size mediated by hepatic lipase and CETP can be prevented by LPL (Newnham et al., 1990).

Relationship between LPL and HDL in vivo

A role for LPL in HDL metabolism has been supported by in vitro evidence. There is a positive relationship between LPL activity and HDL-C, particularly HDL2-C (Taskinen and Kuusi, 1987; Tornvall et al., 1995). Similar results have been found in postheparin plasma (Kuusi et al., 1989; Blades et al., 1993). Other studies have demonstrated that the HDL concentration is related to the fractional removal rate of VLDL and there is an inverse relationship between the magnitude of alimentary lipemia and the concentration of HDL2 (Patsch et al., 1987). Furthermore, enhanced LPL activity and plasma TG

clearance produced by exercise training are also related to elevated HDL-C levels (Thompson, 1990; Podl et al., 1994). Mowri et al. (1994) found that the association between HDL2 and postprandial LPL activity is stronger in A-I/A-II-HDL2 than in A-I-HDL2, indicating a possible role for LPL in the conversion of A-I/A-II-HDL3 into A-I/A-II-HDL2.

In contrast to the above findings, it has also been reported that LPL may play a role in conversion of HDL2 to HDL3 (Deckelbaum et al., 1986). According to these studies, the TG transferred from TG-rich lipoproteins to LDL and HDL (mediated by CETP), would be susceptible to hydrolysis by LPL and HL, resulting in increased levels of small HDL (HDL3) and small LDL. In fact, recent studies have demonstrated an association between LPL activity and small dense LDL (Miesenbock and Patsch, 1992; Karpe et al., 1993).

1.4.5 Hepatic Lipase (HL)

Physical characteristics of HL
Enzymatic functions of HL
Remodelling of HDL by HL
Relationship between HL and HDL in vivo
Comparison of LPL and HL

Physical characteristics of HL

Hepatic lipase (HL) is a glycoprotein with Mr of 53,400 - 60,000 Da which is synthesized in the liver. HL resides on the luminal surface of hepatic endothelial cells, binding to heparan sulfate proteoglycans (Kuusi et al., 1979a). Even though HL is also detected in the rat ovary and adrenal (Jansen et al., 1980), mRNA studies suggest it accumulates there from the circulation rather than by direct synthesis.

Enzymatic functions of HL

Similar to LPL, HL has triglyceride lipase, monoglycerol lipase and phospholipase activities (Kuusi et al., 1982). Anti-HL antibody treatment leads to accumulation TG in VLDL, IDL, and HDL, and to the accumulation of PL and cholesterol in LDL (Goldberg et al., 1982).

HL facilitates the initial uptake of chylomicron remnants by the liver (Sultan et al., 1989; Sultan et al., 1990). HL treatment of chylomicron remnants increases exposure of apo E (Brasaemle et al., 1993), even though apo E is not essential in this uptake (Shafi et al., 1994). In vitro, HL converts larger LDL into small LDL (Musliner et al., 1979; Homma et al., 1985). When HL is deficient, the conversion of IDL to LDL is almost totally inhibited (Demant et al., 1988) and the LDL particles become enlarged and enriched in triglycerides (Goldberg et al., 1982; Auwerkx et al., 1989; Connelly, et al., 1990).

HL is necessary for the hepatic uptake of HDL TG and CE, a process which is dependent on the HL-mediated hydrolysis of HDL PL (Kadowaki et al., 1992). The addition of HL to HDL in incubation of cultured hepatocytes led to the uptake of cholesterol from HDL into the cells, presumably as the lipolysis of PL at the surface of HDL led to an increase in the effective concentration of cholesterol on the particle surface beyond that in the cell membranes (Bamberger et al., 1983). HL may also influence HDL metabolism by altering the metabolism of apo E. Supporting evidence includes the shift of apo E from VLDL to HDL during postheparin lipolysis (Blum, 1982) and the reduced clearance of TG-rich lipoprotein containing apo E in HL-deficient animals (Daggy and Bensadoun, 1986). Familial deficiency of HL in humans, which is caused by a single point mutation in the HL gene, is characterized by an increase in both VLDL remnants and TG-riched HDL (Breckenridge, 1987; Auwerkx et al., 1989).

Remodelling of HDL by HL

Several studies have implicated a direct effect of HL on the HDL subpopulation distribution. Shirai et al. (1981) demonstrated that in vitro HDL2 is the preferred substrate for HL compared to HDL3 or LDL. Mowri et al.(1992, 1994) showed that A-I/A-II-HDL_2 is a better substrate for HL than A-I-HDL_2, and that the inverse correlation of HL activity with A-I/A-II-HDL_2 is stronger than that with A-I-HDL_2. Earlier studies with HDL obtained from rabbits, a species in which activity of HL is naturally very low, have shown HL converts rabbit HDL (size and composition resembling to human HDL2) into particles comparable to human HDL3 (Clay et al., 1989). However, incubation of human HDL with HL does not lead to significant reductions in HDL particle size (Hopkins & Barter, 1986), which may be explained by the fact that human HDL are not normally enriched in triglyceride. Moreover, the fact that rabbit plasma is particularly rich in activity of CETP (Ha & Barter, 1982), which is known to promote an accumulation of triglyceride into the HDL fraction (Hopkins et al., 1985), may also explain the more pronounced effect of HL on rabbit HDL size than that of humans.

The combined effects of CETP and HL on HDL particle size have been investigated (Hopkins & Barter, 1986; Newnham & Barter, 1990). In these studies, preparations of human HDL3 were depleted of CE and enriched in TG by incubation with Intralipid and CETP. When these modified TG-enriched HDL were subsequently incubated with HL, a significant proportion of these TG was hydrolysed and there was a reduction in HDL size from a diameter of 8.6 nm to 7.4 nm (Hopkins & Barter, 1986). In subsequent studies, incubation of human plasma supplemented with additional VLDL, CETP and HL resulted in almost total conversion of the HDL fraction into very small particles with a diameter of 7.4 nm (Newnham & Barter, 1990). Coincident with a reduction in HDL size promoted by HL, a proportion of apolipoproteins is shed from the HDL particles, allowing regeneration of new HDL particles within the plasma compartment (Clay et al., 1992).

Relationship between HL and HDL in vivo

An inverse relationship has been demonstrated between HL activity and both HDL and HDL2 levels (Taskinen and Kuusi, 1987). Injection of HL antibodies into rats showed that as HL is inactivated, HDL2 and chylomicron remnants accumulate (Kuusi et al., 1979b; Sultan et al., 1990). HDL2 PL concentration is also increased (Jansen et al., 1980). This evidence is consistent with the proposition that HL is involved in the conversion of HDL2 to HDL3 through its hydrolysis of TG in the core and PL on the surface of HDL.

The virtual absence of HDL3 in human subjects with a familial deficiency of HL strongly supports the role of HL in the conversion of HDL2 to HDL3 (Demant et al., 1988). Since this rare disease has been only reported in a few families (Hayden et al., 1991), the relationship of HDL to the risk of CHD is uncertain.

Comparison of LPL and HL

The LPL activity in postheparin plasma is about the same as that of HL, about 350 mU/ml and 370 mU/ml respectively (Olivecrona and Olivecona, 1994). LPL and HL are structurally similar, have similar active sites, and both hydrolyze TG and PL. However, they select different types of lipoproteins as their substrate, eg. LPL hydrolyzes lipids mainly in VLDL, whereas HL acts mainly on HDL. This probably reflects the differences in the lipid-binding sites on the two lipases. In addition, HL seems to be more a potent phospholipase than LPL (Rojas et al., 1991; Deckelbaum et al., 1992). The other major difference between these two lipases is the requirement of apo C-II as a cofactor for LPL (Breckenridge et al., 1978); but the role of apo C-II in the activation of HL is uncertain.

In conclusion, LPL and HL seem to have partially overlapping roles in lipoprotein metabolism. LPL action can be viewed as the bulk reaction in lipoprotein metabolism, whereas HL is involved in further degradation and remodelling of remnants formed by

LPL. Low LPL activity and high HL activity alone, or in combination, have been proposed to drive plasma lipoproteins towards an "atherogenic profile" (Olivecrona and Olivecrona, 1994).

1.4.6 Summary of HDL Remodelling in Plasma

As is apparent from the previous sections, HDL undergoes continual modification in plasma in processes mediated by several enzymes and lipid transfer proteins. These plasma factors act primarily on the lipid components of HDL; the alteration in HDL lipid composition frequently leads to changes in HDL particle size. For example, the size of HDL may be increased by LCAT generating cholesteryl esters within HDL; by PLTP transferring phospholipids into HDL; by lipoprotein lipase hydrolysing TG-rich lipoproteins leading to the release and subsequent accumulation of redundant surface material in the HDL fraction. On the other hand, CETP depletes HDL core lipids by transferring CE out of HDL and results in a reduction in HDL particle size. If the HDL particles are enriched with triglyceride, HL may hydrolyses the triglyceride in HDL which in turn reduces HDL particle size.

There is evidence that changes in HDL particle size by these HDL lipid modulating factors may also alter HDL apolipoprotein content. It has been shown that in association with the increase in HDL size, the number of molecules of apo A-I per HDL particle also increases. For example, in the presence of LDL, incubation of reconstituted HDL complexes with LCAT causes an increase in the number of apo A-I molecules in the rHDL from 2 to 3 per particle (Nichols et al., 1985; Nichols et al., 1987). On the contrary, the combined effects of CETP and HL, which include transferring CE out HDL, enriching HDL with triglyceride and subsequent hydrolysing the HDL triglyceride, have been shown to not only reduce HDL particle size but also cause a proportion of apo A-I to be lost from the HDL (Clay et al., 1991; Clay et al., 1992). Although it can be concluded that HDL lipid regulating factors, such as CETP, PLTP, LCAT, LPL and HL,

cause secondary changes in HDL particle size and may also change HDL apolipoprotein contents, the conditions under which HDL changes its apolipoprotein contents are not well defined.

1.5 SCOPE OF THIS THESIS

This thesis examines in depth the effect of remodelling HDL on the metabolism of apo A-I. We propose that apo A-I cycles between HDL (lipid-associated) and a lipid-free pool in plasma. Apo A-I dissociates from HDL as HDL particles decrease in size and surface components become redundant. Once released from HDL, the apo A-I may only remain as lipid-free transiently. It could subsequently (i) enter the interstitial space and interact with cell to aquire cholesterol (Hara and Yokoyama, 1991); (ii) interact with phospholipids which are released from TG-rich lipoprotein undergoing lipolysis (Clay et al., 1992; Clay and Barter, 1996) or are transferred by PLTP (Tollefson et al., 1988b); or (iii) be excreted from the kidney (Horowitz et al., 1992). In the studies described in this thesis, it is postulated that the lipid-free apo A-I also reassociates with HDL by incorporating into the particles as they increase in size. According to this hypothesis, the apo A-I dissociation from or reassociation with HDL depends on the balance between factors which influence HDL size. By performing in vitro studies, the following are tested: (i) apo A-I dissociates from HDL under the effect of CETP following the depletion of HDL core lipid and the reduction in HDL particle size; (ii) the lipid-free apo A-I reincorporates into HDL which are increasing in size as cholesteryl esters are generated by LCAT.

The materials and methods used for experiments during the generation of this thesis are described in the following chapter. In chapter 3 the effects of incubation of HDL and CETP in the presence of VLDL and/or LDL on apo A-I are examined. It is aimed at exploring the conditions in which apo A-I dissociates from HDL and what regulates this

process. It then followed by the characterization of the dissociated apo A-I, which is presented in Chapter 4.

The work in Chapter 5 investigates whether the dissociation of apo A-I is preventable and reversible by LCAT. The reincorporation of apo A-I into HDL particles would indicate the cycling of apo A-I during the remodelling of HDL in plasma. This may imply a way in which apo A-I, the major apolipoprotein of HDL, is preserved in vivo. Other possible fates of the dissociated apo A-I, if it does occur in vivo, are also discussed.

Chapter 6 demonstrates the mechanism by which HDL apo A-I content is increased. Apart from particle fusions between HDL particles as suggested by other researchers, there is also evidence that under certain conditions, lipid-free apo A-I can directly incorporate into pre-existing HDL particles and thus increase the number of molecules per particle.

In the concluding comments (Chapter 7), the regulation of HDL apo A-I content by some plasma factors are discussed in light of the reverse correlation of CHD with plasma levels of HDL and apo A-I.

CHAPTER 2

METHODS AND MATERIALS

2.1 ISOLATION OF PLASMA LIPOPROTEINS

2.2 ISOLATION OF APOLIPOPROTEIN A-I

2.3 PREPARATION OF RECONSTITUTED HDL

2.4 PURIFICATION OF CETP

- 2.4.1 Isolation of CETP
- 2.4.2 CETP activity assay

2.5 PURIFICATION OF LCAT

- 2.5.1 Isolation of LCAT
- 2.5.2 LCAT activity assay

2.6 CHEMICAL ANALYSES

2.7 ELECTROPHORESIS

- 2.7.1 Non-denaturing gradient gel electrophoresis
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- 2.7.3 SDS-polyacrylamide gel electrophoresis
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2.8 SIZE EXCLUSION CHROMATOGRAPHY

2.9 CROSS-LINKING OF APOLIPOPROTEINS

2.10 ELECTRON MICROSCOPY

2.11 CHEMICALS AND REAGENTS

2.1 ISOLATION OF PLASMA LIPOPROTEINS

Blood was collected from healthy, normolipidemic subjects into tubes containing EDTA-Na_2 (final concentration 1 mg/ml) and placed immediately on ice. Samples for isolation of VLDL were collected from subjects who had fasted for 14 hours. Plasma was separated at 4°C by centrifugation at 2,500 r.p.m. for 20 minutes. Lipoprotein fractions were isolated from plasma in the following density intervals: <1.006 g/ml (VLDL), 1.019-1.055 g/ml (LDL), 1.019-1.21 g/ml (LDL plus HDL), 1.07-1.21 g/ml (HDL), 1.13-1.21 g/ml (HDL_3), < 1.21 g/ml (VLDL plus LDL plus HDL). Lipoprotein-deficient plasma (LPDP) was isolated as the fraction of d>1.25 g/ml. Plasma samples were adjusted to the appropriate density with solid KBr (Hatch and Lees, 1968), placed in polyallomer quick-seal tubes (Beckman Instruments, Inc. California, USA) topped up with KBr solutions of appropriate density and sealed. The tubes were ultracentrifuged in 55.2 Ti or 50.3 Ti Beckman rotors in a Beckman L8-70 ultracentrifuge at 4°C, at a speed of 55,000 rpm or 50,000 rpm. The length of ultracentrifugation depends on the density at which the samples being separated: 16 h for density <1.063 g/ml and 24-26 h for any higher density. In each case samples were subjected to two successive spins at the higher density to minimize contamination by other proteins. Supernatants and infranatants in each spin were recovered by tube slicing. All resulting mixtures were extensively dialyzed against Tris-buffered saline (TBS), pH 7.4, containing 0.01 M Tris, 0.15 M NaCl, 0.006% (w/v) NaN_3, and 0.005% (w/v) EDTA-Na_2.

2.2 ISOLATION OF APOLIPOPROTEIN A-I

The human plasma for the isolation of apo A-I, CETP and LCAT was donated by the Transfusion Service at the Royal Adelaide Hospital, South Australia. HDL was isolated from 800 ml of plasma by using a 55.2 Ti rotor at a speed of 55,000 r.p.m. in a Beckman L8-70 ultracentrifuge, with a single 16 hr spin at d1.07 g/ml and two 26 hr

spin at d1.21 g/ml. The isolated HDL were exhaustively dialyzed against a 0.01 M ammonium bicarbonate solution before delipidated (Osborne, 1986). During the delipidation, methanol was first added to the partly lyophilized HDL for 3 h before Chloroform and chilled Diethyl ether were added (methanol:chloroform:ether 1:1:3 (v/v)). The mixture was chilled at -20°C for 30 min, then subjected to centrifugation at 1500 rpm, 4°C for 10 min. The infranatant was subjected to further delipidation using ethanol and chilled ether (1:4 v/v). The latter procedure was repeated 3 times, followed by drying the apo HDL pellet under nitrogen. The resulting apo HDL was subsequently dissolved in 20 mM Tris, pH 8.2, freeze dried, and stored at -20°C before being subjected anion-exchange chromatography for further separation of apolipoproteins.

The anion-exchange chromatography was performed on a 2.6 x 24.0 column of Q Sepharose Fast Flow (Pharmacia LKB Biotechnology, Uppsala, Sweden) attached to a Fast Performance Liquid Chromatography (FPLC) system. The apo A-I and apo A-II were resolved by a modification of the method of Weisweiler (1987). The apo A-I and apo A-II, each of which appeared as a single band after electrophoresis on a 20% SDS-polyacrylamide gradient gel stained with Coomassie R 350. The purified apolipoproteins were dialysed against 0.01 M ammonium bicarbonate (3 x 5 L), lyophilized and stored at -20°C. Prior to use they were reconstituted in 0.01 M Tris-HCl, 3.0 M guanidine-HCl, 0.01% (w/v) EDTA-Na_2 (pH 8.2) for 1-2 hr and then exhaustively dialysed against TBS (5 x 1 L).

2.3 PREPARATION OF RECONSTITUTED HDL

Discoidal rHDL were prepared by the cholate dialysis method from egg PC, unesterified cholesterol, and apo A-I (Matz and Jonas, 1982), at a molar ratio of 89: 10: 1 (PC : UC : apoA-I). Cholesterol was dissolved in chloroform at a concentration of 10 mg/ml. The cholate solution (30 mg/ml) was prepared by dissolving sodium cholate in TBS, pH 7.4. The mixing of lipids and apo A-I was performed in test tubes. Each preparation included

10 to 20 tubes, depending on the amount of rHDL required. As the first step of the procedure, 6.32 μmol of egg PC and 0.71 μmol of cholesterol were drawed into each test tube before being dried under nitrogen. The cholate (6.32 μmol) was then added. TBS, pH7.4 was also added to bring the volume of the mixture to about 0.5 ml. The test tubes were kept in ice and vortexed every 10-15 minutes until the mixture became clear (in 1-2 hours). Apo A-I solution, in an amount equivalent to 0.071 μmol (2 mg) of apo A-I, was added into each test tube which was left in ice for another 2-3 hours. The lipid-cholate-apo A-I mixtures from each test tubes were pooled and dialysed against 5x1 L TBS (pH7.4) over 5 days to remove the cholate. In order to obtain a homogeneous population of discs which are small in size, the mixture was further incubated with LDL, at a protein ratio of 4:1 (apoB : apo A-I). This incubation led to the loss of phospholipids from the rHDL discs to LDL (Jonas et al., 1988), and a reduction in their size. The resulting discs were then isolated from LDL by ultracentrifugation and subjected to size exclusion chromatography. Fractions containing particles of diameter 7.6-7.8 nm were pooled and used in experiments.

2.4 PURIFICATION OF CETP

2.4.1 Isolation of CETP

Each batch of CETP was purified from 2 L human plasma. Plasma proteins were precipitated between 35 and 55% saturation of ammonium sulfate and suspended in 250 ml Milli Q water. This fraction was dialysed against 5L of Milli Q water before being subjected to ultracentrifugation at a density of 1.25 g/ml. The d>1.25 g/ml fraction was subjected to hydrophobic interaction chromatography on a XK 50/60 column containing Phenyl-Sepharose 6 Fast Flow (high substitution) (Pharmacia Biotechnology AB), which had been pre-equilibrated with 1L of 3M NaCL. Unbound proteins were eluted with 0.15M NaCl. The bound hydrophobic proteins were eluted at a flow rate of 10 ml/min with Milli Q water/0.02% NaN_3/0.01% EDTA-Na_2, pH10. Active fractions were pooled and left in 50 mM NaOAc by adding 0.5M NaOAc, pH4.5. The resultant fine precipitate

(denatured proteins) was removed by low speed centrifugation. The supernatant was subjected to cation exchange chromatography on a XK 26/40 column containing CM (Carboxy-Methyl)-Sepharose Fast Flow (Pharmacia Biotechnology AB), which had been pre-equilibrated with 500 ml of 50 mM NaOAc, pH4.5. CETP was eluted from the column at a flow rate of 10 ml/min with a linear 0-0.4 M NaCl at 50 mM NaOAc, pH4.5. Fractions were promptly dialysed against 3X5 L 20 mM Tris, pH7.4. Fractions were then assayed for CETP activity and active fractions were pooled. This pool was subjected to chromatography on a Mono Q HR 5/5 anion exchange column (Pharmacia Biotechnology AB), which had been pre-equilibrated with 10 ml 20 mM Tris, pH7.4. CETP was eluted with the same buffer with a 0-0.5 M NaCl gradient at a flow rate of 1 ml/min. Active fractions were pooled and stored at -70°C in 1 ml aliquots. The CETP appeared as a single band after electrophoresis on a 20% SDS-polyacrylamide gradient gel stained with Coomassie R 350.

2.4.2 CETP activity assay

The CE transfer activity of the purified CETP was determined by measuring its capacity to promote the transfer of radio-labelled CE ([^{3}H] CE) from HDL_3 to LDL (Tollefson et al., 1988b). Ten µl [^{3}H] HDL, 10 µl LDL and 20 µl CETP sample were incubated in the presence of 10 µl DTNB (to inhibit LCAT activity). TBS (pH7.4) was added to make up the total volume to 175 µl and the incubation was conducted at 37°C for 3 h. At the end of incubation, LDL were precipitated with heparin and $MnCl_2$ (Burstein et al., 1970). The non-transferred [^{3}H] CE in HDL remained in the supernatant after a 5 min centrifugation at 1500 g. A 200 µl aliquot of the supernatant was placed into 10 ml of aqueous scintillation cocktail (Ready Safe, BECKMAN, USA) and counted in a BECKMAN LS6000 TA liquid scintillation system for 2 min. CETP activity was expressed as arbitrary units, where one unit is the amount of transfer activity contained in 1 ml of a preparation of pooled human lipoprotein-deficient plasma.

2.5 PURIFICATION OF LCAT

2.5.1 Isolation of LCAT

Leithin:cholesterol acyltransferase was purified by the method described by Mahadevan and Soloff (1983) with some modifications. Two litres of human plasma were initially subjected to precipitation at 35% saturation of ammonium sulfate. Citric acid was added to the supernatant for precipitation of proteins. This precipitate was suspended in 200 ml of H_2O and the pH of the sample was raised to 7.4 by the addition of a saturated solution of Na_2CO_3. It was then dialysed against 2x5 L of H_2O before being ultracentrifuged at a density of 1.25 g/ml. The conditions for this ultracentrifugation was as for HDL isolation described in Section 2.1. The resulting d>1.25 g/ml (lipoprotein-deficient) fraction was recovered and subjected to chromatography in Phenyl-Sepharose 6 Fast Flow column (XK 50/60, high substitution) (Pharmacia Biotechnology AB), which had been pre-equilibrated with 1L of 3M NaCL. After washing the column with 0.15M NaCl, LCAT was eluted with Milli Q water at a flow rate of 10 ml/min. Active fractions were pooled and dialysed against 2x5L of 20 mM Tris, pH7.4, containing 1 ml β-mercaptoethanol/L. The same buffer was used to pre-equilibrate a DEAE-Fast Flow column (Pharmacia Biotechnology AB). The active pool was loaded to this column and eluted with 32% of buffer 20 mM Tris/500mM NaCl, pH7.4, containing 1 ml β-mercaptoethanol/L. Ten ml fractions were collected at a flow rate of 10 ml/min. All chromatographic steps were carried out at room temperature on a fast protein liquid chromatography (FPLC) system (Pharmacia Biotechnology AB). Samples from each fraction was run on SDS gel followed by silver stained to check the purity of the isolated LCAT. A band of Mr 68,000 was found predominant on the gel. Active fractions were mixed with fatty acid free BSA (BSA final concentration 1 mg/ml) and stored in 10 ml aliquots at -80°C.

2.5.2 LCAT activity assay

Activity of LCAT was determined as described by Piran and Morin (1979) using rHDL discs (molar ratio egg-PC:cholesterol:apo A-I 89:10:1) pre-labelled with [^{3}H] cholesterol.

The final concentration of unesterified cholesterol 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 1:10 β-mercaptoethanol, and 55 µl of TBS, pH7.4, were pre-incubated at 37°C under N_2 for 30 min. LCAT samples (12 µl) were added and then further incubated at 37°C under N_2 for 1h. The reaction was stopped by addition of 0.5 ml of 1% digitonin in 95% ethanol. The mixture was vortexed for 15 sec to denature the proteins and extract lipids. Excess cholesterol (25 µl of 5 mg/ml unesterified cholesterol in ethanol) was then added and the mixture was vortexed again. The labelled cholesterol-diginitoinde, along with denatured proteins, was sedimented by centrifugation at 1500 g for 10 min. A 400 µl aliquot was taken from the supernatant which contained the labelled esterified cholesterol. This aliquot was added directly to 10 ml of aqueous scintillation cocktail (Ready safe, BECKMAN, USA) and counted in a BECKMAN LS6000 TA liquid scintillation system for 2 min. LCAT activity was expressed as nmol CE formed/ ml LCAT/h.

2.6 CHEMICAL ANALYSES

All chemical assays were performed on a Cobas Fara Centrifugal Analyzer (Roche Diagnostics, Zurich, Switzerland). Concentrations of total cholesterol, free cholesterol and phospholipid were measured using enzymatic kits from Boehringer Mannheim (Germany). The concentration of esterified cholesterol was calculated as the difference between the concentration of total (esterified plus free) cholesterol and free cholesterol. Protein concentrations were measured using the method of Lowry et al. (1951) adapted for use on the Cobas Fara (Clifton et al., 1988) and using bovine serum albumin as a standard. Concentrations of apo A-I, apo A-II and apo B were measured immunoturbidmetrically at appropriate dilutions using sheep anti-human apo A-I antiserum and calibration serum from Boehringer Mannheim (Germany). The reagent for all the apolipoprotein assays was a solution of saline (0.9% (w/v) NaCl) containing 40% (v/v) polyethylene glycol 600 (Boehringer Mannheim) and 0.025% (v/v) buffer concentrate (Behring).

2.7 ELECTROPHORESIS

2.7.1 Non-denaturing gradient gel electrophoresis

Particle sizes of HDL and rHDL were determined by electrophoresis on 3-35% non-denaturing polyacrylamide gels (Gradipore, Australia), using a Gel Electrophoresis Apparatus (Pharmicia C500/400). Samples were pre-mixed with a solution containing 40% (w/v) sucrose and 0.01% (w/v) bromo-phenol blue before applied onto the gel. The electrophoresis was carried out in Tris (0.09M)-Borate (0.08M)-EDTA-Na2 (0.003M) buffer, pH8.4, at 150-180 volt for a total of 3000 volt-hours. A calibration standard (High Molecular Weight Electrophoresis Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden) containing thyroglobulin (Stokes' radius 8.50 nm), ferritin (6.1 nm), lactate dehydrogenase (4.08 nm) and bovine serum albumin (3.55 nm) was also subjected to electrophoresis for later calculation of particle size.

When electrophoresis was completed, the gels were put into 10% (w/v) sulphosalicylic acid for 1 hour to fix the protein (hence the whole particle) on the gel. The gels were stained for 3 h in 0.04% (w/v) Coomassie G-250 in 3.5% (v/v) perchloric acid and then de-stained in 5% (v/v) acetic acid for 16-24 h. Scanning of gels were performed with an Ultroscan XL laser densitometer (LKB, Bromma, Sweden) attached to a computer installed with the GelScan XL Software Package (Pharmacia LKB Biotechnology). The HDL populations were resolved into peaks and the relative area of each peak was estimated. Assuming an inverse logarithmic relationship between particle size and migration distance on the gel, the particle size of each population was determined by comparing the relative migration of the peak with that of the known protein standards.

2.7.2 Immunoblot analysis

After non-denaturing gradient gel electrophoresis as described above, without fixing and staining the gel, the separated proteins were electrophoretically transferred from the gel to

nitrocellulose membranes (pore size 0.45 μm, Hybond-C extra, Amersham, Netherlands) on a Multiphore II electrophoresis unit (Pharmacia Biotechnology AB, Uppsala, Sweden). The anodic transfer buffer was 0.025 M Tris, 0.2 M glycine, and 20% (v/v) methanol, pH 8.3. The cathodic transfer buffer was 0.025 M Tris, 0.2 M glycine, and 0.003 M SDS, pH 8.3. The transfer was carried out at 90 mA for 8 h at room temperature. After transfer, the membrane was kept at 4°C overnight in 0.2 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.02% (w/v) NaN_3, and 0.01% (w/v) EDTA-Na_2 (phosphate-buffer saline, PBS), with 0.1% (v/v) tween 20 (PBS-T) to block unbound membrane sites. The membrane was then incubated for 1.5 h at 37°C with sheep anti-human apo A-I antiserum (Boehringer Mannheim, Germany) diluted 1:300 with PBS-T. After washing with PBS-T (3 x 10 min), the membrane was incubated for 1 h at 37°C with donkey anti-sheep and goat-IgG conjugated to horseradish peroxidase (Silenus Laboratories Pty. Ltd., Hawthorn, Australia) diluted 1:500 with PBS-T. The membrane was then washed with PBS-T (2 x 10 min), and finally in PBS alone for 10 min. Human apo A-I was detected by treating the membrane with 0.1 M sodium acetate, pH 5.0, containing 0.6% (v/v) hydrogen peroxide, 5% (v/v) dimethylformamide, and 0.1% (w/v) 3-amino-9-ethyl carbazole.

2.7.3 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed either on the PhastSystem (Pharmacia Biotechnology AB, Uppsala, Sweden) or carried in the same apparatus as for non-denaturing gel electrophoresis but using SDS buffer. In the PhastSystem, 20% homogenous polyacrylamide gel and SDS buffer strips (Pharmacia) were used. For molecular weights references, a calibration kit for low molecular weight proteins (Pharmacia Biotechnology AB, Uppsala, Sweden), including Phosphorylase b (subunit MW 94,000), Bovine Serum Albumin (67,000), Ovalbumin (43,000), Carbonic Anhydrase (30,000), Soybean Trypsin Inhibitor (20,100) and a-Lactalbumin (14,400), was used on the electrophoresis. The electrophoresis at the PhastSystem was for 95 volt hours. The gel was stained with 0.1% (w/v) PhastGel Blue R (350) (Pharmacia)

containing 10% (v/v) acetic acid, destained with methanol: acetic acid: H_2O (30:10:60 v/v/v) and preserved with glycerol: acetic acid: H_2O (10:10:80 v/v/v).

For cross-linking studies, SDS-polyacrylamide gel electrophoresis was carried out on 3-35% polyacrylamide gels (Gradipore, Australia), using a SDS buffer containing 0.04M Tris, 0.02M sodium acetate, 0.002M EDTA-Na_2, 0.2% SDS, pH7.4. Before the samples were loaded, the gel was pre-equilibrated in the buffer for 1 hour at 70 volts. The electrophoresis was at 300 volts for 10 min, followed by 30-60 min at 150 volts. The gel was fixed in isopropanol: acetic acid: H_2O (25:10: 65, v/v/v) for 1 hour, stained overnight with 0.1% (w/v) Coomassie Blue R-250 containing 25% (v/v) methanol and 10% (v/v) acetic acid. For destaining the gel, a solution of methanol: acetic acid: H_2O (25: 10: 65, v/v/v) was used.

2.7.4 Agarose gel electrophoresis

Electrophoresis of lipoproteins and apolipoproteins on agarose gel was carried out in a Bio-Rad Mini Sub gel electrophoresis system (Hercules, CA). The running buffer, Barbital Buffer (pH8.6), contained 10 mM/L barbitone and 50 mM/L barbitone sodium. The 0.6% agarose gel was prepared by dissolving (by heating) 0.18 g agarose in 30 ml running buffer. When cool, but still in solution, the mixture was poured into a gel tray where a sample well template was placed. It was left at room temperature for about 25 min until the gel became firm, and then left standing at 4°C for 5 min. Ten to 15 µl samples (containing about 20 µg protein) were pre-mixed with tracking dye (40% (w/v) sucrose and 0.01% (w/v) bromo-phenol blue) before being placed into sample wells on the gel. The electrophoresis was run at 100 volt for 1 h at room temperature. The gel was fixed for 10 min with a 60/30/10 (v/v/v) ethanol/water/acetic acid solution, stained (30 min), destained (overnight) and scanned as for non-denaturing gradient gel. The electrophoretic mobility of particles was calculated by dividing the electrophoretic velocity

(migration distance (μm) / Time (s)) by the electrophoretic potential (voltage (v) / length of gel (cm)) (Sparks and Phillips, 1992).

$$\text{Mobility} = \frac{\text{Migration distance } (\mu m) \ / \ \text{Time (s)}}{\text{Voltage (v)} \ / \ \text{Length of gel (cm)}}$$

2.8 SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography was used to separate lipoprotein particles of different size. Samples were loaded in a volume of 200 μl (containing 1-2 mg protein) onto a prepacked Superose™ 6 HR 10/30 column (Pharmacia-LKB, Uppsala, Sweden) connected to FPLC system. Lipoproteins were eluted at room temperature with TBS, pH7.4, at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and stored at 4°C for subsequent analyses.

2.9 CROSS-LINKING OF APOLIPOPROTEINS

The number of apo A-I molecules associated with rHDL was determined by cross-linking (Staros, 1982; Rye, 1989). Apolipoproteins on the intact rHDL particles were cross-linked with the bifunctional cross-linking reagent bis (sulfosuccinimidyl) suberate (BS) (Pierce Chemical Co., Rockford, IL). The monomer of lipid-free apo A-I and its self-associated oligomers were used as standards. All samples were adjusted to a protein concentration of 0.5 mg/ml and dialysed against 50mM sodium phosphate buffer, pH7.4. A solution of 10mM Bis (sulfosuccinimidyl) suberate (BS) was prepared by dissolving the powder in the same phosphate buffer. After the dialysis, 200 μl of the sample was mixed with 50 μl BS and left at room temperature for 30 min. The mixture was then dialysed overnight against 1 L of SDS sample buffer, containing 0.01M Tris, 0.001M EDTA-Na_2, and 1% (w/v) SDS, pH8.0. The samples were then incubated at 37°C for 45 min before a 20 μl aliquot was applied to SDS-polyacrylamide gel electrophoresis as

described in section 2.6.3 of this chapter. The gel was scanned and the number of apo A-I molecules per rHDL particle was determined by referring to the migration positions of monomers and self-associated oligomers of apo A-I.

2.10 ELECTRON MICROSCOPY

Electron microscopy was performed as described previously (Rye, 1990) with minor modification. Samples were diluted to a protein concentration of 0.1 mg/ml with a solution containing 125mM ammonium acetate, 2.6mM ammonium hydrogen carbonate, and 0.26mM EDTA-Na_2, pH7.4. The samples were dialysed against 2X1 L of the same buffer and then negatively stained with 2% (w/v) sodium phosphotungstate (pH7.4) by placing equal volumes of sample and stain on a Butvar-coated 300 mesh copper grid (Probing & Structure Pty. Ltd., Australia). The sample and stain were drawn off with filter paper after a few minutes, leaving a thin film which was dried at room temperature prior to examination under the electronmicroscope (Phillips CM200 Transmission Electron Microscope).

2.11 CHEMICALS AND REAGENTS

Acetic acid glacial	BDH Chemicals 10001
Agarose	Sigma Chemicals A-6013
3-Amino-*9*-Ethylcarbazole	Sigma Chemicals A-5754
Albumin, bovine, essentially fatty acid free	Sigma Chemicals A-6003
Ammonium acetate	Ajax Chemicals D3247
Ammonium hydrogen carbonate	BDH Chemicals 10302
Ammonium sulphate	BDH Chemicals 10033
Anti-human-apolipoprotein-A-I-antiserum	Boehringer Mannheim 726478
Anti-human-apolipoprotein-A-II-	

antiserum	Boehringer Mannheim 726486
Anti-human-apolipoprotein-B-antiserum	Boehringer Mannheim 726494
Anti-sheep/Goat Ig. Affinity isolated HRP conjugated raised in Monkey	Silenus Lab. UAH
Barbitone	BDH Chemicals 10415
Barbitone sodium	BDH Chemicals 10365
Boric acid	BDH Chemicals 10058
Bromophenol blue	BDH Chemicals 44305
Buffer concentrate for apolipoprotein determination	Behring OUEC 40/41
Calibration serum for apolipoprotein	Boehringer Mannheim 837237
Calibrator for automated systems	Boehringer Mannheim 759350
Chloroform	BDH Chemicals 10077
Cholesterol (5-cholesten-3β-ol)	Sigma Chemicals C-8667
[1a,2a(n)-^{3}H] Cholesterol	Amersham International TRK 330 B70
Cholesterol reagent (CHOD-PAP)	Boehringer Mannheim 1442341
Cholesterol standards	Boehringer Mannheim 709905
Cholic acid (sodium salt)	Sigma Chemicals C-1254
Citric acid	BDH Chemicals 10081
Coomassie brilliant blue G-250	Bio-Rad 161-0406
Coomassie brilliant blue R-250	Bio-Rad 161-0400
Coomassie brilliant blue R-350 (PhastGel Blue R-350)	Pharmacia 17-0518-01
Diethyl ether	BDH Chemicals 10094
Digitonin	Sigma Chemicals D-5628
Dimethylformamide	BDH Chemicals 10322
DTNB (5,5'-Dithio-bis[2-nitrobenzoic acid])	Sigma Chemicals D-8130
EDTA-Na_2 (Ethylenediaminetetra-acetic acid disodium salt)	BDH Chemicals 10093

Egg PC (L–α-Phosphatidylcholine)	Sigma Chemicals P-5388
Ethanol	BDH Chemicals 10107
Folin & Ciocalteu's phenol reagent	Sigma Chemicals F-9252
Formaldehyde sodium	BDH Chemicals 10113
Free cholesterol reagent	Boehringer Mannheim 310328
Glutaraldehyde	Ajax Chemicals 698
Glycerol	BDH Chemicals 10118
Glycine	Ajax Chemicals 1083
Guanidine (aminomethanamidine) hydrochloride	Sigma Chemicals G-3272
Heparin (sodium salt)	Sigma Chemicals H-9399
Hydrogen peroxide	BDH Chemicals 10366
Manganous chloride	Ajax Chemicals 1127
2 -mercaptoethanol	Merck-Schuchardt 805740
Methanol	BDH Chemicals 10158
Perchloric acid	BDH Chemicals 101764
Phospholipids reagent	Boehringer Mannheim 691844
Polyethylene glyco 6000	Boehringer Mannheim 240907
Potassium bromide	BDH Chemicals 10195
Precinorm L (Special control serum for lipids	Boehringer Mannheim 781827
Propan-2-ol (Iso-propyl alcohol)	BDH Chemicals 10224
Silver nitrate	Ajax Chemicals 449
Sodium acetate	BDH Chemicals 10236
Sodium azide	Sigma Chemicals S-2002
Sodium bromide	BDH Chemicals 30116
Sodium carbonate	BDH Chemicals 10240
Sodium chloride	BDH Chemicals 10241
Sodium dodecyl sulphate (SDS)	BDH Chemicals 44244

Sodium hydrogen carbonate	BDH Chemicals 10247
Sodium dihydrogen orthophosphate	BDH Chemicals 10245
di -Sodium hydrogen othophosphate	BDH Chemicals 10249
Sucrose	BDH Chemicals 10274
5 -Sulphosalicylic acid	BDH Chemicals 103464
Triglycerides reagent	Boehringer Mannheim 877557
Tris (Tris[hydroxymethyl]amion-methane)	Sigma Chemicals T-1378
Tungstophosphoric acid	BDH Chemicals 10287
Tween-20	BDH Chemicals 66368

CHAPTER 3

DISSOCIATION OF APO A-I FROM HDL DURING LIPID TRANSFER MEDIATED BY CETP

3.1 INTRODUCTION

3.2 METHODS

3.3 RESULTS

- Evidence that apo A-I dissociates from HDL
- Factors influencing the dissociation of apo A-I from HDL
  - *Duration of incubation*
  - *Concentration of CETP*
  - *Concentration of VLDL*
  - *Concentration of LDL*
  - *Concentration of HDL*

3.4 DISCUSSION

3.1 INTRODUCTION

The protective effect of HDL against CHD (Rubin et al., 1991; Plump et al., 1994) has been suggested due to its involvement in the pathway of reverse cholesterol transport. Since most of the tissue in the body are exposed to interstitial fluid rather than plasma, the transfer of tissue cholesterol to plasma HDL must be mediated by particles which are small enough to enter the interstitial space to pick the cholesterol. In fact, evidence exists that cellular cholesterol is delivered initially to a particles which contain apo A-I as its sole protein (Castro and Fielding, 1988). They differ from plasma HDL in that they are much smaller in size and that they possess a prebeta rather than an alpha mobility on agarose gel electrophoresis (Castro and Fielding, 1988). The origin of these primary acceptors of cellular cholesterol is not clear. One of the possibilities is that they are generated from apo A-I which dissociates from the plasma HDL fraction.

In chapter one, the remodelling of HDL which takes place during their circulation in plasma has been discussed. Several factors are known operating in plasma to modify the lipid composition of HDL. These include LCAT which catalyses the esterification of cholesterol in HDL (Glomset, 1968), CETP which transfers cholesteryl esters from HDL to other lipoproteins in exchange for either triglyceride or cholesteryl esters (Barter et al., 1982) and hepatic lipase which hydrolyses HDL triglyceride and phospholipids (Shirai et al., 1981). These factors all have substantial effects on the concentration and composition of HDL lipids and on HDL particle size (Hopkins et al., 1985; Rajaram and Barter, 1986; Clay et al., 1990). Under certain circumstances, the remodelling of HDL results in the reduction in HDL particle size following the loss of a substantial proportion of their lipids and a proportion of their main protein constituent, apolipoprotein (apo) A-I (Clay et al., 1991; Clay et al., 1992).

The dissociation of apo A-I from HDL has been recently described in studies in which human plasma was supplemented with exogenous CETP, hepatic lipase and VLDL and

incubated in vitro at 37°C (Clay et al., 1991). These incubation conditions led to a reduction in HDL particle size and a coincident dissociation of a proportion of the apo A-I from the HDL fraction. The dissociation of apo A-I was maximal after two to three hours of incubation. However, with continuation of the incubation beyond three hours, the dissociated apo A-I progressively returned to the HDL density range, such that after eight hours virtually all of the apo A-I was again recovered in the HDL fraction (Clay et al., 1992). While the mechanism responsible for this reversibility was not determined, the simple fact that it occurred meant that the process of dissociation could not be investigated in isolation from the metabolic events which led to the return of the dissociated apo A-I to the HDL density range.

In the present study we investigate the dissociation of apo A-I under conditions where it is shed from HDL and is not further metabolized. Under these circumstances the apo A-I is recoverable in a form identical to that in which it dissociates. The results presented in this chapter were from the investigation into factors which regulate the dissociation of apo A-I from HDL. Whereas the studies of the characterization of the dissociated apo A-I is presented in Chapter 4.

3.2 METHODS

Isolation of lipoproteins

Plasma was obtained from healthy, normolipidemic subjects, as described in the general methods. Samples for isolation of VLDL were collected from subjects who had fasted for 14 h as the supernatant after ultracentrifugation at a density of 1.006 g/ml (d<1.006 g/ml). The fractions of density 1.019-1.21 g/ml (containing a mixture of LDL and HDL), 1.019-1.055 g/ml (containing LDL) and 1.13-1.21 g/ml (containing HDL3) were isolated ultracentrifugally as described in the Chapter 2. After their isolation all lipoprotein fractions were dialysed extensively against Tris-buffered saline, pH 7.4

(TBS) containing 0.01 M Tris, 0.15 M NaCl, 0.006% (w/v) NaN3 and 0.005% (w/v) EDTA.

Incubations

Incubation mixtures were placed in sealed plastic tubes and either kept at 4 ^{o}C or incubated at 37 ^{o}C in a shaking water bath. The incubations were terminated by placing the tubes on ice. At the end of the incubations, an aliquot of each mixture was adjusted to d 1.25 g/ml with KBr and subjected to ultracentrifugation at 4 ^{o}C at 100,000 rpm for 16 h in a Beckman 100.2 rotor in a Beckman TL-100 ultracentrifuge. The fraction of d<1.25 g/ml fraction was recovered and assayed for lipids and apolipoproteins. HDL particle size distribution in the d<1.25 g/ml fraction was determined by nondenaturing polyacrylamide gradient gel electrophoresis. In some experiments an aliquot of the total incubation mixture was subjected directly to non denaturing polyacrylamide gradient gel electrophoresis and immunoblotted for apo A-I. Aliquots of incubation mixtures were also subjected to size-exclusion chromatography before 0.5 ml fractions were collected and assayed for apo A-I, apo A-II, total cholesterol, and phospholipids.

Statistical analyses

All statistical analyses were performed using Student's t test for paired samples. The accepted level of significance was taken to be $P < 0.05$.

3.3 RESULTS

Evidence that apo A-I dissociates from HDL

Dissociation of apo A-I from HDL was demonstrated by non-denaturing gradient gel electrophoresis followed by immunoblot analysis for apo A-I (Fig. 3-1), by size-exclusion chromatography (Fig. 3-2) and by ultracentrifugation (Table 3.1).

Mixtures of human LDL and HDL (the plasma fraction of d 1.019-1.21 g/ml) were either kept at 4°C or incubated at 37°C in the absence or presence of CETP. After the incubation, an aliquot of each mixture was subjected to gradient gel electrophoresis without prior ultracentrifugation. The particle size distribution of apo A-I, as determined by immunoblot analysis, is shown in Fig.3-1. In the samples kept at 4°C (Fig. 3-1, track A) or incubated at 37°C in the absence of CETP (Fig. 3-1, track B), the apo A-I was identified only in particles within the size range of HDL_2 and HDL_3. By contrast, in the samples incubated at 37°C with CETP (Fig. 3-1, Track C), not only was most of the apo A-I now associated with a subpopulation of HDL that was smaller than the original HDL_3 but a proportion of the apo A-I migrated considerably further than albumin (Stokes' diameter = 7.1) to a position identical to that of purified, lipid-free apo A-I (Fig. 3-1, Track D).

In other experiments, mixtures of HDL and LDL were incubated for 24 h at 37 °C in the absence or presence of CETP and then separated by size exclusion chromatography. Figure 3-2 shows the elution profiles of apo A-I, apo A-II, phospholipids, and cholesterol in a representing experiment. In samples incubated in the absence of CETP (Fig. 3-2, Control), apo A-I co-eluted from the column with cholesterol, phospholipid, and apo A-II. The highest concentration of each constituent was in fraction 27. After incubation with CETP, the elution of all HDL constituents was delayed, consistent with reduction in HDL size as shown by gradient gel electrophoresis (Fig. 3-1). Whereas the phospholipid, cholesterol, and apo A-II still co-eluted from the column (peak in fraction 28), the elution of apo A-I was delayed even further and did not peak until fraction 30. A significant proportion of the apo A-I was recovered in fractions 31 and 32, which were essentially free of lipids and apo A-II. As a consequence, the molar ratios of phospholipid-apo A-I and of cholesterol-apo A-I were close to zero in fraction 31 and 32. (Note that albumin elutes from this column with a peak between fractions 29 and 30).

Fig. 3-1. Photograph of an immunoblot showing the size distribution of apo A-I. The human plasma fraction of d 1.019-1.21 g/ml was incubated under various conditions before being subjected (without prior ultracentrifugation) to gradient gel electrophoresis on 3-40% polyacrylamide gels as described under Chapter 2. The size distribution of apo A-I was determined by immunoblot analysis as described. Track A shows a sample kept at 4 ^{o}C. Track B shows the effects of incubating the sample (895 nmol total cholesterol and 114 µg apo A-I in a volume of 123 µl) at 37 ^{o}C for 24 h. Track C shows the effects of incubation in the presence of CETP at a final concentration of 4.4 units/ml. Track D shows a sample of purified apo A-I. Numbers refer to the Strokes' diameters of high-molecular-weight protein standards.

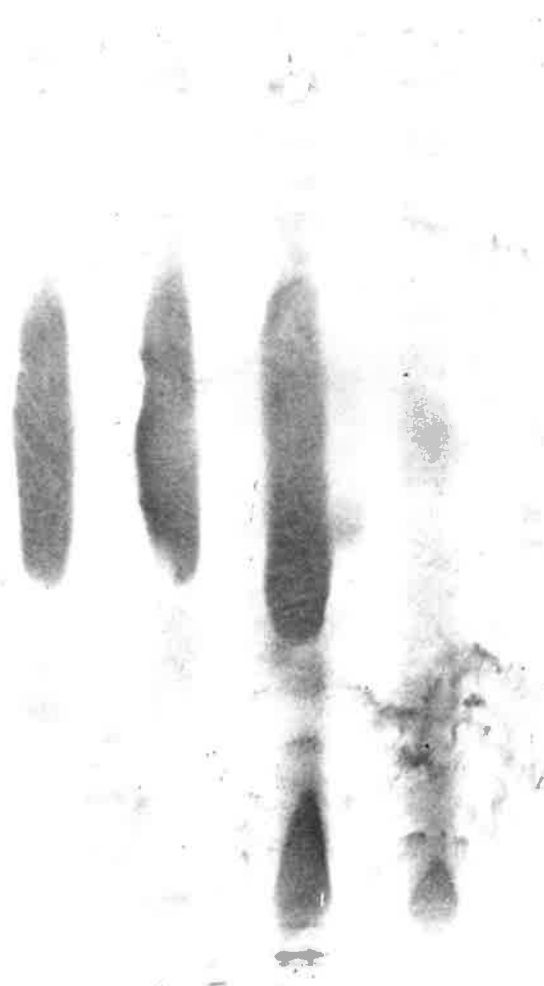
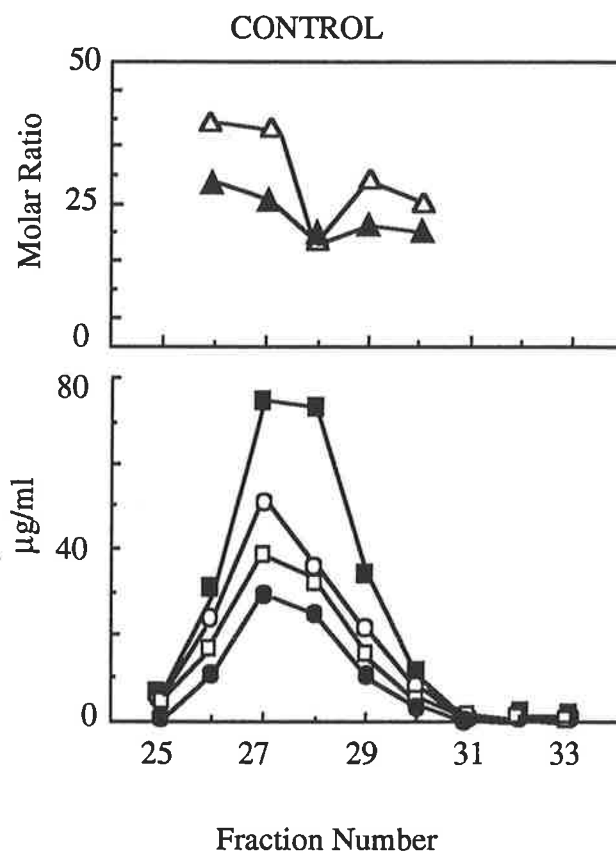

A
B
C
D
17.0
12.2
8.16
7.10

Fig. 3-2. Fractionation of HDL by size-exclusion chromatography. The plasma fraction of d 1.019-1.21 g/ml (993 nmol total cholesterol and 170 µg apo A-I in a final volume of 0.6 ml) was incubated for 24 h at 37 °C in the absence (Control) or presence (CETP) of CETP (final concentration 4.8 units/ml). Incubations were stopped by placing samples on ice. Mixtures were then subjected to size-exclusion chromatography on a Superose 6HR 10/30 column. The lipoproteins were eluted with TBS at a flow rate of 30 ml/h and fractions of 0.6 ml were collected and assayed for apo A-I, apo A-II, total cholesterol, and phospholipids. As apo A-I and apo A-II were measurable only in fractions 25-32 and as the recoveries of each apolipoprotein from the column were 85-90%, only fractions 24-33 are shown. The lower panels show the distribution of apo A-I (—■—), apo A-II (—●—), total cholesterol (—□—), and phospholipids (—○—). The upper panels show the molar ratios of phospholipid:apo A-I (—▲—), and cholesterol:apo A-I (—△—) in the eluted fractions.


CONTROL
Molar Ratio
50
25
0
µg/ml
80
40
0
25
27
29
31
33
Fraction Number

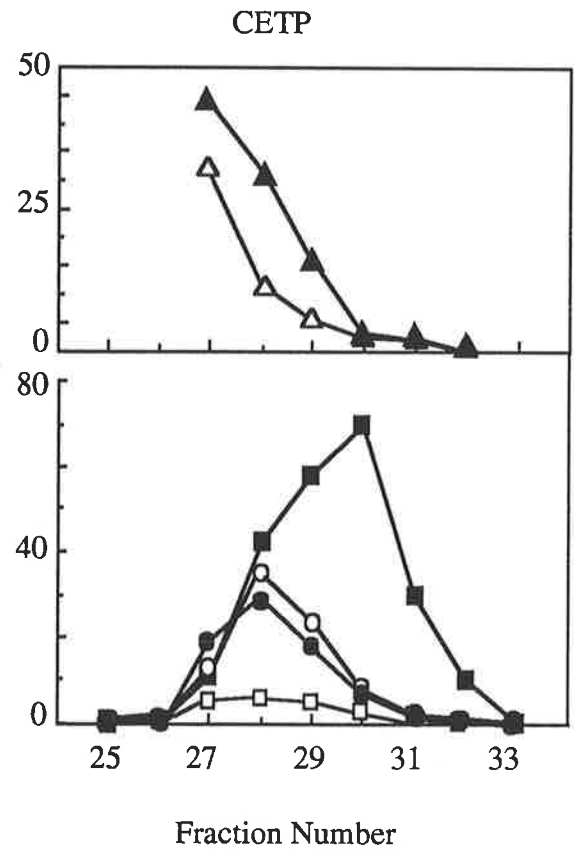

CETP
50
25
0
80
40
0
25
27
29
31
33
Fraction Number

Table 3.1 Concentrations of Constituents in the Fraction of d<1.25 g/ml

Additions	Apo A-I (μg/ml)	Apo A-II (μg/ml)	Cholesterol (nmol/ml)
None	372 ± 195	150 ± 67	2324 ± 1140
CETP	284 ± 145[a]	141 ± 60[a]	2360 ± 1151[ns]

Values represent mean ± SD of seven experiments. The human plasma fraction of d 1.019-1.21 g/ml (containing a mixture of LDL and HDL) was incubated at 37 °C for 24 h in the absence or presence of CETP (mean concentration 4.6 units/ml). After incubation, the mixture was subjected to ultracentrifugation to recover the fraction of d<1.25 g/ml as described the Methods section. Concentrations are expressed per ml incubation mixture.

[ns] not significant

[a] Significance of difference from sample incubated in the absence of CETP: $p < 0.05$.

To quantitate how much of the apo A-I had dissociated from HDL, the incubation mixtures were subjected to ultracentrifugation to separate the lipoprotein fraction ($d<1.25$ g/ml) from the lipoprotein-deficient fraction ($d>1.25$ g/ml). Recovery of constituents in the fraction of $d<1.25$ g/ml is shown in Table 3.1. Compared with incubating HDL and LDL in the absence of CETP, incubation of HDL and LDL in the presence of CETP resulted in a mean loss of 23% of the apo A-I from the fraction of $d<1.25$ g/ml ($p<0.05$). This loss of apo A-I from the lipoprotein fraction was accompanied by a small but significant ($p<0.05$) loss of apo A-II but no loss of cholesterol from the 1.25 g/ml supernatant (Table 3.1). The results shown in Table I are from incubations performed in the absence of albumin. In four of the experiments, replicate samples were incubated in the presence of 1% BSA; the extent of dissociation of apo A-I was identical to that in the corresponding sample incubated in the absence of albumin (result not shown).

Factors influencing the dissociation of apo A-I from HDL

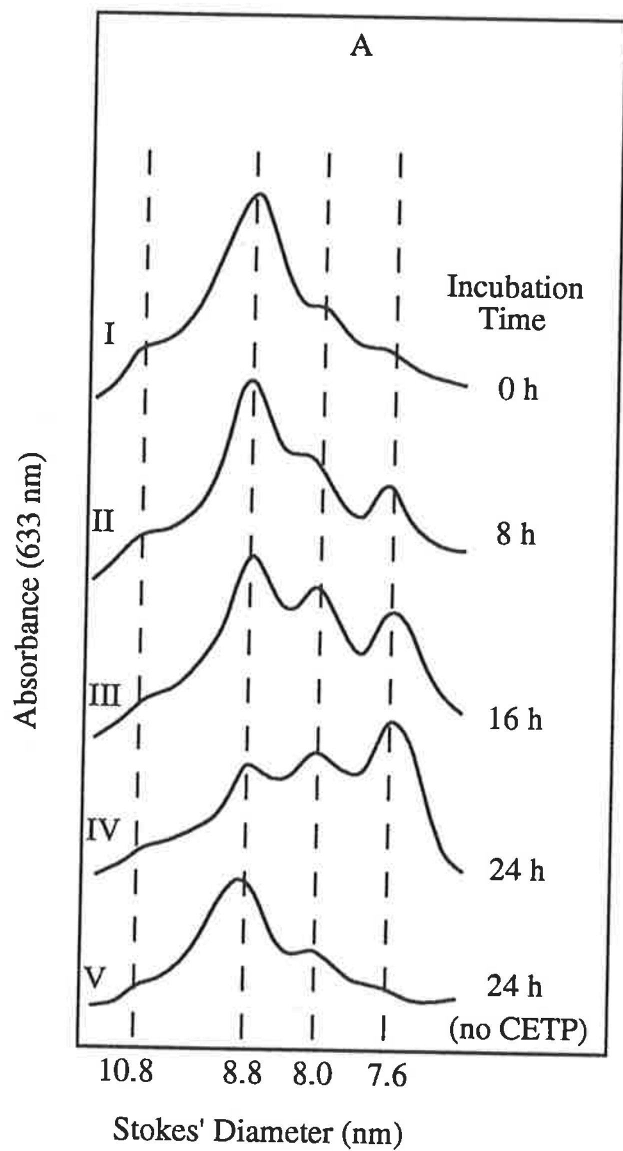
Duration of incubation (Fig. 3-3)

Mixtures of LDL plus HDL (the plasma fraction of d 1.019-1.21 g/ml) and CETP were incubated at 37 ^{o}C for varying periods. The loss of apo A-I from the fraction of $d<25$ g/ml was approximately linear with time up to 24 h (Fig. 3-3B) and coincided with the progressive conversion of HDL$_{3a}$ (diameter 8.8nm) into HDL$_{3c}$ (diameter 7.6nm) (Fig. 3-3A, profiles I-IV). Incubation for 24 h in the absence of CETP had no effect on HDL size (Fig. 3-3A, profile V) and was not associated with a loss of apo A-I from the d 1.25 g/ml supernatant (result not shown).

Concentration of CETP (Fig. 3-4)

Mixtures of LDL and HDL (the plasma fraction of d 1.019-1.21 g/ml) were incubated at 37 ^{o}C for 24 h in the presence of varying concentrations of CETP. Incubation in the absence of CETP (Fig. 3-4A; profile I) had no effect on HDL particle size distribution and did not promote dissociation of apo A-I as judged by comparison with a sample kept at 4°C (result not shown). However, the presence of CETP in the incubation was

Fig. 3-3. Effects of incubation time on changes to HDL particle size and dissociation of apo A-I. The d 1.019-1.21 g/ml fraction of plasma (20 μl containing 600 nmol of total cholesterol and 80 μg of apo A-I) was either kept at 4 °C in the presence of CETP (final concentration 4.6 units/ml) (panel A, profile I), incubated at 37 °C in the absence of CETP (profile V), or supplemented with CETP (4.6 units/ml) and incubated at 37 °C for 8, 16, 24 h (profiles II-IV). The final incubation volume was 0.12 ml. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and were electrophoresed on a 3-30% non denaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apo A-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apo A-I recovered in the d 1.25 g/ml supernatant of the experimental and control (incubated for 24 h in the absence of CETP) incubations. The dissociation is expressed as a percentage of the apo A-I in the 1.25 g/ml supernatant of the control sample.


A
Absorbance (633 nm)
Incubation
Time
I
0 h
II
8 h
III
16 h
IV
24 h
V
24 h
(no CETP)
10.8
8.8
8.0
7.6
Stokes' Diameter (nm)

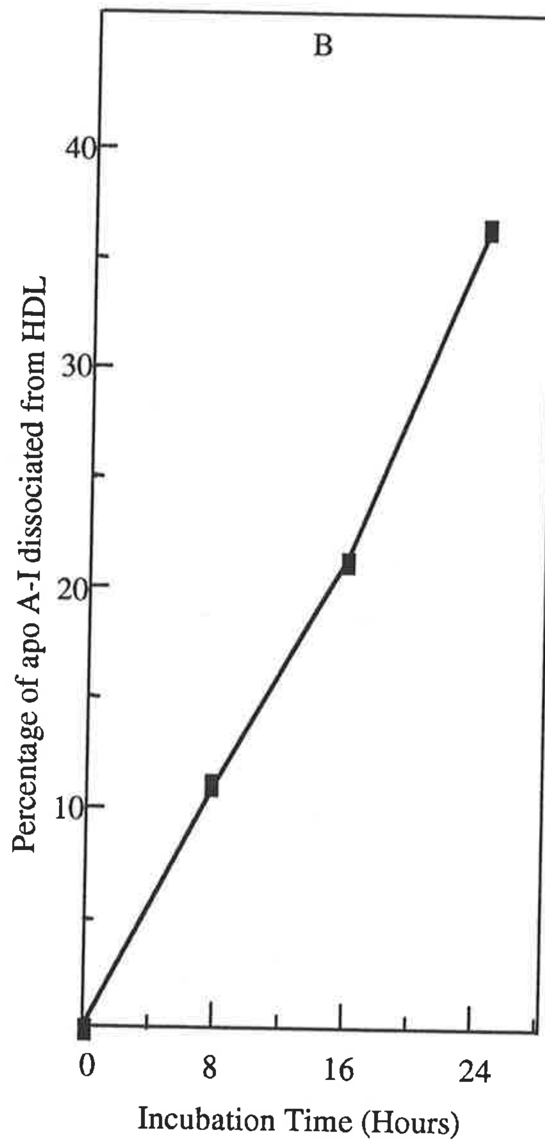
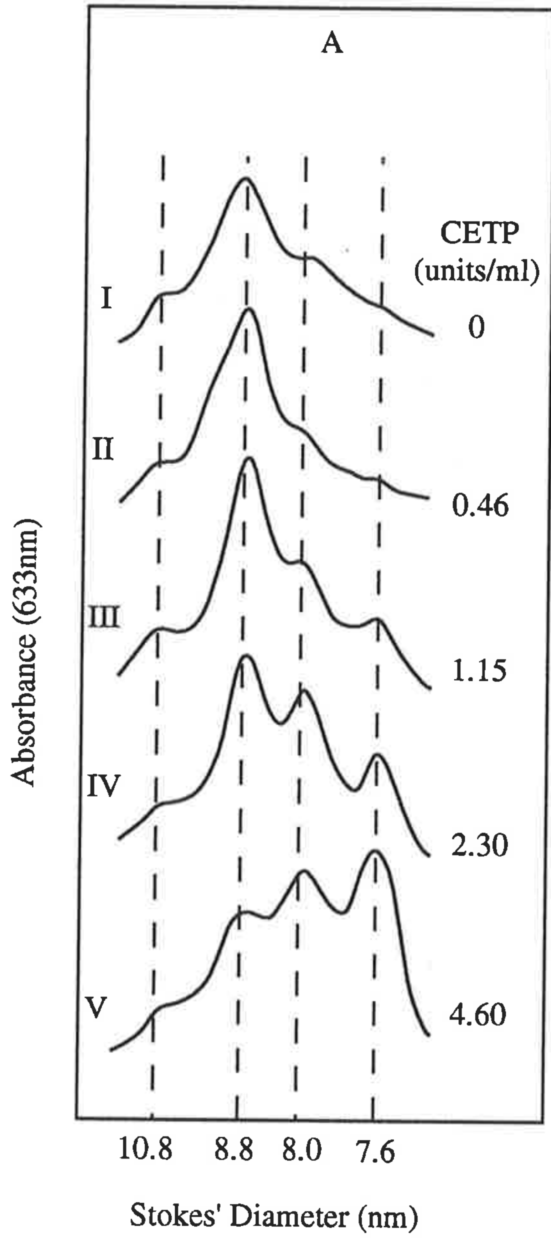
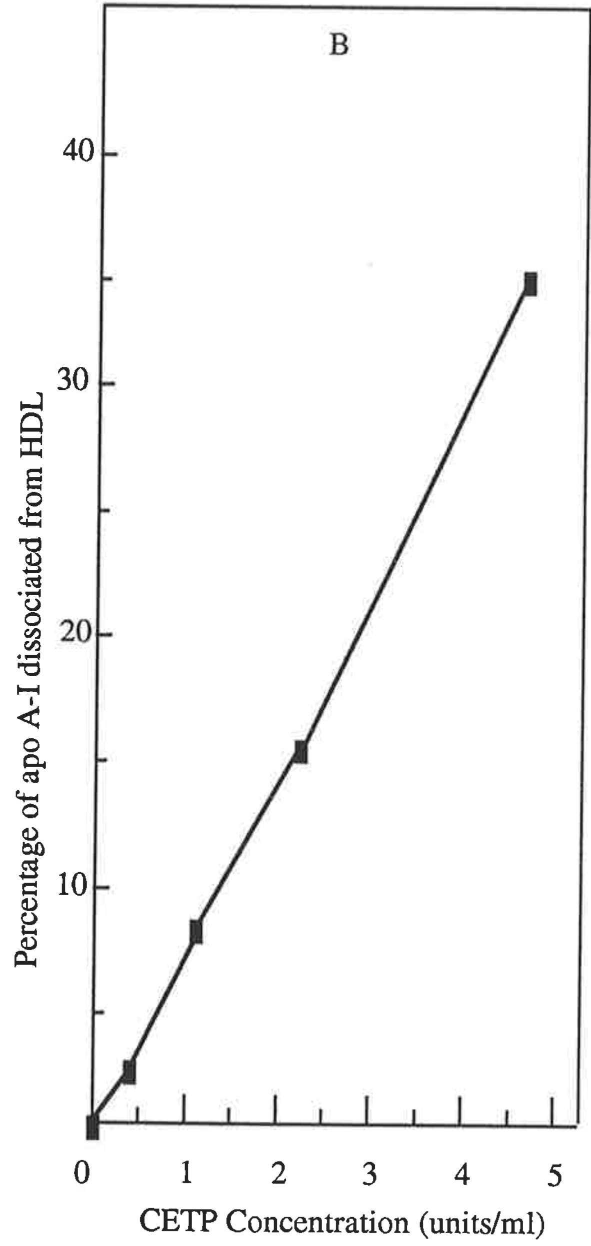

B
Percentage of apo A-I dissociated from HDL
40
30
20
10
0
8
16
24
Incubation Time (Hours)

Fig. 3-4. Effect of CETP concentration on changes to HDL particle size and dissociation of apo A-I. The d 1.019-1.21 g/ml fraction of plasma (40 µl containing 1200 nmol of total cholesterol and 160 µg of apo A-I) was incubated at 37 °C for 24 h in the absence of CETP (panel A, profile I) or in the presence of CETP (20, 50, 100, and 200 µl containing respectively 0.11, 0.28, 0.55, and 1.10 units of CETP) (profiles II-V). The final incubation volume was adjusted to 0.24 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and were electrophoresed on a 3-30% non denaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apo A-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apo A-I recovered in the d 1.25 g/ml supernatant of the experimental and control (containing no CETP) incubations. The dissociation is expressed as a percentage of the apo A-I in the d 1.25 g/ml supernatant of the control sample.


A
CETP
(units/ml)
I
II
III
IV
V
0
0.46
1.15
2.30
4.60
Absorbance (633nm)
10.8
8.8
8.0
7.6
Stokes' Diameter (nm)


B
40
30
20
10
0
1
2
3
4
5
Percentage of apo A-I dissociated from HDL
CETP Concentration (units/ml)

accompanied by a progressive conversion of HDL_{3a} to HDL_{3c} (Fig. 3-4A, profiles II-V) and a loss of apo A-I from the 1.25 g/ml supernatant that was dependent on the concentration of CETP (Fig. 3-4B).

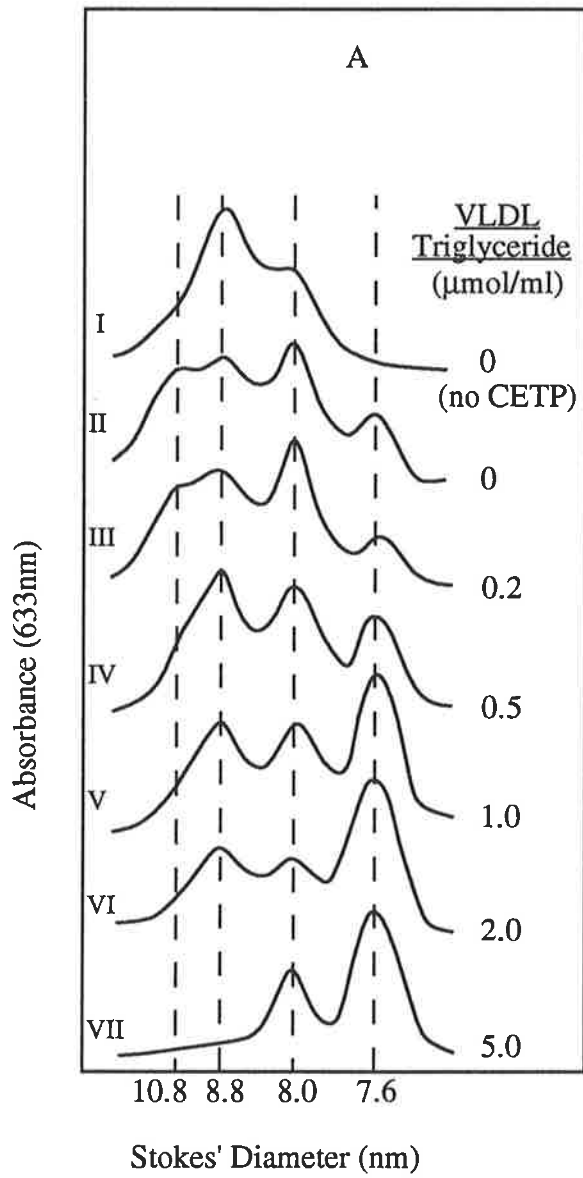
Concentration of VLDL (Fig. 3-5)

Mixtures HDL_3 and CETP were incubated at 37 °C for 24 h in the presence of varying concentrations of VLDL. Taking the incubation of HDL_3 in the absence of both CETP and VLDL as the reference point (Fig. 3-5A, profile I), incubation in the presence of CETP but in the absence of VLDL (Fig. 3-5A, profile II) resulted in the conversion of a proportion of the HDL_{3a} (diameter 8.8 nm) into both smaller $HDL_{3b,3c}$ particles (diameter 8.0 and 7.6 nm) and larger HDL_2 particles (diameter 10.8 nm) as reported previously (Barter et al., 1990 c); under these conditions there was no the loss of apo A-I from the 1.25 g/ml supernatant. The addition of VLDL to the incubation mixture resulted in a progressive decrease in the conversion to larger HDL_2 particles, a progressive increase in the conversion to smaller HDL_{3c} particles (Fig.3-5A, profiles III-VII) and a loss of apo A-I from the 1.25 g/ml supernatant that was dependent on the concentration of VLDL (Fig. 3-5B). As reported previously (Barter et al., 1990 a), the reduction in HDL particle size that occurred during incubation with CETP and VLDL was associated with (and was probably secondary to) a CETP-mediated net reduction in the core lipid content of HDL.

Concentration of LDL (Fig. 3-6)

Mixtures of HDL_3 (d 1.13-1.21 g/ml) and CETP were incubated in the presence of varying concentrations of LDL (d 1.09-1.055 g/ml). By comparison with the control incubation of HDL_3 which was carried out with no additions (Fig. 3-6A, profile I), incubation in the presence of CETP but no LDL (Fig. 3-6A; profile II) resulted in the conversion of the HDL_{3a} into both larger and smaller particles as outlined above. Under these conditions there was minimal loss of apo A-I from the 1.25 g/ml supernatant. However, when LDL was added to the incubation mixture, as was observed with the

Fig. 3-5. Effect of VLDL concentration on changes to HDL size and dissociation of apo A-I. The plasma fraction of d 1.13-1.21 g/ml (HDL_3) was incubated alone at 37 ^{o}C for 24 h (panel A, profile I) or after addition of CETP (profile II) or CETP plus VLDL (profile III-VII). All incubations included 26 μl of HDL_3 (containing 81 nmol total cholesterol, 114 μg apo A-I). CETP was present at a final concentration 2.7 units/ml and VLDL at triglyceride concentrations of 0, 0.2, 0.5, 1.0,2.0, and 5.0 mmol/L. The final incubation volume was adjusted to 0.2 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and electrophoresed on a 3-30% non denaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apo A-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apo A-I recovered in the d1.25 g/ml supernatants of the experimental and control (containing equivalent concentrations of VLDL but no CETP) incubations. The dissociation is expressed as a percentage of the apo A-I in the d 1.25 g/ml supernatant of the control samples.


A
VLDL
Triglyceride
(µmol/ml)
I
II
III
IV
V
VI
VII
0
(no CETP)
0
0.2
0.5
1.0
2.0
5.0
Absorbance (633nm)
10.8
8.8
8.0
7.6
Stokes' Diameter (nm)

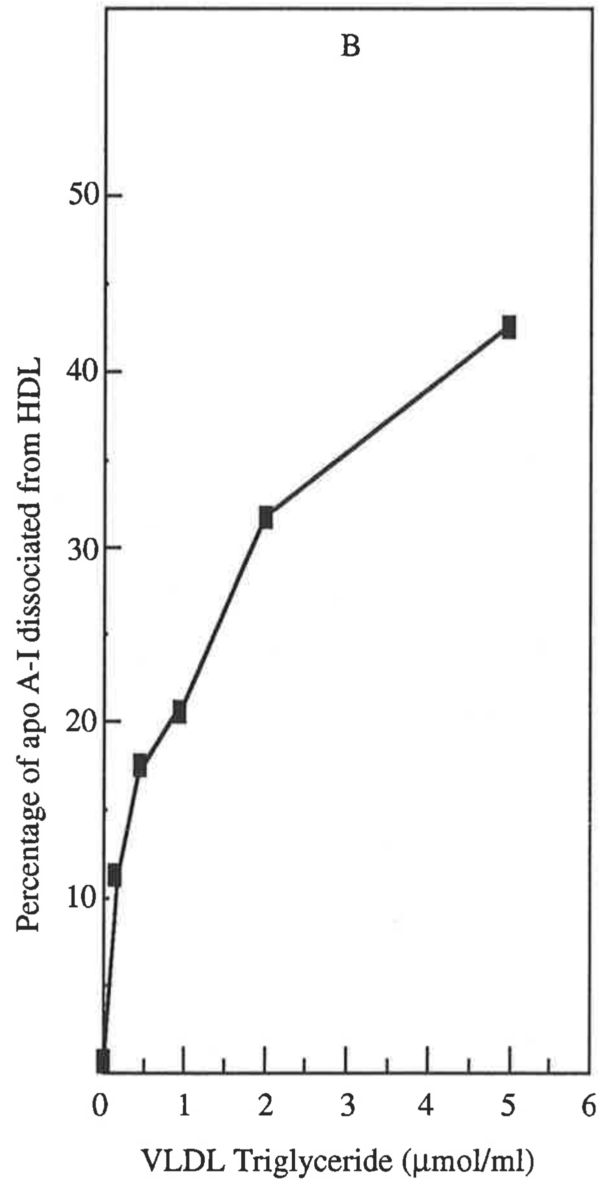
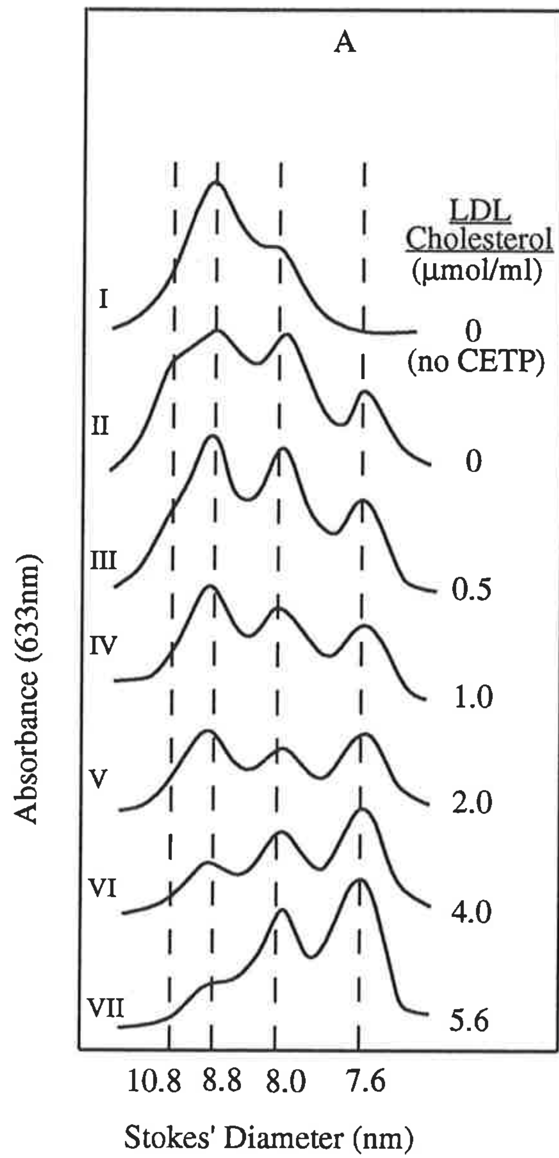
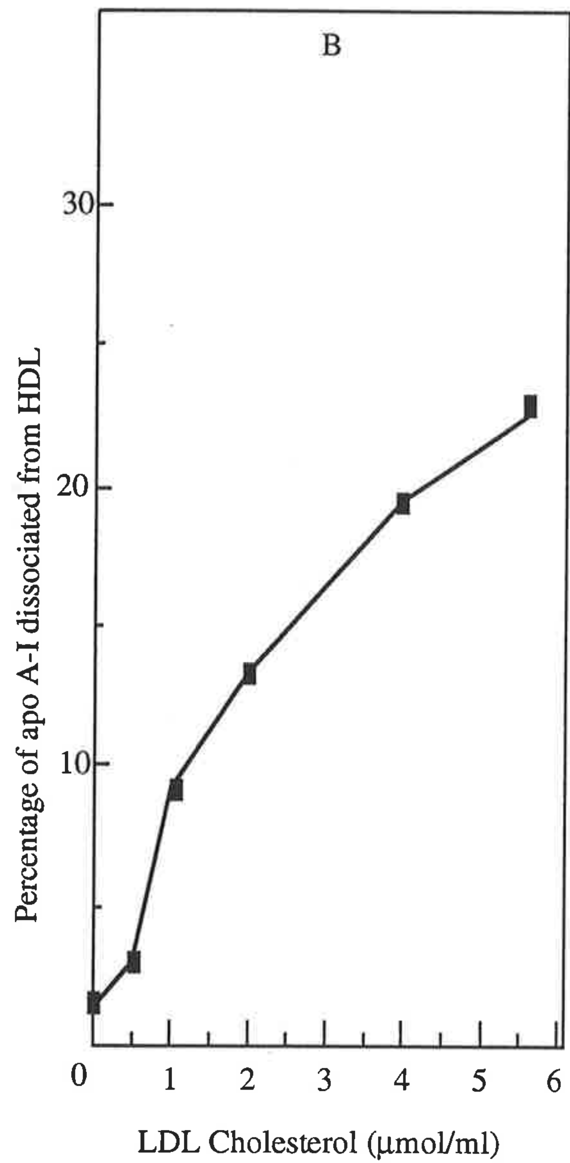

B
50
40
30
20
10
Percentage of apo A-I dissociated from HDL
0
1
2
3
4
5
6
VLDL Triglyceride (µmol/ml)

Fig. 3-6. Effect of LDL concentration on changes to HDL size and dissociation of apo A-I. The plasma fraction of d 1.13-1.21 g/ml (HDL_3) was incubated alone at 37 ^oC for 24 h (panel A, profile I) or with CETP (profile II) or CETP plus LDL (profile III-VII). All incubations included 52 µl of HDL_3 (containing 162 nmol total cholesterol, 228 µg apo A-I). CETP was present at a final concentration 2.7 units/ml and LDL at cholesterol concentrations of 0, 0.5, 1.0, 2.0, 4.0, and 5.6 mmol/L. The final incubation volume was adjusted to 0.4 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and electrophoresed on a 3-30% non denaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apo A-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apo A-I recovered in the d1.25 g/ml supernatants of the experimental and control (containing equivalent concentrations of LDL but no CETP) incubations. The dissociation is expressed as a percentage of the apo A-I in the d 1.25 g/ml supernatant of the control samples.


A
LDL
Cholesterol
(μmol/ml)
I
II
III
IV
V
VI
VII
0
(no CETP)
0
0.5
1.0
2.0
4.0
5.6
Absorbance (633nm)
10.8
8.8
8.0
7.6
Stokes' Diameter (nm)


B
Percentage of apo A-I dissociated from HDL
30
20
10
0
1
2
3
4
5
6
LDL Cholesterol (μmol/ml)

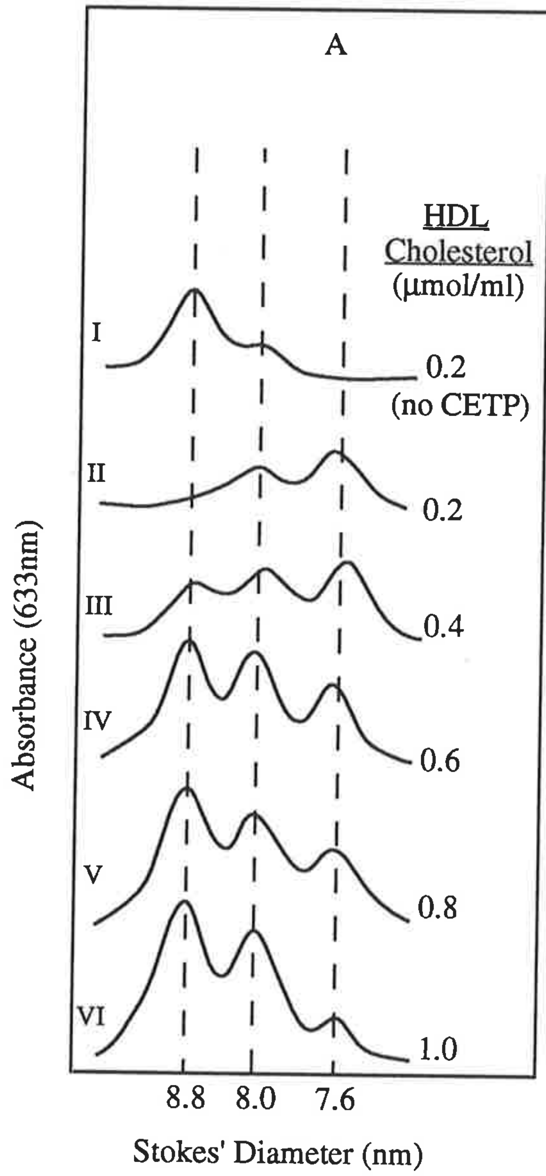
addition of VLDL, there was a progressive reduction in the conversion to larger HDL_2-like particles, a progressive increase in conversion to smaller HDL_{3c} particles (Fig. 3-6A, profiles III-VII) and a concentration-dependent loss of apo A-I from the 1.25 g/ml supernatant (Fig. 3-6B).

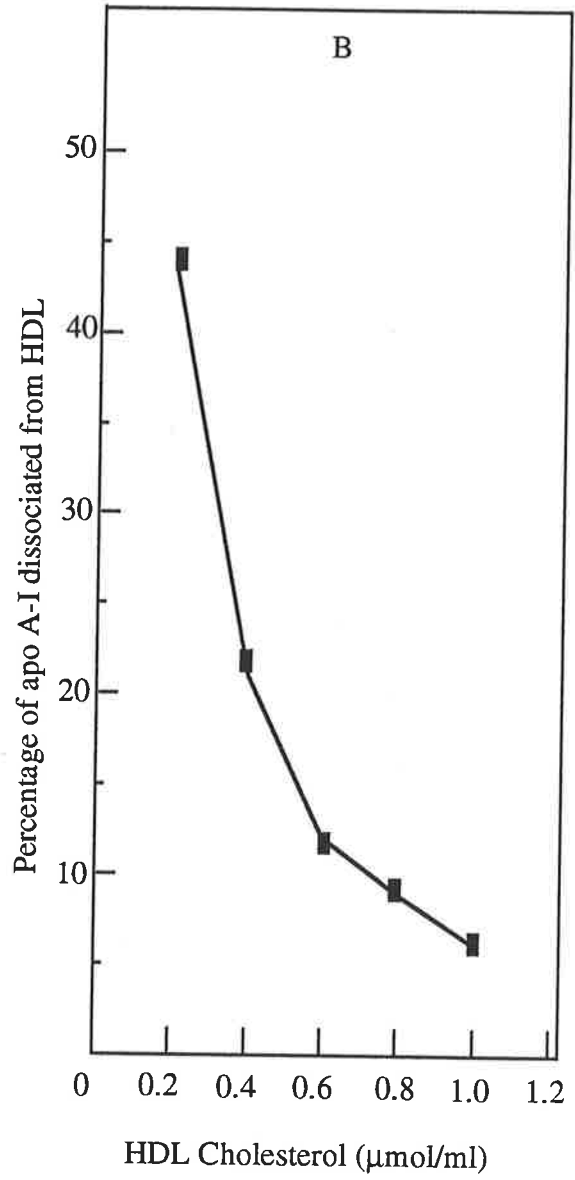
Concentration of HDL (Fig. 3-7)

To determine whether varying the concentration of HDL affected the dissociation of apo A-I, mixtures containing fixed concentrations of LDL and CETP were incubated in the presence of varying concentrations of HDL_3. For each concentration of HDL_3, there was also a control incubation that contained HDL_3 and LDL but no CETP. In these control incubations there were no changes to the particle size distribution of HDL and no evidence of a loss of apo A-I from the 1.25 g/ml supernatant compared with a sample kept at 4 ^oC (result not shown). The losses of apo A-I from the 1.25 g/ml supernatant shown in Fig. 3-7B have been calculated as the difference between the samples that were incubated with CETP and the control samples that were incubated in the absence of CETP.

When HDL_3 were incubated with LDL and CETP, both the reduction in HDL size and the dissociation of apo A-I from HDL were inversely related to the concentration of HDL. At an HDL_3 cholesterol concentration of 0.2 mM there was a quantitative conversion of the HDL_{3a} to HDL_{3c} (Fig. 3-7A, profile II). This was accompanied by a loss of 44% of the apo A-I from the 1.25 g/ml supernatant (Fig. 3-7B). As the concentration of HDL_3 increased there was a progressive reduction in the conversion to smaller HDL_{3c} particles (Fig. 3-7A, profiles III-VI) and a progressive decrease in the percentage of apo A-I lost from the d 1.25 g/ml supernatant (Fig. 3-7B). At an HDL_3 cholesterol concentration of 1.0 mM only 6% of the apo A-I was lost.

Fig. 3-7. Effect of HDL concentration on changes to HDL size and dissociation of apo A-I. A preparation of LDL was supplemented with varying amounts of HDL_3 and incubated at 37 °C for 24 h in the absence (panel A, profile I) or presence (profile II-VI) of CETP. Incubation mixtures included 37 µl LDL (containing 814 nmol total cholesterol), 60 µl CETP (final concentration 2.7 units/ml) and HDL_3 at cholesterol concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mmol/L. The final incubation volume was adjusted to 0.2 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and electrophoresed on a 3-30% non denaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apo A-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apo A-I recovered in the d1.25 g/ml supernatants of the experimental and control (containing equivalent concentrations of HDL but no CETP) incubations. The dissociation is expressed as a percentage of the apo A-I in the d 1.25 g/ml supernatant of the control samples.


A
HDL
Cholesterol
(µmol/ml)
I
II
III
IV
V
VI
0.2
(no CETP)
0.2
0.4
0.6
0.8
1.0
Absorbance (633nm)
8.8
8.0
7.6
Stokes' Diameter (nm)


B
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
1.2
Percentage of apo A-I dissociated from HDL
HDL Cholesterol (µmol/ml)

3.4 DISCUSSION

The present study demonstrates that a CETP-mediated reduction in HDL particle size is accompanied by the dissociation of apo A-I from HDL. In the earlier studies in which apo A-I was shown to dissociate from HDL, the incubations contained all constituents of human plasma plus additional CETP, hepatic lipase and VLDL (Clay et al., 1991; Clay et al., 1992). Under those conditions the appearance of lipoprotein-unassociated apo A-I was transient, with a return of the apo A-I to the HDL density range coinciding with the appearance of discoidal HDL in the incubation mixture (Clay et al., 1992). In these studies, it was postulated that the initial dissociation of apo A-I from HDL was a simple consequence of the shedding of redundant surface constituents from particles of reduced size. The subsequent return of the apo A-I to the HDL density range was explained as the formation of complexes between the dissociated apo A-I and phospholipids and unesterified cholesterol had been released from the surface of VLDL during lipolysis of VLDL triglyceride by hepatic lipase. However, regardless of the mechanism, the simple fact that the apo A-I did return to the HDL fraction made it difficult either to quantitate the dissociation or to characterize the dissociated apo A-I.

This problem was circumvented in the present study by using incubation conditions in which further metabolic processing of the dissociated apolipoprotein was unlikely. We took advantage of earlier observations that the core lipid content and the particle size of HDL are reduced by incubation with CETP plus either VLDL or LDL (Barter et al., 1990 c). It has now been shown that under such conditions there is also a dissociation of apo A-I from HDL fraction. Both the reduction in HDL size and the dissociation of apo A-I from HDL were slower than when the incubation also contained hepatic lipase (Clay et al., 1991; Clay et al., 1992) but they were otherwise comparable. The major advantage of this approach was that the dissociated apo A-I did not subsequently return to the HDL fraction and was therefore available for quantification and characterization. Under these conditions, the dissociation of apo A-I was linear with time up to 24 hours. It increased

with increasing incubation concentrations of CETP, VLDL and LDL but decreased as the concentration of HDL increased.

The dissociation of apo A-I in the present study was demonstrated by three independent techniques and could not be dismissed as an artefact of ultracentrifugation. Furthermore, it was totally dependent on the presence of CETP in the incubation mixture. However, CETP alone was not sufficient. As reported previously (Barter et al., 1990 c), when HDL are incubated with CETP in the absence of other lipoprotein fractions there is a conversion of the original particles into new populations of both larger and smaller particles. While there may have been a redistribution of apo A-I between different HDL particles, there was no evidence of a loss of apo A-I from the total HDL fraction. However, when either VLDL or LDL were added to the mixture of HDL and CETP, formation of the larger conversion products was suppressed while conversion to the smaller HDL particles was enhanced in a concentration-dependent fashion. This enhancement of the conversion of the original HDL to smaller particles coincided with a concentration-dependent dissociation of apo A-I from the HDL fraction. By contrast, increasing the concentration of HDL decreased both the reduction in HDL particle size and the dissociation of apo A-I. Thus, in all experiments, the dissociation of apo A-I from HDL coincided with a reduction in HDL size.

Considering that the initial cellular cholesterol acceptors apo A-I-containing particles which are much smaller than the bulk HDL in plasma, it is possible to speculate that the significance of the dissociation of apo A-I from HDL is to provide particles which are small enough to enter the interstitial space to pick the cholesterol. However, this process may also have an impact on the metabolic function of HDL and on the regulation of apo A-I concentration in plasma.

Subjects with elevated concentrations of triglyceride-rich lipoproteins are known to have decreased concentrations of plasma apo A-I (Rao et al., 1980; Saku et al., 1985; Hong et

al., 1988). They are also known to have an increase in the plasma concentration of lipoprotein-unassociated apo A-I (Ishida et al., 1987; Neary and Gowland, 1987). It has been suggested that the lipoprotein-unassociated apo A-I in such subjects represents a pool of the apolipoprotein which is especially susceptible to dissociation from HDL and that its subsequent rapid clearance by the kidney explains why the plasma apo A-I concentration is low in some hypertriglyceridemic patients (Horowitz et al., 1992). Such a conclusion is supported by the observation in the present study that the dissociation of apo A-I increased as the concentration of VLDL increased. It is interesting to note that the relationship between lipoprotein concentration and the dissociation of apo A-I from HDL in vitro in the present studies (a positive relationship for VLDL and LDL and a negative relationship for HDL) is the same as the relationship between lipoprotein concentrations and the development of coronary heart disease. The implications of this observation are unknown, although it is tempting to speculate that a dissociation of apo A-I from HDL may in some way predispose to the development of atherosclerosis.

CHAPTER 4

CHARACTERIZATION OF THE APO A-I DISSOCIATED FROM HDL

4.1 INTRODUCTION

4.2 METHODS

4.3 RESULTS

Lipids and apolipoproteins composition

SDS-polyacrylamide gel electrophoresis

Agarose gel electrophoresis

4.4 DISCUSSION

4.1 INTRODUCTION

As described in Chapter 3, remodelling of HDL under certain circumstances, such as depleting HDL cholesterol esters to LDL by CETP, results in a reduction in HDL size and the coincided dissociation of apo A-I from HDL. While the HDL particle itself after CE depletion have previously been partially characterized (Barter et al., 1990 c), there is little information regarding the apo A-I that dissociated from HDL. It is not clear whether the apo A-I dissociates as lipid-free apolipoprotein or as a component of a lipid-protein complex or both. Neither is how their electrophoresis mobility differ from the alpha-migrating bulk HDL in plasma.

In this Chapter, the dissociated apo A-I particles were characterized following the incubation of HDL plus LDL in the presence of CETP. The concentrations of lipids and apolipoproteins were measured in these dissociated particles. Their molecular weight and electrophoresis mobility were also determined.

4.2 METHODS

The fraction of density 1.019-1.21 g/ml (containing a mixture of LDL and HDL) was incubated at 37 ^{o}C for 24 h in the absence or presence of CETP. Following incubation, the incubation mixtures were adjusted to density 1.25 g/ml and subjected to ultracentrifugation at 4 ^{o}C in TL 100.4 rotor (100,000 rpm, 16 h). The fractions of d >1.25 were subsequently recovered and concentrated 10-fold by lyophilization. Results in Table II are from two separate experiments, each was duplicate in samples.

SDS-polyacrylamide gel electrophoresis and agarose gel electrophoresis were performed as described in Chapter 2.

4.3 RESULTS

Lipids and apolipoproteins composition (Table 4.1)

Mixtures of HDL and LDL were incubated at 37 ^{o}C for 24 h in the presence and absence of CETP. After incubation, the fraction of d>1.25 g/ml was recovered by ultracentrifugation and concentrated 10-fold by lyophilization. The recovery of constituents in the 1.25 g/ml infranatant of two experiments is shown in Table 4.1. After incubation of mixtures of HDL and LDL in the absence of CETP there was minimal recovery of either apolipoproteins or lipids in the fraction of d>1.25 g/ml. The amount of apo A-I recovered represented less than 1% of that in the total incubation mixture. In contrast, after incubation of HDL in the presence of both LDL and CETP, there was a substantial recovery of apo A-I in the d>1.25 g/ml fraction. Even in the presence of CETP, however, there was a minimal recovery of apo A-II, phospholipid or cholesterol in the 1.25 g/ml infranatant. Expressing the recoveries of constituents in molar terms, it is apparent in each experiment that each mole of apo A-I is associated with, at most, only one mole of phospholipid and one mole of cholesterol. In other words, the apo A-I recovered in the 1.25 g/ml infranatant is comparable to that recovered in the size-exclusion chromatography fractions 31 and 32 (Fig. 3-2, Chapter 3) in that it was essentially lipid-free.

SDS-polyacrylamide gel electrophoresis (Fig. 4-1)

Aliquots of the 1.25 g/ml infranatant recovered following the incubation of HDL, LDL and CETP were also subjected to SDS-polyacrylamide gel electrophoresis (Fig. 4-1). Only two bands were apparent (Fig. 4-1, profile A): one with a molecular weight of 28,000 which was identical to purified apo A-I (Fig. 4-1, profile B), while the other, with a molecular weight of 71,000, was identical to purified CETP (Fig. 4-1, profile C).

Table 4.1 Recovery of Apolipoproteins and Lipids in the Lipoprotein Depleted Fraction of d> 1.25 g/ml

	Apo A-I (μg)		Apo A-II (μg)		Phospholipid (μg)		Cholesterol (nmol)	
Experiments	(-CETP)	(+CETP)	(-CETP)	(+CETP)	(-CETP)	(+CETP)	(-CETP)	(+CETP)
A	10.0	150.0	0	3.8	1.2	4.6	0.8	6.3
B	5.4	42.3	0	0.9	0.5	1.1	0.2	1.4

The human plasma fraction of d 1.019-1.21 g/ml (containing a mixture of LDL and HDL) was incubated at 37 ^{o}C for 24 h in the absence or presence of CETP. In experiment A, the volume of the incubation was 3.6 ml, which included 0.6 ml of the d 1.019-1.21 g/ml fraction (11 μmol cholesterol and 2.6 mg apo A-I) and either 3 ml of a preparation of CETP to provide a final CETP concentration of 4.6 units/ml or 3 ml TBS. In experiment B, the volume of the incubation was 2.1 ml, which included 0.3 ml of the d 1.019-1.21 g/ml fraction (4 μmol cholesterol and 0.5 mg apo A-I) and either 1.8 ml of a CETP preparation (final incubation concentration of CETP 4.0 units/ml) or 1.8 ml TBS. After incubation, the mixtures were subjected to ultracentrifugation. The fraction of d>1.25 g/ml (the lipoprotein-depleted fraction)was recovered, concentrated 10-fold, and assayed for apolipoproteins and lipids. Values represent the total mass of each constituent in the lipoprotein-depleted fraction. Analysis of the lipoprotein fraction of d<1.25 g/ml indicated that in the incubations containing CETP, the loss of apo A-I was 22% and 19%, respectively, in experiments A and B when compared to the incubations that were conducted in the absence of CETP.

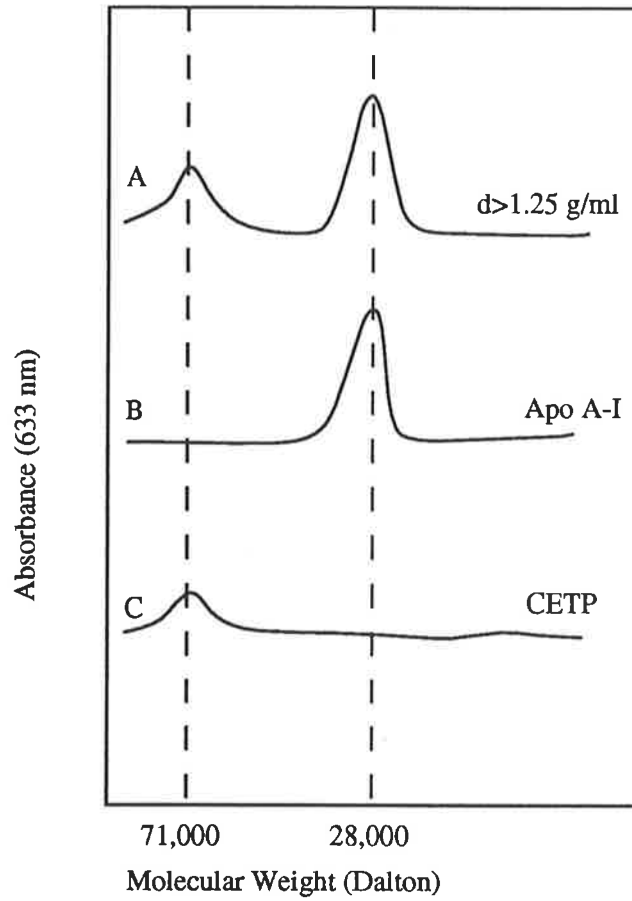


Fig. 4-1. Determination of the molecular weights of proteins in the d>1.25 g/ml fraction by SDS-polyacrylamide gel electrophoresis. The human plasma fraction of d 10.19-1.21 g/ml was incubated at 37 °C for 24 h in the presence of CETP. Final incubation volume was 3.6 ml, which included 0.6 ml of the 10.19-1.21 g/ml fraction (containing 11 µmol cholesterol and 2.6 µg apo A-I) and 3 ml CETP (final concentration 4.6 units/ml). After incubation, lipoproteins were separated from the lipoprotein-deficient fraction by ultracentrifugation at a density of 1.25 g/ml. The lipoprotein-deficient fraction (d< 1.25 g/ml) was concentrated 10-fold by lyophilization and electrophoresed on a 20% homogeneous SDS gel. The profiles were obtained by laser densitometric scanning of the stained gel. The molecular weights of proteins in the fraction of d>1.25 g/ml (profile A) were determined by reference to low molecular weight standards. Profiles B and C represent samples of purified human apo A-I and CETP, respectively.

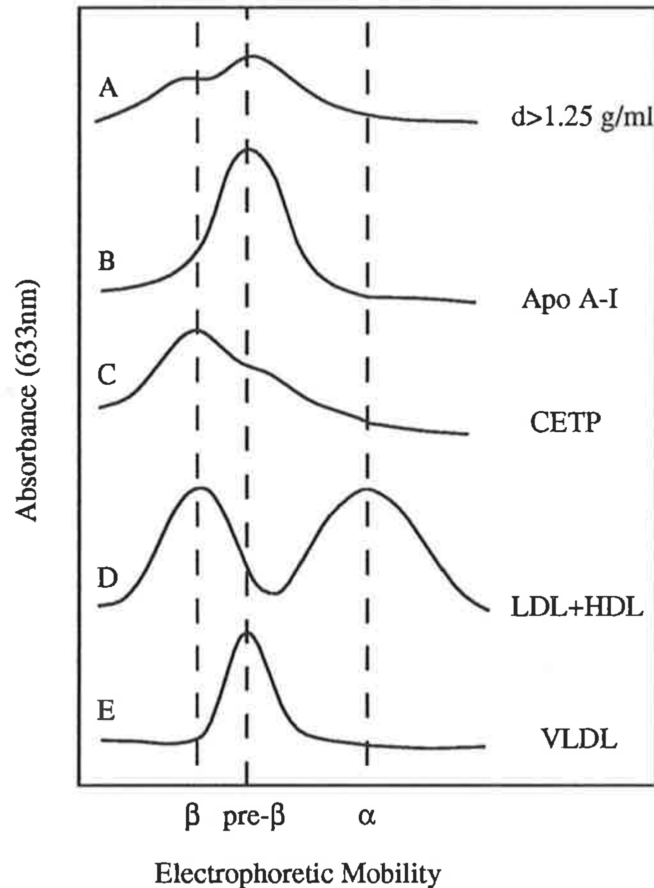


Fig. 4-2. Determination of mobility of proteins in the d>1.25 g/ml fraction by agarose gel electrophoresis. The human plasma fraction of d 10.19-1.21 g/ml was incubated at 37 °C for 24 h in the presence of CETP. Final incubation volume was 3.6 ml, which included 0.6 ml of the 10.19-1.21 g/ml fraction (containing 11 μmol cholesterol and 2.6 μg apo A-I) and 3 ml CETP (final concentration 4.6 units/ml). After incubation, lipoproteins were separated from the lipoprotein-deficient fraction by ultracentrifugation at a density of 1.25 g/ml. The lipoprotein-deficient fraction (d< 1.25 g/ml) was recovered, concentrated 10-fold by lyophilization, and subjected to agarose gel electrophoresis (profile A). Electrophoresis of purified apo A-I (profile B), purified CETP (profile C). LDL plus HDL in the fraction of d 1.019-1.21 g/ml (profile D), and a preparation of VLDL (profile E) was also performed on the same gel. The profiles were obtained by laser densitometric scanning of the stained gels.

Agarose gel electrophoresis (Fig. 4-2)

The 1.25 g/ml infranatant was also subjected to agarose gel electrophoresis (Fig. 4-2). Two bands with beta and prebeta mobilities were apparent (Fig. 4-2, profile A). These equated with the prebeta mobility of purified apo A-I (Fig. 4-2, profile B) and the predominant beta mobility of CETP (Fig. 4-2, profile C). The beta and alpha mobilities of LDL and HDL respectively in the plasma fraction of d 1.019-1.25 g/ml (Fig. 4-2, profile D) and the prebeta mobility of isolated VLDL (Fig. 4-2, profile E) are also shown.

4.4 DISCUSSION

Following the incubation of HDL with LDL and CETP, the dissociation of apo A-I recovered in the fraction of d>1.25 g/ml (Table 4.1) and by size-exclusion chromatography (Fig. 3-2) was essentially free of lipid. However, the possibility that each molecule of apo A-I was associated with one or possibly two molecule of either phospholipid or cholesterol can not be ruled out. This result was similar to that observed previously when size-exclusion chromatography was used to fractionate mixtures of HDL and LDL that had been incubated with CETP in the presence of nonesterified fatty acids (Barter et al., 1990 b). As is the case with purified apo A-I, the dissociated apo A-I had a prebeta mobility on agarose gel electrophoresis (Fig. 4-2), an observation that raises questions regarding its relationship to the prebeta-migrating apo A-I known to exist in human plasma (Kunitake et al., 1985).

It has been reported by several workers that up to 10% of the apo A-I in human plasma exists in prebeta-migrating particles rather than in the alpha-migrating bulk HDL (Kunitake et al., 1985; Neary et al., 1987; Duval et al., 1989; O'Kane et al., 1992). Activity of CETP (Kunitake et al., 1992) and of lipases (Neary et al., 1991; Miller, 1992) have been implicated in the release of prebeta apo A-I from HDL, while LCAT has been shown to reconvert prebeta apo A-I to alpha-migrating particles (Neary et al., 1991; Miida et al., 1992). LCAT has also been implicated in the dissociation of apo A-I from

HDL in a process that may involve fusion of HDL particles (Nichols et al., 1987). Apo A-I (possibly lipid-free) is displaced from HDL by the addition of apo A-II in vitro (van Tornout et al., 1981) and also by the dilution of HDL in a solution of 0.15 M NaCl (Pownall et al., 1978). A proportion of the prebeta-migrating apo A-I found in unmodified human plasma exists in particles which are smaller and denser than the bulk HDL and, in this sense, may be classified as lipoprotein-unassociated apo A-I. While there have been very few studies of the composition of lipoprotein-unassociated, prebeta-migrating apo A-I, at least one report has indicated that the apo A-I is a component of a particle which is demonstrably not lipid-free (Kunitake et al., 1985). It should be noted, however, that prebeta mobility of apo A-I indicates no more than the fact that it resides in particles which are more negatively charged than those in the bulk HDL fraction. For example, purified, lipid-free apo A-I has a prebeta mobility, as do reconstituted discoidal HDL (rHDL) which contain significant amounts of lipid (Sparks et al., 1992). Thus, at some point during the conversion of discoidal HDL to spheroidal HDL there must be a change in the conformation of apo A-I that exposes negatively charged amino acid residues and consequent changes the mobility of the particle. It follows that there exists a spectrum of prebeta particles in human plasma, ranging from lipid-free apo A-I at one end to complex particles in a stage of transition between discoidal and spheroidal HDL at the other. Indeed, several distinct populations of prebeta-migrating HDL have been identified in human plasma (Castro and Fielding, 1988).

The fact that lipid-free apo A-I has not been identified in human plasma is not surprising in view of the high affinity of this apolipoprotein for phospholipids and the ready availability of phospholipids in plasma. For example, phospholipids are continually being released from VLDL and chylomicrons that are undergoing lipolysis. Even in the absence of lipolysis, it is possible that plasma factors such as the phospholipid transfer protein are capable of delivering phospholipids from a range of lipoproteins to lipid-free apo A-I. This may explain why the smallest of the prebeta-migrating, apo A-I-containing particles so far identified in fresh human plasma (designated prebeta-1 HDL) contain a

single molecule of apo A-I particles combined with substantial amounts of phospholipids (Castro and Fielding, 1988).

The origin of prebeta-1 HDL is unknown. This is not a trivial issue, as such particles have been implicated as the initial acceptors of cell cholesterol in the pathway of reverse cholesterol transport (Castro and Fielding, 1988). It is possible that prebeta-1 HDL are formed as a consequence of the binding of phospholipids to the lipid-free apo A-I which dissociates from HDL in the CETP-mediated process observed in the present study. If this is so, it follows that the process of apo A-I dissociation may be of important in the pathway of reverse cholesterol transport. However, it is possible that the major significance of the dissociation relates to its role in the regulation of apo A-I concentration in plasma.

CHAPTER 5

INCORPORATION OF APO A-I INTO HDL DURING HDL-CHOLESTEROL ESTERIFICATION

5.1 INTRODUCTION

5.2 METHODS

5.3 RESULTS

Prevention of the apo A-I dissociation by lipoprotein-deficient plasma

Return of the dissociated apo A-I to the lipoprotein fraction

Studies using lipoprotein-deficient plasma

Studies using purified LCAT

Incorporation of purified, lipid-free apo A-I into HDL

Role of LCAT in the incorporation of lipid-free apo A-I into HDL

5.4 DISCUSSION

5.1 INTRODUCTION

In Chapter 3 and Chapter 4, it was demonstrated that reduction in HDL size following the depletion of HDL core lipids by CETP is accompanied by a dissociation of lipid-free apo A-I from HDL. This finding is consistent with previous studies, where the depletion of HDL lipids was carried out by using both CETP and hepatic lipase (Clay et al., 1991; Clay et al., 1992). It implicates that plasma factors which modify HDL lipid composition and HDL particle size also influence HDL apolipoprotein composition. That is, when the core lipid content (cholesteryl esters plus triglyceride) of HDL is reduced by CETP and/or hepatic lipase, apo A-I dissociates from HDL in a lipid-free state following the reduction in HDL size.

This Chapter investigates if the dissociation of apo A-I from HDL is reversible in a process of increasing HDL particle size. After the HDL size was reduced and a proportion of apo A-I had dissociated, the size of the HDL is subsequently increased by incubation with purified LCAT or the lipoprotein-deficient plasma (containing LCAT), and the return of the dissociated apo A-I to the lipoprotein fraction was monitored. In some experiments, the dissociated apo A-I was removed before the addition of LCAT and replaced by purified, lipid-free apo A-I. It was found that, in both the cases, the apo A-I, either the dissociated or the exogenous added lipid-free apo A-I, was reassociated with the HDL density fraction, indicating the incorporation of apo A-I into the HDL. These results implicate the existence of a cycle in which the apo A-I in plasma alternates between association with HDL and a lipid-free state. It also suggests that this cycle is regulated by factors operating in plasma, such as CETP, hepatic lipase and LCAT.

5.2 METHODS

Isolation of lipoprotein-deficient plasma (d> 1.25 g/ml fraction)

Lipoprotein-deficient plasma was isolated ultracentrifugally as the fraction of d>1.25 g/ml from plasma obtained from healthy, normolipidemic subjects. The ultracentrifugation was performed in a Beckman TL-100 ultracentrifuge at 4°C, using a Beckman TL 100.2 rotor at a speed of 100,000 rpm for 16 h. Before use, The isolated lipoprotein-deficient plasma was dialysed against Tris-buffered saline (TBS), pH 7.4, containing 0.01 M Tris, 0.015 M NaCl, 0.006% (w/v) NaN_3 and 0.005% (w/v) EDTA-Na_2.

Experimental conditions

Incubation mixtures were placed in sealed plastic tubes and either kept at 4°C or incubated at 37 oC in a shaking water bath. The incubations were terminated by placing the tubes on ice. To measure the concentration of lipoprotein-associated apo A-I, mixtures were adjusted to a density of 1.25 g/ml with solid KBr and subjected to a single ultracentrifugal spin at 4°C at 100,000 rpm for 16 h in a Beckman LA 100.2 rotor in a Beckman TL-100 ultracentrifuge. By using only a single spin at a density of 1.25 g/ml, the recovery of lipoprotein-associated apo A-I was maximized. It has been shown previously that when unmodified HDL are subjected to these conditions, virtually all of the apo A-I is recovered in the fraction of d< 1.25 g/ml. Following its recovery, the fraction of d< 1.25 g/ml was assayed for the concentrations of lipids and apolipoproteins in the total lipoprotein fraction and subjected to non-denaturing polyacrylamide gradient gel electrophoresis to monitor HDL particle size.

Statistical analyses

The significance of changes in concentration of apo A-I was determined by Student's t-test for paired samples.

5.3 RESULTS

Prevention of the apo A-I dissociation by lipoprotein-deficient plasma (Fig. 5-1)

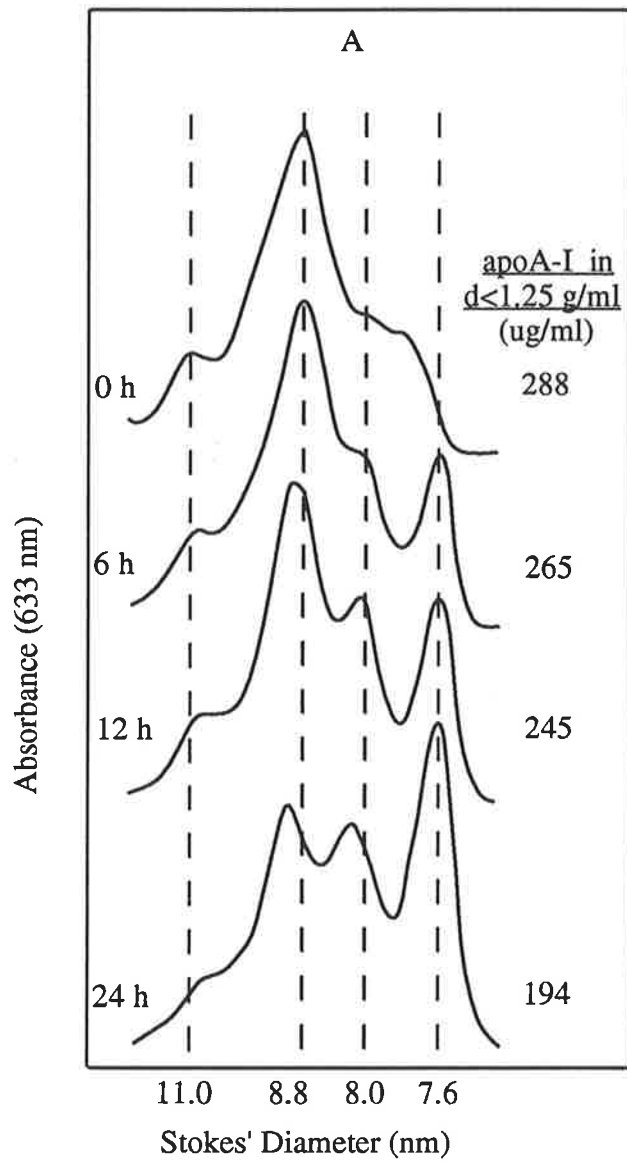
A mixture of VLDL, LDL and HDL was isolated as the plasma fraction of d< 1.21 g/ml and either kept at 4°C or incubated with CETP at 37 °C for 6, 12 or 24 h. As reported in Chapter 3, these incubation conditions resulted in the progressive conversion of a proportion of the HDL into a population of small particles of diameter 7.6 nm and a coincident reduction in the concentration of lipoprotein-associated apo A-I (Fig. 5-1A). The dissociation of apo A-I from HDL was assessed as the loss of apo A-I from particles of d< 1.25 g/ml (Fig. 5-1A). If, however, the incubation mixture contained lipoprotein-deficient plasma in addition to the CETP, only minor changes to HDL size were apparent (Fig. 5-1B) and there was no reduction in the concentration of lipoprotein associated apo A-I. The incubation conditions used in this and the experiment described below did not change the concentrations of total cholesterol, phospholipids or apo A-II in the fraction of d< 1.25 g/ml (results not shown).

Return of the dissociated apo A-I to the lipoprotein fraction

Studies using lipoprotein-deficient plasma (Fig. 5-2)

The experiments described above demonstrated that the presence of lipoprotein-deficient plasma was able to prevent the reduction in HDL size and the dissociation of apo A-I from the HDL. Experiments were also conducted to determine whether the dissociation of apo A-I could be reversed after the event. To this end, in an initial incubation comparable to that described above, the plasma fraction of d< 1.21 g/ml (containing VLDL, LDL and HDL) was either kept at 4°C (Fig. 5-2, 0h) or incubated with CETP at 37 °C for 24 h. This incubation resulted in the conversion of a proportion of the HDL into a population of small particles of diameter 7.6 nm (Fig. 5-2, 24 h) and the loss of about 25% of the apo A-I from the lipoprotein-containing fraction of d< 1.25 g/ml. Aliquots of the incubation mixtures were then supplemented with either the lipoprotein-deficient fraction of plasma (d>1.25 g/ml) or with an equivalent volume of TBS and

Fig. 5-1. Effects of lipoprotein-deficient plasma on the reduction in HDL size and the dissociation of apo A-I from HDL mediated by CETP. The plasma fraction of d< 1.21 g/ml (40 μl containing 965 nmol total cholesterol and 192 μg apo A-I) was supplemented with CETP (final concentration of 4.5 units/ml) and either 400 μl TBS (Panel A) or 400 μl of the lipoprotein-deficient (d> 1.25 g/ml) fraction of plasma (Panel B). The final incubation volumes were 600 μl. Mixtures were incubated at 37°C for the times shown before being placed on ice. After incubation, lipoproteins in the fraction of d< 1.25 g/ml were recovered by ultracentrifugation, assayed for apo A-I concentration and subjected to non-denaturing gradient gel electrophoresis. Profiles show the particle size distribution of HDL obtained by laser densitometric scans of stained gels.


A
apoA-I in
d<1.25 g/ml
(ug/ml)
0 h
288
6 h
265
12 h
245
24 h
194
Absorbance (633 nm)
11.0
8.8
8.0
7.6
Stokes' Diameter (nm)

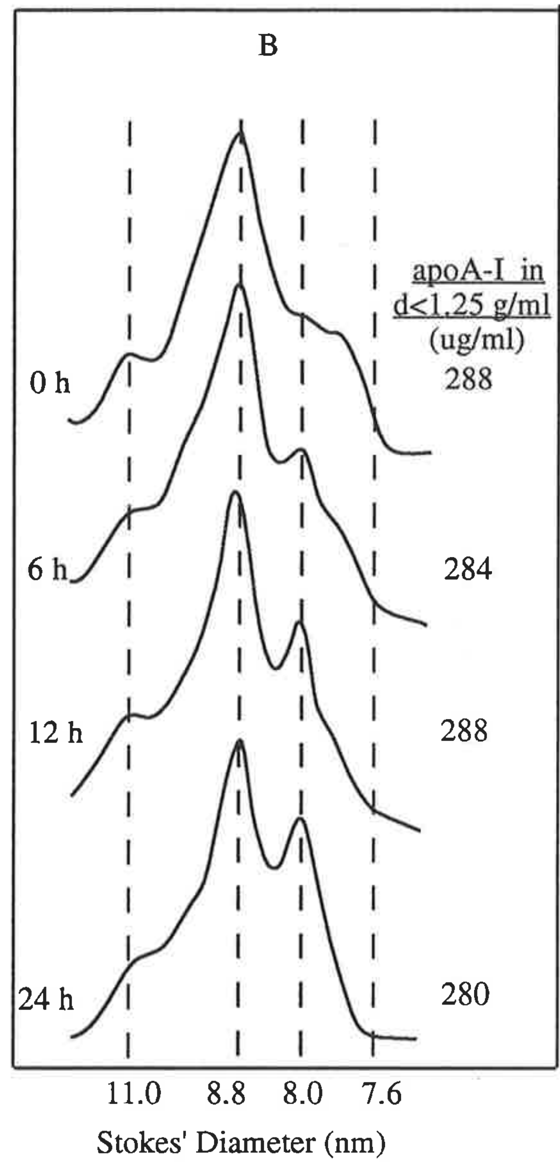
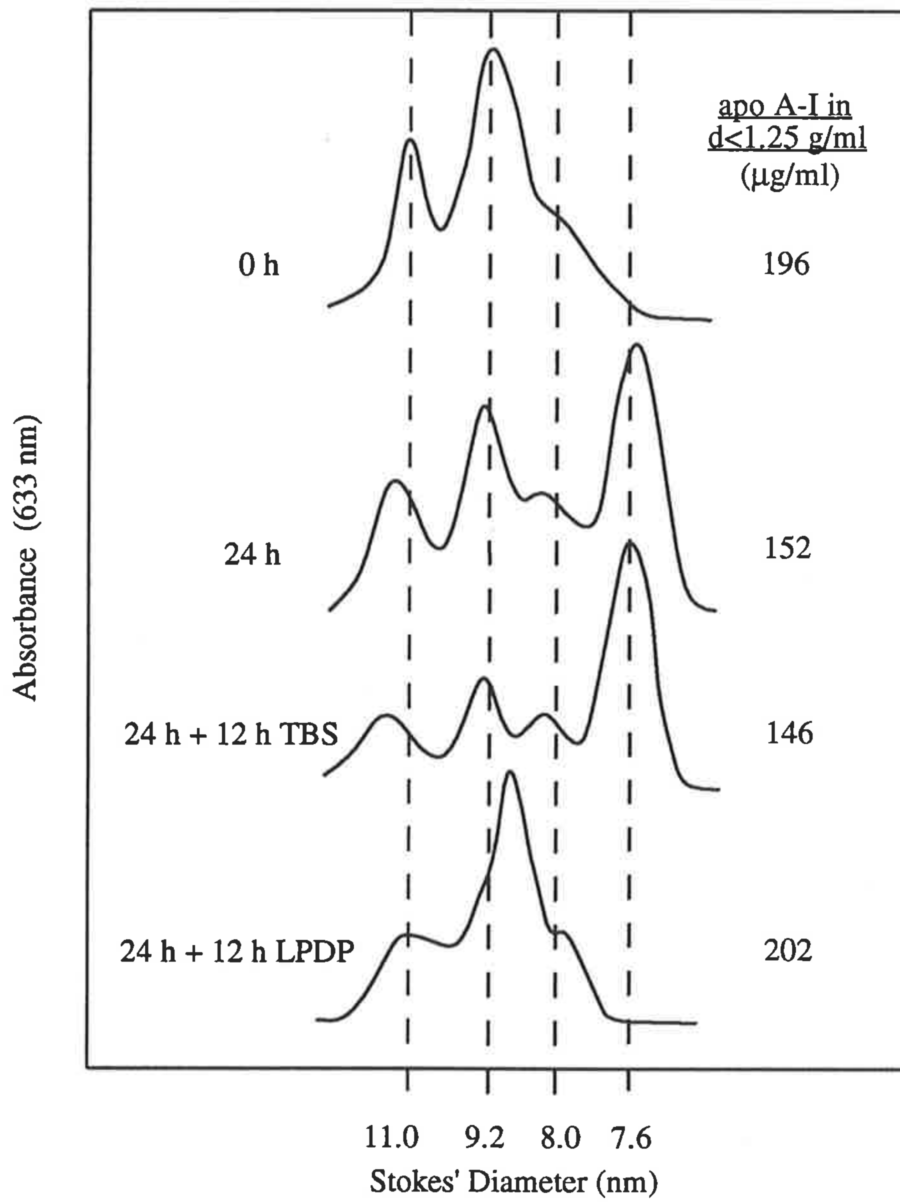

B
apoA-I in
d<1.25 g/ml
(ug/ml)
0 h
288
6 h
284
12 h
288
24 h
280
11.0
8.8
8.0
7.6
Stokes' Diameter (nm)

Fig. 5-2. Ability of lipoprotein-deficient plasma to reverse the reduction in HDL size and the dissociation of apo A-I. The plasma fraction of d< 1.21 g/ml (15 μl containing 432 nmol total cholesterol and 144 μg apo A-I) was supplemented with CETP (final concentration 6 units/ml) in an incubation volume of 200 μl. The mixture was either kept at 4 °C (0 h) or incubated for 24 h at 37 °C (24 h). After 24 h of incubation, aliquots were supplemented with either 500 μl TBS (24h + 12h TBS) or 500 μl of the lipoprotein-deficient (d> 1.25 g/ml) fraction of plasma (24h + 12h LPDP) and incubated at 37 °C for a further 12 h. The volume during this second incubation was 700 μl. After the incubations, lipoproteins in the fraction of d< 1.25 g/ml was recovered by ultracentrifugation, assayed for apo A-I concentration and subjected to non-denaturing gradient gel electrophoresis. Profiles show the particle size distribution of HDL obtained by laser densitometric scans of stained gels.



apo A-I in
d<1.25 g/ml
(µg/ml)
0 h
196
24 h
152
24 h + 12 h TBS
146
24 h + 12 h LPDP
202
Absorbance (633 nm)
11.0
9.2
8.0
7.6
Stokes' Diameter (nm)

incubated at 37 ^{o}C for a further 12 h. When this second incubation contained TBS, there was no further change in either HDL particle size (Fig. 5-2, 24 h + 12 h TBS) or the amount of apo A-I associated with the fraction of d< 1.25 g/ml. When, however, the incubation was supplemented with lipoprotein-deficient plasma for the last 12 h of incubation, the small HDL were reconverted into larger HDL (Fig. 5-2, 24 h + 12 h LPD plasma) and all of the apo A-I which had been lost returned to the fraction of d< 1.25 g/ml.

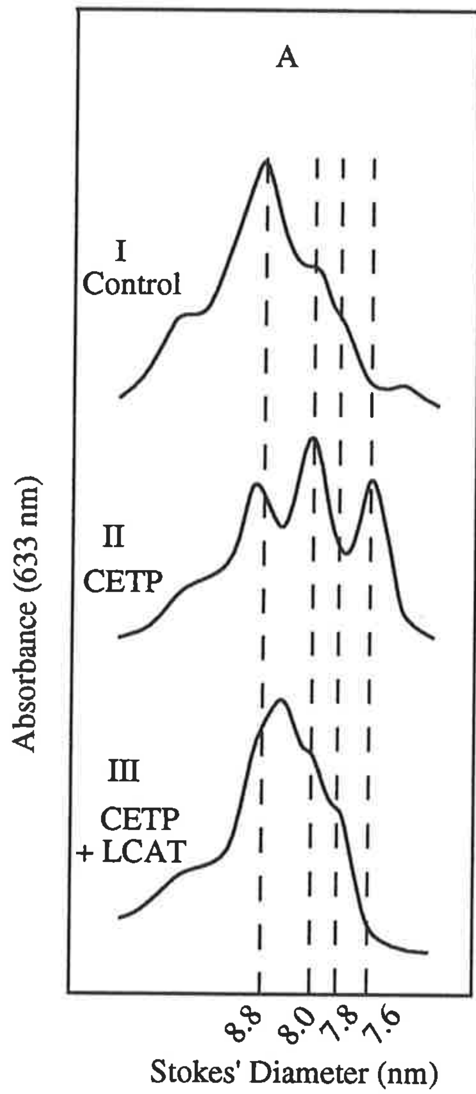
Studies using purified LCAT (Fig. 5-3)

The return of the dissociated apo A-I to the lipoprotein fraction was also studied by adding purified LCAT. Similar to the studies described above, incubation of HDL, LDL and VLDL (the plasma fraction d<1.21 g/ml) in the presence of CETP resulted in a marked reduction in HDL particle size, with an appearance of a substantial population of HDL_{3c} (Fig. 5-3A, profile II). There was also a loss of 33% of the apo A-I from the d 1.25 g/ml supernatant (Fig. 5-3B). When the mixture was subsequently supplemented with bovine serum albumin and LCAT in an amount sufficient to esterify 30% of the free cholesterol in the mixture during 12 h of incubation, the HDL size distribution (Fig. 5-3A, profile III) returned towards that in a nonincubated sample (profile I). Coincident with this increase in HDL size, most of the dissociated apo A-I returned to the d 1.25 g/ml supernatant (Fig. 5-3B). The presence of albumin but no LCAT in the second 12 h incubation had no effect on the HDL particle size and did not return the dissociated apo A-I to the d 1.25 g/ml supernatant (result not shown).

Incorporation of purified, lipid-free apo A-I into HDL (Fig. 5-4, Table 5.1)

These experiments were conducted to determine whether exogenous, purified, lipid-free apo A-I is incorporated into HDL which are increasing in particle size. To this end, HDL were first reduced in size and depleted of apo A-I by incubating the plasma fraction of d< 1.21 g/ml with CETP for 24 h as described above. The modified lipoproteins were separated ultracentrifugally from the CETP as the fraction of d< 1.21 g/ml before being

Fig. 5-3. Reversal of the reduction in HDL particle size and return of the dissociated apo A-I to the lipoprotein fraction during incubation with LCAT. A mixture of HDL, LDL, and VLDL (20 µl of the plasma fraction of $d < 1.21$ g/ml containing 482 nmol cholesterol and 155 nmol triglyceride) was supplemented with CETP (final concentration of 3.1 units/ml) in a final volume of 180 µl and incubated at 37 °C for 24 h. After this incubation, one aliquot was placed on ice, while another was supplemented with bovine serum albumin (final concentration of 20 mg/ml) and LCAT to give a final mixture volume of 580 µl and incubated for an additional 12 h. The amount of LCAT added was sufficient to esterify 30% of the unesterified cholesterol present in the incubation mixture. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and were electrophoresed on a 3-30% non denaturing gradient gel. Panel A presents the particle size distribution of HDL. Profile I shows a sample kept at 4 °C. Profile II shows the results of 24 h of incubation with CETP. Profile III shows the result of 24 h of incubation with CETP followed by a further 12 h of incubation with LCAT. Panel B shows the recovery of apo A-I in the d 1.25 g/ml supernatant under the various conditions.


A
I
Control
II
CETP
III
CETP
+ LCAT
Absorbance (633 nm)
8.8
8.0
7.8
7.6
Stokes' Diameter (nm)

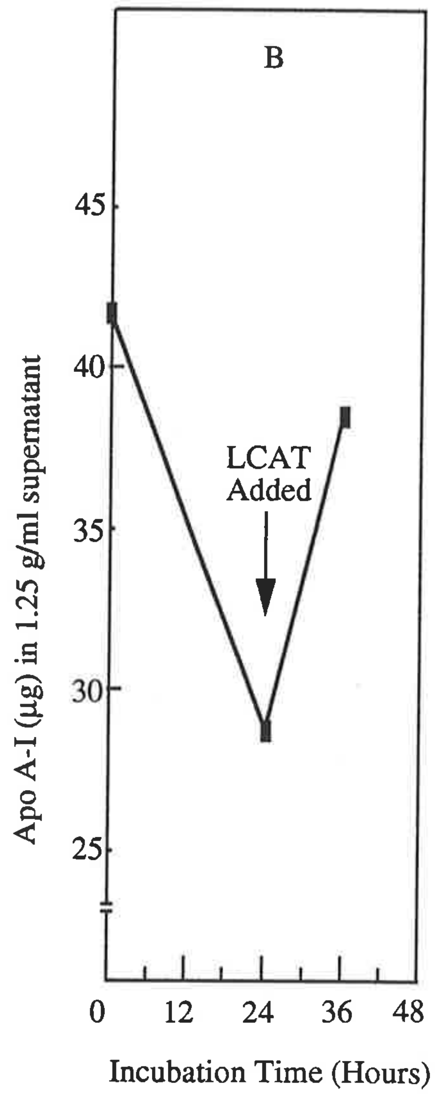

B
45
40
35
30
25
LCAT
Added
Apo A-I (µg) in 1.25 g/ml supernatant
0
12
24
36
48
Incubation Time (Hours)

Fig. 5-4. Effects lipid-free apo A-I on HDL size. The plasma fraction of d< 1.21 g/ml was mixed with CETP and incubated for 24 h as described in the legend to Fig. 5-1. The modified lipoproteins were then recovered by ultracentrifugation as the fraction of d< 1.21 g/ml. Aliquots of this mixture (100 µl containing 450 nmol total cholesterol and 89 µg apo A-I) were supplemented with 500 µl of the lipoprotein-deficient (d> 1.25 g/ml) fraction of plasma and either kept at 4 °C (profile I) or incubated at 37 °C for 12 h (profile III). Other aliquots were supplemented with both lipoprotein-deficient plasma and 38 µg lipid-free apo A-I and either kept at 4 °C (profile II) or incubated for 12 h at 37 °C (profile IV). The final incubation volumes were 600 µl. After the incubations, the fraction of d< 1.25 g/ml was recovered and subjected to non-denaturing gradient gel electrophoresis to monitor HDL particle size. The profiles represent laser densitometric scans of the gels from a single experiment which is representative of four separate experiments.

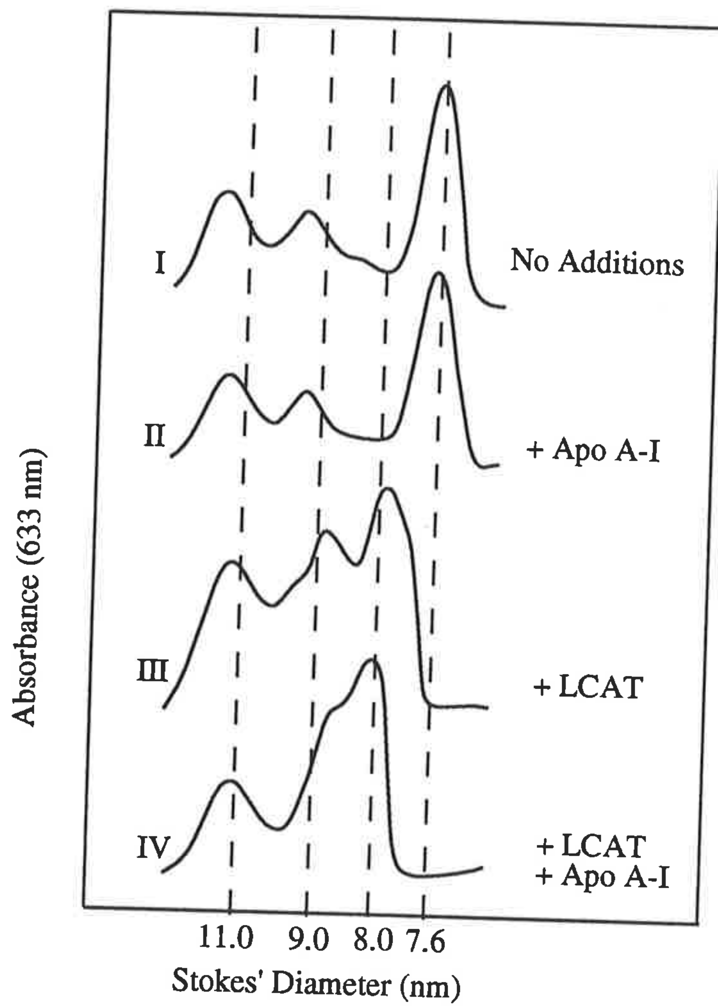

I
No Additions
II
+ Apo A-I
III
+ LCAT
IV
+ LCAT
+ Apo A-I
Absorbance (633 nm)
11.0
9.0
8.0
7.6
Stokes' Diameter (nm)

Table 5.1 Incorporation of Lipid-free ApoA-I into Lipoproteins

Temperature	ApoA-I added	Concentration in fraction of d<1.25 g/ml	
		TC[a] (nmol/ml)	ApoA-I (μg/ml)
4°C	-	689 ± 103	94 ± 17
4°C	+	707 ± 117	98 ± 18
37°C	-	695 ± 131	107 ± 19 *
37°C	+	705 ± 129	124 ± 21**

Plasma lipoprotein of d< 1.21 g/ml were mixed with CETP and incubated at 37 °C for 24 h as described in the legend to Fig. 5-1. The modified lipoproteins were reisolated by ultracentrifugation as the fraction of d< 1.21 g/ml and supplemented with 500 μl of the lipoprotein-deficient (d>1.25 g/ml) fraction of plasma. Samples were further supplemented with either TBS or TBS containing 30 μg lipid-free apo A-I and then either kept at 4 °C of incubated for 12 h at 37 °C. The final volume was 600 μl. After this incubation, the fraction of d>1.25 g/ml was recovered by ultracentrifugation and assayed for total cholesterol and apo A-I. Values represent the mean±SD of four separate experiments, each of which was performed in duplicate.

[a] TC denotes total cholesterol.

* $p < 0.05$ compared to those kept at 4 °C; ** $p < 0.001$ compared to all others.

combined with lipoprotein-deficient plasma and subjected to further 12 h of incubation in the absence or the presence of exogenous, lipid-free apo A-I. Following this second incubation, the HDL (plus VLDL and LDL) were recovered as the ultracentrifugal fraction of d< 1.25 g/ml. In mixtures which were kept at 4°C, the modified HDL consisted several populations, the most prominent of which contained small particles of diameter 7.6 nm (Fig. 5-4, profile I). At 4°C, the addition of exogenous, lipid-free apo A-I had no effect on either the HDL size distribution (Fig. 5-4, profile II) or the concentration of apo A-I in the fraction of d< 1.25 g/ml (Table 5.1). When the mixture was incubated at 37 ^{O}C, the HDL particles of diameter 7.6 nm were converted into larger particles whether or not lipid-free apo A-I was present (Fig. 5-4, profiles III and IV). In the 37 ^{O}C incubations conducted in the absence of exogenous, lipid-free apo A-I, there was an 11% increase in the concentration of apo A-I in the fraction of d<1.25 g/ml (Table 5.1), possible reflecting an incorporation into HDL of apo A-I present as a contaminant in the lipoprotein-deficient plasma. However, when the mixture was incubated at 37 ^{O}C in the presence of exogenous, lipid-free apo A-I, the concentration of apo A-I in the d< 1.25 g/ml fraction increased by 29% over that in the 4°C controls and by 16% over that in samples incubated at 37 ^{O}C in the absence of added lipid-free apo A-I (Table 5.1). This 16% increase represented an incorporation into the fraction of d< 1.25 g/ml of 34% of the lipid-free apo A-I which had been added. The increases in concentration of apo A-I were confined to lipoproteins in the density range 1.063-1.25 g/ml, with no apo A-I detectable in particles of d< 1.063 g/ml in any of the incubations (results not shown).

Role of LCAT in the incorporation of lipid-free apo A-I into HDL (Table 5.2)

Further studies were conducted to define the effects of LCAT and the consequent increase in HDL size on the incorporation of lipid-free apo A-I into HDL. As in the experiments described above, the particle size of HDL was first decreased by incubating the plasma fraction of d< 1.21 g/ml in the presence of CETP. The d< 1.21 g/ml fraction was then reisolated by ultracentrifugation, supplemented with bovine serum albumin and either kept at 4°C or incubated at 37 ^{O}C in the presence of lipid-free apo A-I alone, LCAT alone

or apo A-I plus LCAT. Incubation in the absence of LCAT did not increase HDL particle size. However, when the incubation mixture contained LCAT, the size of the HDL increased in a manner comparable to that observed in incubations with lipoprotein-deficient plasma as shown in profiles III and IV in Figure 5-4 (result not shown). The concentration of apo A-I in the fraction of d< 1.25 g/ml did not change when lipid-free apo A-I and LCAT were added to samples kept at 4°C. Nor was there a change in the concentration of apo A-I in the d< 1.25 g/ml fraction in 37°C samples supplemented with lipid-free apo A-I in the absence of LCAT or with LCAT in the absence of added apo A-I (Table 5.2). However, when incubations were conducted at 37°C in the presence of both LCAT and apo A-I, there was a 36% increase in the concentration of apo A-I in the fraction of d< 1.25 g/ml (Table 5.2).

To determine whether pre-existing HDL are necessary for the LCAT-mediated incorporation of apo A-I into the lipoprotein fraction, preparations of LCAT and lipid-free apo A-I were mixed with the plasma fraction of d< 1.063 g/ml (containing LDL and VLDL) and incubated in the presence and absence of HDL which had been reduced in particle size as described above. In the absence of the HDL there was no incorporation of apo A-I into the lipoprotein fraction of d< 1.25 g/ml, while in the presence of the HDL there was an incorporation of apo A-I which was comparable to that shown in Table 5.2 (result not shown).

To determine whether the LCAT-mediated incorporation of apo A-I into HDL was dependent on an increase in particle size, unmodified HDL which had not been reduced in size or depleted of apo A-I were incubated with LCAT and lipid-free apo A-I. The lipoproteins in the unmodified plasma fraction of d< 1.21 g/ml (HDL, VLDL and LDL) were isolated by ultracentrifugation, mixed with LCAT and bovine serum albumin and incubated at 37°C for 24 h in the presence and absence of purified, lipid-free apo A-I. LCAT promoted only minor increases in HDL size whether or not lipid-free apo A-I was present. When lipid-free apo A-I was added to the mixture, there was only a minimal (<

Table 5.2 Effects of LCAT on the Incorporation of Lipid-free Apo A-I into Lipoproteins

Temperature	LCAT added	Apo A-I added	Apo A-I in d<1.25 g/ml (Percent of control)
4 °C	+	+	100 ± 5
37 °C	-	+	100 [a]
37 °C	+	-	105 ± 2
37 °C	+	+	136 ± 18

Plasma lipoprotein of d< 1.21 g/ml were mixed with CETP and incubated at 37 °C for 24 h as described in the legend to Fig. 5-1. The modified lipoproteins were reisolated by ultracentrifugation as the fraction of d< 1.21 g/ml. Aliquots of this fraction (85 µl containing 370 nmol total cholesterol, 92 nmol of unesterified cholesterol and 70 µg apo A-I) were supplemented with bovine serum albumin (final concentration 20 mg/ml) and either kept at 4 °C in the presence of LCAT and lipid-free apo A-I (30 µg), or incubated in the presence of lipid-free apo A-I alone, LCAT alone or both apo A-I and LCAT for 12 h at 37 °C. The amount of LCAT added was sufficient to esterify 50 nmol cholesterol during the 12 h of incubation. Final volumes were 300 µl. Following the incubation, the mixtures were subjected to ultracentrifugation to recover the fraction of d< 1.25 g/ml which was assayed for apo A-I. Values are expressed relative to control samples kept at 4 °C with no added LCAT and no added lipid-free apo A-I.

[a] This value is the mean of two experiments.

5%) increase in the concentration of apo A-I in the fraction of d< 1.25 g/ml (result not shown).

5.4 DISCUSSION

Studies in Chapter 3 and 4 have clearly demonstrated that a CETP-mediated reduction in HDL size promotes the dissociation of substantial amounts of lipid-free apo A-I from HDL. It is not known whether a comparable dissociation of lipid-free apo A-I from HDL occurs in vivo. If it does, the dissociated apo A-I could have several potential fates: (i) It could enter the interstitial space and function as an acceptor of cellular cholesterol (Hara and Yokoyama, 1991), (ii) it could be excreted through the kidney (Horowitz et al., 1992) or (iii) it could interact with the phospholipids released from triglyceride-rich lipoproteins undergoing lipolysis to form discoidal structures (Clay et al., 1992; Clay and Barter, 1996). Alternatively, just as lipid-free apo A-I dissociates from HDL when they lose core lipids and become smaller, it is possible that lipid-free apo A-I may reincorporate into HDL which are increasing in size as cholesteryl esters are generated by LCAT. This would indicate the existence of a cyclic dissociation and reassociation of apo A-I with HDL. The "association" component of this cycle has been confirmed in vitro in the present studies by establishing that there is a net mass incorporation of purified, lipid-free apo A-I into HDL which are increasing in size during incubation with LCAT. The fact that the exogenous apo A-I appeared in the HDL fraction only when the incubation mixtures already contained HDL, demonstrated that the apo A-I was incorporated into pre-existing HDL particles and was not formed into new particles.

Incorporation of exogenous, lipid-free apo A-I into HDL has been observed previously in incubations of human whole plasma (Hopkins and Barter, 1989). In these earlier studies, the presence of exogenous apo A-I inhibited changes in HDL size (Hopkins and Barter, 1989). This inhibition of HDL size change by exogenous, lipid-free apo A-I was not observed in the present studies in which an LCAT-mediated increase in the size of

very small HDL was unaffected by the addition of exogenous, lipid-free apo A-I. The mechanism by which apo A-I inhibited the changes in HDL size in the whole plasma studies is not known. It is possible, however, that the changes in HDL size which occur in whole plasma incubations conducted in the absence of exogenous apo A-I are secondary to fusion and subsequent remodelling of HDL particles. As discussed below, it may be argued that such fusion of HDL particles does not take place when lipid-free apo A-I is present.

The capacity of LCAT to increase the size of HDL is well documented (Rajaram and Barter, 1986). It has also been reported that LCAT-mediated increases in HDL particle size are accompanied by an increase in the number of apo A-I molecules per HDL particle (Nichols et al., 1985). This has been best demonstrated in studies using reconstituted HDL (rHDL). In such studies, the generation of cholesteryl esters by LCAT promotes the conversion of discoidal rHDL containing two molecules of apo A-I into small spherical rHDL which also contain two molecules of apo A-I (Nichols et al., 1985). Further generation of cholesteryl esters by LCAT results in the conversion of the small spherical rHDL into larger particles containing three molecules of apo A-I (Nichols et al., 1985). The fact that this increase in the number of apo A-I molecules per particle is achieved in incubations which do not contain lipid-free apo A-I has led to the conclusion that there must be some form of particle fusion (Nichols et al., 1985). It has been postulated that fusion of two small particles which each contain two molecules of apo A-I generates an unstable intermediate containing four molecules of apo A-I. Subsequent dissociation of one molecule of apo A-I from the fusion product would generate a more stable three-apo A-I rHDL. This conclusion has been supported experimentally by the observation that conversion of rHDL containing two apo A-I molecules into larger particles containing three molecules of apo A-I coincides with a loss of apo A-I from particles of d< 1.20 g/ml (Nichols et al., 1985).

In the presents studies, which have used native rather than reconstituted HDL, there was no evidence that an LCAT-mediated increase in HDL size led to a dissociation of apo A-I. In fact, the opposite was observed. So long as lipid-free apo A-I was present, LCAT-mediated increases in HDL size were accompanied by an increase in the concentration of HDL apo A-I. These apparently conflicting results may indicate that an increase in the apo A-I content of an HDL particle can be achieved by more than one mechanism. For example, if there is no source of lipid-free apo A-I, particle fusion may occur. However, if lipid-free apo A-I is present, its direct incorporation into the expanding HDL may be energetically preferable to HDL particle fusion. It has been shown previously that an incorporation of apo A-IV into HDL coincides with an LCAT-mediated increase in HDL size (Castro and Fielding, 1988). It is possible that the incorporation of apo A-I and apo A-IV into HDL is achieved by a similar mechanism which is dependent on an increase in the surface area available for accommodating the apolipoproteins.

Most of the apo A-I in plasma is a component of spherical HDL and has alpha mobility on agarose gel electrophoresis. However, a small proportion of the apo A-I in human plasma has a prebeta mobility (Nichols et al., 1985) comparable to that of lipid-free apo A-I (Lefevre et al., 1989; Chapter 1). Indeed, some of the prebeta-migrating apo A-I in plasma exists in particles which are smaller and denser than the alpha-migrating HDL, although such particles have been shown to contain phospholipids (Nichols et al., 1985; Rye and Barter, 1994). As it was suggested in Chapter 1, these small, prebeta-migrating complexes of apo A-I and phospholipids have their origin as lipid-free apo A-I which has dissociated from HDL. In support of this, prebeta apo A-I has been shown to be generated in plasma by CETP (Kunitake et al., 1992) and to be reconverted into alpha-migrating particles by LCAT (Neary et al., 1991; Miida et al., 1992). It is therefore tempting to speculate that such interconversions of pre-beta and alpha-migrating apo A-I may reflect a cyclical process comparable to the dissociation and reassociation of lipid-free apo A-I from HDL which has been demonstrated in the present studies.

In conclusion, these studies demonstrated the existence in vitro of a cyclic process in which lipid-free apo A-I dissociates from and reassociates with HDL depending on whether the HDL are decreasing or increasing in size. If such a process were to operate in vivo, it would follow that a balance between factors which decrease and increase HDL size may influence not only the particle size distribution of HDL but also the concentration of HDL apo A-I. Furthermore, given that dissociated apo A-I which does not reassociate with HDL may be excreted through the kidney (Horowitz et al., 1992), factors which determine the balance between dissociation and reassociation of apo A-I may also be important determinants of the total concentration of apo A-I in plasma.

CHAPTER 6

EFFECT OF LCAT ON THE INCORPORATION OF LIPID-FREE APO A-I INTO RECONSTITUTED HIGH DENSITY LIPOPROTEIN (R-HDL)

6.1 INTRODUCTION

6.2 METHODS

6.3 RESULTS

Effects of LCAT on the size and composition of rHDL

Incorporation of lipid-free apo A-I into rHDL

Time course of the incorporation of lipid-free apo A-I into rHDL

Concentration-dependence of the incorporation of lipid-free apo A-I into rHDL

Effect of LCAT activity on the incorporation of lipid-free apo A-I into rHDL

6.4 DISCUSSION

6.1 INTRODUCTION

Like other lipoproteins, plasma HDLs undergo continuous changes in their composition and structure under both normal and pathologic status. They may also change in relate to sex, age, nutrition, smoking, and physical exercise. It is now known that heterogeneity of HDL has important implications in their physiological function (Castro and Fielding, 1988) and in their abilities to protect against the development of atherosclerosis (Johansson et al., 1991; Schultz et al., 1993). Much of the heterogeneity of HDL is the consequence of remodelling of the lipoproteins by plasma factors (Barter, 1990). As described in the previous chapters, when HDL interact with triglyceride rich lipoproteins and the cholesteryl ester transfer protein (CETP) there is a reduction in the HDL particle content of cholesteryl esters, a decrease in HDL size and a dissociation of apolipoprotein (apo) A-I from the HDL. Conversely, when lecithin:cholesterol acyltransferase (LCAT) increases the cholesteryl ester content of HDL, there is an associated increase in HDL size and an increase in the number of molecules of apo A-I per particle (Nichols et al., 1985).

A mechanism for the LCAT-mediated increase in the number of apo A-I molecules per particle has been proposed by Nichols and co-workers (Nichols et al., 1985). According to their view, as LCAT increases the cholesteryl ester content of HDL, it also promotes particle fusion to form larger HDL particles in which the number of molecules of both cholesteryl esters and apo A-I are increased. However, studies presented in Chapter 5 let us to have an alternate view, which holds that an LCAT-mediated increase in HDL cholesteryl esters is accompanied by the direct incorporation of lipid-free apo A-I into the HDL particle. The experiments described in this chapter seek to differentiate between these two proposed mechanisms.

In order to overcome the problem of the HDL heterogeneity, discoidal reconstituted high density lipoproteins (rHDL) was used in the studies described in this chapter. These reconstitute micellar particles of defined chemical composition, with the overall size and

density of HDL (Jonas, 1986; Jonas et al., 1989) provide a simplified object for the studies. The preparation of rHDL is based on the dispersion of lipids in sodium cholate, allowing the formation of particles containing apolipoprotein, phosphatidylcholines (PC) and unesterified cholesterol. The resulting complexes are discoidal particles with various diameters, and have the same discoidal morphology for the native discoidal HDL (Jonas, 1986). The resulted discoidal complexes can be further transformed into spherical rHDL particles.

Studies have shown that the synthetic discoidal complexes are suitable ligands of cell surface receptor binding (Lalazar et al., 1989) or as cholesterol acceptors from cells (Steinmetz et al., 1990; Jonas et al., 1994). In the LCAT reaction study, it has been shown that apo A-I and apo C-I are the best activator for LCAT (Jonas et al., 1984), and the enzyme activation by apo A-I depends on the structure of the apolipoprotein in the complexes (Jonas et al., 1984; Jonas and McHugh, 1984). Starting with a homogeneous population of HDL particles, it was possible to study the precursor-product relations resulted from the lipid transfer. Rye and Barter (1994) have shown that the interaction of rHDL with CETP is analogous to that of native HDL. Furthermore, they have studied the role of apolipoproteins in the regulation of this process (Rye et al., 1992; Rye and Barter, 1994) and the effect of CETP on rHDL composition, size and structure (Rye et al., 1992; Rye et al., 1995).

In the present study, the discoidal rHDL was incubated with LCAT in the presence of low density lipoproteins as a source of additional UC for the LCAT reaction. The discoidal rHDL acquired a substantial amount of cholesteryl esters (CE), increased in size, and was converted into spherical particle. As a consequence, the number of the apo A-I molecules per particle from two to three. In some experiments, additional lipid-free apo A-I was also present in the incubation. Based on the total amount of rHDL-associated apo A-I and the number of the apo A-I molecules per particle, the particle number of rHDL itself was estimated. By comparing rHDL particles numbers before

and after the incubation, one can determine whether fusion of rHDL particles or direct incorporation of the lipid-free apolipoprotein into the rHDL have been occurred. If the increase in the number of the apo A-I molecules per particle from two to three is due to particle fusions, there would be a reduction in total number of rHDL particles. On the other hand, if it is due to the direct incorporation of the lipid-free apolipoprotein into the rHDL, that is, every rHDL particle obtains one exogenous lipid-free apo A-I molecule, the amount of rHDL-associated apo A-I would increase approximately 50% and the process does not reduce the number of HDL particles.

6.2 METHODS

Preparation of reconstituted HDL

Discoidal rHDL were prepared from egg phosphatidylcholine (PC), unesterified cholesterol (UC) and apo A-I by the cholate dialysis method (Matz and Jonas, 1982; Jonas et al., 1988). Before use, the discoidal rHDL were incubated at 37 oC for 24 hours with LDL at a protein ratio of 4:1 (apo B : apo A-I) and then subjected to size exclusion chromatography on a prepacked Superose 6 HR 10/30 column (Pharmacia LKB Biotechnology). This approach provided a homogeneous population of discoidal rHDL of diameter 7.9 nm as determined by non-denaturing polyacrylamide gradient gel electrophoresis. The molar ratio of PC : UC : apo A-I was 33:7:1.

Isolation of LCAT

LCAT was isolated from human plasma and assayed for activity as described in Chapter 2. No apo A-I was detected in the purified LCAT. The LCAT activity was expressed as nmol cholesterol esterified per hour per ml of the LCAT solution. The LCAT samples used in this study esterified 91 nmol cholesterol per hour per ml.

Experimental conditions

Details of individual incubations are provided in the legends to the Tables and Figures. Incubation mixtures were placed in sealed plastic tubes and either kept at 4 ^{o}C or incubated at 37 ^{o}C in a shaking water bath. Incubations were terminated by placing the tubes on ice. After incubation, samples were subjected to ultracentrifugation to recover the fraction of d< 1.25 g/ml (total lipoproteins) or the fraction of 1.07 g/ml< d< 1.25 g/ml (rHDL). When isolating the 1.07-1.25 g/ml fraction, samples were subjected to two successive spins at the lower density to ensure complete removal of LDL. An absence of contamining LDL was confirmed by the findings that: (i) apo B was unmeasurable in an assay that is quantitative at concentrations down to 6 μg per ml, (ii) there were no beta-migrating particles detectable when the samples were subjected to agarose gel electrophoresis and (iii) there were no detectable particles of LDL size when the samples were subjected to gradient gel electrophoresis on 3-35% non-denaturing gels. The 1.07-1.25 g/ml fraction was assayed for lipids and apo A-I to determine rHDL composition. It was also subjected to cross-linking to determine the number of molecules of apo A-I per particle, to agarose gel electrophoresis and to electron microscopy. The fraction of d< 1.25 g/ml was assayed for apo A-I to determine the concentration of lipid-associated apo A-I (as distinct from lipid-free apo A-I which has a density > 1.25 g/ml). It was established in preliminary experiments that the fraction of d< 1.07 g/ml did not contain apo A-I under any of the experimental conditions used. Thus, the concentration of apo A-I in the fraction of d< 1.25 g/ml equates with that associated with rHDL. The fraction of d< 1.25 g/ml was also subjected to non-denaturing gradient gel electrophoresis to monitor rHDL size.

Chemical analyses

As described in the "Methods and Materials" chapter, concentrations of total cholesterol, free cholesterol and phospholipid were measured using enzymatically on a Cobas Fara Centrifugal Analyser. The concentration of esterified cholesterol was calculated as the difference between the concentrations of total (esterified plus free) cholesterol and

unesterified cholesterol. Concentration of apo A-I was measured immunoturbidmetrically using sheep anti-human apo A-I antiserum and calibration serum. The assays were standardised using appropriate dilutions of apo calibration serum. These assays have the capacity to quantitate apo A-I and apo B down to concentrations of 10 μg/ml and 6 μg/ml, respectively.

6.3 RESULTS

Effects of LCAT on the size and composition of rHDL (Fig.6-1, Table 6.1)

Prior to being incubated with LCAT, the discoidal rHDL had an apparent of diameter of 7.9 nm and a molar ratio of PC: UC: apo A-I of 33:7:1 (Fig. 6-1A). They contained two molecules of apo A-I per particle as determined by cross-linking (Table 6.1) and appeared as stacked discs in electron micrographs (result not shown). When incubated for three hours at 37 ^oC with LCAT plus LDL (as a source of additional UC for the LCAT reaction), the generation of cholesteryl esters (CE) coincided with the rHDL being converted into smaller particles with a diameter of 7.7 nm and a molar ratio of PL: CE: UC: apo A-I of 26:11:6:1 (Fig. 6-1A). Most of these particles still contained two molecules of apo A-I per particle (Fig. 6-2) but now contained a substantial amount of CE and were probably spherical. Beyond three hours, the 7.7 nm particles were converted progressively into particles of diameter 8.8 nm. After 24 hours, virtually all of the rHDL had a diameter of 8.8 nm (Fig. 6-1A) and a molar ratio of PL: CE: UC: apo A-I of 16:23:3:1 (Fig. 6-1A). There was also an increase from two to three apo A-I molecules per particle as determined by cross-linking (Fig. 6-2). These particles appeared as spheres in electron micrographs (result not shown). The surface charge of the particles was assessed by agarose gel electrophoresis. The non-incubated, discoidal rHDL had an electrophoretic mobility (prebeta) identical to that of lipid-free apo A-I (result not shown). The spherical rHDL formed during 24 hours of incubation at 37 ^oC with LCAT and LDL had an increased electrophoretic mobility compared to that of

discoidal rHDL (Table 6.1). The spherical rHDL migrated to an alpha position comparable to that of native HDL.

When the incubation mixtures of rHDL, LDL and LCAT were supplemented by the addition of an amount of lipid-free apo A-I equivalent to 50% of that present in the rHDL, virtually identical results were obtained. The changes in particle size and composition were the same as those in the incubations conducted without the added lipid-free apo A-I (Fig. 6-1B). As in the absence of lipid-free apo A-I, 24 hours of incubation converted the prebeta-migrating, discoidal rHDL into alpha-migrating, spherical particles in which the number of apo A-I molecules per particle was increased from two to three.

When these incubations were conducted in the presence of LDL but in the absence of LCAT there was no change in the size or composition of the original rHDL, whether or not lipid-free apo A-I was added to the incubation mixture (result not shown).

As stated above, when discoidal rHDL were incubated with LCAT and LDL, the number of apo A-I molecules per particle increased from two to three, regardless of whether the incubation was supplemented with lipid-free apo A-I (Table 6.1). In the absence of added lipid-free apo A-I, this increase in the number of apo A-I molecules occurred without any alteration in the concentration of rHDL-associated apo A-I (Table 6.2). This indicated that the number of rHDL particles must have been reduced by one third (Table 6.1). In the presence of lipid-free apo A-I, on the other hand, the increase from two to three in the number of apo A-I molecules per particle was accompanied by a 44% increase in the concentration of rHDL-associated apo A-I (Table 6.2), indicating that under these conditions the number of rHDL particles was essentially unchanged (Table 6.1).

Figure 6-1. Changes in size and composition of rHDL during incubation with LCAT in the absence and in the presence of added lipid-free apo A-I. Incubations were conducted at 37 ^{o}C for 0 to 24 h. Incubation mixtures contained discoidal rHDL (apo A-I final concentration 0.1 mg/ml), LDL (apo B final concentration 0.4 mg/ml), LCAT (2.3 ml), bovine serum albumin (18 mg/ml final concentration) and β-mercaptoethanol (final concentration 14 mM). In the incubations shown in panel B, the mixtures were supplemented by the addition of lipid-free apo A-I at a final concentration of 0.05 mg/ml, equivalent to 50% of the apo A-I in the discoidal rHDL. The final incubation volume was 2.65 ml. After incubation, the fraction of density 1.07-1.25 g/ml was isolated by ultracentrifugation and assayed for composition. The d< 1.25 g/ml fraction was also recovered and subjected to gradient gel electrophoresis as described in the methods. The profiles showing the particle size distribution of rHDL were obtained by laser densitometric scans of stained gels.

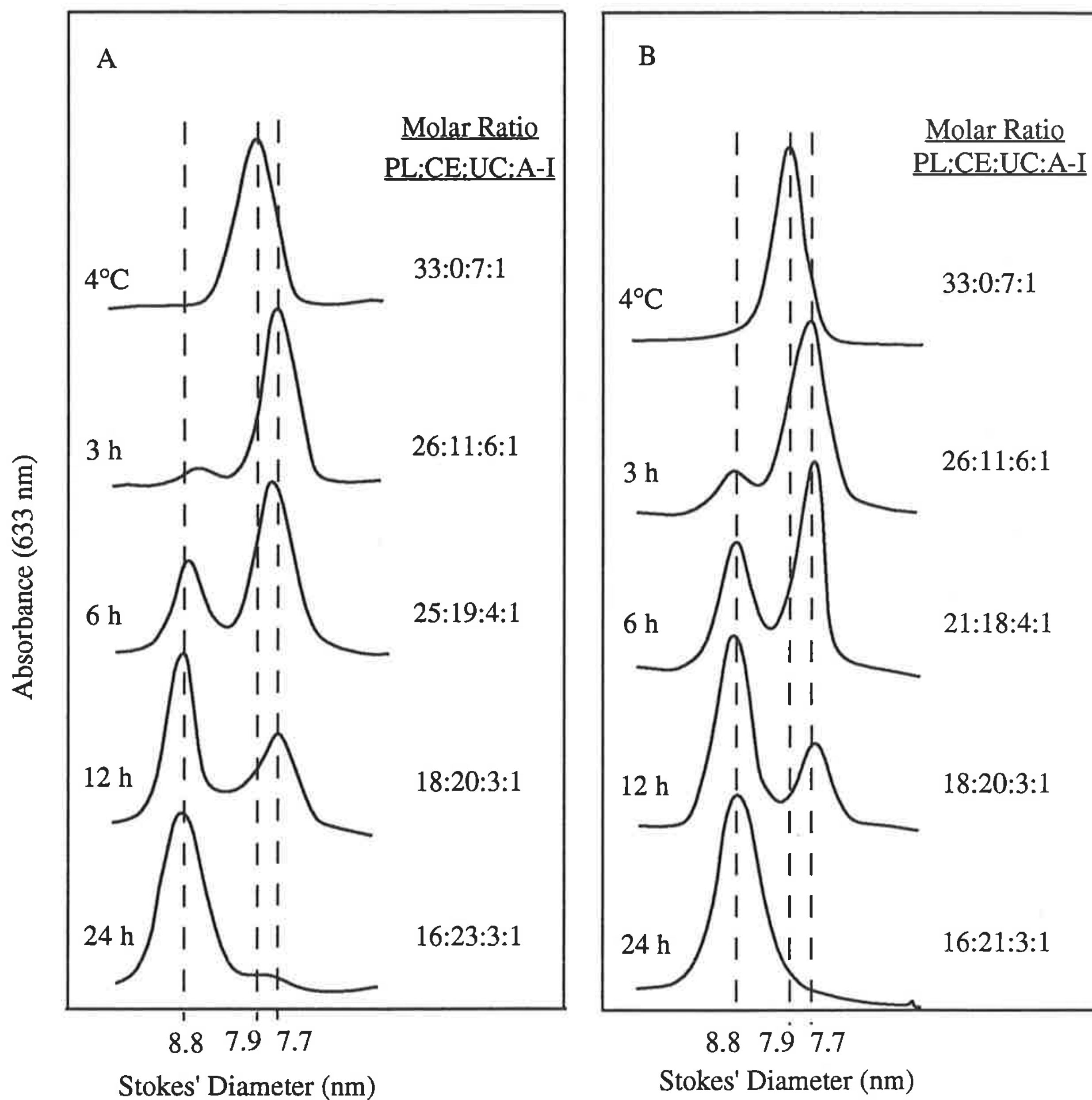

No lipid-free apo A-I added
Lipid-free apo A-I added
A
Molar Ratio
PL:CE:UC:A-I
4°C
33:0:7:1
3 h
26:11:6:1
6 h
25:19:4:1
12 h
18:20:3:1
24 h
16:23:3:1
B
Molar Ratio
PL:CE:UC:A-I
4°C
33:0:7:1
3 h
26:11:6:1
6 h
21:18:4:1
12 h
18:20:3:1
24 h
16:21:3:1
Absorbance (633 nm)
8.8 7.9 7.7
Stokes' Diameter (nm)
8.8 7.9 7.7
Stokes' Diameter (nm)

Figure 6-2. Cross-linking of rHDL. Aliquots of rHDL were maintained at 4 ^{o}C or incubated at 37 ^{o}C under various conditions. At the end of the incubations, the rHDL were isolated by ultracentrifugation as described in the legend to Table 6.1 and cross-linked as described in the Chapter 2. The cross-linked samples were then subjected to electrophoresis on a 3-35% SDS gradient gel. Track 1 shows cross-linked, lipid-free apo A-I. Track 2 and 3 represent, respectively, rHDL which were either maintained at 4 ^{o}C or incubated for 24 h at 37 ^{o}C with no additions. The rHDL in track 4 and 5 were mixed with LDL and LCAT, as in Table 6.1, and incubated at 37 ^{o}C for 3h and 24 h, respectively. Track 6 shows rHDL following incubation at 37 ^{o}C for 24 h with LDL, LCAT and exogenous lipid-free apo A-I as in Table 6.1.

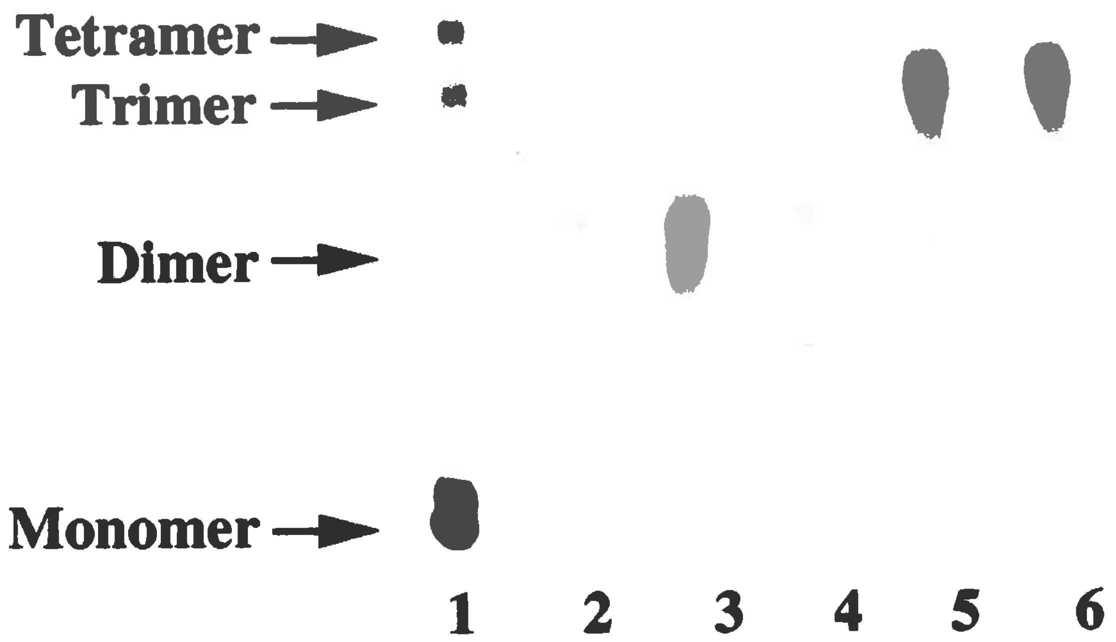

Tetramer
Trimer
Dimer
Monomer
1
2
3
4
5
6

Table 6.1 All incubations were for 24 h and contained rHDL (final apo A-I concentration 0.1 mg/ml), LDL (final apo B concentration 0.4 mg/ml), LCAT (2.3 ml), bovine serum albumin (final concentration 18 mg/ml) and β-mercaptoethanol (final concentration 14 mM). The final incubation volume was 2.65 ml. After incubations, the rHDL were recovered by ultracentrifugation in the density interval 1.07-1.25 g/ml. The results represent the means of three experiments.

[a] The numbers of apo A-I molecules per rHDL particle were determined by cross-linking. The numbers of molecules of other constituents were calculated relative to apo A-I.

[b] Electrophoretic mobility was calculated as described in Chapter 2.

[c] Determined by electron microscopy.

[d] The relative numbers of rHDL particles were calculated from the relative concentrations of rHDL-associated apo A-I as shown in Table 6.2 and the number of apo A-I molecules per rHDL particle.

[e] The amount of added lipid-free apo A-I was equivalent to 50% of the apo A-I in the discoidal rHDL.

[f] Not determined.

Table 6.1 Characterization of the rHDL Formed During Incubation with LCAT and LDL in the Presence and Absence of Lipid-free Apo A-I.

Incubation Conditions	Molecules of constituents / particle				Electrophoretic mobility [b]	Morphology[c]	Relative number of particles [d]
	PL	UC	CE	Apo A-I [a]			
4 ^{o}C (no lipid-free apo A-I)	68	14	1	2	0.43	discoidal	100
4 ^{o}C (+ lipid-free apo A-I)	66	15	1	2	*f*	*f*	99
37 ^{o}C (no lipid-free apo A-I)	52	9	67	3	0.52	spherical	62
37 ^{o}C (+ lipid-free apo A-I)[e]	53	8	63	3	0.52	spherical	96

Incorporation of lipid-free apo A-I into rHDL (Table 6.2)

While the addition of lipid-free apo A-I had no observable effect on the composition, size and electrophoretic mobility of the rHDL formed during incubation of discoidal rHDL with LCAT and LDL (Table 6.1, Fig. 6-1), there was, nevertheless, an obvious incorporation of the lipid-free apo A-I into the HDL fraction (Table 6.2). When incubations were conducted in the absence of added lipid-free apo A-I, the concentration of rHDL-associated apo A-I did not change (Table 6.2). However, when exogenous, lipid-free apo A-I was included in the incubation mixture in an amount equivalent to 50% of that in the discoidal rHDL, there was a 44% increase in the concentration of rHDL-associated apo A-I (Table 6.2). This apparent quantitative incorporation of lipid-free apo A-I into rHDL was supported by the observation in samples incubated at 37 ^{o}C in the presence of LCAT that > 90% of the apo A-I in the total incubation mixtures was recovered in the ultracentrifugal fraction of d< 1.25 g/ml, with no measurable apo A-I being found in the fraction of d< 1.25 g/ml, whether or not the samples had been supplemented with exogenous, lipid-free apo A-I (result not shown). In fact, measurable amounts of apo A-I were recovered in the d< 1.25 g/ml fraction only in those samples that were supplemented with exogenous apo A-I and either kept at 4 ^{o}C or incubated at 37 ^{o}C in the absence of LCAT. Under these circumstances more than 90% of the added lipid-free apo A-I was recovered in the d> 1.25 g/ml fraction.

Time course of the incorporation of lipid-free apo A-I into rHDL (Fig.6-3)

The time course of incorporation of exogenous, lipid-free apo A-I into rHDL during incubation with LCAT and LDL (Fig. 6.3) roughly paralleled the formation of the 8.8 nm particles (Fig.6-1B). After 12 hours of incubation, when about one third of the rHDL had been converted into particles with a diameter of 8.8 nm (Fig. 6-1B), the concentration of rHDL-associated apo A-I had increased by about 20%, while after 24 hours, when all of the rHDL had been converted to 8.8 nm particles, the rHDL-associated apo A-I concentration was increased by more than 40% (Fig. 6-3).

Table 6.2 Effect of LCAT on rHDL-associated Apo A-I

	Recovery of Apo A-I in d<1.25 g/ml Fraction (Percentage of Control)[a]		
	4 °C	37 °C	37 °C
	+ LCAT + apo A-I[b]	+ LCAT - apo A-I[b]	+ LCAT + apo A-I[b]
Experiments			
1.	100	98	143
2.	98	98	148
3.	103	102	150
4.	102	103	138
5.	98	99	141
6.	94	105	144
Mean±SD	99.2 ± 3.3	100.8 ± 2.9	144.0 ± 4.4

Discoidal rHDL (final apo A-I concentration 0.1 mg/ml), LDL (final apo B concentration 0.4 mg/ml), LCAT (2.1 ml), bovine serum albumin (final concentration 18 mg/ml) and β-mercaptoethanol (final concentration 14 mM) were incubated at 37 °C for 24 h. The final incubation volume was 2.5 ml. After incubations, the rHDL were recovered by ultracentrifugation in the density interval 1.07-1.25 g/ml.

[a] The amount of apo A-I associated with rHDL is expressed as a percentage of that in a control sample kept at 4 °C with no additions.

[b] Lipid-free apo A-I was added (final concentration 0.05 mg/ml) in an amount equivalent to 50% of the apo A-I in the discoidal rHDL.

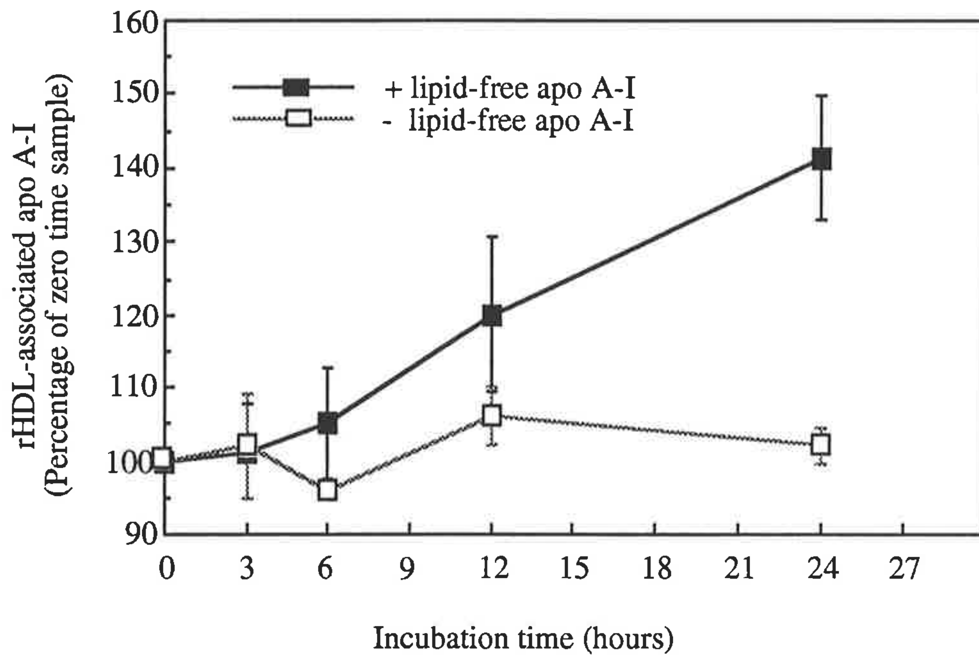


Figure 6-3. Time course of the incorporation of lipid-free apo A-I into rHDL during incubation with LCAT. Incubation conditions were as described in the legend to Figure 6-1. After incubation, rHDL-associated apo A-I was recovered as the ultracentrifugal fraction of d< 1.25 g/ml. Concentrations of rHDL-associated apo A-I are expressed as percentages of those in samples kept at 4 °C with no additions. The data points and bars represent the respective means and standard deviations of four experiments.

Concentration-dependence of the incorporation of lipid-free apo A-I into rHDL (Fig. 6-4)

To determine whether the concentration of lipid-free apo A-I is a limiting factor in its incorporation into rHDL, mixtures of discoidal rHDL, LCAT and LDL were supplemented with increasing amounts of apo A-I. At concentrations up to about 50% of that in the discoidal rHDL, there was a quantitative incorporation of the lipid-free apo A-I into rHDL (Fig. 6-4). At this point, virtually all of the rHDL had been converted from particles containing two molecules of apo A-I into particles containing three molecules of apo A-I (Fig. 6-2). Increasing the concentration of the exogenous apo A-I to levels above 50% of that initially in rHDL did not further increase its incorporation (Fig. 6-4). Under these conditions the surplus was recovered in the lipoprotein-free fraction of d> 1.25 g/ml. Thus, the amount of lipid-free apo A-I is a limiting factor in its incorporation into rHDL only when present at concentrations less than 50% of that initially in the rHDL.

Effect of LCAT activity on the incorporation of lipid-free apo A-I into rHDL (Fig. 6-5)

Not only was the incorporation of exogenous, lipid-free apo A-I into rHDL during incubation with LDL dependent on the presence of LCAT but the magnitude of the incorporation was a function of the amount of LCAT added (Fig. 6-5). In incubations that did not contain added lipid-free apo A-I, however, the concentration of rHDL-associated apo A-I was uninfluenced by the presence of LCAT.

6.4 DISCUSSION

The effects of LCAT on rHDL in the present study are qualitatively consistent with that presented in Chapter 5. In each case LCAT increased both the cholesteryl ester content and the size of HDL and also promoted the incorporation of lipid-free apo A-I into HDL. By taking advantage of the homogeneity of the rHDL, we were also able to demonstrate in the present studies that the number of molecules of apo A-I per particle increased during the incubation with LCAT (Table 6.1).

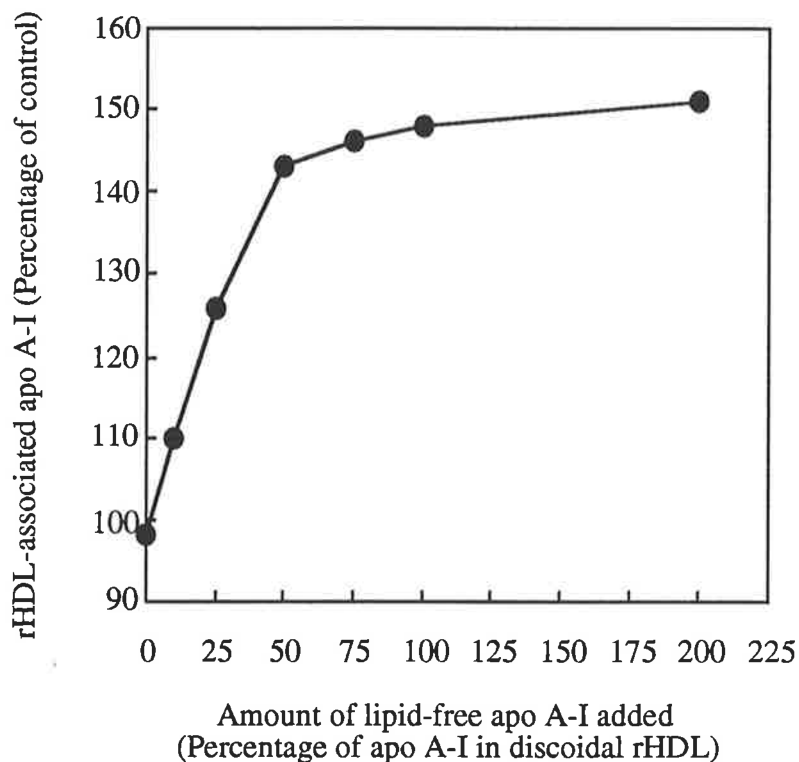


Figure 6-4. Concentration dependence of the incorporation of lipid-free apo A-I into rHDL during incubation with LCAT. Incubations were conducted at 37 °C for 24 hours. Incubation mixtures contained discoidal rHDL (final apo A-I concentration 0.1 mg/ml), LDL (final apo B concentration 0.4 mg/ml), LCAT (0.85 ml), bovine serum albumin (final concentration 18 mg/ml) and b-mercaptoethanol (final concentration 14 mM). Exogenous, lipid-free apo A-I was added to the mixture at concentrations ranging from 0 to 0.2 mg/ml, equivalent to 0 to 200% of that in the discoidal rHDL. The final incubation volume was 1.0 ml. After incubation, rHDL-associated apo A-I was recovered as the ultracentrifugal fraction of d< 1.25 g/ml. Concentrations of rHDL-associated apo A-I are expressed as percentages of those in samples kept at 4 °C with no additions. Each point represents the mean of two experiments.

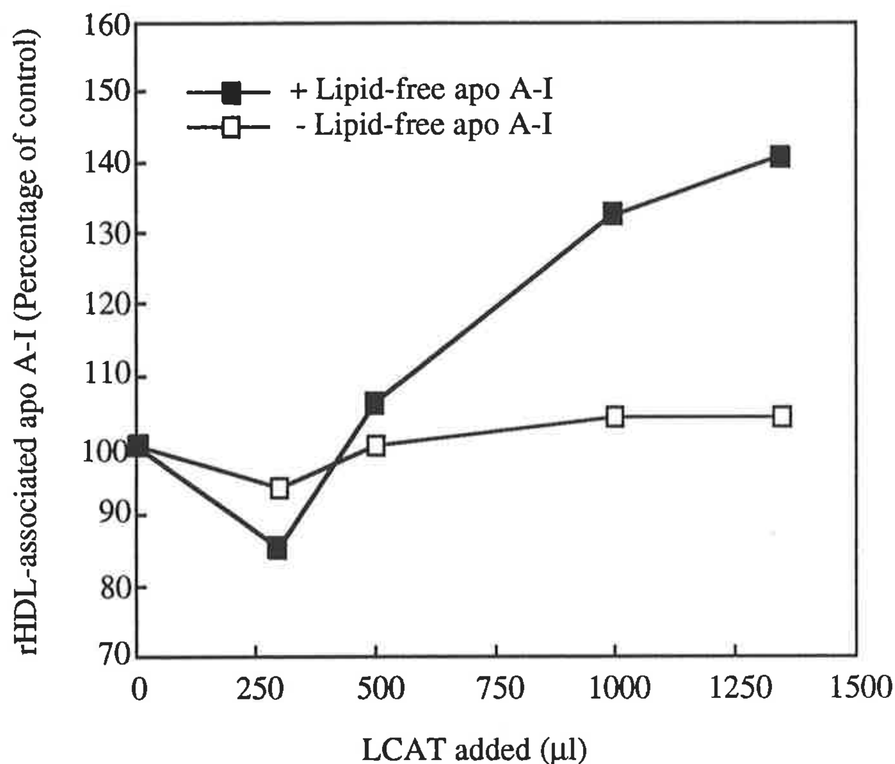


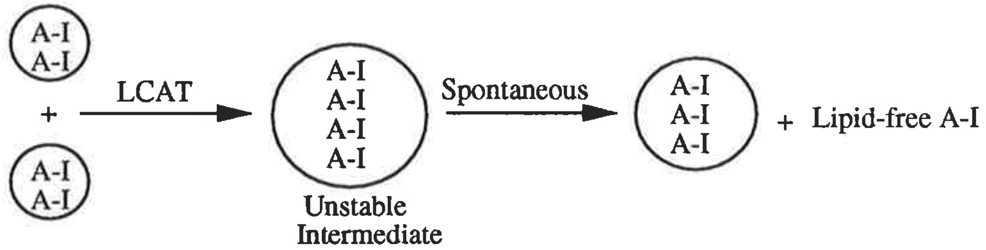
Figure 6-5. Effects of LCAT activity on the incorporation of lipid-free apo A-I into rHDL. Incubations were conducted at 37 °C for 24 hours. The incubation mixtures contained discoidal rHDL (final apo A-I concentration 0.1 mg/ml), LDL (final apo B concentration 0.4 mg/ml), bovine serum albumin (final concentration 18 mg/ml) and b-mercaptoethanol (final concentration 14 mM). Half of the mixture contained no added lipid-free apo A-I, while the other was supplemented by the addition of lipid-free apo A-I at a final concentration of 0.05 mg/ml, equivalent to 50% of that in the discoidal rHDL. LCAT was added in amounts ranging from 0-1.350 ml. The final incubation volume was 1.57 ml. After incubation, rHDL-associated apo A-I was recovered as the ultracentrifugal fraction of d< 1.25 g/ml. Concentrations of rHDL-associated apo A-I are expressed as percentages of that in a sample kept at 4°C with no additions. The points are means obtained from duplicate incubations in a single experiment.

In confirmation of previous studies reported by Nichols et al (1985), discoidal rHDL containing two molecules of apo A-I per particle are initially converted by LCAT into small spherical rHDL which still have two apo A-I molecules per particle. These small spherical rHDL are subsequently converted by LCAT into larger spherical rHDL with three molecules of apo A-I per particle. In the absence of a source of additional apo A-I, an increase from two to three in the number of apo A-I molecules per particle must be accompanied by a reduction in the number of HDL particles. Indeed, it has been postulated that LCAT promotes a fusion of small, spherical, two apo A-I-containing HDL particles into half the number of larger HDL particles, each containing four molecules of apo A-I (Nichols et al., 1985). It has been further suggested that these larger HDL are unstable and spontaneously shed a molecule of apo A-I to form more stable particles, each of which now contains three molecules of apo A-I (Nichols et al., 1985). The end result of this process is a halving of the number of HDL particles and the appearance of one quarter of the apo A-I in a lipoprotein-free form. While such a result has been reported (Nichols et al., 1985), it has not been confirmed in the present studies.

When rHDL were incubated with LCAT in the absence of added lipid-free apo A-I in the present studies, an increase in number of apo A-I molecules per particle from two to three was confirmed. However, in contrast to the previous report (Nichols et al., 1985), the number of particles was reduced by only one third rather than one by half and there was no appearance of lipid-free apo A-I. One interpretation of the present result is that any apo A-I that might have dissociated from unstable fusion products was subsequently incorporated into other rHDL which were increasing in size, thus providing an alternate mechanism for increasing the number of apo A-I molecules per particle from two to three. If such a process of direct incorporation of apo A-I were to be energetically more favourable than particle fusion, it follows that lipid-free apo A-I would not accumulate and the number of HDL particles would be reduced, as illustrated in Fig. 6-6C and as

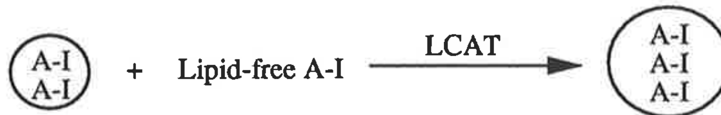
Figure 6-6. Hypothetical models of the mechanisms by which LCAT increases the number of apo A-I molecules per HDL particle from two to three. **A. Fusion Model.** LCAT mediates the fusion of small HDL particles containing two molecules of apo A-I to form half the number of larger but unstable particles which contain four molecules of apo A-I. These larger particles spontaneously shed a molecule of apo A-I to form more stable HDL which now contain three molecules of apo A-I per particle; a by-product of this process is the release of 25% of the apo A-I into a lipid-free pool. **B. Direct Incorporation Model.** LCAT mediates a direct incorporation of lipid-free apo A-I into HDL, increasing the number of molecules of apo A-I per particle from two to three without a reduction in the number of HDL particles. **C. Combination of the Fusion and Direct Incorporation Models.** This combination increases the number of apo A-I molecules per HDL particle from two to three and decreases the number of HDL particles by one third.

A. Particle fusion alone



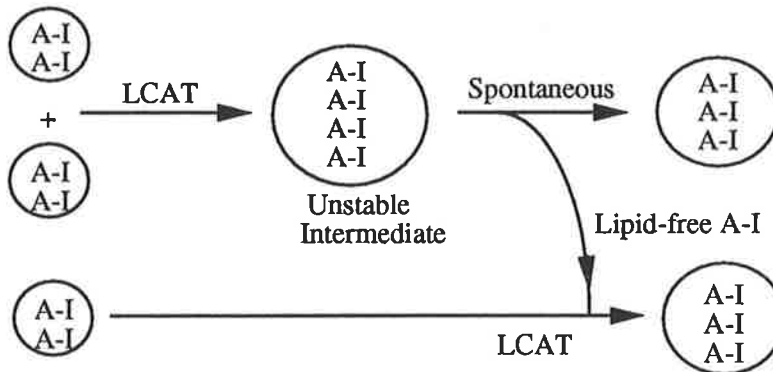
[Number of HDL particles reduced by one half]

B. Direct incorporation alone



[Number of HDL particles unchanged]

C. Combination of fusion and direct incorporation



[Number of HDL particles reduced by one third]

observed (Table 6.1), by one third rather than by the one half predicted by fusion alone (Fig. 6-6A).

The validity of this proposed process of direct incorporation of lipid-free apo A-I into HDL was tested in the present study by adding exogenous lipid-free apo A-I to the incubation mixtures. If direct incorporation of apo A-I into small rHDL particles were to take precedence over particle fusion during interaction with LCAT, the addition of exogenous lipid-free apo A-I would abolish particle fusion. Under these circumstances, the increase in rHDL apo A-I content would occur without a decrease in the number of HDL particles (Fig. 6B). This is precisely what was found experimentally (Table 6.1). Thus, the results of the present study are consistent with the existence of two mechanisms by which the apo A-I content of HDL particles can be increased during interactions with LCAT, with evidence suggesting that a direct incorporation of lipid-free apo A-I takes precedence over particle fusion.

There are several potential sources of lipid-free apo A-I in plasma. In addition to that which may be released from unstable fusion products as outlined above, lipid-free apo A-I may be secreted directly from the liver and intestine (Eisenberg, 1984) or released from HDL by the action of lipases (Clay et al., 1992; Miller, 1992). As shown in previous chapters, lipid-free apo A-I also dissociates from native HDL following a CETP-mediated reduction in their cholesteryl ester content and particle size. The fact that this dissociated, lipid-free apo A-I is reincorporated into the depleted HDL during their subsequent interaction with LCAT has led to the postulation of a cycle in which alternating CETP-mediated decreases and LCAT-mediated increases in the cholesteryl ester content and the size of HDL particles are accompanied by the cycling of apo A-I between HDL and a lipid-free pool.

These studies contribute substantially to our understanding the remodelling of HDL by LCAT. They also have several clinical implications. The existence of a process of direct

incorporation of lipid-free apo A-I into HDL provides a means for retaining in the HDL fraction apo A-I that might otherwise be lost from the circulation by excretion in urine (Segal et al., 1979; Horowitz et al., 1992). It also provides a means for maintaining HDL particle numbers and thus for transporting larger amounts of cholesteryl esters that might otherwise be transferred to potentially atherogenic apo B-containing lipoproteins.

CHAPTER 7

CONCLUDING COMMENTS

The powerful prediction of coronary artery disease by decreased plasma levels of HDL cholesterol (HDL-C) (Miller and Miller, 1975; Gordon and Rifkind, 1989) and the strong inverse correlation between plasma levels of apo A-I and CAD (Brunzell et al., 1984; Miller, 1987) have stimulated a sustained interest in the metabolism and regulation of HDL. While the apparent anti-atherogenic effect of HDL (Rubin et al., 1991; Plump et al., 1994) has been suggested due to involvement in reverse cholesterol transport, the initial step in this pathway, eg., the transfer of cholesterol from tissues to HDL, remains obscure. It has recently been suggested that the initial cellular cholesterol acceptor in the interstitial space is a population of prebeta-migrating particles which contain apo A-I as its sole protein. It is well documented that a small proportion of apo A-I in human plasma exists as a component of small, prebeta-migrating particles (Kunitake et al., 1985). However, the metabolic interrelationship between these prebeta-migrating apo A-I and the alpha-migrating apo A-I in the main HDL fraction is not known.

Based on the proposition that one of the origins of the prebeta-migrating apo A-I is from plasma HDL, this thesis investigates the regulation of the main HDL apolipoprotein, apo A-I, by examining the effects of lipid transfer and cholesterol esterification of lipoproteins. CETP mediates the net transfers of cholesteryl esters from HDL to VLDL and of triglyceride from VLDL to HDL. However, the triglyceride transfer is less than that of cholesteryl esters. This results in a reduction in the core lipid content and particle size of the HDL (Barter et al., 1990a; Chapter 3). Similarly, incubation of HDL and LDL in the presence of CETP causes a reduction in the core lipid content and particle size of the HDL (Chapter 3). Coincident with the reduction in HDL particle size, there is a dissociation of up to one-third of the apo A-I from the HDL fraction (Chapter 3). As in the case of the reduction in HDL core lipids and particle size, the dissociation of apo A-I is linear with incubation time (up to 24 h); the percentage of apo A-I which dissociates from HDL correlates positively with the concentrations of VLDL, LDL and CETP in the incubation mixture and negatively with the concentration of HDL (Chapter 3).

The dissociated apo A-I is essentially free of cholesterol and phospholipids and is not associated with other lipoproteins (Chapter 4). When subjected to agarose gel electrophoresis, the dissociated apo A-I migrates to a prebeta position (Chapter 4) which is identical to that of purified, lipid-free apo A-I and is distinct from the alpha migration of the bulk HDL in plasma.

If the lipid-free apo A-I does dissociate from HDL in vivo, it has several potential fates: (i) the apo A-I may enter the interstitial space and function as an acceptor of cellular cholesterol (Hara and Yokoyama, 1991); (ii) it may be excreted through the kidney (Horowitz et al., 1992); (iii) it may interact with the phospholipids released from triglyceride-rich lipoproteins undergoing lipolysis to form apo A-I-phospholipid discs within plasma (Clay et al., 1992; Clay and Barter, 1996); (iv) alternatively, just as lipid-free apo A-I dissociates from HDL when they lose core lipids and become smaller, it is possible that lipid-free apo A-I may reincorporate into HDL which are increasing in size as a result of acquiring additional cholesteryl esters generated by LCAT.

This thesis has tested the final possibility. It shows that cholesterol esterification by LCAT within HDL plays an important role in the incorporation of lipid-free apo A-I into HDL (Chapter 5). HDL was first reduced in size by lipid transfer under the effect of CETP. Subsequent incubation of the modified HDL with LCAT in the presence of LDL (as a source of unesterified cholesterol) resulted in the increase in HDL particle size and, if a source of lipid-free apo A-I was also present in the incubation, a substantial incorporation of the added apo A-I was incorporated into HDL fraction. The subsequent result was that the concentration of HDL apo A-I increased by more than 35%. The LCAT-mediated appearance of lipid-free apo A-I in the HDL fraction only occurred when the incubation mixtures already contained HDL, indicating that the apo A-I was incorporated into pre-existing HDL particles and was not formed into new particles.

In fact, the LCAT-mediated increase in cholesteryl ester content of HDL and increase in HDL size was accompanied by an increase in the number of molecules of apo A-I per particle (Nichols et al., 1985; Chapter 6). It has been proposed that the LCAT-mediated increase in the number of apo A-I molecules per HDL particle may be achieved by particle fusion (Nichols et al., 1985). In the present study, the incubations conducted in the absence of added lipid-free apo A-I did not cause changes in the concentration of HDL-associated apo A-I, indicating that the increase from two to three molecules of apo A-I per particle was achieved at the expense of a one third reduction in the number of rHDL particles in a process that must have involved particle fusion. In contrast, when the incubation mixture was supplemented by the addition of lipid-free apo A-I, there was an increase in the concentration of HDL-associated apo A-I of approximately 50%, indicating that under these conditions, the increase from two to three in the number of apo A-I molecules per rHDL particles was achieved by a direct incorporation of lipid-free apolipoproteins without fusion of HDL particles.

Therefore, the LCAT-mediated increase in the number of apo A-I molecules per HDL particle may be achieved by either of two mechanisms. In the absence of lipid-free apo A-I, there is particle fusion and a consequent reduction in the number of HDL particles. In the presence of lipid-free apo A-I, the increase in number of apo A-I molecules per particle is achieved by a direct incorporation of the lipid-free apolipoprotein into the HDL. This latter process does not reduce the number of HDL particles.

In conclusion, this thesis has provided in vitro evidence of a cyclic dissociation and reassociation of apo A-I with HDL in processes which are regulated by plasma factors that modify HDL lipids. If it does occur in vivo, it may implicate that plasma factors which determine the dissociation and reassociation of apo A-I with HDL may determine whether the apo A-I will be lost (eg., excreted through kidney) or preserved during HDL remodelling. It follows that these plasma factors may also be important determinants of the total concentration of apo A-I in plasma.

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