THE SUBSTRATE SPECIFICITIES OF HEPATIC LIPASE AND ENDOTHELIAL LIPASE FOR HIGH DENSITY LIPOPROTEIN PHOSPHOLIPIDS: A COMPARATIVE STUDY.

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A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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March 2003

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Revised Kinetic parameters for HL- and EL-mediated phospholipid hydrolysis in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.

Spherical rHDL	Enzyme	Constituent Hydrolysed	V_{max}	$K_m(app)$	Catalytic efficiency
			nmol NEFA/mL HL/h	*μmol/L PL*	$V_{max}/K_m(app)$
(POPC)rHDL	HL	Phospholipid	800.0 ± 42.5	2327.0±195.3	0.34
(PLPC)rHDL	HL	Phospholipid	346.3 ± 12.9	1250.0 ± 87.9	0.28
(PAPC)rHDL	HL	Phospholipid	147.4 ± 20.4	936.0 ± 265.7	0.16
(PDPC)rHDL	HL	Phospholipid	365.1 ± 60.21	1993.0 ± 421.4	0.18
(POPC)rHDL	EL	Phospholipid	133.5 ± 12.4	208.7 ± 52.64	0.64
(PLPC)rHDL	EL	Phospholipid	146.3 ± 18.76	294.9 ± 90.63	0.50
(PAPC)rHDL	EL	Phospholipid	184.0 ± 23.46	274.2 ± 85.88	0.67
(PDPC)rHDL	EL	Phospholipid	209.5 ± 16.33	108.6 ± 30.46	1.93

Revised Kinetic parameters for HL- and EL-mediated triglyceride hydrolysis in spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL

Spherical rHDL	Enzyme	Constituent Hydrolysed	V_{max}	$K_m(app)$	Catalytic efficiency
			nmol NEFA/mL HL/h	*μmol/L PL*	$V_{max}/K_m(app)$
[^{3}H]TO-(POPC)rHDL	HL	Triglyceride	1270.0 ± 141.1	470.7 ± 77.98	2.70
[^{3}H]TO-(PLPC)rHDL	HL	Triglyceride	1777.0 ± 148.1	677.8 ± 77.22	2.62
[^{3}H]TO-(PAPC)rHDL	HL	Triglyceride	1133.0 ± 188.8	533.1 ± 125.8	2.12
[^{3}H]TO-(PDPC)rHDL	HL	Triglyceride	1527.0 ± 293.5	885.4 ± 225.7	1.72
[^{3}H]TO-(POPC)rHDL	EL	Triglyceride	6.2 ± 0.3	8.1 ± 1.1	0.77
[^{3}H]TO-(PLPC)rHDL	EL	Triglyceride	9.7 ± 0.3	6.6 ± 0.6	1.47
[^{3}H]TO-(PAPC)rHDL	EL	Triglyceride	41.4 ± 1.9	47.2 ± 3.3	0.88
[^{3}H]TO-(PDPC)rHDL	EL	Triglyceride	21.9 ± 1.0	22.9 ± 2.0	0.96

SUMMARY

Hepatic lipase (HL) and endothelial lipase (EL) are both members of the triglyceride lipase gene family that preferentially hydrolyses high density lipoproteins (HDL) and are involved in the *in vivo* metabolism and regulation of HDL. Both HL and EL hydrolyses HDL phospholipids. The main HDL phospholipid species are phosphatidylcholines, with the four most abundant being, 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 1-palmitoyl-2-linoleoyl phosphatidylcholine PLPC, 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC) and 1-palmitoyl-2-docosahexanoyl phosphatidylcholine (PDPC). The aim of this thesis was to determine if HL and EL have different substrate specificities for HDL phospholipids.

In order to carry out these studies, it was important to use HDL that varied systematically in their phospholipid composition, but which were comparable in all other respects. This was achieved by using well-defined, homogenous preparations of spherical reconstituted HDL (rHDL). Although HL and EL both have phospholipase A_1 activity, the results showed that HL preferentially hydrolysed rHDL phospholipids with shorter, less saturated *sn*-2 acyl esters, while EL preferentially acted on rHDL phospholipids with longer, more unsaturated *sn*-2 acyl esters.

To determine if HDL phospholipid composition also affects HL- and EL-mediated triglyceride hydrolysis, the rHDL were enriched with radiolabelled triglycerides. As triolein was the only triglyceride constituent in all the rHDL, differences in the rate of triglyceride hydrolysis were attributed to the different phospholipid composition of the rHDL. These studies also gave an indication as to how rHDL phospholipid composition

regulated the interactions of HL and EL with the rHDL surface. The results showed that while rHDL phospholipids have a major impact on the interactions of EL with the rHDL surface, this was not the case for the interaction of rHDL and HL.

In most of the studies in this thesis formation of nonesterified fatty acids (NEFA) was determined directly using a mass assay. As this approach is not highly sensitive, a novel spectroscopic technique was also developed, whereby NEFA formation was measured as the binding of NEFA to the fluorescent probe AcryloDan-derivatised Intestinal Fatty Acid Binding Protein (ADIFAB). This approach has the advantage of requiring shorter incubation times and smaller amounts of substrates. The results obtained using this approach agreed well with the results that were obtained by measuring NEFA mass.

In conclusion, the studies in this thesis show that HL and EL have completely different substrate specificities for rHDL phospholipids. HL preferentially hydrolyses rHDL with POPC and PLPC, while EL preferentially hydrolyses rHDL with PAPC and PDPC. The studies also indicate the interactions of EL, but not HL, with the surface of rHDL is regulated by the phospholipid composition of the particles. These findings indicate that HDL phospholipid hydrolysis levels are regulated by the complementary activities of HL and EL.

DECLARATION

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

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

MyNgan Duong.

ACKNOWLEGEMENTS

I wish to thank my principal supervisor, Associate-Professor Kerry-Anne Rye for her expert supervision throughout this work, which was performed at the Lipid Research Laboratory, Hanson Institute in Adelaide. Her generous support and encouragement have been invaluable to this work.

I would also like to thank my co-supervisor, Professor Philip Barter, for his enthusiastic council and insightful guidance.

My gratitude to my colleges for their friendship and advice; Dr. Moira Clay, Dr. Paul Baker, Dr. Neil Hime, Richard Bright, Jane Roberts, Maria Psaltis, Kate Drew, Dr. Stephen Nicholls, Dr. Patrick Kee, Dr. Daniela Caiazza, Diana Pyle, Dr. Dale Ashby, Dr. Anisa Jahangiri, Kevin Wee, Angel Liang, Nongnuch Settasatian and Mary Marucci.

I thank the Helpman Scholarship in Cardiovascular Research for supporting me during my studies.

Finally, my heartfelt thanks to my parents and family for their patience and support that have never wavered throughout this work.

PUBLICATIONS AND ABSTRACTS

Publications

Duong M, Psaltis M, Rader D, Marchadier D, Barter PJ, and Rye KA. (2003) Evidence that hepatic lipase and endothelial lipase have different substrate specificities for high density lipoproteins. *In the process of being submitted.*

Rye KA, Duong M, Psaltis M, Curtiss L, Bonnet D, Stocker R and Barter PJ. (2002) Evidence that phospholipids play a key role in pre-β apoA-I formation and high density lipoprotein remodeling. *Biochemistry* 41:12538-12545.

Other Publications

Settasatian N, Duong M, Curtiss LK, Ehnholm C, Jauhiainen M, Huuskonen J and Rye KA. (2001) The mechanism of the remodeling of high density lipoproteins by phospholipid transfer protein. *Journal of Biological Chemistry* 276(29): 26898-905.

Rye KA and Duong M. (2000) Influence of phospholipid depletion on the size, structure, and remodeling of reconstituted high density lipoproteins. *Journal of Lipid Research* 41(10): 1640-50.

Abstracts Presented

MN.Duong, PJ. Barter and K-A. Rye. 1997. The influence of phospholipids on the size and structure of spherical, reconstituted high density lipoproteins. *XIth International Symposium of Atherosclerosis, Paris, France.*

MN.Duong, W. van Amelsfort, H-Q. Liang and K-A. Rye. 1998. The influence of phospholipid concentration on the size, structure and function of reconstituted high density lipoproteins (HDL). *XXIVth Annual Scientific Meeting of the Australian Atherosclerosis Society, Cairns, Queensland, Australia.*

MN.Duong and K-A. Rye. 2000. The influence of phospholipid acyl chain composition on hepatic lipase-mediated hydrolysis of high density lipoprotein phospholipid. *XIIth International Symposium of Atherosclerosis, Stockholm, Sweden.*

MN. Duong, D. Rader, PJ. Barter and K-A. Rye. 2001. Evidence that endothelial lipase and hepatic lipase have opposing substrate specificities. *XXVIth Annual Scientific Meeting of the Australian Atherosclerosis Society, Fremantle, Western Australia.*

ABBREVIATIONS

α-helices	amphipathic helices
ABCAI	ATP binding cassette transporter
apo	apolipoprotein
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CAD	coronary artery diseases
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
d	density
DETAPAC	Diethylenetriamine pentaacetic acid
DTNB	5,5'-Dithio-bis(nitrobenzoic acid)
EDTA-Na_2	ethylenediaminetetraacetic acid, disodium salt
EL	endothelial lipase
FED	fish-eye disease
FH	familial hypercholesterolemia
FLD	familial LCAT deficiency
FPLC	fast performance liquid chromatograpy
GPLRP2	guinea pig pancreatic lipase-related protein 2
HDL	high density lipoproteins
HL	hepatic lipase
hPL	human pancreatic lipase
IDL	intermediate density lipoproteins

LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoproteins
LPL	lipoprotein lipase
lyso-PC	lysophosphatidylcholines
NEFA	nonesterified fatty acid
NF-κB	nuclear factor κB
PAPC	1-palmitoyl-2-arachidonyl phosphatidylcholine
PDPC	1-palmitoyl-2-docosahexanoyl phosphatidylcholine
PLA_2	phospholipase A_2
PLPC	1-palmitoyl-2-linoleoyl phosphatidylcholine
PLTP	phospholipid transfer protein
PON	paraoxonase
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
RCT	reverse cholesterol transport
rHDL	reconstituted HDL
SAA	serum amyloid A
SDS	sodium dodecyl sulphate
SR-BI	scavenger receptor class B type I
TBS	Tris-buffered saline
TC	total cholesterol
TG	triglycerides
TO	triolein
UC	unesterified cholesterol
VLDL	very low density lipoproteins

CHAPTER 1 INTRODUCTION

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SCOPE OF THIS THESIS

LIPOPROTEINS

Lipids are essential for maintaining the integrity and normal function of cells within the human body. The term 'lipids' includes phospholipids, triglycerides, nonesterified fatty acids (NEFA), cholesteryl esters (CE), and unesterified cholesterol. Being insoluble in water, lipids are packaged into lipoproteins for transport in the blood. Structurally, lipoproteins are large spherical complexes consisting of hydrophobic, neutral lipids (triglycerides and CE) surrounded by a monolayer of phospholipids, unesterified cholesterol and apolipoproteins (apo) (Fig. 1.1). Apolipoproteins are amphipathic, with hydrophobic as well as hydrophilic properties and are responsible for the water solubility of lipoproteins {Segrest *et al.*, 1992}. Apolipoproteins also confer stability to lipoproteins and regulate their interactions with plasma factors.

Epidemiological studies have shown that coronary heart disease (CHD) is correlated with plasma lipid levels {Gordon, 1990}. With CHD events increasing in Western Societies, the regulation of plasma lipid and lipoprotein levels is an area that is being investigated intensely. Low density lipoproteins (LDL) and triglyceride-rich lipoproteins are known to be atherogenic, while high density lipoproteins (HDL) are well recognised to be protective against atherosclerosis.

1.1 PLASMA LIPID TRANSPORT

The human body requires lipids for a variety of functions. These functions include maintaining cell membrane integrity, fulfilling energy requirements, participating in biochemical pathways, the synthesis of steroid hormones and the production of bile.

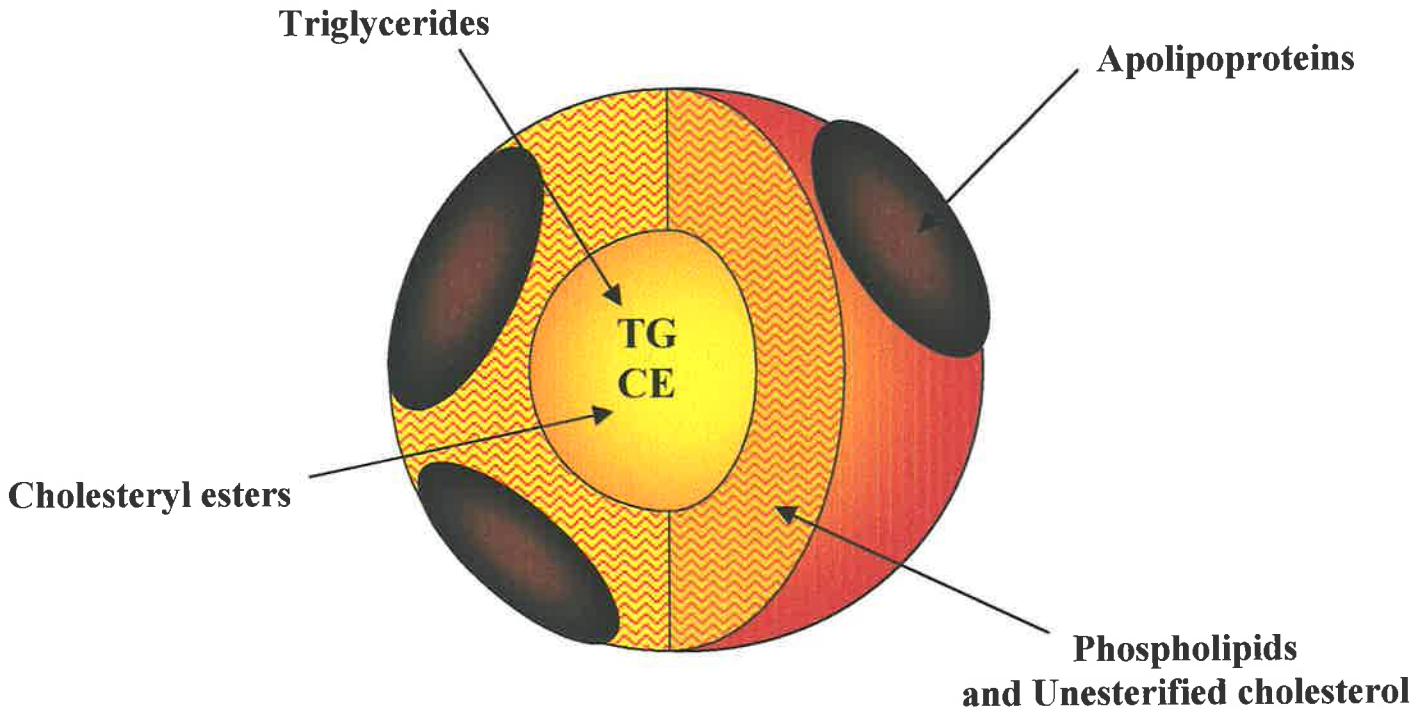

Figure 1.1. Lipoprotein structure.

The body has several methods for delivering lipids to appropriate areas via lipoproteins.

Dietary triglycerides and cholesterol are absorbed by mucosal cells in the small intestine, assembled into chylomicrons and secreted into the lymphatic system {Ginsberg, 1990; Grundy, 1986}. From the mesenteric lymphatics, chylomicrons enter the venous system via the thoracic duct, where they aquire apolipoproteins such as apoC and apoE from HDL. The triglycerides in chylomicrons are hydrolysed when they interact with lipoprotein lipase (LPL) in the capillary beds. As the core lipid content of the chylomicrons decreases, chylomicron remnants are generated. During this process, the chylomicrons shed redundant phospholipids, unesterified cholesterol and apolipoproteins from their surface. These constituents are transferred to HDL {Redgrave and Small, 1979}. The triglycerides that are hydrolysed by LPL are taken up by muscle and adipose tissues as fatty acids and glycerides. These are used either as an energy source by muscle cells, or stored as triglycerides as energy reserves in adipocytes. Chylomicron remnants are taken up by the liver via a receptor-mediated process with apoE as the ligand {Brown and Goldstein, 1986}. The CE in the chylomicron remnants are either excreted in the bile or released back into the plasma as newly synthesized lipoproteins.

Very low density lipoproteins (VLDL) are triglyceride-rich lipoproteins that are assembled in the hepatocytes {Shelness and Sellers, 2001}. VLDL perform the same function as chylomicrons and deliver endogenous triglycerides and cholesterol from the liver to peripheral tissues for storage or for use as an energy source {Ginsberg, 1990}. LPL hydrolyses VLDL triglycerides to produce intermediate low density lipoproteins (IDL), and then low density lipoproteins (LDL) {Berneis and Krauss, 2002}. Hepatic

lipase (HL), to a lesser extent, also hydrolyses triglycerides and phospholipids in VLDL and IDL to produce LDL.

HDL remove excess cholesterol from peripheral cells, and transport it to the liver for excretion in a process termed reverse cholesterol transport (RCT) {Barter and Rye, 1996}. In the first step of RCT, unesterified cholesterol and phospholipids are transferred from peripheral cells to lipid-poor apoA-I and nascent, discoidal HDL. This process is regulated by an integral membrane protein, the ATP binding cassette transporter (ABCA1) {Oram and Vaughan, 2000; Attie *et al.*, 2001; Oram 2002; Oram and Lawn, 2001}. In the second step, the unesterified cholesterol in HDL is esterified by lecithin:cholesterol acyltransferase (LCAT). The resulting CE are sequestered in the HDL core and the discoidal HDL are converted into spherical particles {Barter *et al.*, 1985; Francone *et al.*, 1989}. The third step of RCT involves the transfer of CE from HDL to other lipoproteins by cholesteryl ester transfer protein (CETP) {Barter *et al.*, 1982}. The final step of RCT involves the uptake of CE by the liver, via by the scavenger receptor (SR-BI) {Trigatti *et al.*, 2000}, or via receptor-mediated pathways where apoB and apoE are the ligands {Brown *et al.*, 1981}.

1.2 CLASSIFICATION OF LIPOPROTEINS

Lipoproteins vary widely in size, composition, function and density. They are classified on the basis of their physical-chemical characteristics such as, hydrated density, into chylomicrons, VLDL, IDL, LDL and HDL (Table 1.1). Each lipoprotein fraction is classified further into a number subpopulations (Table 1.2).

Table 1.1. The physical-chemical properties of human plasma lipoproteins[a]

Properties	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/mL)	< 0.95	0.95 – 1.006	1.006 – 1.019	1.019 – 1.063	1.063 – 1.21
Electrophoretic mobility	Origin	Pre-β	pre-β to β	β	α
Mean Particle Mass (10^6 Da)	1,000	4.5 - 100	4.0	3.0	0.2 – 0.4
Mean Diameter (nm)	100 – 1,000	30 – 70	25	20	8 – 10
Major Lipids	Exogenous TG	Endogenous TG	Endogenous TG, CE	CE	Phospholipids, CE
Major Apolipoproteins	B-48, C, E	B-100, C, E	B-100, E	B-100	A-I, A-II, C
Main Synthesis Sites	Intestine	Liver, intestine	Intravascular	Intravascular	Intestine, liver Intravascular

VLDL, Very low density lipoproteins; IDL, Intermediate density lipoproteins; LDL, Low density lipoproteins; HDL, High density lipoproteins; TG, triglycerides; CE, cholesteryl esters. [a]Sourced from Patsch and Gotto *et al.*, 1986; Ginsberg, 1990; Gotto *et al.*,2000.

Table 1.2. Chemical composition (% mass) and densities of lipoprotein subspecies[a]

Lipoproteins		Densities (g/mL)	Composition (% mass)				
			Protein	CE	UC	TG	PL
VLDL	VLDL-1	<1.006	11	8	6	58	17
	VLDL-2	1.006 – 1.010	18	24	9	29	22
IDL	IDL-1	1.008 – 1.022	17	35	10	16	21
	IDL-2	1.013 – 1.019	17	37	11	13	21
LDL	LDL-I	1.019 – 1.023	18	43	9	7	22
	LDL-II	1.023 – 1.028	19	45	10	4	23
		1.028 – 1.034	21	45	9	3	22
	LDL-III	1.034 – 1.041	22	46	8	3	21
		1.041 – 1.044	24	44	7	3	21
	LDL-IV	1.044 – 1.051	26	42	7	5	19
		1.051 – 1.060	29	40	7	6	18
HDL	HDL_2	1.063 – 1.125	40	17	5	5	33
	HDL_3	1.125 – 1.21	55	13	4	3	25

[a]Sourced from Eisenberg, 1984; Berneis and Krauss, 2002.

1.3 STRUCTURE AND COMPOSITION OF LIPOPROTEINS

SURFACE CONSTITUIENTS

PHOSPHOLIPIDS

Phospholipids comprise 17 to 30% of the total lipoprotein surface mass {Berneis and Krauss, 2002; Eisenberg, 1984}. The phospholipids are orientated with their polar headgroup in contact with the aqueous plasma environment and the hydrophobic acyl chains facing into the centre of the particles. Phospholipids are the most polar of the lipids. They consist of a glycerol backbone with a phosphate headgroup, which is often associated with other charged groups, at the *sn*-3 position and two uncharged fatty acyl chains at the *sn*-1 and *sn*-2 positions.

Monolayer studies show that acyl chain unsaturation and length {Stubbs *et al.*, 1981; Slotte, 1995} and headgroup charge {Scherer and Seelig, 1989; Seelig *et al.*, 1987} determines the chemical and physical properties of a lipid-water interface. Phospholipids also influence the surface charge of lipoproteins {Zhang *et al.*, 1998}.

APOLIPOPROTEINS

Apolipoproteins are also located on the lipoprotein surface. They are structural proteins that consist of a number of amphipathic α-helixes separated by proline-punctuated β-turns {Segrest *et al.*, 1992}. Apolipoproteins associate with lipids and provide solubility and stability to the lipoproteins {Segrest *et al.*, 2000}. Apolipoproteins also play a critical role in lipoprotein metabolism by acting as cofactors for several of the plasma factors that are involved in lipoprotein metabolism. They are also ligands for receptors that direct lipoproteins to target tissues.

There are five main classes of apolipoproteins ; A (I, II, IV, V), B, C (I, II, III), D and E (Table 1.3). ApoF, H and J have been reported but little is known about their function or physiological relevance with regards to lipoprotein metabolism.

ApoA-I is the major protein in HDL, and comprises 60 to 70% of the total protein mass of the particle {Patsch and Gotto, 1987; Benoit *et al.*, 1999}. It has a molecular mass of 28,300 Da and is secreted by the small intestinal mucosa and liver cells as a pre-pro-protein. Once in the circulation, the pro-protein is cleaved to produce mature apoA-I {Fielding and Fielding, 1995}. About 90% of the apoA-I in the plasma is associated with HDL, and approximately 5% is in a lipid-free or lipid-poor form called pre-β HDL {Fielding and Fielding, 1995; Rothblat *et al.*, 1992}. Pre-β HDL are the precursors of the mature, spherical HDL that are present in normal human plasma. Most of the apoA-I in HDL is derived from either the liver or intestine, but about 20% is transferred from nascent intestinal chylomicrons {Tailleux and Fruchart, 1996}. The primary structure of apoA-I contains 22 amino acid repeats which form α-helical domains {Frank and Marcel, 2000}. The number of amphipathic helices, which determines structural stability, varies from 6 to 10 per apoA-I molecule, according to the number of apoA-I and phospholipid molecules on the lipoprotein surface {Segrest *et al.*, 2000; Frank and Marcel, 2000}. The affinity of apoA-I for lipid increases as the number of α helixes increases {Segrest *et al.*, 1992}. In discoidal HDL, it is accepted that apoA-I stabilises the phospholipid/unesterified cholesterol bilayers by forming an annulus around the particles and shielding the phospholipid acyl chains from water. However, there are several theories as to how the α-helices in apoA-I are arranged in discoidal HDL {Klon *et al.*, 2002}. In the 'picket fence' model the long axis of the α-helices are parallel to

Table 1.3. Properties and functions of major human plasma apolipoproteins.

Apolipoprotein	Molecular weight (Daltons)	Site of Synthesis	Major lipoprotein carriers	Functions
ApoA-I	28,300	Liver, intestine	HDL, CM	LCAT activation; HDL receptor ligand; cholesterol efflux stimulation[a, b]
ApoA-II	17,414	Liver, intestine	HDL, CM	HDL receptor ligand; modulation HL activity[a]
ApoA-IV	46,000	Intestine	HDL, CM	LCAT activation; modulation of LPL activity; HDL receptor ligand[a, b]
ApoB-100	550,000	Liver	VLDL, IDL, LDL	Assembly and secretion of VLDL; LDL receptor ligand[c]
ApoB-48	264,000	Intestine	CM	Assembly and secretion of CM[c]
ApoC-I	6,600	Liver	CM, VLDL, HDL	LCAT activation; inhibition of TG-rich particle uptake by liver[b, d]
ApoC-II	8,850	Liver	CM, VLDL, HDL	LCAT and LPL activation[a, b]
ApoC-III	8,800	Liver	CM, VLDL, HDL	LPL and CETP inhibition; inhibition of TG-rich particle uptake by liver[b, d]
ApoD	19,000	Liver, intestine, brain, placenta, adrenals, pancreas, spleen	HDL	Transport of lipids, steroids, bilrubin[a]
ApoE	34,100	Ubiquitous, mainly in liver, macrophages, steroidogenic organs	CM, VLDL, HDL	LDL- and apoE-receptor ligand; cholesterol efflux promotion[b, e]

CM, Chylomicrons; TG, triglyceride; LCAT, Lecithin:cholesterol acyltransferase; LPL, Lipoprotein lipase; HL, Hepatic lipase. Source from [a]von Eckardstein *et al.*, 1994; [b]Tailleux and Fruchart, 1996; [c]Ginsberg, 1990; [d]Shachter, 2001; [e]Curtiss and Boisvert, 2000.

the HDL phospholipid acyl chains {Brasseur *et al.*, 1990}. In the "belt' model the α-helices are perpendicular to the HDL phospholipid acyl chains {Segrest *et al.*, 1999; Maiorano and Davidson, 2000}, while in the 'hairpin' model the helices are also orientated in this manner but each apoA-I molecule folds back on itself at helix 5 so that helical repeats 1-4 are antiparallel to those of 6-10 {Roger *et al.*, 1998}.

ApoA-I also has other important roles in lipoprotein metabolism besides its structural function in HDL. ApoA-I is a cofactor for the LCAT-mediated esterification of cholesterol {Jonas, 1991}, it is a ligand for receptors such as SR-BI {Xu *et al.*, 1997} and may play a role in regulating HDL clearance from the plasma {Braschi *et al.*, 1999}. ApoA-I is also able to promote the efflux of cholesterol from cells such as fibroblasts, macrophages, endothelial cells and hepatocytes, {Fournier *et al.*, 1996; Oram and Yokoyama, 1996; Rothblat *et al.*, 1999}.

ApoA-II is the second most abundant apolipoprotein in HDL, comprising up to 20% of the total protein mass {von Eckardstein *et al.*, 1994}. In humans, it is secreted mainly from the liver and is isolated from the plasma as a dimer consisting of two 77-amino acid chains linked by a disulphide bridge {Brewer *et al.*, 1972}. ApoA-II is highly conserved in closely related species such as humans and monkeys but in species such as the mouse, where it exists as a monomer, human apoA-II shares only 55% sequence homology with mouse apoA-II {Blanco-Vaca *et al.*, 2001}. ApoA-II also contains α helices that stimulate cholesterol and phospholipid efflux from various cells such as macrophages and fibroblasts {Segrest *et al.*, 1992; Oram and Yokoyama, 1996}. ApoA-II has a higher affinity for lipid than apoA-I, and readily displaces apoA-I from HDL. Apart from helping to maintain the structural integrity of HDL, little is known

about the precise role of apoA-II in HDL metabolism although it is believed to modulate RCT {Tailleux *et al.*, 2002}. In animals, such as the dog, rabbit, chicken and cow, apoA-II is not present in detectable quantities. Given that apoA-II deficiency in animals and humans is associated with relatively normal plasma lipoprotein profiles, it appears that apoA-II is not needed for HDL assembly, regulation and metabolism {Deeb *et al.*, 1990}.

ApoA-IV is minor constituent of HDL and chylomicrons. Most of the apoA-IV in plasma is not associated with lipoproteins {Ginsberg, 1990}. It is synthesised by the small intestine as a 46 kDa gycoprotein and is polymorphic in the general population {Green *et al.*, 1980}. The physiological role of apoA-IV is unknown, but several functions have been suggested, including a role in RCT by virtue of its ability to activate LCAT and promote cellular cholesterol efflux {Tailleux and Fruchart, 1996}. Studies of overexpression of human apoA-IV in mice provide support for this role {Duverger *et al.*, 1996; Tangirala *et al.*, 1999; Benoit *et al.*, 1999}. In these animals atherosclerotic lesions were reduced from 56-85% compared to the controls {Duverger *et al.*, 1996; Tangirala *et al.*, 1999; Benoit *et al.*, 1999}. HDL that were isolated from the apoA-IV transgenic mice also have enhanced cholesterol efflux compared to HDL from control animals {Duverger *et al.*, 1996}. A study of human apoA-IV expression in apoE-deficient mice suggests that apoA-IV may also act as an antioxidant {Ostos *et al.*, 2001}.

ApoA-V is a recently identified minor HDL apolipoprotein. It is synthesised by the liver and its physiological significance is not known. Overexpression of apoA-V decreases plasma triglyceride and cholesterol levels in mice {van der Vliet *et al.*, 2002}.

ApoB is associated with several classes of lipoproteins including chylomicrons, IDL, LDL and VLDL. ApoB-100 is the largest apolipoprotein with a molecular mass of 550 kDa and has several very hydrophobic domains which function as lipid-binding sites {Kane *et al.*, 1980}. As apoB has a high affinity for lipids, it does not transfer between lipoproteins. Two main forms of apoB are synthesised in humans. ApoB-100 is the main apolipoprotein found in VLDL, IDL and LDL and is produced by the liver {Davis and Hui, 2001; Chang *et al.*, 1999}. ApoB-48 is found only in chylomicrons and its remnants and is secreted by the small intestine {Feher and Richmond, 2001}. ApoB-48 and apoB-100 are transcribed from the same gene {Young, 1990}. ApoB-100 results from the translation of the full length apoB mRNA, while apoB-48 comprises a fragment that consist of 48% of the amino terminal in apoB-100 {Young, 1990; Hedrick *et al.*, 2001}. ApoB-48 is the product of an in-frame translational stop codon {Young, 1990; Hedrick *et al.*, 2001}. As the LDL binding domain is missing in apoB-48, its sole function appears to be the packaging and secretion of chylomicrons from the intestine {Ginsberg, 1990}. ApoB-100 plays a crucial role in the uptake of LDL, VLDL and IDL via the LDL receptor. Other subspecies of apoB are apoB-74 and apoB-26 which have respective molecular weights of 407 and 126 kDa. ApoB-74 and apoB-26 are most probably derived from apoB-100 {Herbert *et al.*, 1983}.

ApoC-I comprises about 10% of the total protein mass in VLDL and 2% in HDL {Herbert *et al.*, 1983}. It activates LCAT and inhibits CETP activity {Shachter, 2001}. ApoC-I is also a potent inhibitor of VLDL-, LDL-receptor and LDL receptor-related protein binding {Shachter, 2001}.

ApoC-II is associated with VLDL and accounts for up to 10% of its total protein mass {Herbert *et al.*, 1983}. This apolipoprotein is also associated with IDL, chylomicrons and HDL. ApoC-II is a potent activator of LPL {Olivecrona and Bentgsson-Olivecrona, 1993}. Hence, apoC-II deficiency is characterised by severe hypertriglyceridemia {Durrington and Sniderman, 2000; Feher and Richmond, 2001}.

ApoC-III is the most abundant of the apoC apolipoproteins. Approximately 25% of the apoC-III in plasma is associated with VLDL, while 60% resides in HDL {Curry *et al.*, 1980}. This apolipoprotein accounts for about 50% of the total protein mass in VLDL and 2% in HDL {Herbert *et al.*, 1983}. It is also found in small quantities in chylomicrons and IDL. ApoC-III exists in three isoforms, with either 0, 1 or 2 sialic acid residues in the carboxyl-terminal {Herbert *et al.*, 1983}. It inhibits LPL and HL activity as well as uptake of VLDL remnants and chylomicrons by the liver {Shachter, 2001}.

ApoD is another minor HDL apolipoprotein. About 60% of plasma apoD resides in HDL, with the remainder being complexed to VLDL, IDL and LDL. Little is known about its function though it has been suggested that apoD might be involved in CE transfer from HDL to triglyceride-rich lipoproteins {Ginsberg, 1990}.

ApoE is associated with all lipoprotein classes. Receptor-mediated clearance of chylomicron and VLDL remnants from the plasma is mediated primarily by apoE {Linton *et al.*, 1998; Curtiss and Boisvert, 2000}. ApoE also promotes cholesterol efflux from peripheral cells {Zhu *et al.*, 1998}. ApoE is expressed in most tissue types with the liver, macrophages and steroidogenic organs having the highest expression

levels {von Eckardstein *et al.*, 1994}. ApoE is antiatherogenic. This is demonstrated clearly in the apoE deficient mouse, which readily develops atherosclerosis even on a normal chow diet {Zhang *et al.*, 1992}. ApoE exists as three major isoforms, apoE-2, apoE-3 and apoE-4 that are determined by three alleles. The most common allelic form, apoE-3, is associated with normal lipoprotein metabolism. The frequency of this allele in the apoE gene is 0.783 {Zannis *et al.*, 1981}. ApoE-2, in which a cysteine residue is substituted for an arginine at position 158, is the most common mutation and is present in 90% of diagnosed type III hyperlipoproteinaemia patients {Durrington and Sniderman, 2000}. Approximately 1% of the population is homozygous for this isoform {Feher and Richmond, 2001}. As apoE-2 does not bind to the LDL receptor, it is associated with the accumulation of cholesterol-enriched VLDL {Durrington and Sniderman, 2000}. However, this defect in lipoprotein metabolism is usually not so severe that hyperlipoproteinaemia develops unless it is accompanied by other disorders such as diabetes, obesity or hypothyroidism {Durrington and Sniderman, 2000}. The apoE-4 mutation is a rarer genotype than apoE-2 and is considered a risk factor for Alzheimer's disease {Mahley and Ji, 1999; Durrington and Sniderman, 2000}. The apoE-4 isoform has an arginine substituted for cysteine at position 112 {Mahley and Ji, 1999}.

UNESTERIFIED CHOLESTEROL

Unesterified cholesterol is a more polar molecule than CE and is located in the surface of lipoproteins. It is important for regulating membrane fluidity and stability {Small, 1997}. The unesterified cholesterol in HDL is derived from several sources, including nascent HDL, cell membranes and triglyceride-rich lipoproteins {Eisenberg, 1984}. LPL-mediated hydrolysis of VLDL enhances the transfer of unesterified cholesterol to

HDL and LDL {Perret *et al.*, 1983}. The esterification of unesterified cholesterol in HDL by the action of LCAT establishes a concentration gradient that causes unesterified cholesterol to move from cell membranes and other lipoproteins to HDL.

CORE LIPIDS

CHOLESTERYL ESTERS

CE is the major core lipid in LDL and HDL. CE is sequestered in the core of lipoproteins due to its water-insoluble nature. CETP mediates transfers of CE between HDL, LDL and VLDL {Barter *et al.*, 1982; Barter and Rye, 1994}. In the case of HDL and VLDL, these transfers are accompanied by the transfer of triglycerides in the opposite direction, from VLDL to HDL. The CETP-mediated transfer of CE out of HDL has been reported to enhance LCAT activity {Eisenberg, 1984}.

TRIGLYCERIDES

Triglycerides are nonpolar molecules that consist of a glycerol backbone with a fatty acid acyl chain in each position. Triglycerides are the major core lipid of VLDL and chylomicrons. Dietary (or exogenous) triglycerides are transported in the circulation as a constituent of chylomicrons and their remnants {Grundy, 1986}. Endogenous triglycerides are synthesised in the liver and transported in the plasma by VLDL and IDL {Ginsberg, 1990}. The triglycerides in lipoproteins are hydrolysed by LPL and HL into fatty acids and monoglycerides which, in turn, are broken down into glycerol and fatty acids by tissue lipases {Goldberg *et al.*, 2000}. These are either taken up by cells for respiration, or resynthesised for storage in adipocytes.

1.4 LIPOPROTEIN METABOLISM

ApoA-CONTAINING LIPOPROTEINS

HDL are very heterogenous and constitute 30 to 35% of the total circulating lipoproteins {Tailluex and Fruchart, 1996}. They originate either in the liver or intestine and are the smallest and densest of all lipoprotein classes {von Eckardstein *et al.*, 1994}. The core of HDL is rich in CE {Skinner, 1994}. The main HDL apolipoproteins are apoA-I and apoA-II {Brewer *et al.*, 1972; von Eckardstein *et al.*, 1994}. The plasma factors LCAT, CETP and PLTP, which remodel lipoproteins, are also associated with HDL.

One of the main functions of HDL is to accept and transport cellular cholesterol from extrahepatic tissues to the liver for recycling or removal in the process of RCT {Barter and Rye, 1994}. There are several subspecies of HDL that vary in their ability to carry out this function.

The initial acceptor of cellular cholesterol is a discoidal particle with pre-β mobility {Castro and Fielding, 1988}. Pre-β HDL is essentially lipid-poor or lipid-free apoA-I. Pre-β HDL contains small amounts of phospholipids and unesterified cholesterol. About 5% of the apoA-I in human plasma exists in this form {Phillips *et al.*, 1998; Fielding and Fielding, 1995}. Pre-β HDL are either secreted from the liver and intestine, or assembled in the plasma from apoA-I that has dissociated from mature HDL during remodeling by plasma factors {Clay *et al.*, 1992; Liang *et al.*, 1994; von Eckardstein *et al.*, 1996} (Fig. 1.2). In the plasma, this apoA-I rapidly acquires phospholipids and unesterified cholesterol, either through the activity of ABCA1, or

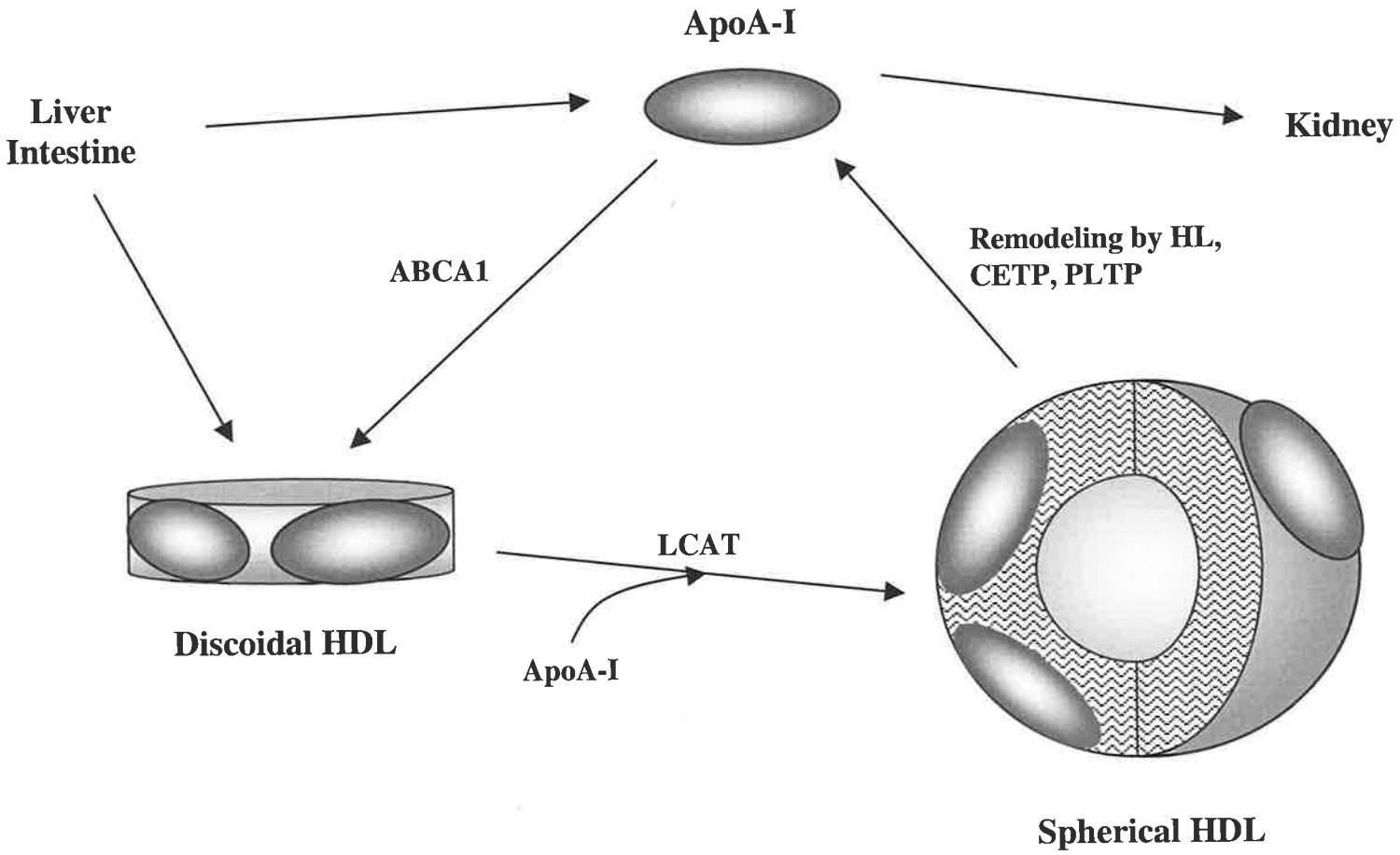

Figure 1.2. The cycling of apoA-I between the different subspecies of HDL.

from the LPL-mediated hydrolysis of triglyceride-rich lipoproteins, to become pre-β HDL {Attie *et al.*, 2001}. Lipidation occurs rapidly as phospholipids and unesterified cholesterol are acquired from cells and other lipoproteins. The unesterified cholesterol that is acquired from cells is esterified by LCAT, the enzyme that generates all CE in plasma {Jonas, 1991}. HDL is the main substrate for LCAT. The CE that are formed by the action of LCAT partition from the surface into the core of HDL and convert the discs into small spherical HDL, and then into larger spherical HDL particles {Francone *et al.*, 1989; Rye *et al.*, 1999}. Many factors constantly act on HDL to modify their size and composition and increase their heterogeneity. All of the constituents in HDL can be catabolized independently. For example, CE are selectively removed from HDL by the SR-BI that is expressed in the liver and steroidogenic organs {Trigatti *et al.*, 2000}. This reduces the size of the HDL particle {Webb *et al.*, 2002}. Triglycerides are hydrolysed by LPL and HL {Deckelbaum *et al.*, 1990; Jackson *et al.*, 1986}, in a process that reduced the size of the particles and mediates the dissociation of lipid-free or lipid poor apoA-I {Rye *et al.*, 1999}.

The spherical HDL in human plasma are classified on the basis of their apolipoprotein compositions into three main subpopulations, a minor subpopulation that contains only apoA-II {Bekaert *et al.*, 1992} and two main subpopulations: HDL that contains apoA-I and apoA-II, A-I/A-II HDL, and HDL that contains apoA-I but not apoA-II, A-I HDL {Cheung and Albers, 1984}. A-I/A-II HDL accounts for more than half of the plasma HDL yet its origins are poorly understood {Rye *et al.*, 1999}. Evidence from *in vitro* studies suggests that A-I/A-II HDL are assembled in the plasma and not secreted as intact particles {Clay *et al.*, 2000}. Both apoA-I and apoA-II are secreted from the liver into the circulation as nascent discoidal particles {Blanco-Vaca *et al.*, 2001; Tailleux *et*

al., 2002}. The mechanism by which A-I/A-II HDL are generated from discoidal (A-I)rHDL and discoidal (A-II)rHDL may involve remodeling by LCAT {Clay *et al.*, 2000}. Clay *et al.* (2000) have shown that the formation of A-I/A-II HDL *in vitro* involves an LCAT-mediated particle fusion {Clay *et al.*, 2000}. Displacing apoA-I from A-I HDL with lipid-poor apoA-II may also generate A-I/A-II HDL {Durbin and Jonas, 1997}.

Most A-I HDL are the size and density of HDL_2, while A-I/A-II HDL are the size and density of HDL_3 {Fruchart *et al.*, 1992}. There have been reports of differences in the metabolism and function of A-I HDL and A-I/A-II HDL. For example, HL preferentially hydrolyses triglycerides in A-I/A-II HDL {Mowri *et al.*, 1992}. This may account for the lower triglyceride content and smaller particle size of the A-I/A-II HDL in comparison to A-I HDL {Rye *et al*, 1999}. The plasma factors, CETP and LCAT are found associated with A-I HDL but not A-I/A-II HDL {Cheung *et al.*, 1986}. The lack of these plasma factors may contribute to the lower ability of A-I/A-II HDL to promote cellular cholesterol efflux from extrahepatic cells {Barbaras *et al.*, 1987; Lagrost *et al.*, 1995}.

ApoB-CONTAINING LIPOPROTEINS

Chylomicrons ($d < 0.95$ g/mL) are the largest apoB-containing lipoproteins. They contain apoB-48 as well as apoA-I, apoE, apoA-IV and apoC. After they are secreted into the plasma chylomicrons acquire apoE, apoC-II and apoC-III from HDL {Ginsberg, 1990}. Chylomicrons transport dietary triglycerides and cholesterol from the small intestine to peripheral cells where lipid absorption occurs {Grundy, 1986}.

Chylomicrons have a short half-life and are rapidly cleared from the plasma. In the circulation, their triglycerides are hydrolysed by LPL {Jackson, 1983}. LPL is concentrated in the capillary beds of skeletal muscles, adipose tissue and myocardial tissue, all of which have high energy expenditure or are known to be fat storage sites {Merkel *et al.*, 2002}. As the triglycerides in chylomicrons are hydrolysed some of their surface constituents become redundant and are transferred to nascent HDL and small HDL {Ginsberg, 1990}. The resulting chylomicron remnants are removed from the circulation by LDL receptors and the LDL receptor-related protein and in the liver {Goldberg *et al.*, 2000}. Evidence in mice that overexpress SR-BI indicates that SR-BI also has a role in clearing chylomicron remnants and apoB-containing lipoproteins from the plasma {Wang *et al.*, 1998}.

VLDL is secreted from the liver and transports endogenous triglycerides and CE {Bamberger and Lane, 1988}. VLDL contains one apoB-100 per particle as well as apoE and apoC. Most of the apoE and apoC are acquired from HDL after secretion {Mahley *et al.*, 1984}. The size of VLDL is regulated by the availability of triglycerides. In conditions of obesity, increased alcohol consumption or type II diabetes mellitus, which result in excess triglyceride synthesis, very large, triglyceride-rich VLDL are generated {Ginsberg, 1990}. When triglyceride availability is reduced, VLDL particle size is also reduced. VLDL can either be removed from the plasma at this stage by LDL receptor related proteins, or remain in the circulation to be further catabolized {Grundy, 1986}.

The VLDL that remain in the circulation are acted upon by LPL {Preiss-Landl *et al.*, 2002}. This decreases the size and increases the density of the particles and converts

them into IDL {Berneis and Krauss, 2002; Grundy, 1986}. IDL contain equal amounts of triglycerides and CE, and are either taken up directly from the plasma and catabolised by the liver, or remain in the plasma and are catabolized into LDL {Ginsberg, 1990; Grundy, 1986}.

LDL are CE-rich lipoproteins that contain one molecule of apoB {Ginsberg, 1990}. LDL is a ligand for the LDL receptor. The number of LDL receptors expressed by cells is regulated by cellular cholesterol levels and determines LDL residence time in the plasma {Brown and Goldstein, 1986}. The LDL receptor serves a dual purpose in LDL metabolism. Firstly, it limits LDL production by removing IDL from the circulation {Brown *et al.*, 1981}. Secondly, by determining LDL uptake through regulation of cellular cholesterol levels, it also controls the rate of LDL degradation {Brown and Goldstein, 1986}. The major site of LDL removal is the liver which accounts for 75–85% of the LDL that is removed from plasma {Grundy, 1986}. The concentration of LDL in the sinusoids of the liver is much higher than in the interstitual fluid. Hence, availability of LDL to the receptors on hepatocytes is higher than those on other cell types {Grundy, 1986}.

1.5 LIPOPROTEINS AND ATHEROSCLEROSIS

ATHEROSCLEROSIS

The initial stages of atherosclerosis involve the formation of fatty streaks that later develop into atheroma or plaques {Steinberg, 1988; Steinberg and Witztum 1990; Ross, 1993}. Fatty streaks usually form in branch points of arteries, at sites of high shear stress and turbulence, such as the aortic arch {Libby *et al.*, 2002; Collins and Cybulsky,

2001}. At these sites damage to the endothelium is most likely to occur as the result of oxidation, glycation or hypertension {Nilsson *et al.*, 2002; Libby *et al.*, 2002; Durrington and Sniderman, 2000; Assman *et al.*, 1999}. LDL in the plasma penetrate into the endothelium and accumulate in the intima at these sites. Monocytes are also attracted to these sites of injury, penetrate into the vessel wall and undergo phenotypic transition into macrophages {Steinberg, 1988; Ross, 1993}. These macrophages then take up LDL and are converted into foam cells, which accumulate to form fatty streaks {Steinberg and Witztum, 1990}. Macrophages also release chemotactic and growth factors that initiate an inflammatory response to attract additional LDL and monocytes that leads to lesion progression {Collins and Cybulsky, 2001; Reape and Groot, 1999; Ross, 1999; Viedt *et al.*, 2002}.

PROATHEROGENIC LIPOPROTEINS

In animals and humans the lipoproteins that are known to be atherogenic include apoB100-containing LDL, IDL and chylomicron remnants. There are two main theories as to why apoB-containing lipoproteins are proatherogenic. The first is that they interact with proteoglycans and other matrix proteins via apoB {Steinberg *et al.*, 1989; Sartipy *et al.*, 1999}. This causes the lipoproteins to accumulate in the blood vessel wall {Goldstein *et al.*, 1979}. The longer an LDL particle resides in the artery wall, the more likely it is to undergo oxidative modification and be taken up by macrophages. Proteoglygan-lipoprotein complexes are also more rapidly taken up by macrophages {Steinberg and Witztum, 1990}. This leads to the development of fatty streaks and plaque formation.

The other theory as to why apoB-containing lipoproteins are atherogenic relates to chemical or compositional properties that are independent of apoB and modify the particles in ways that make them atherogenic {Rudel and Kesaniemi, 2000}. These changes include oxidation, aggregation (initiated by phospholipases and sphingomyelinases) and other enzymatic alterations {Goldberg *et al.*, 2000}. The hydrolysis of triglycerides and phospholipids in apoB-containing particles can also produce biologically active lipids, such as nonesterified fatty acids (NEFA), glycerides and lysophosphatidylcholines (lyso-PC). Lyso-PC and NEFA induce the expression of proatherogenic factors, such as adehesion molecules and proteoglycans that are associated with the development of atherosclerotic lesions {McIntyre *et al.*, 1999; Navab *et al.*, 2002}.

IDL is regarded as an atherogenic lipoprotein. It accumulates under conditions of dysbetalipoproteinaemia {Durrington and Snidermann, 2000}. In cholesterol fed animals IDL becomes enriched with cholesterol and apoE {Steinberg and Witztum, 1990}. As the clearance of these particles by the apoE receptor is impaired, they accumulate in the plasma {Durrington and Snidermann, 2000}. It is well documented that IDL are taken up very rapidly by macrophages in a process that leads to the accumulation of cholesterol and the development of foam cells {Goldstein *et al.*, 1980}.

The evidence that LDL are atherogenic is indisputable. Familial hypercholesterolemia (FH), which is caused by a single defective gene involving the LDL receptor, is characterised by premature atherosclerosis that is related to the resulting elevated plasma LDL levels {Steinberg and Witztum, 1990}. Mice and rabbits that are genetically modified to express high plasma levels of LDL, either by knocking out the

LDL receptor, or by overexpression of apoB-100, also develop atherosclerotic lesions {Goldberg *et al.*, 2000}. It is now understood that the LDL involved with the development of fatty streaks have to be chemically or structurally altered before macrophages are able to take them up at a rate fast enough to produce foam cells {Goldstein *et al.*, 1979}. Unmodified normal LDL are slowly, if rarely taken up by macrophages. The LDL that initiate fatty streak formation are oxidised and are taken up 3 to 10 times faster by monocytes/macrophages than normal LDL {Steinberg and Witztum, 1990}. Oxidation of LDL occurs when free radicals interact with the polyunsaturated phospholipids in LDL and form phospholipid hydroperoxides {Thomas, 2000; Durrington *et al.*, 2001}. The hydroperoxides react with and change the apoB binding properties, allowing the uptake of the oxidised LDL by macrophages through scavenger receptors {Thomas, 2000}. Several conditions may cause LDL oxidation including smoking, glycation reactions and oxidative biochemical pathways {Feher and Richmond, 2001; Durrington and Sniderman, 2000}.

It is uncertain whether the VLDL are atherogenic. The finding that VLDL are more potent inducers of nuclear factor-κB (NF-κB) than LDL, suggests that they may be atherogenic {Dichtl *et al.*, 1999}. NF-κB plays an important role in regulating inflammatory immune responses by activating numerous genes, including those involved in the expression of adehesion molecules, cytokines and growth factors {Nilsson *et al.*, 2002; Collins and Cybulsky, 2001}.

ANTIATHEROGENIC LIPOPROTEINS

It is generally accepted that HDL are anti-atherogenic. Epidemiological data and prospective studies show a strong correlation between low plasma HDL concentrations

and CHD {Gordon, 1990}. ApoE- or LDL receptor-deficient mice, that are susceptible to atherosclerosis, exhibit a 70-46% regression of fatty streak formation when HDL levels were increased by introducing recombinant adenovirus encoding for human apoA-I {Benoit *et al.*, 1999; Tangirala *et al.*, 1999}.

1.6 ANTIATHEROGENIC PROPERTIES OF HDL

There have been several proposed mechanisms as to how HDL protect against atherosclerosis. These include the involvement of HDL in RCT as well as their antioxidant and anti-inflammatory properties.

REVERSE CHOLESTEROL TRANSPORT (RCT)

HDL plays a pivotal role in RCT. This process involves the removal of excess cholesterol from peripheral tissues and its transport by HDL to the liver, where it is either excreted or recycled *de novo* into other lipoproteins. RCT consists of four main steps: (i) efflux of cellular cholesterol to pre-β HDL, (ii) esterification of the cholesterol by LCAT, (iii) the transfer of the CE from HDL to other lipoproteins by CETP, and (iv) uptake of the CE by the liver either by receptor-mediated mechanisms, or selectively by SR-BI {Barter and Rye, 1996; Barter, 1993; Trigatti *et al.*, 2000} (Fig. 1.3).

Three mechanisms of cellular cholesterol efflux to HDL have been proposed {Rothblat *et al.*, 1999; Fielding and Fielding, 1995}. The first mechanism involves the passive diffusion of cholesterol out of cells into acceptor particles in the extracellular fluid. This movement of cholesterol is determined by a concentration gradient and does not

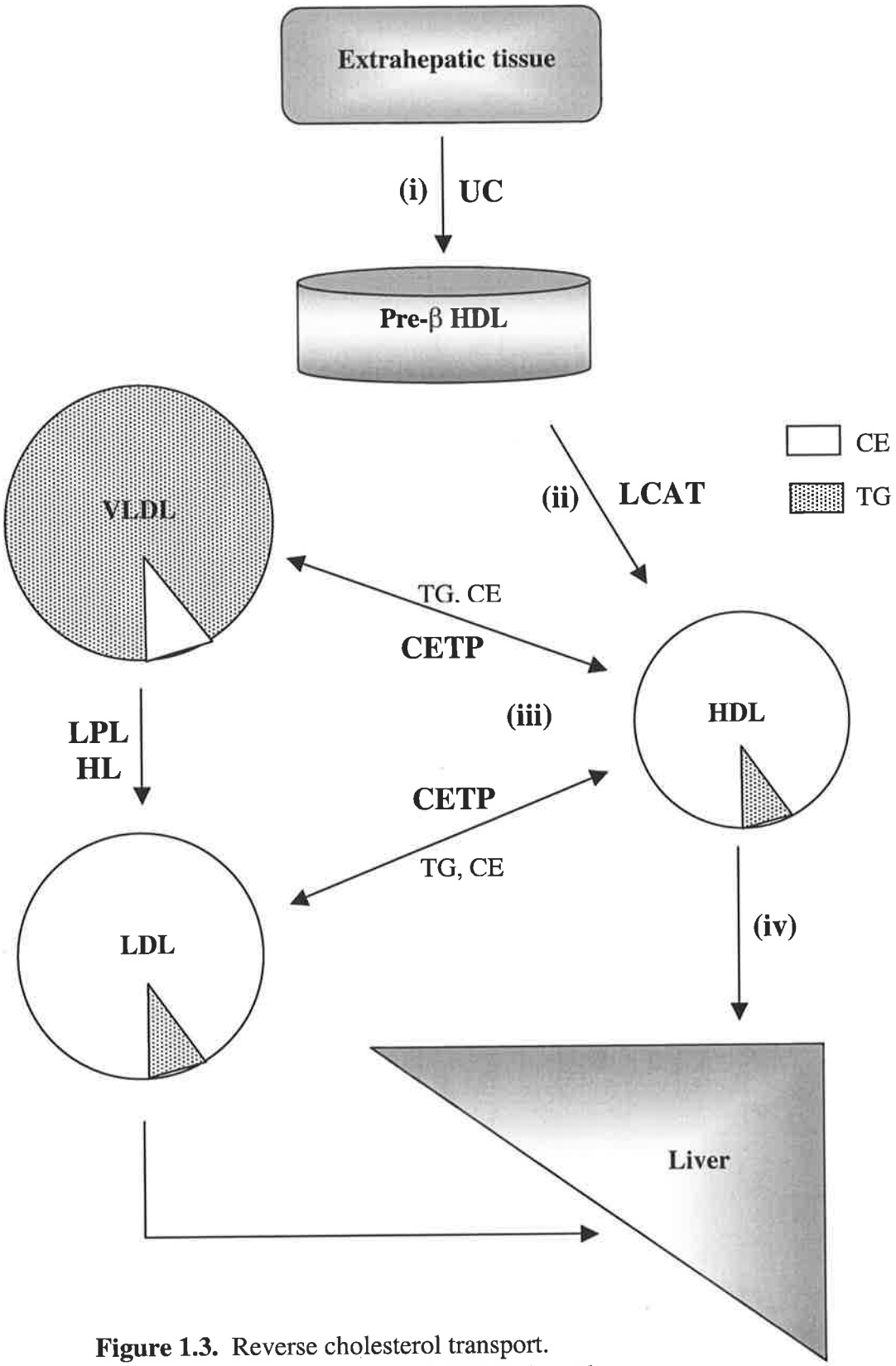

Figure 1.3. Reverse cholesterol transport. Adapted from Barter and Rye, 1996; Stein and Stein, 1999.

involve direct contact between the cell and acceptor particle {Johnson *et al.*, 1991; Rothblat *et al.*, 1992}. In the second mechanism, the presence of a cholesterol acceptor mediates the cholesterol efflux via direct contact between the acceptor and cells {Mendez, 1997}. This contact involves the α-helical domains of the acceptor (lipid-poor apoA-I or pre-β HDL) interacting with the lipid domains in the cell membranes. Both phospholipid and cholesterol efflux occur in this microsolubilisation process {Davidson *et al.*, 1994; Gillotte *et al.*, 1998}. The third mechanism of cholesterol efflux is mediated by SR-BI {Ji *et al.*, 1997; Rothblat *et al.*, 1999}. As this is a more efficient process, larger lipoproteins can act as acceptors. ApoA-I is the principal ligand for SR-BI, with the α-helix being the structural motif that is required for receptor recognition {Williams *et al.*, 2000}. The conformation of apoA-I in HDL also regulates the lipoprotein binding to hepatic cells and SR-BI {Leblond and Marcel, 1991; de Beer *et al.*, 2001}. It is proposed that SR-BI forms a hydrophobic channel through which cholesterol can move down a concentration gradient that forms between the cell membrane and HDL {Rodrigueza *et al.*, 1999; Trigatti *et al.*, 2000}.

Once cellular cholesterol efflux has occurred, the unesterified cholesterol on the pre-β HDL is esterified by LCAT {Jonas, 1991}. This process results in the pre-β HDL being converted into small, then large spherical HDL as CE are progressively sequestered into the particle core. CE are also transferred, by CETP, out of the HDL to other lipoproteins, such as VLDL and LDL {Barter *et al.*, 1982}. The esterification and transfer of CE out of HDL establishes a 'cholesterol sink' so that the direction of cholesterol efflux occurs down a concentration gradient, from cells to HDL {Rothblat *et al.*, 1999}.

The CE is eventually delivered to the liver either, directly by HDL or, after being transferred to LDL (or VLDL) by CETP. The liver takes up CE by two mechanisms: (i) there are the receptor-mediated pathways, which involve the binding of HDL and LDL to apoE and LDL receptors in a process that involves uptake of the whole lipoprotein {Brown *et al.*, 1981; von Eckardstein *et al.*, 2000}, and (ii) the selective delivery of HDL CE via SR-BI to hepatocytes. In the latter case, the HDL particle remains intact but decreases in size {Rodrigueza *et al.*, 1999; von Eckardstein *et al.*, 2001}.

Although, it is indisputable that HDL is mainly responsible for the movement of cholesterol from extrahepatic cells to the liver, there have been studies that have questioned its role as the regulator of cholesterol flux in RCT {Osono *et al.*, 1996; Jolley *et al.*, 1998; Alam *et al.*, 2001}. In these studies, RCT is quantified *in vivo* as the rate of cholesterol acquisition by extrahepatic cells. This relationship is based on the assumption that, in a steady-state system, the rate of acquisition of cholesterol by cells, from lipoproteins and *de novo* synthesis, is balanced by rate of cholesterol transport to the liver for removal {Spady, 1999}. Studies using this methodology have compared the rate of RCT with the level of HDL in the mouse {Osono *et al.*, 1996; Jolley *et al.*, 1998; Alam *et al.*, 2001}. The results showed that neither plasma HDL, nor apoA-I levels, are related to cholesterol flux from extrahepatic tissue to the liver {Osono *et al.*, 1996; Jolley *et al.*, 1998}. The enhancement of factors that affect RCT, such as overexpression of SR-BI, LCAT, 7α-hydroxylase (mediates the conversion of cholesterol to bile) and apoA-I, also failed to increase cholesterol flux throughout the entire pathway {Alam *et al.*, 2001}. So, although HDL is widely accepted as being antiatherogenic, there is evidence suggesting that RCT is not the underlying mechanism of this protective effect.

ANTIOXIDANT PROPERTIES OF HDL

HDL has been shown to protect LDL from oxidative damage {Parthasarathy *et al.*, 1990}. The mechanism of this process is not clear but relates to the capacity of HDL to act as a scavenger for lipid peroxides {Bowry *et al.*, 1992; Parthasarathy *et al.*, 1990} and the ability of HDL, and its associated proteins, to reduce oxidised lipids {Garner *et al.*, 1997; Christison *et al.*, 1995; Vohl *et al.*, 1999}.

Bowry *et al.* (1992) observed in fasting subjects that lipid hydroperoxides in plasma HDL are taken up and catabolised by hepatocytes and the perfused rat liver at a faster rate than unoxidised lipids. The lipid hydroperoxides are transferred from LDL to HDL by CETP {Christison *et al.*, 1995}. However, there was no such uptake or catabolism of oxidised lipids in LDL by the perfused liver {Christison *et al.*, 1996}.

HDL-associated proteins may also have antioxidant activity. For example, the methionine residues in apoA-I and apoA-II have been shown to reduce phospholipid hydroperoxides to the corresponding hydroxides {Garner *et al.*, 1997}.

Paraoxonase (PON) is another HDL protein that reportedly has antioxidant properties. PON is a calcium dependent esterase that has hydrolytic activity against organophosphates {Durrington *et al.*, 2001}. LCAT has been reported to block LDL oxidation *in vitro* as well, probably by metabolising hydroperoxide products {Vohl *et al.*, 1999}. PON and LCAT hydrolyse the oxidised phospholipids and CE hydroperoxides, that are transferred from LDL to HDL, to produce non-atherogenic products which can be transported, via HDL, to the liver for removal {Mackness *et al.*, 2000}.

LDL receptor deficient PON knockout mice have shown increased susceptibility to atherosclerosis {Shih *et al.*, 1998}. The HDL and LDL in these mice are also more prone to oxidation than HDL and LDL from control animals. Platelet-activating factor acetylhydrolase (PAFAH) is another protein that may be involved in hydroperoxide degradation {Watson *et al.*, 1995}. The enzymatic activity of PAFAH resembles that of PON. However, although PAFAH is present in both LDL and HDL, its activity on LDL may differ from that on HDL {Mackness and Durrington, 1995}. The activity of PAFAH on LDL has been reported to prevent LDL oxidation, but the antioxidant properties of HDL-associated PAFAH have not been established {Durrington *et al.*, 2001}. In PON1-knockout mice, PAFAH activity is unaffected, but the HDL isolated from these animals fails to protect LDL from oxidation {Shih *et al.*, 1998}.

ANTI-INFLAMMATORY PROPERTIES OF HDL

It is recognised that atherosclerosis is primarily an inflammatory disorder {Ross, 1999}. There is compelling evidence that inflammation initiates and promotes the development of atheroma {Collins and Cybulsky, 2001; Libby *et al.*, 2002; Reape and Groot, 1999}. Epidemological studies have shown that patients with high basal levels of the inflammatory marker, C-reactive protein have a two-fold increase in predicted future vascular events {Ridker, 2002; Nilsson *et al.*, 2002}. It is generally accepted that one of the ways that HDL protects against atherosclerosis is through its anti-inflammatory properties. There are several methods by which HDL can inhibit or suppress the inflammatory responses of the body.

HDL is able to inhibit pro-inflammatory responses by preventing LDL oxidation and scavenging the oxidised lipids which initiate these responses {Bowry *et al.*, 1992;

Parthasarathy *et al.*, 1990}. HDL and apoA-I also bind to lipopolysaccharides and endotoxins and prevent the release of TNF-α {Shah *et al.*, 2001}.

HDL may also protect against atherosclerosis by inhibiting expression of endothelial adhesion molecules {Cockerill *et al.*, 1995; Ashby *et al.*, 1998}. Adhesion molecules initiate fatty streak formation by capturing and recruiting macrophages {Reape and Groot, 1999; Ross, 1999}. Vascular cell adhesion molecule expression is down regulated when endothelial cells are preincubated with HDL *in vitro* {Cockerill *et al.*, 1995; Ashby *et al.*, 1998; Baker *et al.*, 1999}. The inhibitory effect of HDL on adhesion molecules expression has also been demonstrated *in vivo* in the mouse and pig {Dimayuga *et al.*, 1999; Cockerill *et al.*, 2001}. An intact HDL particle, with both phospholipids and apolipoproteins, is required to produce this inhibitory effect {Ashby *et al.*, 1998}, with rHDL containing 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) and 1-palmitoyl-2-arachidonyl phosphatidylcholine (PAPC) inhibiting adehesion molecule expression by 95 and 70%, respectively {Baker *et al.*, 1999; Baker *et al.*, 2000}. One of the mechanisms by which HDL inhibit adhesion molecule expression is thought to be through inhibition of the sphingosine kinase pathway {Xia *et al.*, 1999; Barter *et al.*, 2002}.

Given the anti-inflammatory nature of HDL, it is serendipidous that HDL cholesterol and plasma apoA-I levels are decreased in many chronic and acute inflammatory states {Tietge *et al.*, 2002; Baussermann *et al.*, 1988}. It has been observed that patients suffering chronic inflammatory diseases such as rheumatoid arthritis are at greater risk of CVD {Wolfe *et al.*, 1994}. Two acute-phase proteins that are secreted during inflammatory states have been implicated in modifying HDL cholesterol levels {Barter,

2002; Tietge *et al.*, 2002; Clifton *et al.*, 1985}. These are serum amyloid A (SAA) protein and secretory phospholipase A_2 ($sPLA_2$).

SAA is synthesised in the liver and is released into the circulation in response to physical trauma {Clifton *et al.*, 1985; Barter, 2002}. Most of the SAA in plasma is transported as a constituent of HDL. SAA displaces apoA-I from HDL *in vitro* {Clifton *et al.*, 1985; Ashby *et al*, 2001}. HDL cholesterol is also decreased by SAA as it inhibits the esterification of cholesterol by LCAT {Steinmetz *et al.*, 1989}. The antioxidant properties of HDL are compromised by high levels of SAA as well {van Lenten *et al.*, 1995}. However, SAA does not inhibit the ability of HDL to reduce adehesion molecule expression in TNF-α-stimulated cells {Ashby *et al.*, 2001}.

PLA_2 have the ability to remodel rHDL *in vitro* into smaller particles without the loss of lipid-poor apoA-I {Rye and Duong, 2000}. In double transgenic mice that expressed human PLA_2 and apoA-1, HDL particles were also smaller with low concentrations of phospholipids and CE but enriched with triglycerides {Tietge *et al.*, 2002}. When $sPLA_2$ is overexpressed in mice, there is an increased in proatherogenic lyso-PC levels which may be the result of $sPLA_2$ activity on HDL and LDL {Leitinger *et al.*, 1999}. Furthermore, the HDL isolated from the $sPLA_2$ transgenic mice were unable to prevent LDL oxidation in cultured cells {Leitinger *et al.*, 1999}. The reduction of PON in the HDL isolated from these animals may be the reason for the lack of oxidative protection.

1.7 HDL PHOSPHOLIPIDS

The main phospholipid in plasma HDL, phosphatidylcholine, comprises 81% of the total phospholipids in HDL (Fig. 1.4). Sphingomyelin, phosphatidylethanolamine and phosphatidylinositol are minor phospholipid species in HDL that respectively comprise 14%, 2.3% and 2.8% of the total phospholipids {Zhang *et al.*, 1998}. The phosphatidylcholines in HDL are derived from nascent HDL, triglyceride-rich lipoproteins that are being lipolysed by LPL, and cell membranes. Triglyceride-rich lipoproteins, especially chylomicrons and VLDL, provide 4 to 8 g (5-10 mmol) of phospholipid per day to HDL {Eisenberg, 1984}. The amount of phosphatidylcholine transferred to HDL is offset by the amount that is hydrolysed by the *sn*-2 acyl esterase and transferase activity of LCAT which also forms 5–10 mmol of CE per day {Eisenberg, 1984}. A proportion of HDL phospholipids are also transferred to other lipoproteins {Patsch and Gotto, 1987}. The rate of these transfers is at least 5 times faster than apolipoprotein exchange {Eisenberg, 1984}. Phospholipid transfers are facilitated by PLTP and CETP {Tall, 1986; Nishida and Nishida, 1997; Kawano *et al.*, 2000}. A small proportion of HDL phospholipid exchange also occurs by spontaneous diffusion {Eisenberg, 1984}. Enzymes such as endothelial lipase (EL), HL and LPL are also continually hydrolysing HDL phospholipids as it circulates in the plasma.

In human HDL, the most abundant phosphatidylcholines are PLPC (34.4 %) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) (13%), followed by PAPC (9.2%) and 1-palmitoyl-2-docosahexanoyl phosphatidylcholine (PDPC) (3.7%) {Subbaiah and Monshizadegan, 1988} (Table 1.4). Given that a large proportion of the phospholipids in HDL come from chylomicrons, diet has a large influence on HDL phospholipid composition {Myher *et al.*, 1981; Sola *et al.*, 1990}. For example, diets rich in

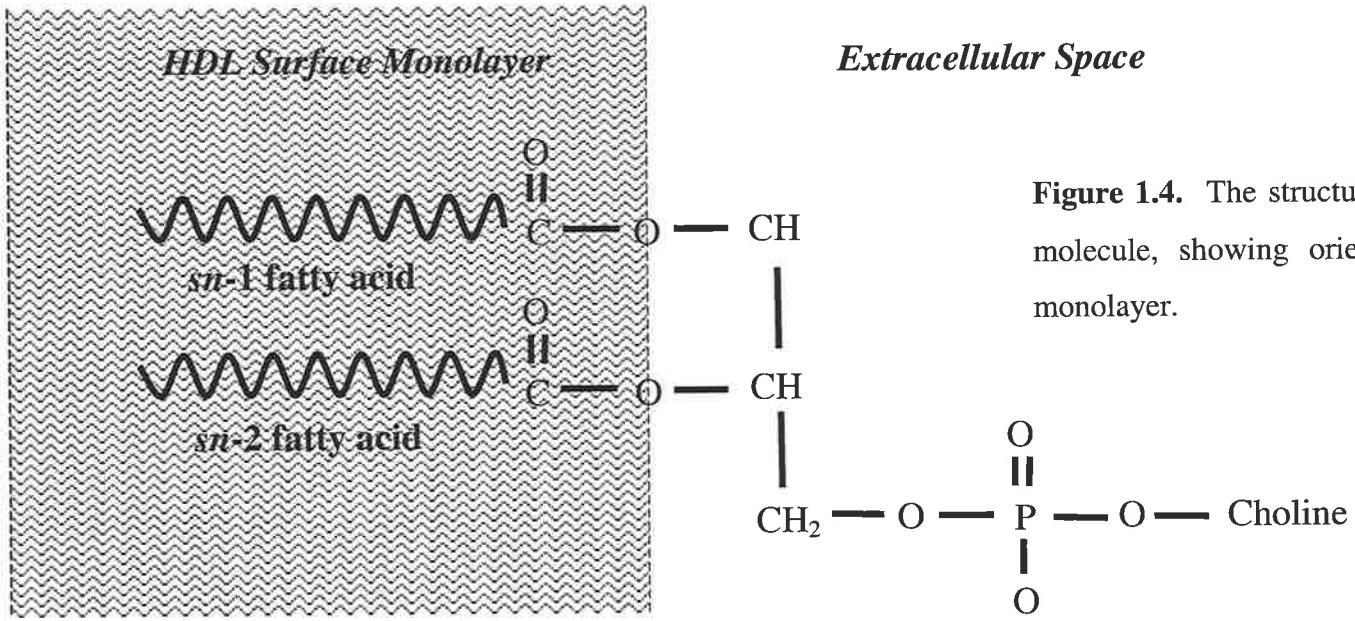

Figure 1.4. The structure of a phosphatidylcholine molecule, showing orientation in the HDL lipid monolayer.

Table 1.4. The main phosphatidylcholines in HDL and their acyl fatty acid moiety[a].

PC	*sn*-1/*sn*-2	*sn*-1 fatty acid	*sn*-2 fatty acid	Composition (mol%)
POPC	16:0/18:1	palmitic	oleic	13.0
PLPC	16:0/18:2	palmitic	linoleic	34.4
PAPC	16:0/20:4	palmitic	arachidonic	9.2
PDPC	16:0/22:6	palmitic	docosahexanoic	3.7

[a]Sourced from Subbaiah and Monshizadegan, 1988; Zhang *et al.*, 1998.

monounsaturated fatty acids, such as olive oil- or safflower oil-rich diet, enrich HDL with POPC and PLPC, while diets high in PUFA enrich HDL with PDPC {Sola *et al.*, 1990; Gillotte *et al.*, 1998}.

1.8 PLASMA FACTORS THAT REMODEL HDL.

Remodeling describes the processes where HDL undergo changes in structure, size and composition. These processes are mediated by a number of plasma factors.

1.8.1 LECITHIN:CHOLESTEROL ACYLTRANSFERASE (LCAT)

LCAT is a 47 kDa glycoprotein that is synthesised in the liver {Jonas, 1991}. It becomes active in the circulation, where it associates mainly with HDL, which is its preferred substrate {Francone *et al.*, 1989: Jonas, 1991}. LCAT is responsible for generating all of the CE in the plasma {Glomset, 1968}. It mediates the transfer of a fatty acid from the *sn*-2 position of phosphatidylcholine to unesterified cholesterol, forming CE and lyso-PC. The CE is sequestered into the HDL core while lyso-PC dissociates from the lipoprotein and binds to albumin. ApoA-I is the main cofactor of LCAT {Cho *et al.*, 2001}. Other apolipoproteins that activate LCAT, include apoA-IV, apoC-I and apoC-II {von Eckardstein *et al.*, 1994; Shachter, 2001; Tailleux and Fruchart, 1996}. Evidence of the importance of LCAT in HDL metabolism comes from studies of LCAT overexpression in mice, which accumulate large CE-enriched, apoE-containing HDL_1 and have reduced concentrations of pre-β HDL {Berard *et al.*, 1997; Fournier *et al.*, 1999}. *In vitro* studies have shown that the mechanism by which LCAT remodels HDL involves direct incorporation of lipid-poor apoA-I into discoidal, nascent HDL {Liang *et al.*, 1996}. In the absence of lipid-poor apoA-I, the LCAT-mediated

remodeling of HDL involves particle fusion and the dissociation of lipid-poor apoA-I {Nichols *et al.*, 1985, Liang *et al.*, 1994}.

LCAT deficiency has been reported in humans {Jonas, 1991; Santamarina-Fojo *et al.*, 2000}. Partial LCAT deficiency results in fish-eye disease (FED) {Kuivenhoven *et al.*, 1997}. Complete absence of LCAT activity results in familial LCAT deficiency (FLD) {Kuivenhoven *et al.*, 1997}. The clinical manifestations of these disorders include cloudy corneas, reduced levels of plasma HDL, as well as apoA-I and apoA-II {Santamarina-Fojo *et al.*, 2000; Kuivenhoven *et al.*, 1997}. FLD is further characterised by hypertriglyceridemia, haemolytic anaemia and renal disease. All these symptoms are associated with the accumulation of unesterified cholesterol in cell membranes. In both FED and FLD, nascent CE-rich HDL are not converted to mature, spherical CE-enriched HDL. This results in increased catabolism of the nascent HDL and very low HDL levels.

In 1968, Glomset proposed that LCAT activity plays a central role in RCT whereby its ability to esterify cholesterol in HDL generates a concentration gradient that promotes the efflux of cholesterol from peripheral cells to HDL {Glomset, 1968}. This is considered to be antiatherogenic, and is supported by studies of patients with angiographically documented CAD and survivors of myocardial infarction {Solajic-Bozicevic *et al.*, 1994}. In these subjects, LCAT activity is reduced by 24-50% {Solajic-Bozicevic *et al.*, 1994}. Mice transgenic human for LCAT also have increased cellular cholesterol efflux {Berard *et al.*, 1997; Fournier *et al.*, 1999}.

However, the relationship between LCAT activity and atherosclerosis is complicated by the fact that the CE that it generates are transferred by CETP to the atherogenic apoB-containing lipoproteins {Santamarina-Fojo *et al.*, 2000}. The potentially proatherogenic effects of LCAT are evidenced by the finding that the reduction in atherosclerotic lesions in human LCAT transgenic rabbits fed a high cholesterol diet is abolished in the presence of defective LDL receptors {Brousseau *et al.*, 2000}. In mice, which lack CETP, overexpression of LCAT resulted in either increased atherosclerosis or there was no marked change from controls {Berard *et al.*, 1997}. Although plasma HDL levels are elevated in these transgenic mice, the composition and function of these particles are abnormal, because CETP-mediated transfer of CE to apoB-containing lipoproteins does not occur and RCT is impaired {Berard *et al.*, 1997}. When CETP is coexpressed with LCAT in these mice, the atherosclerosis that was induced by a high cholesterol diet was significantly reduced {Foger *et al.*, 1999}. Hence, it appears that CETP plays a crucial role in the antiatherogenic effects of LCAT.

1.8.2 CHOLESTERYL ESTER TRANSFER PROTEIN (CETP)

CETP is a hydrophobic glycoprotein with a molecular mass of about 74 kDa {Hesler *et al.*, 1987}. The majority of the CETP in plasma is associated with HDL {Francone *et al.*, 1989}. CETP transfers CE and triglycerides between plasma lipoproteins and accounts about a third of the phospholipid transfer activity in the plasma {Kawano *et al.*, 2000}. The bi-directional transfer of CE and triglycerides between HDL, LDL and VLDL that is mediated by CETP results in an equilibrium of these core lipids between all the plasma lipoproteins *in vivo* {Barter and Rye, 2001; Quintao, 1995}. Given that CE is the predominate core lipid in HDL, there is a net movement of CE out of HDL

into VLDL, and a net mass transfer of triglyceride out of VLDL into HDL {Barter and Rye, 2001}.

CETP also remodels HDL in a process that reduces the size of the particle and is accompanied by the dissociation of lipid-poor, pre-β migrating apoA-I {Barter *et al.*, 1990; Liang *et al.*, 1994; Rye *et al.*, 1995}. The activity of CETP can generate HDL that are triglyceride-enriched. These particles are the preferred substrates for HL {Shirai *et al.*, 1981; Clay *et al.*, 1992}. The HL-mediated hydrolysis of HDL effectively reduces particle size and promotes the dissociation of lipid-poor apoA-I {Clay *et al.*, 1992; Liang *et al.*, 1994}.

Triglyceride-enrichment of HDL by CETP also enhances HDL-PLTP interactions {Rye *et al.*, 1998; Settasatian *et al.*, 2001}. When CETP is incubated with HDL in the absence of triglyceride-rich lipoproteins, HDL fusion and remodeling occur {Rye *et al.*, 1997}. This generates small particles in the absence of the dissociation of lipid-poor apoA-I {Rye *et al.*, 1997}.

The role of CETP in atherosclerosis is unclear. Through its ability to generate pre-β HDL, which promotes cellular cholesterol efflux, activity of CETP is potentially antiatherogenic. However, as CETP transfers CE from HDL to VLDL and LDL, it also has the potential to be proatherogenic.

Studies in rabbits, a species with high CETP activity, suggest that it may be atherogenic. Inhibition of CETP in the rabbit increases plasma HDL cholesterol levels, reduces LDL

concentrations and promotes regression of atherosclerotic lesions {Sugano *et al.*, 1998; Okamato *et al.*, 2000}.

However, when mice were used in transgenic experiments the results were conflicting depending on the metabolic background. As mice are naturally CETP-deficient, HDL are the main carriers of plasma cholesterol. In apoE knockout/LDL receptor knockout mice that develop atherosclerosis, the introduction of human CETP worsened the disease state {Plump *et al.*, 1999}. This suggests that CETP is atherogenic when LDL or remnant clearance is impaired. However, when CETP is introduced to mice expressing human LCAT, mean aortic lesion areas were reduced {Foger *et al.*, 1999}. The absence of CETP in the human LCAT transgenic mice cause the impaired hepatic uptake of HDL CE and accumulation of abnormal HDL, which was reversed with the introduction of CETP. Reductions in atherosclerotic lesions were also reported in mice transgenic for human apoC-III and human CETP {Hayek *et al.*, 1995}. These animals have high levels of triglyceride-rich remnants. The introduction of CETP decreased the concentration of the remnants and promoted cellular cholesterol efflux, by generating pre-β HDL and increasing the delivery of HDL CE to the liver, which helps to establish a 'cholesterol sink'.

Humans with CETP deficiency have large CE-enriched HDL, similar to that of the mice. There are at least nine known gene mutations and three polymorphisms of the CETP gene that lead to CETP deficiency {Hirano *et al.*, 2000}. The two most common forms of CETP deficiency are a null mutation, IN14 and a missense mutation at exon 15, D442:G {Chiba *et al.*, 1997; Zhong *et al.*, 1996}. IN14 is present in about 2% of the Japanese population and homozygotes for this mutation have no detectable CETP

activity or mass {Inazu *et al.*, 1994}. D442:G is present in 7% of the Japanese population {Inazu *et al.*, 1994}. Individual who are homozygous for D442:G have only partial CETP deficiency {Inazu *et al.*, 1994}. Studies of human CETP deficiency indicated that CHD is related to HDL cholesterol concentrations {Zhong *et al.*, 1996; Moriyama *et al.*, 1998}. CETP deficiency has no impact on CHD risk when HDL cholesterol levels are either high or low {Zhong *et al.*, 1996; Moriyama *et al.*, 1998}. In individuals with normal HDL cholesterol (41-60 mg/dL), by contrast, CETP deficiency is proatherogenic {Zhong *et al.*, 1996}. These studies suggest that CETP is cardioprotective when HDL levels are in the normal range. In other words, the ability of CETP to promote cholesterol efflux from peripheral tissues may out-weigh its ability to increase levels of the proatherogenic VLDL and LDL. Hence, when HDL cholesterol levels are normal, CETP deficiency is associated with CHD. When HDL cholesterol levels are high, CETP-mediated transfer of CE occurs primary between HDL populations rather than to VLDL and LDL. CETP deficiency in this case would not change the status of CHD risk and the high HDL levels remain cardioprotective. However, when HDL levels are low, CETP is no longer the rate-limiting factor in generating pre-β HDL, especially when HL activity is normal. Under these circumstances, the proatherogenic effect of low HDL cholesterol is greater than the ability of CETP to enhance pre-β HDL formation in the first step of RCT {Fielding and Havel, 1996}. The absence of CETP activity then would make no difference and CHD risk remains high. A further study on human CETP deficiency reported that in some cases CHD was coupled with high levels of HDL {Hirano *et al.*, 1995}. However, these patients were found to have consistently low levels of HL activity as well. Therefore, depending on HDL level, and the metabolic background, CETP can be either antiatherogenic or proatherogenic.

1.8.3 PHOSPHOLIPID TRANSFER PROTEIN (PLTP)

PLTP is a hydrophobic glycoprotein that is associated with HDL. Most of the circulating PLTP is derived from hepatocytes and adipocytes, although its mRNA is expressed by most tissue types {van Tol, 2002}. Plasma PLTP has a molecular mass of about 81 kDa {Huuskonen *et al.*, 1998}. Together with CETP, lipopolysaccharide-binding protein and bactericidal/permeablility-increasing protein, PLTP belongs to the lipopolysaccharide binding/lipid transfer protein family {Huuskonen *et al.*, 2001}. Based on the crystallographic structure of bactericidal/permeablility-increasing protein, and its close amino acid homology to PLTP, the structure of PLTP is predicted to be a two domain, boomerang-shaped molecule with two hydrophobic clusters that contain lipid binding domains {Huuskonen *et al.*, 1999}. Site-directed mutagensis of key amino acid residues at these lipid-binding pockets result in significant loss of specific phospholipid transfer activity {Huuskonen *et al.*, 1999}.

PLTP plays an important role in lipoprotein metabolism. It mediates the transfer of phospholipids between lipoprotein particles and remodels HDL to generate both larger and smaller particles {Tu *et al.*, 1993; von Eckardstein *et al.*, 1996; Lusa *et al.*, 1996; Nishida and Nishida, 1997; Settassatian *et al.*, 2001}. PLTP also mediates the transfer of unesterified cholesterol between lipoproteins {Nishida and Nishida, 1997}. The PLTP-mediated remodeling of HDL is accompanied by the dissociation of lipid-poor pre-β migrating apoA-I {Tu *et al.*, 1993; Settassatian *et al.*, 2001; Huuskonen *et al.*, 2000}. This leads to the formation of pre-β HDL and enhanced cellular cholesterol efflux {von Eckardstein *et al.*, 1996}. The process responsible for generating large particles appears to be one of fusion, rather than net lipid transfer or particle aggregation {Lusa *et al.*, 1996; Settassatian *et al.*, 2001}. When HDL are enriched with

triglycerides, the remodeling of HDL, as well as phospholipid transfers, are markedly enhanced {Rye *et al.*, 1998; Settassatian *et al.*, 2001}. It is proposed that the increase in PLTP-mediated triglyceride-enriched rHDL remodeling is due to a destabilisation of apoA-I in the rHDL {Settassatian *et al.*, 2001}. When phospholipid transfers are reduced by either chemical modification, or site-directed mutagenesis of PLTP, HDL remodeling is also reduced {Huuskonen *et al.*, 2000}.

The importance of PLTP *in vivo* has been demonstrated in PLTP knockout mice {Jiang *et al.*, 1999}. These animals have markedly decreased levels of HDL phospholipids, apoA-I and cholesterol due to the impaired transfer of surface components from VLDL to HDL {Jiang *et al.*, 1999}. These mice also have low plasma HDL concentrations, as a consequence of hypercatabolism of phospholipid-depleted HDL. When these same PLTP deficient mice are placed on a high fat diet, phospholipids, unesterified cholesterol, CE, as well as apoA-VI and apoE accumulate in their VLDL and LDL {Jiang *et al.*, 1999; Qin *et al.*, 2000}. These studies strongly support a role for PLTP in the transfer of phospholipids, from triglyceride-rich lipoproteins into HDL.

The ability of PLTP to produce pre-β HDL during the remodeling of HDL is potentially antiatherogenic {Tu *et al.*, 1993; von Eckardstein *et al.*, 1996}. This is consistent with mice overexpressing PLTP (2.5 to 4.5x the levels found in controls) having a 2- to 3-fold increase in pre-β HDL formation {van Haperen *et al.*, 2000}. The plasma from these transgenic animals was also more efficient than the plasma of wild-type mice in preventing the accumulation of intracellular cholesterol in macrophages. PLTP also has the ability to mediate the transfer of the antioxidant α-tocopherol between lipoproteins

and endothelial cells and prevent LDL oxidation {Kostner *et al.*, 1995; Desrumaux *et al.*, 1999}.

1.8.4 Triglyceride Lipase Gene Family

LPL, pancreatic lipase, HL, EL and lipase H are all members of the triglyceride lipase gene family. These lipases are derived from a common evolutionary origin and share structural similarities {Hide *et al.*, 1992}. The determination of the structure of pancreatic lipase by X-ray crystallography has given important information about members of this gene family {Winkler *et al.*, 1990}. Given the high amino acid sequence homology of LPL, EL and HL to pancreatic lipase (~30% in the carboxyl-terminal and ~50% in the amino-terminal domain), the conservation of disulphide bridges, and the similarity of their lipolytic functions, it is generally accepted that these lipases all have the same basic 3D structure {Derewenda and Cambillau, 1991; Hide *et al.*, 1992; Wong and Schotz, 2002}.

The tertiary structures of these lipases features an α/β hydrolase fold {Schrag and Cygler, 1997; Derewenda, 1994}. Other highly conserved structural features included the catalytic triad, the Ser-His-Asp residues that are found in the active site, cysteine residues which form disulphide bridges, the lid and surface loop(s) near the active site, as well as the adjacent lipid binding regions {Jaye *et al.*, 1999; Brady *et al.*, 1990; Wong and Schotz, 2002}.

Pancreatic lipase, LPL, HL, EL and lipase H all consist of two domains, a carboxyl-domain (C-terminal) and the larger amino-domain (N-terminal) {Winkler *et al.*, 1990; Jin *et al.*, 2002; Hill *et al.*, 1996; Wong *et al.*, 1991; Jaye *et al.*, 1999}. The N-terminal

domain is generally associated with catalytic activity, while the C-terminal domain has lipid binding and anchoring functions {Wong *et al.*, 1994; Wong *et al.*, 1991; Dichek *et al.*, 1993; Hill *et al.*, 1996; Davis *et al.*, 1992}. The lipid-, heparin-, and receptor-binding functions are all located in the C-terminal domain {Lookene *et al.*, 1997; Hill *et al.*, 1998; Sendak *et al.*, 2000; Salinelli *et al.*, 1996; Dichek *et al.*, 1993}. Although the two domains have their own discrete functions it is believed that they communicate with each other {Wong and Schotz, 2002}. In general, the amino acid sequence homology between lipases is greater in the N-terminal domain than in the C-terminal {Wong and Schotz, 2002}.

In the lipase gene family the surface loops and the lid region are thought to determine substrate specificity {Faustinella *et al.*, 1992; Henderson *et al.*, 1993; Kobayashi *et al.*, 1996; Dugi *et al.*, 1995}. The phospholipase and triglyceride lipase activities of LPL and HL were reversed when the lids of LPL and HL were exchanged to generate chimeras {Dugi *et al.*, 1995}. When these chimeras were expressed in HL-deficient mice, the plasma phospholipid concentration varied according to the lid {Kobayashi *et al.*, 1996}. Mice expressing a LPL/HL-lid chimera had a marked decrease of 81% in plasma phospholipid concentration, while those expressing a HL/LPL-lid chimera saw a 32% decline in plasma phospholipids {Kobayashi *et al.*, 1996}. The lid is also proposed to play a role in lipid binding {Dugi *et al.*, 1992}.

All members of the triglyceride lipase gene family share a common mechanism whereby they are activated upon contact with a lipid-water interface {Derewenda *et al.*, 1992}. This process is called interfacial activation {Verger and de Haas, 1976; Marangoni, 1994}. When a lipase binds to an interface it initiates a series of

conformational changes around the active site, whereby the lid and surface loops rearrange and allow the substrates to access to the catalytic site {Keiper *et al.*, 2001; Egloff *et al.*, 1995; Derewenda *et al.*, 1992}.

1.8.5 LIPOPROTEIN LIPASE (LPL)

LPL is a glycoprotein with a molecular mass of about 55 kDa {Jackson, 1983}. It is synthesised by adipocytes, macrophages, cardiac and skeletal myocytes and is mostly bound to the capillary endothelium by HSPG {Olivecrona and Bengtsson-Olivercrona, 1993}. While some LPL is found in the plasma associated with lipoproteins, this LPL is inactive {Karpe *et al.*, 1998}.

The activity of LPL is sensitive to salt and it is active in the dimeric, but not the monomeric form {Olivecrona *et al.*, 1985; Osborne *et al.*, 1985}. The cofactor of LPL is apoC-II {Wong *et al.*, 1997}. ApoC-II binding is thought to require the interaction of both domains of LPL {Hill *et al.*, 1998; Kobayashi *et al.*, 2002}. LPL is an obligate dimer and its hydrolytic activity involves the interaction of the lipid binding domain in the C-terminal of one LPL subunit with the C-terminal of the other subunit in a head-to-tail manner {Wong *et al.*, 1997}.

LPL has high triglyceride lipase activity and low phospholipase activity {McCoy *et al.*, 2002}. The rate of LPL-mediated phospholipid hydrolysis is 1% of that for triglycerides {Jackson, 1983}. It hydrolyses triglycerides in chylomicrons, VLDL and other triglyceride-rich lipoproteins and is involved in energy distribution and storage in adipocytes and muscle cells {Jackson, 1983; Merkel *et al.*, 2002; Preiss-Landl *et al.*, 2002}.

When LPL hydrolyses triglycerides in apoB-containing lipoproteins, apolipoproteins and phospholipids dissociate from the particles and are incorporated into HDL {Ginsberg, 1990}. The finding that overexpression of LPL in transgenic mice increases plasma HDL cholesterol levels and markedly reduces VLDL and plasma triglyceride concentration are consistent with this mechanism {Clee *et al.*, 1997; Shimada *et al.*, 1996}. Additionally, very low plasma HDL cholesterol levels and severe hypertriglyceridemia are evident in LPL gene knockout mice {Coleman *et al.*, 1995}.

LPL also acts as a ligand for lipoproteins by binding simultaneously to lipoproteins and cell surfaces via HSPG and/or receptors {Williams *et al.*, 1992; Olivecrona and Bengtsson-Olivecrona, 1993; Medh *et al.*, 2000}. This binding is reduced by treatment with heparitinase {Williams *et al.*, 1992}. LPL facilitates apoB-containing lipoprotein cellular uptake by enhancing lipoprotein binding to receptors {Medh *et al.*, 2000; Rumsey *et al.*, 1992}. LPL also facilitates the cellular uptake and degradation of LDL that occurs independently of receptors {Williams *et al.*, 1992}. This LPL-enhanced LDL degradation occurs at much slower rate than uptake of LDL by the classic LDL-receptor pathway {Rumsey *et al.*, 1992}.

While the role of LPL in lipoprotein metabolism is potentially antiatherogenic, its bridging/ligand function is regarded as proatherogenic {Clee *et al.*, 2000}. The atherogenic nature of LPL, to some extent, is dependent on the site of expression. Expression of LPL activity in muscle or adipose tissues is antiatherogenic as it is associated with cardioprotective lipid profiles of high plasma levels of HDL and low levels of apoB-containing lipoproteins {Preiss-Landl *et al.*, 2002}. High expression of LPL in endothelial wall is considered proatherogenic because of its ability to promote

lipid deposition in the endothelium, which leads to the formation of fatty streaks {Clee *et al.*, 2000; Babaev *et al.*, 2000}. This is achieved either through enhanced recruitment of LDL into the subendothelial space or by the byproducts of LPL lipolysis increasing endothelial permeability {Rutledge *et al.*, 1997}.

The macrophage is the primary source of LPL in the endothelium {Babaev *et al.*, 2000}. Studies in transgenic mice show that an increase in macrophage-derived LPL expression is accompanied by an increase in atherosclerotic lesion size {Clee *et al.*, 2000; Babaev *et al.*, 2000}. Hence, the role of LPL in atherogenesis is dependent on the balance between LPL expression in macrophages and those in muscle and adipose cells.

1.8.6 LIPASE H

Jin *et al.* (2002) have recently cloned a new member of the triglyceride lipase gene family called lipase H. This lipase has a molecular mass of 63 kDa and is expressed in the pancreas, lung and intestine. Lipase H exhibits 46% amino acid identity with LPL and EL {Jin *et al.*, 2002}. Lipase H is highly conserved among different species, with the mouse and human enzymes sharing 85.4% identity. The catalytic triad (Ser^{144}-Asp^{178}-His^{249}) and the cysteine residues involved in protein folding are also conserved in lipase H, as are two stretches of hydrophobic residues adjacent to the active site. The regions bordering the lid domain and the positively charged regions in the N-terminal domain are also conserved in lipase H. These positively charged residues, combined with the ability of lipase H to bind to heparin suggest that like LPL, HL and EL, it also anchors itself to the cell surfaces by HSPG. The two areas of hydrophobic residues near the active site suggest that lipase H interacts with lipoproteins. The lid of lipase H is comprised of 12 residues and shares little homology with the lids of other lipases. This

is consistent with lipase H having different substrate specificity from LPL, HL and EL. The substrates of lipase H have yet to be identified.

1.8.7 HEPATIC LIPASE (HL)

1.8.7.1 Structure and Function

HL is a 65 kDa glycoprotein that is synthesised in the endoplasmic reticulum of hepatocytes {Jansen *et al.*, 1978; Bensadoun and Berryman, 1996}. Like LPL, catalytically active HL exists as a dimer {Hill *et al.*, 1996}. The N-terminal of HL contains the catalytic triad as well as the lid and the β5 and β9 surface loops that surround the active site {Dugi *et al.*, 1995; Wong and Schotz, 2002}. The C-terminal domain of HL contains the lipid-, heparin- and receptor-binding sites {Hill *et al.*, 1996; Davis *et al.*, 1992; Wong and Schotz, 2002}.

In humans, HL is mostly found bound to the endothelium of hepatic sinusoids by HSPG {Perret *et al.*, 2002}. HL is also found in the adrenals and other steroid-producing organs, such as the ovaries and testis {Verhoeven and Jansen, 1994}. However, as Northern blots for HL mRNA are negative in these tissues, it is believed that HL is transported from hepatocytes via the circulation to heparin-sensitive sites in these organs {Hixenbaugh *et al.*, 1989}. Recently, HL mRNA has been detected in murine peritoneal macrophages and human monocyte-derived macrophages {Gonzalez-Navarro *et al.*, 2002}. This finding raises the possibility that HL may have local effects, especially in arterial lesions, in promoting the progression of atherosclerosis. Like LPL, HL is able to promote LDL and monocyte recruitment into the subendothelial space {Clee *et al.* 2000; Aviram *et al.*, 1988; Babaev *et al.*, 2000; Rutledge *et al.*, 1997; Donner *et al.*, 1998}. This may contribute to foam cell formation {Babaev *et al.*, 2000;

Rutledge *et al.*, 1997}. A large proportion of the cellular uptake of HL, and its subsequent degradation, is also mediated by HSPG and the LDL receptor-related protein {Kounnas *et al.*, 1995}.

Several factors, such as cellular cholesterol levels, regulate HL synthesis {Busch *et al.*, 1990; Perret *et al.*, 2002}. In hepatoma cells, an inverse relationship was observed between cellular cholesterol levels, HL mRNA levels, and activity {Busch *et al.*, 1990}. When hepatoma cells were incubated with mevinolin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor that prevents cellular cholesterol synthesis, production of HL and HMG-CoA reductase transcripts were stimulated. This effect was reversible in hepatoma cells that had been incubated with mevalonic acid, a mevinolin antagonist {Busch *et al.*, 1990}. It has been suggested that HL and SR-BI act synergistically to regulate cellular cholesterol levels {Lambert *et al.*, 1999}. This is supported by the similar tissue distribution of HL and SR-BI, with high levels of expression of both proteins in the liver and steroidogenic organs {Acton *et al.*, 1996; Verhoeven and Jansen, 1994}. HL is reported to enhance the SR-BI-mediated selective uptake of HDL CE in embryonic kidney cells 3-fold while, in HL-knockout mice, SR-BI expression is upregulated in response to low cellular cholesterol levels {Lambert *et al.*, 1999; Wang *et al.*, 1996}.

Hormones also regulate HL activity. Estrogens decrease HL activity, while anabolic steroids elevate it {Cohen *et al.*, 1999}. While pre-menopausal women have lower HL activities than men, the activity level increases with the onset of menopause {Tikkanen *et al.*, 1982}. Glucocorticoids also play a role in regulating HL activity where, the

administration of corticotrophins markedly reduced HL activity in men {Berg and Nilsson-Ehle, 1994}.

1.8.7.2 HL in HDL Metabolism

HL has both phospholipase A_1 and triglyceride lipase activities {McCoy *et al.*, 2002}. While HL hydrolyses the phospholipids and triglycerides in all types of lipoproteins {Olivercrona and Bengtsson-Olivercrona, 1993; Medh *et al.*, 1999; Landin *et al.*, 1984}, it exhibits a clear preference for HDL over LDL and triglyceride-rich lipoproteins {Shirai *et al.*, 1981}. HL also remodels HDL into smaller particles in a process that is accompanied by the dissociation of apoA-I {Deckelbaum *et al.*, 1986; van Tol *et al.*, 1980; Groot *et al.*, 1981; Clay *et al.*, 1992}. Both the lipid-poor apoA-I and small HDL particles are found to stimulate cholesterol efflux from cells {Castro and Fielding, 1988; Agnani and Marcel, 1993; Fournier *et al.*, 1996}. Moreover, HDL that have been preincubated with HL and reisolated, also deliver cholesterol to liver cells more efficiently than untreated HDL {Bamberger *et al.*, 1983}.

HL activity in humans varies widely. This is a consequence of polymorphisms in the HL gene {Cohen *et al.*, 1994; Cohen *et al.*, 1999}. This allelic variation is responsible for up to 25% of the variability in plasma HDL cholesterol levels in the general population {Cohen *et al.*, 1994}. Genetic variation in HL activity also influences HDL cholesterol and HDL subclass distribution in humans {Shohet *et al.*, 1999; Guerra *et al.*, 1997; Jansen *et al.*, 1997; De Oliveira e Silva *et al.*, 1999; Bersot *et al.*, 1999; Couture *et al*, 2000; Grundy *et al.*, 1999}.

The importance of HL in HDL metabolism has been demonstrated in animal models, and in cases of human HL deficiency. Transgenic rabbits {Barbagallo *et al.*, 1999} and mice {Busch *et al.*, 1994} overexpressing human HL have reduced plasma levels of HDL cholesterol and apoB-containing lipoproteins. The HDL subclass distribution is also altered, with the HDL in the transgenic animals being smaller than HDL from control animals. In mice, this effect correlated with a 42% decrease in cholesterol accumulation in the aorta compared to controls {Busch *et al.*, 1994}.

Adenovirus-infected mice expressing catalytically inactive HL also have decreased HDL cholesterol levels {Dugi *et al.*, 2000}. This was attributed to the noncatalytic ligand activity of HL, which involves the formation of a bridge between HDL and HSPG and mediates HDL uptake. HL is reported to perform the same function for apoB-containing lipoproteins, by mediating the removal of VLDL, IDL and LDL from the plasma in transgenic mice and subjects with HL deficiency {Dichek *et al.*, 2001; Dichek *et al.*, 1998; Zambon *et al.*, 2000}.

Some cases of total HL deficiency in humans have been reported {Hegele *et al.*, 1993}. The HDL and LDL in these individuals are large and enriched with triglycerides and phospholipids. There is also impaired catabolism of lipoprotein remnants in these subjects. Several patients with HL deficiency have HDL cholesterol levels in the ninetieth percentile despite also having hypertriglyceridemia {Connelly *et al.*, 1990}. Mice with HL deficiency also have elevated levels of triglyceride- and phospholipid-rich HDL {Homanics *et al.*, 1995}. The mice, however, do not display an accumulation of remnants suggesting that there are other compensatory mechanisms for their removal in this species.

1.8.7.3 HL and Atherosclerosis

The role of HL in the development of atherosclerosis is unclear. Animal studies have indicated that HL is either pro-atherogenic, or has no effect on CHD risk.

The notion that HL is potentially atherogenic is consistent with the finding that its activity lowers plasma HDL concentrations {Jansen *et al.*, 2002; Santamarina-Fojo *et al.*, 1998}. As mentioned earlier, pre-menopausal women have low levels of HL activity {Tikkanen *et al.*, 1982}. This may account for their higher HDL levels and lower incidence of atherosclerosis, compared to age-matched men {Applebaum-Bowden *et al.*, 1985}. High HL activities are also associated with smoking, type 2 diabetes, FH and inactivity, all of which increase the risk of developing atherosclerosis {Jansen *et al.*, 2002}.

Several studies have also reported that low HL activity is associated with atherosclerosis. HL deficiency in humans is characterised by premature atherosclerosis {Hegele *et al.*, 1993}. However, as other lipoprotein abnormalities such as LDL receptor defects are often associated with this condition, it is unclear whether HL deficiency causes atherosclerosis directly. A common HL allele variant, the T-allele, is responsible for lowering HL activity by 15-30% {Shohet *et al.*, 1999}. While this is associated with increased HDL cholesterol, a higher frequency of this allele is found in CAD patients than in controls {Jansen *et al.*, 1997: Shohet *et al.*, 1999}. This suggests that HL activity may be cardioprotective. There are also other HL allele variants that lower HL activity and are found in relatively high frequency in CAD patients {Su *et al.*, 2002; Pihlajamaki *et al.*, 2000; Guerra *et al.*, 1997}.

The results of animal studies are also conflicting. In cholesterol-fed transgenic mice that overexpress HL, aortic accumulation of cholesterol decreased by 42%, despite a reduction in plasma HDL cholesterol levels {Busch *et al.*, 1994}. HL deficiency in the apoE-deficient mice, by contrast, reduced atherosclerotic susceptibility despite an elevation in plasma cholesterol levels {Mezdour *et al.*, 1997}.

It has been proposed that these seemingly conflicting results are due to the fact that there is an optimal level of HL activity which is antiatherogenic {Jin *et al.*, 2002}. High HL activities increase the risk of atherosclerosis by lowering HDL levels, while low HL activities compromise remnant clearance from the plasma and increase CHD risk. Another possible explanation is that low HL activity has little impact on CHD risk unless it is combined with another lipid abnormality {Jansen *et al.*, 2002}. In cases where CETP activity is low or absent, HL activity is inversely related to atherogenic risk {Hirano *et al.*, 1995}. Thus, human subjects with both low CETP and HL activities are at increased CHD risk {Hirano *et al.*, 1995}.

1.8.8 ENDOTHELIAL LIPASE (EL)

1.8.8.1 Structure and Function

EL is a 68 kDa glycoprotein that shares 45% with LPL and 40% homology HL {Jaye *et al.*, 1999}. EL is expressed as a pro-protein with a hydrophobic 18-residue secretory signal peptide that is cleaved before the protein is glycosylated and secreted {Jaye *et al.*, 1999; Hirata *et al.*, 1999}. EL has at least one isoform, a truncated 40 kDa protein that is secreted along with non-glycosylated and glycosylated EL {Jaye *et al.*, 1999}. The functional profile and physiological relevance of EL remains unknown. Six new

variants of EL isoforms have recently been identified in human subjects with high HDL cholesterol levels {deLemos *et al.*, 2002}.

Jaye *et al.* (1999) cloned EL from the THP-1 human monocyte cell line. In the same year Hirata *et al.* (1999) cloned EL from human umbilical vein endothelial cells. EL is synthesised by several tissues including macrophages, hepatocytes, steroidogenic organs and the placenta. However, EL is unique amongst the triglyceride lipase gene family in that it is expressed in endothelial cells. In the endothelium, EL expression is upregulated in response to cytokines and mechanical stimuli {Hirata *et al.*, 2000}. EL has high phospholipase A_1 but low triglyceride lipase activity {McCoy *et al.*, 2002; Choi *et al.*, 2002; Jin *et al.*, 2002}.

Several functional domains are highly conserved among EL, HL and LPL {Jaye *et al.*, 1999}. The conserved regions of the enzymes include the catalytic triad, consisting of Ser-Asp-His residues, as well as the lipid binding domains (residues 163-172 and 272-281 in EL) and heparin-binding sites {Hirata *et al.*, 1999}. The conservation of the lipid and heparin binding sites implies that EL interacts with lipoproteins directly and binds to the endothelial surface via HSPG in same manner as HL and LPL. EL has 10 cysteine residues that are completely conserved among HL, LPL and pancreatic lipase {Hirata *et al.*, 1999}. As these residues are involved in the formation of the disulfide bonds that are responsible for protein folding, they determine the lipase's tertiary structure {van Tilbeurgh *et al.*, 1994}. As such EL is likely to have the $\alpha\beta$ hydrolase folds that are present in HL and LPL.

The enzymatic activity of EL however, is very different from that of HL and LPL {McCoy *et al.*, 2002}. This is probably because the amino acids in the EL lid region have minimal homology with the corresponding regions of other lipases {Hirata *et al.*, 1999; Jaye *et al.*, 1999}. EL is unlike LPL in that it does not require a cofactor {Jaye *et al.*, 1999; Hirata *et al.*, 1999}. It is however, similar to LPL in that it is a salt sensitive enzyme {McCoy *et al.*, 2002}.

1.8.8.2 EL in HDL Metabolism

HDL phospholipids are the main substrate of EL {Jaye *et al.*, 1999; McCoy *et al.*, 2002}. To this end, circulating HDL cholesterol and apoA-I levels in wild-type and human apoA-I transgenic mice decreased significantly after being injected with recombinant adenovirus encoding human EL cDNA {Jaye *et al.*, 1999}. EL deficiency in mice, on the other hand, markedly increases HDL cholesterol levels {Choi *et al.*, 2002}.

Adenoviral vector-mediated transfer of the EL gene into chow-fed LDL-receptor deficient mice also reduces VLDL and LDL cholesterol, suggesting that these lipoproteins are also substrates for EL {Jaye *et al.*, 1999}. Choi *et al.* (2002) have also reported that EL increases LDL and chylomicron remnant uptake by cultured cells {Choi *et al.*, 2002}.

1.8.8.3 EL and Atherosclerosis

The role of EL in atherosclerosis is not known. However, its effect on HDL levels and its upregulation in response to endothelial stimuli suggest that it might be proatherogenic.

EL mRNA levels are increased when human umbilical vein and coronary artery endothelial cells were subjected to fluid shear stress and cyclic stretch {Hirata *et al.*, 2000}. EL expression is also upregulated by inflammatory cytokines such as TNF-α and IL-1β {Hirata *et al.*, 2000}. LPL and HL are known to serve a bridging function where apoB-containing particles are taken up by cells in a noncatalytic manner {Clee *et al.*, 2000; Dichek *et al.*, 1998; Dugi *et al.*, 2000; Dichek *et al.*, 2001}. It is possible that EL may have a similar function. Indeed, there is some evidence to suggest that EL acts as a nonlipolytic ligand for apoB lipoprotein uptake {Choi *et al.*, 2002}. As EL is expressed in endothelial cells, it therefore follows that it may promote LDL accumulation in the artery wall. In this local fashion, EL may function as a proatherogenic factor. Given that EL also reduces HDL levels *in vivo*, the possibility that it is proatherogenic is reasonably high.

SCOPE OF THIS THESIS

As described in section 1.8.7 and 1.8.8, the phospholipase activity of HL and EL decreases HDL levels. The phospholipid composition of HDL varies widely according to the dietary intake of lipids {Sola *et al.*, 1989; Myher *et al.*, 1981}. For this reason it is important to know if HL and EL have the same substrate specificities for HDL phospholipids.

The main aims of this thesis are to determine if (i) HL and EL have different substrate specificities for HDL phospholipids, (ii) if phospholipids regulate the interactions of HL and EL with the surface of HDL, and (ii) how HDL phospholipid composition affects HL- and EL-mediated triglyceride hydrolysis.

These goals could not be achieved by using HDL isolated from human plasma. These preparations vary in size and charge, as well as apolipoprotein and phospholipid composition. In order to overcome these problems a new technique for preparing spherical rHDL, in which phospholipid composition is strictly controlled, has been used {Rye *et al.*, 2002}. Four preparations of spherical rHDL that contained either POPC, PLPC, PAPC or PDPC as the only phospholipid constituent were used for the studies described in this thesis. As these rHDL were comparable in size, had similar core lipid/apolipoprotein molar ratios and contained apoA-I as the sole apolipoprotein they were ideal models for comparing the substrate specificities of HL and EL for HDL phospholipids. The rHDL were also used to determine how the phospholipids affect the ability of HL and EL to hydrolyse HDL triglycerides and the interactions of HL and EL with the rHDL surface.

CHAPTER 2 MATERIALS AND METHODS

2,1 ISOLATION OF APOLIPOPROTEIN A-I

2.2 PREPARATION OF RECONSTITUTED HDL THAT CONTAIN DIFFERENT PHOSPHOLIPIDS

2.2.1 Discoidal rHDL

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2.5 ISOLATION OF CETP

2.6 CHEMICAL ANALYSIS

2.7 ELECTROPHORESIS

2.7.1 Non-denaturing gradient gel electrophoresis

2.7.2 SDS-polyacrylamide gel electrophoresis

2.8 CHEMICALS AND REAGENTS

2.1 ISOLATION OF APOLIPOPROTEIN A-I

ApoA-I was isolated from samples of pooled, autologously donated human plasma (Gribbles Pathology, Adelaide, Australia). Total HDL were isolated from plasma by ultracentrifugation in the $1.07 < d < 1.21$ g/mL density range. Three consecutive spins were carried out at 55,000 rpm, in a 55.2 Ti rotor, using an Optima LE-80K Ultracentrifuge (Beckman Coulter, Fullaton, CA). The first spin was for 16 hr at a density of 1.07 g/mL. The $d > 1.07$ g/mL fraction was collected by tube slicing then subjected to two consecutive 26 hr spins at a density of 1.21 g/mL. All of these procedures were carried out at 4 °C. The isolated HDL were dialysed extensively against a 10 mmol/L ammonium bicarbonate solution, lyophilised for 3 hr then delipidated as described by Osborne (1986). The resulting apoHDL was isolated and dried under a stream of N_2. The apoHDL were then dissolved in 20 mmol/L Tris, pH 8.2, pooled, freeze dried and stored at –20 °C until required.

ApoA-I was isolated from the apoHDL by chromatography on an XK 26/40 Q Sepharose Fast Flow column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) attached to a Fast Performance Liquid Chromatography (FPLC) system (Amersham Pharmacia Biotech AB). ApoA-I and apoA-II were resolved using a modification of the method of Weisweiler (1987) {Rye, 1990}. The apoA-I appeared as a single band when subjected to electrophoresis on a homogenous 20% SDS-polyacrylamide PhastGel (Amersham Pharmacia Biotech) and Coomassie R-350 staining. The isolated apoA-I and apoA-II were dialysed against 20 mmol/L ammonium bicarbonate (3 x 5 L), then lyophilised and stored in an air-tight container at –20 °C. The apolipoproteins were reconstituted in 10 mmol/L Tris-HCl, 3000 mmol/L guanidine-HCl, 0.01% (w/v) EDTA-Na_2 for 1-2 hr and dialysed extensively against TBS (5 x 1 L), prior to use.

2.2 PREPARATION OF rHDL THAT CONTAIN DIFFERENT PHOSPHOLIPIDS

2.2.1 Discoidal rHDL

Cholesterol (10 mg/mL) in chloroform:methanol (2:1 (v/v)) and sodium cholate (30 mg/mL) in 10 mmol/L Tris-buffered saline (TBS) (10 mmol/L Tris, 150 mmol/L NaCl), pH 7.4, containing 50 μmol/L diethylenetriamine-pentaacetic acid (DETAPAC), 0.006% NaN_3 (w/v) and 10 μmol/L butylated hydroxytoluene (BHT) were prepared. Discoidal rHDL contained either POPC, PLPC, PAPC or PDPC (Avanti Polar Lipids, Alabaster, AL, USA) as the only phospholipid, UC and apoA-I were prepared by the cholate dialysis method {Matz and Jonas, 1982}. The initial molar ratio of phospholipid: UC: apoA-I was 110:5:1. The phospholipids and UC were added to clean glass test tubes and dried down under N_2. Sodium cholate (molar ratio cholate:phospholipid 1:1) and TBS were added so that the total volume was 0.5 mL. The mixtures were kept on ice and vortexed every 15 min for 1-2 hr until the solutions became optically clear. ApoA-I (2 mg/ tube) was added and the mixtures were left to stand on ice for a further 2 hr, then pooled and dialysed against TBS with DETAPAC and BHT (pH 7.4) (5 x 1 L) over 5 days. Chelex 100 resin (Bio-Rad, Hercules, CA) (2 g/L) was added to the TBS during the dialysis to prevent inadvertent oxidation of the lipids. The composition and size of the discoidal rHDL was determined and they were stored under argon, in the presence of Chelex until use.

2.2.1 Spherical rHDL

Spherical rHDL were prepared by incubating the individual discoidal rHDL with LCAT and UC dissolved in ethanol {Rye *et al.*, 2002}. In a typical incubation the discoidal (POPC)rHDL (final UC concentration 0.21 mmol/L) were mixed with fatty acid-free

BSA (final concentration 40 mg/mL), β-mercaptoethanol (final concentration 4.0 mmol/L) and LCAT (2.2 mL of a preparation that esterified 1.3 μmol CE/mL LCAT/h). The volume of the incubation mixture was 9.9 mL. The incubation was placed in a shaking waterbath at 37 °C. After 30 min, 44.7 μL of 25 mmol/L UC in ethanol and an additional 1.4 mL of LCAT was added. Fatty acid-free BSA and β-mercaptoethanol were also added to maintain their respective final concentrations at 40 mg/mL and 4.0 mmol/L. These additions were repeated at 30 min intervals, for a total of 7 hr, then incubated without other additions for a further 17 hr. The final volume of the incubation mixture was 36.5 mL. The rHDL were isolated by ultracentrifugation in the$1.07 < d < 1.21$ g/mL density range. The first spin was carried out at 55,000 rpm for 16 hr. The $d > 1.07$ g/mL fraction was collected by tube slicing, adjusted to a density of 1.21 g/mL, then subjected to a further two 16 hr spins at 100,000 rpm. The rHDL were collected by tube slicing as the fraction of $d < 1.21$ g/mL. All the ultracentrifugation was done at 4 °C. The rHDL were dialysed against (3 x 1 L) TBS with DETAPAC, BHT, and Chelex, before use. The amount of LCAT required to convert the discoidal rHDL into spherical rHDL varied according to the phospholipid composition of the discoidal rHDL {Rye *et al.*, 2002}. This reflects the different substrate specificities of the discoidal rHDL for LCAT {Parks and Gebre, 1997}.

2.3 ISOLATION OF HL

HL was isolated from pooled samples of postheparin plasma. The plasma was obtained from patients injected with a bolus of 25,000 IU of heparin prior to percutaneous transluminal coronary angioplasty (Cardiovascular Investigation Unit, Royal Adelaide Hospital). Postheparin plasma was isolated by centrifugation at 3,000 rpm for 10 min at

4 °C and stored at –70 °C before use. HL was isolated from the thawed plasma by Heparin Sepharose Fast Flow chromatography (Amersham Pharmacia Biotech) using a HR 10/30 column that had been pre-equilibrated with 300 mL of 5.0 mmol/L sodium barbital 150 mmol/L NaCl buffer, pH 7.4. Prior to loading onto the column, the thawed plasma was mixed with a 5.0 mmol/L sodium barbital, 450 mmol/L NaCl buffer (pH 7.4) (1:1, (v:v)). The diluted plasma was loaded onto the column and the column was washed with 300 mL of 5.0 mmol/L sodium barbital 300 mmol/L NaCl (pH 7.4) to remove unbound proteins. The HL was eluted with a linear gradient of 400-1,300 mmol/L NaCl in 5.0 mmol/L sodium barbital (pH 7.4). Fractions (8.0 mL) were collected. Heparin (50 IU) was added to each fraction. The fractions were dialysed against TBS (3 x 2 L) to which heparin (6,250 IU/L) was added. The activity of HL in each fraction was assessed (see below) and the active fractions were pooled and concentrated 30-fold. Heparin (1:10 (v/v)) was added to stabilise the concentrated HL. The activities of the pooled samples were determined and the HL was stored at –70 °C in aliquots until use.

HL activity was assessed using spherical (POPC)rHDL as the substrate. (POPC)rHDL (final phospholipid mass 20 nmol) were incubated, at 37 °C for 1 hr, with fatty acid-free BSA (final concentration 20 mg/mL) and a constant amount of HL (25 µL). The final volume of the incubation mixture was 140 µL. The mass of nonesterified fatty acid (NEFA) formed was quantitated using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan). The activities of the HL preparations ranged from 32 to 122 nmol NEFA generated/mL HL/h.

2.4 ISOLATION OF LCAT

LCAT was isolated from pooled samples of human plasma. The plasma (2 L) was precipitated at 35% saturation of ammonium sulphate. The precipitated proteins were removed by centrifugation. The supernatant was then treated with 1,000 mmol/L citric acid (125 mL). The proteins that were precipitated by the citric acid were resuspended in 100 mL of water and the pH adjusted with Na_2CO_3, to pH 7.4. The solution was dialysed against water (2 x 5 L) then ultracentrifugated at 55,000 rpm, 4 °C for 26 hr at a density of 1.25 g/mL. The d > 1.25 g/mL fraction was recovered by tube slicing. The d > 1.25 g/mL fraction was subjected to an additional spin at 55,000 rpm and the d > 1.25 g/mL fraction was recovered by tube slicing. The d > 1.25 g/mL fractions were pooled and subjected to chromatography on an XK 50/60 column containing Phenyl-Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) that had been pre-equilibrated with 1 L of 3,000 mmol/L NaCl. The column was washed with 150 mmol/L NaCl to remove unbound proteins. LCAT was eluted from the column with Milli Q water (0.02% (w/v) NaN_3, 0.01% (w/v) EDTA-Na_2). Fractions (10 mL) were collected and assayed for activity (see below). The active fractions were pooled and dialysed against 20 mmol/L Tris (2 x 5 L) pH 7.4 containing 1 mL β-mercaptoethanol/L. The pooled fractions were loaded onto a XK 26/40 column packed with DEAE Sepharose Fast Flow (Amersham Pharmacia Biotech) that had been prequilibrated with 500 mL of 20 mmol/L Tris (2 x 5 L) pH 7.4 containing 1 mL β-mercaptoethanol/L. The LCAT was eluted with 20 mmol/L Tris/500 mmol/L NaCl buffer, pH 7.4, containing 1 mL β-mercaptoethanol/L. Ten mL fractions were collected. Fatty acid-free BSA (500 µL) (final concentration 20 mg/mL) was added to each fraction. The fractions were assessed for activity (see below). The purity of the LCAT preparations was checked by SDS gel electrophoresis and silver staining. The LCAT was stabilised with fatty acid-

free BSA (final concentration 1 mg/mL) and concentrated 10-fold by ultrafiltration (Amicon, Danvers, MA). The concentrated LCAT was stored in aliquots (10 mL) at – 70 °C until use.

LCAT activity was determined using [^{3}H]UC-labelled POPC/UC/apoA-I discoidal rHDL as the substrate {Piran and Morin, 1979}. The discoidal rHDL (25 μL of final UC concentration 0.13 nmol/μL), fatty acid-free BSA (50 μL, final concentration 10 mg/mL), β-mercaptoethanol (5 μL of a 1:10 dilution) and TBS (47 μL) pH 7.4, were incubated at 37 °C under N_2, for 30 min. The final volume of incubation mixtures was 147 μL. LCAT (20 μL) was added and the incubation was continued for a further 1 hr. The d > 1.25 g/mL fraction of human plasma (20 μL) was used as a positive control. The reaction was stopped by addition of 0.5 mL of 1% digitonin in 95% ethanol. The mixtures were vortexed for 15 sec. Cholesterol (25 μL of 5 mg/mL UC in ethanol) was added, the mixtures were vortexed again, then centrifuged at 1,500 x g for 10 min. A 400 μL aliquot of the supernatant was removed and mixed with 10 mL of aqueous scintillation cocktail (Ready safe, Beckman, USA). The radioactivity of the samples was assessed using a Beckman LS6000 TA liquid scintillation counter. The assay was linear as long as less than 30% of the [^{3}H]UC was esterified. The activities of the LCAT preparations ranged from 995 to 1590 nmol CE generated/ml LCAT/h.

2.5 ISOLATION OF CETP

CETP was isolated from of pooled samples of human plasma. The pooled samples (2 L) were subjected to precipitation with ammonium sulphate. The proteins that precipitated from the plasma between 35 and 55% saturation of ammonium sulphate

were resuspended in 100 mL of water and dialysed against Milli Q water (1 x 5 L). The precipitated proteins were adjusted to a density of 1.25 g/mL and subjected to ultracentrifugation at 55,000 rpm, for 26 hr. The d > 1.25 g/mL fraction was recovered by tube slicing and subjected to another 26 hr spin at 55,000 rpm. The d > 1.25 g/mL fraction was recovered by tube slicing. The procedures were carried out at 4 °C. The d > 1.25 g/mL fractions were pooled and loaded onto a XK 50/30 column containing a Macroprep Hydrophobic Interaction gel (Bio-Rad Laboratories, Hercules, CA, USA) that had been pre-equilibrated with 500 mL of 3,000 mmol/L NaCl solution. Unbound proteins were eluted with 3,000 mmol/L NaCl. The bound proteins were eluted with Milli Q water (0.02% NaN_3, 0.01% EDTA-Na_2). The fractions (10 mL) were dialysed against Milli Q water (3 x 5 L) and assayed for CETP activity (see below). The active fractions were pooled, adjusted to 50 mmol/L NaOAc (pH 4.5) and loaded onto a XK 26/40 column containing CM (Carboxy-Methyl) Sepharose Fast Flow (Amersham Pharmacia Biotech) pre-equilibrated with 500 mL of 50 mmol/L NaOAc (pH 4.5). Bound proteins were eluted from the column with a linear 0-400 mmol/L NaCl gradient in 50 mmol/L NaOAc buffer, pH 4.5 and dialysed against 20 mmol/L Tris, pH 7.4 (3 x 5 L). The fractions (10 mL) were assayed for CETP activity. The active fractions were pooled and loaded on a Mono Q HR 5/5 anion exchange chromatography column (Amersham Pharmacia Biotech), which had been pre-equilibrated with 10 mL of 20 mmol/L Tris (pH 7.4). CETP was eluted with a linear 0-500 mmol/L NaCl gradient in 20 mmol/L Tris buffer, pH 7.4. The fractions (10 mL) were assayed for CETP activity and pooled. The CETP appeared a single band when subjected to SDS-polyacrylamide gel electrophoresis and stained with Commassie R-350. The CETP was stored at –70 °C until use.

CETP activity was assessed as the transfer of [^{3}H]CE to LDL from [^{3}H]CE-HDL_3 {Burstein *et al.*, 1970; Tollefson *et al.*, 1988}. The incubations consisted of [^{3}H]CE-HDL_3 (10 μL, final total cholesterol concentration 80 nmol/mL), LDL (10 μL, final total cholesterol concentration 240 nmol/mL), the LCAT activity inhibitor DTNB (1.42% (w/v) in TBS) (10 μL), CETP (50 μL) and TBS, pH 7.4. The final volume of the incubation mixtures was 175 μL. The d > 1.25 g/mL fraction of human plasma (50 μL) was used as a positive control. The mixtures were incubated at 37 °C for 3 hr. The reactions were stopped by placing the incubation mixtures on ice. Fatty acid-free BSA (50 μL of 5% solution) was added and the LDL was precipitated by addition of 25 μL of heparin and $MnCl_2$ (1:1 (v/v)) solution. The LDL were sedimented by centrifugation at 1,500 x g for 5 min. The [^{3}H]CE-HDL remaining in the supernatant (200 μL) were added to scintillation fluid (10 mL) and counted in a Beckman LS6000 TA liquid scintillation counter. The assay was linear as long as less than 30% of the [^{3}H]CE was transferred. The activity of CETP was expressed as arbitrary units, where one unit is equivalent to the amount of transfer activity in 1 mL of a preparation of pooled human lipoprotein-deficient plasma. The activities of the CETP preparations varied from 22.7 to 48.2 units of activity/mL.

2.6 CHEMICAL ANALYSIS

A Hitachi 902 automatic analyser (Roche Diagnostics, Mannhein, Germany) was used for all chemical analyses.

Phospholipid concentrations were determined using a modification of the method described by Takayama *et al.* (1977). A buffer solution (500 mmol/L TrisHCl, 21.25

mmol/L Phenol, 5.44 mmol/L $CaCl2.2H_2O$, pH 7.8) was made up with water to a final volume of 50 mL. The reagent consisted of the buffer (800 μL), phospholipase D (800 IU), choline oxidase (11.2 IU), peroxidase (17.75 IU), 4-aminoanti-pyrene (80 μL of 12 mg/mL), Triton X-100 (3.2 mL of 5 g/mL) and water (3 mL). The final volume of the reagent was 8 mL. Choline chloride (15 mmol/L) in 5 g/mL Triton X-100 was used as the standard.

Total cholesterol assays were carried out as described {Rye *et al.*, 1996} using a commercially available kit (Roche Diagnostics).

Unesterified cholesterol was measured as described {Stahler *et al.*, 1977}. Buffer 1 (pH 7.7) consisted of Na_2HPO_4 (360 mmol/L), $NaH_2PO_4.2H_2O$ (41.9 mmol/L), phenol (20 mol/L), methanol (3.75 mL) and water. Buffer 2 (pH 7.7) was Na_2HPO_4 (360 mmol/L), $NaH_2PO_4.2H_2O$ (41.9 mmol/L), 4-aminoanti-pyrene (0.41 mg/mL), polyoxyethylene-9-lauryl ether (200 μL), methanol (3.75 mL) and water. The final volumes of buffer 1 and 2 were both 50 mL. The enzyme reagent contained cholesterol oxidase (12 IU/mL), peroxidase (8 IU/mL) and Tris-HCl (10 mmol/L, pH 7.4). The reagent for the assay contained buffer 1, buffer 2, and the enzyme reagent in the ratio of 1:1:0.01 (v/v/v). The standards were the same as for the total cholesterol assay.
CE concentrations were determined as the difference between unesterified cholesterol and total cholesterol concentrations.

Triglyceride concentrations were assayed as described {Wahlefeld, AW., 1974}. A buffer concentrate (pH 7.6) containing Tris (1500 mmol/L), $MgSO_4.7H_2O$ (180 mmol/L), EDTA-Na_2 (100 mmol/L), 4-chlorophenol (30 mmol/L), potassium

ferrocyanide (1.3 mg), sodium cholate (30 mmol/L), Triton X-100 (3 mL) and water (final volume 50 mL) was prepared. Reagent 1 consisted of the buffer concentrate (2 mL), peroxidase (3 IU), L-glycerin-3-phosphate-oxidase (100 IU), adenosine-5'-triphosphate (11 mg) and water (final volume 20 mL). Reagent 2 contained the buffer concentrate (1 mL), lipase (final concentration 30 IU), ampyrone (1.42 mg) and water (final volume 10 mL). Prior to use, glycerokinase (1 mg/mL) was added to reagent 1 (10 μL/1 mL reagent 1). The standards for the triglyceride assay were obtained from Roche Diagnostics.

The bichichioninic acid assay was used to quantitate protein concentrations {Smith *et al.*, 1985). Reagent A was the bichinchoninic acid solution (50 mL) mixed with Triton X-100 (500 μL). Reagent B was a copper (II) sulphate pentahydrate solution (4% (w/v)). Reagent A and reagent B were mixed together (800:16 (v/v)). A fatty-acid free BSA solution (1.0 mg/mL) was used as the standard.

NEFA concentrations were determined using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan).

2.7 ELECTROPHORESIS

2.7.1 Non-denaturing gradient gel elcetrophoresis

rHDL particle size was determined by non-denaturing gel electrophoresis. The gels were prepared as described by Rainwater *et al.* (1992). Samples (10 μg protein) were mixed with tracking dye (40% (w/v) sucrose, 0.01% (w/v) bromophenol blue) and loaded onto 3-35% non-denaturing polyacrylamide gels. High molecular weight

standards (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), were also applied to the gels. The gels electrophoresed for 3,000 Vh in a Gel Electrophoresis Apparatus (Pharmacia C500/400), filled with Tris-Borate Buffer (90 mmol/L Tris, 80 mmol/L borate, 3.0 mmol/L EDTA-Na_2, pH 8.4).

When the electrophoresis was complete, the gels were fixed with sulphosalicylic acid (10% w/v) for 1 hr, stained for 3 hr with Coomassie G-250 (0.04% (w/v), 3.5% (v/v) perchloric acid and destained overnight in acetic acid (5% (v/v)). The gels were stored in plastic bags with 1-2 mL of a preserving solution (0.002% (w/v) NaN_3). Particle sizes were determined by scanning the gels with a JX-610 scanner sharp and comparing the migration of the particles to that of the protein standards of known diameter.

2.7.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacylamide gel electrophoresis was carried out on a PhastSystem (Amersham Pharmacia Biotech). Samples were mixed (1:1 (v/v)) with an SDS sample buffer (0.025% (w/v) bromophenol blue, 0.035% (w/v), EDTA-Na_2, 1.21 mg/mL Tris, and 25 mg/mL SDS) then boiled for 3 min. The samples, as well as the low molecular weight standards, were loaded onto 20% homogenous polyacylamide gels and electrophoresed for 95 Vh. The low molecular weight calibration kit (Amersham Pharmacia Biotech) contained Phosphorylase b (MW 94,000), BSA (MW 67,000), Ovalbumin (MW 43,000), Carbonic Anhydrase (MW 30,000), Soybean Trypsin Inhibitor (MW 20,100) and α-Lactalbumin (MW 14,400). After electrophoresis, the gels were stained with 0.1% (w/v) Phastgel Blue R (350) (Amersham Pharmacia Biotech) in 10% (v/v) acetic

acid and destained with methanol:acetic acid:water (30:10:60). The gels were preserved with glycerol:acetic acid:water (10:10:80).

2.8 CHEMICALS AND REAGENTS

ADIFAB	Molecular Probes A-3880
Acetic acid glacial	BDH Chemicals 100015N
Adenosine 5' triphosphate, disodium salt (ATP)	Sigma Chemicals A-7699
4-Aminoantipyrine	Sigma Chemicals A-4382
Ammonium bicarbonate	BDH Chemicals 103025E
Ammonium sulphate	BDH Chemicals 10033.3B
β-Arachidonoyl-γ-palmitoyl-L-α-phosphatidylcholine (PAPC)	Avanti Polar Lipids 850459
Barbitone, sodium	BDH Chemicals 103654E
Bichinchoninic acid	Sigma Chemicals B-9643
Boric acid	BDH Chemicals 10058.3R
Bovine serum albumin	Sigma Chemicals A-8022
Bovine serum albumin, fatty acid-free (BSA)	Sigma Chemicals A-6003
Bromophenol blue	BDH Chemicals 44305
Butylated hydroxy toluene (BHT)	Sigma Chemicals B-1378
Chelex 100 resin	Bio-Rad 142-2832

Chloroform	BDH Chemicals 10077.6B
4-Chlorophenol	Sigma Chemicals C-4914
Cholesterol, unesterified	Sigma Chemicals C-8667
[1α, 2α(n)-^{3}H]cholesterol	Amersham Pharmacia TRK330
Cholesterol oxidase	Sigma Chemicals C-5421
Cholic acid, sodium salt	Sigma Chemicals C-1254
Choline oxidase	Sigma Chemicals C-5896
Citric acid	BDH Chemicals 277814N
Coomassie Brilliant Blue G-250	Bio-Rad 161-0406
Coomassie Brilliant Blue R-350 (PhastGel)	Amersham Pharmacia 17-051801
Copper (II) sulphate pentahydrate	Sigma Chemicals C-2284
Diethylenetriamine pentaacetic acid (DETAPAC)	Sigma Chemicals D-1133
Diethyl ether	UNILAB Analytical Reagents 465
Digitonin	Sigma Chemicals D-5628
5,5'-Dithio-bis(nitrobenzoic acid) (DTNB)	Sigma Chemicals D-8130
β-Docosahexanoyl-γ-palmitoly-L-α-phosphatidylcholine (PDPC)	Avanti Polar Lipids 8504461
Ethanol	BDH Chemicals 10107.2500P
Ethylenediaminetetraacetic acid, disodium salt (EDTA-Na_2)	BDH Chemicals 10093.5V
Glycerokinase	Roche Diagnostics 127-159
L-glycerin-3-phosphate oxidase	Roche Diagnostics 775-797

Glycerol	BDH Chemicals 10118.6M
Glycine	BDH Chemicals 10119.CU
Guanidine (aminomethanamidine) hydrochloride	Sigma Chemicals G-4505
Heparin, sodium salt	Sigma chemicals H-3393
n-Hexane	BDH Chemicals 152496G
High molecular weight standard electrophoresis calibration kit	Amersham Pharmacia 17-0445-01
20% Homogenous polyacrylamide gel	Amersham Pharmacia 17-0624-01
Hydrochloric acid	BDH Chemicals 103078R
Iodine	Sigma Chemicals I-3380
β-Linoleoyl-γ-palmitoly-L-α-phosphatidylcholine (PLPC)	Avanti Polar Lipids 850458
Lipase	Sigma Chemicals L-9518
Low molecular weight standard electrophoresis calibration kit	Amersham Pharmacia 17-0446-01
Magnesium sulphate	BDH Chemicals 10151
Manganous chloride	Ajax Chemicals D3247
β-Mercaptoethanol	Merck Chemicals 805740
Methanol	BDH Chemicals 10158.BG
NEFA kit	Wako Pure Chemicals 279-75401
β-Oleoyl-γ-palmitoly-L-α-phosphatidylcholine (POPC)	Avanti Polar Lipids 850457
Palmitic acid	Sigma Chemicals P-9767
Perchloric acid	BDH Chemicals 101754W

Peroxidase	Roche Diagnostics 413-470
Phenol	Sigma Chemicals P-5566
Phospholipase D	Sigma Chemicals P-8023
Polyoxyethylene-9-lauryl ether	Sigma Chemicals P-9641
Potassium bromide	BDH Chemicals 101954F
Potassium ferrocyanide	Sigma Chemicals P-9387
Scintillation cocktail fluid	Beckman Coulter 141349
SDS buffer strips	Amersham Pharmacia 17-0516-01
Silver nitrate	Ajax Chemicals 449
Sodium acetate	Sigma Chemicals S-8625
Sodium azide	Sigma Chemicals S-2002
Sodium bromide	BDH Chemicals 301164S
Sodium carbonate	BDH Chemicals 10240.4H
Sodium dihydrogen orthophosphate	BDH Chemicals 10248
Sodium dodecyl sulphate (SDS)	BDH Chemicals 442442F
di-Sodium hydrogen orthophosphate	BDH Chemicals 30158.4L
Sodium hydroxide	BDH Chemicals 10242.4X
Sucrose	BDH Chemicals 10274.4B
5-Sulphosalicylic acid	BDH Chemicals 103464A
Total cholesterol reagent kit	Roche Diagnostics 2016630
Triolein	Sigma Chemicals T-7140
[^{3}H]Triolein	NEN 215817
Tris (hydroxymethyl) aminomethane	Sigma Chemicals T-1378
Triton X-100	Merck Chemicals 30632
Tween 20	Bio-Rad 170-653

Urea	BDH Chemicals 10290.BG

CHAPTER 3

HYDROLYSIS OF PHOSPHOLIPIDS IN SPHERICAL (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL AND (PDPC)rHDL BY HL AND EL.

3.1 INTRODUCTION

3.2 METHODS

3.3 RESULTS

Physical properties of spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.

Kinetics of HL-mediated phospholipid hydrolysis in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.

Kinetics of EL-mediated phospholipid hydrolysis spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.

3.4 DISCUSSION

3.1 INTRODUCTION

HL plays an important role in the metabolism and regulation of lipoproteins. It functions to (i) catalyse the hydrolysis of phospholipids and triglycerides in apoB-containing lipoproteins to produce LDL and apoB-containing remnants, (ii) promote the receptor-mediated cellular uptake of LDL, (iii) hydrolyse HDL phospholipids and triglycerides to produce smaller HDL particles {Jackson, 1983; Santamarina-Fojo et al., 1998; Thuren, 2000; Shirai *et al.*, 1981; van Tol *et al.*, 1980} and facilitate the uptake of HDL triglyceride and CE by the liver {Kadowaki *et al.*, 1992; Bamberger *et al.*, 1983; Rinninger *et al.*, 1998; Marques-Vidal *et al.*, 1994; Marques-Vidal *et al.*, 1990}. The latter process is accompanied by the dissociation of lipid-poor or lipid-free apoA-I from the HDL {Barrans *et al.*, 1994; Clay *et al.*, 1992}. The lipid-poor apoA-I promotes the cellular cholesterol efflux in the first step of RCT {Castro and Fielding, 1988; Fournier *et al.*, 1996}.

Although HL acts on all lipoproteins, HDL is its preferred substrate {Shirai *et al.*, 1981}. It has both triglyceride lipase and phospholipase activity {McCoy et al., 2002}. Hence, HL deficiency in humans and mice lacking the HL gene have high levels of HDL which are enriched with phospholipids and triglycerides {Homanics *et al.*, 1995; Lambert *et al.*, 2000; Hegele *et al.*, 1993}. On the other hand, overexpression of human HL in mice and rabbits results in decreases in HDL cholesterol levels as well as particle size {Barbagallo *et al.*, 1999; Busch *et al.*, 1994}.

EL is another enzyme which is involved in HDL remodeling. EL clearly interacts with HDL *in vivo*, because its overexpression in mice markedly decreases plasma HDL

levels {Jaye *et al.*, 1999}. This effect of EL on HDL concentration is due to its ability to hydrolyse HDL phospholipids {McCoy *et al.*, 2002}.

The aim of this chapter is to determine if HL and EL hydrolyse different HDL phospholipids. The ability of HL and EL to hydrolyse different HDL phospholipids was elucidated by using rHDL that were comparable in size, had similar lipid/apolipoprotein molar ratios, contained apoA-I as the only apolipoprotein and either, POPC, PLPC, PAPC or PDPC as the only phospholipid constituent. The results show clearly that HL and EL have different specificities for HDL phospholipid, with HL preferentially hydrolysing the phospholipids in (POPC)rHDL and (PLPC)rHDL and, EL preferentially hydrolysing the phospholipids in (PAPC)rHDL and (PDPC)rHDL.

3.2 METHODS

Preparation of spherical rHDL

The spherical rHDL were prepared as described in Chapter 2. Briefly, discoidal rHDL containing either POPC, PLPC, PAPC, or PDPC, UC and apoA-I were prepared by the cholate dialysis method {Matz and Jonas, 1982}. The discoidal rHDL were converted into spherical rHDL by incubation with LCAT and UC as described in detailed in chapter 2. The resulting spherical rHDL were isolated by sequential ultracentrifugation in the $1.07 < d < 1.21$ g/ml density range and dialysed against TBS (pH 7.4) containing, diethylenetriamine-pentaacetic acid (50 μmol/L) and BHT (final concentration 10 μmol/L). The spherical rHDL were stored under argon at 4 °C in the presence of Chelex 100 Resin until use.

Kinetic Studies

All incubations were carried out under N_2, in stoppered tubes, in a shaking water bath maintained at 37 °C. Varying concentrations of rHDL phospholipid (0.1-2.0 mmol/L for HL incubations and 0.1-1.0 mmol/L for EL) were mixed with BSA (final concentration 20 mg/mL), TBS and a constant amount of either HL (24 μL of a preparation that generated 32 nmol NEFA/mL HL/h) or EL (125 μL of a preparation that generated 60 nmol NEFA/mL EL/h). The final volume of the incubation mixtures that contained HL was 120 μL, and 250 μL for EL. When the incubations were complete the tubes were placed immediately on ice.

Phospholipid hydrolysis was determined by quantitating NEFA mass, assuming that one molecule of NEFA is produced for each molecule of phospholipid hydrolysed.

Kinetic parameters, V_{max} and $K_m(app)$, were derived from the line of best fit from linear regression analysis of a Lineweaver-Burk double reciprocal plot of the rate of phospholipid hydrolysis *versus* the concentration of rHDL phospholipid. V_{max} was determined as the reciprocal of the *y*-intercept, and $K_m(app)$ the product of the V_{max} and the slope. The catalytic efficiencies were determined as $V_{max}/K_m(app)$.

Other techniques

Phospholipid protein, NEFA, total cholesterol and unesterified cholesterol concentrations were quantitated as described in Chapter 2. CE concentrations were determined as the difference between unesterified cholesterol and total cholesterol concentrations. rHDL particle size was determine by 3-35% non-denaturing gradient gel electrophoresis.

3.2 RESULTS

Physical Properties of Spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (Fig. 3.2, Table 3.I) :

The preparations were comparable in terms of their CE/apoA-I and phospholipid/apoA-1 molar ratios (Table 3.I). The (POPC)rHDL, (PLPC)rHDL, and (PDPC)rHDL each contained a single, monodisperse population of particles with diameters ranging from 8.8 nm for the (POPC)rHDL to 9.2 nm for the (PDPC)rHDL (Fig. 3.1). Most of the (PAPC)rHDL were 9.1 nm in diameter. This preparation also contained a minor population of larger particles 10.3 nm in diameter.

Kinetics of HL-mediated Phospholipid Hydrolysis in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (Fig.3.2, Table 3.II):

Varying concentrations of (POPC)rHDL (closed circles), (PLPC)rHDL (closed triangles), (PAPC)rHDL (open circles) and (PDPC)rHDL (open triangles) (0.1–2.0 mmol/L phospholipid) were incubated at 37 ^{0}C for 1 hr with a constant amount of HL (Fig. 3.2). The mass of NEFA generated was measured directly and plotted as a function of rHDL phospholipid concentration (Fig. 3.2A).

Lineweaver-Burk double reciprocal plots of the kinetic data are shown in Fig. 3.2B. Kinetic parameters were derived from the plots and are shown in Table 3.II. These results are representative of 3 separate experiments. The V_{max} for (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL phospholipid hydrolysis was 0.769, 0.435, 0.115 and 0.176 μmol NEFA/mL HL/h, respectively. The respective $K_m(app)$ for (PAPC)rHDL and (PDPC)rHDL was 0.58 and 0.83 mmol/L phospholipid,

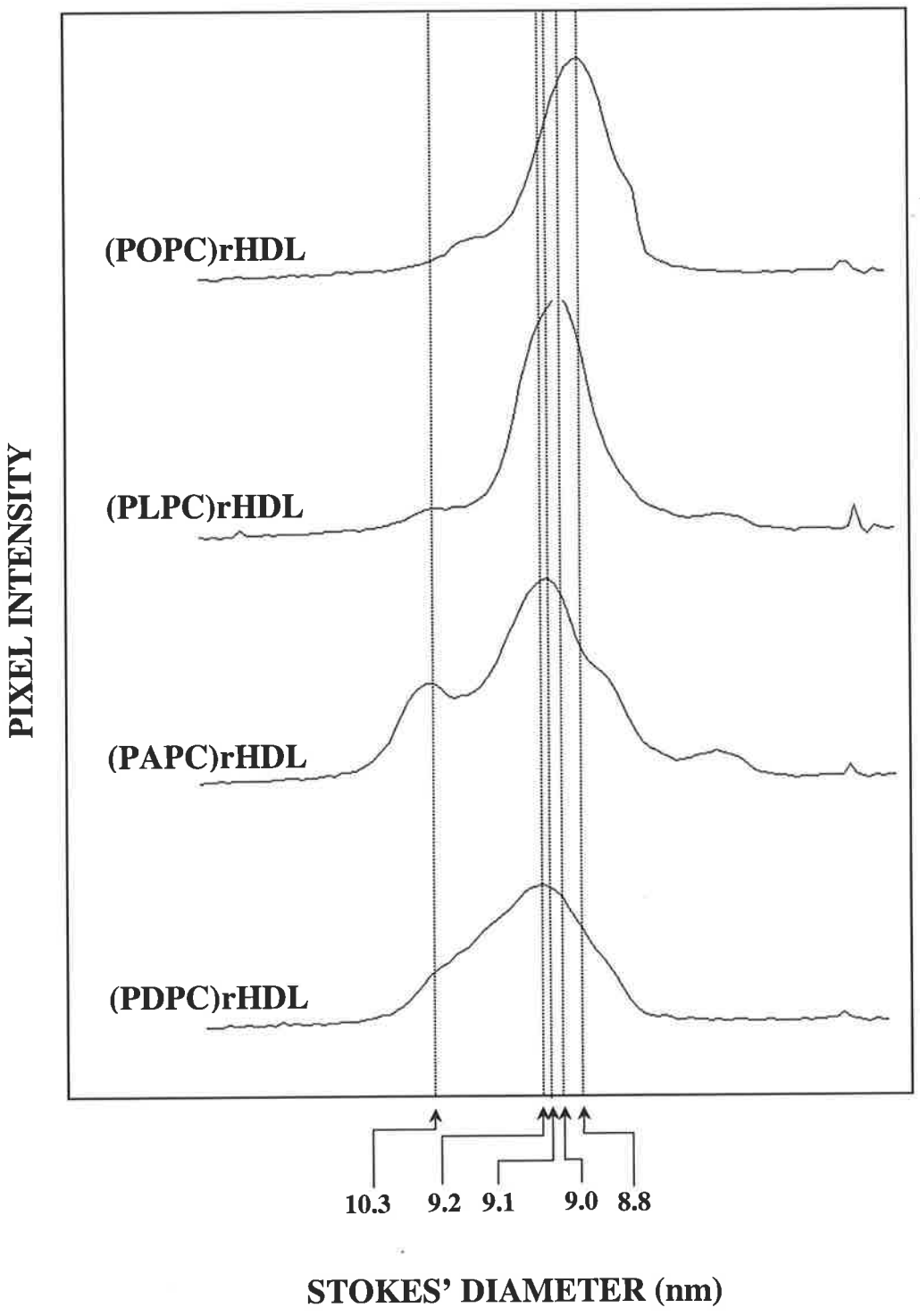


Figure 3.1. Size distribution of spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL. Spherical rHDL were subjected to non-denaturing 3-35% polyacrylamide gradient gel electrophoresis and stained with Coomassie Blue as described in Chapter 2.

Table 3.I. *Physical properties of spherical rHDL.*

Spherical rHDL	Stokes' Diameter[a]	Stoichiometry[b]			
		PL	UC	CE	A1
	nm	*mol/mol*			
(POPC)rHDL	8.8	25.2	0.8	19.2	1.0
(PLPC)rHDL	9.0	34.5	1.5	21.6	1.0
(PAPC)rHDL	9.1	34.6	3.1	20.2	1.0
(PDPC)rHDL	9.2	25.8	2.2	17.0	1.0

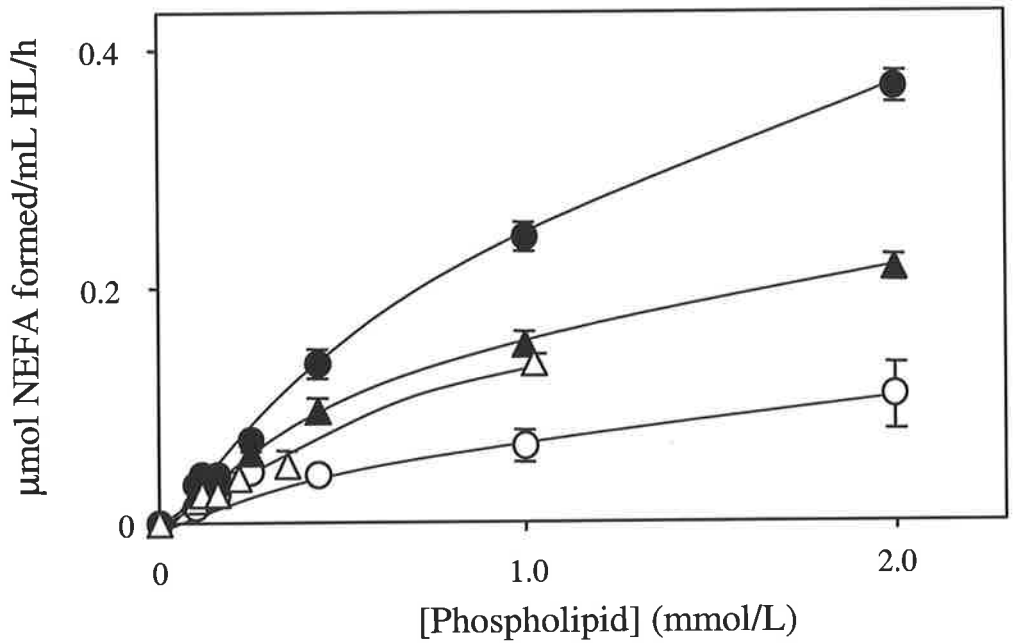
Spherical rHDL were prepared by adding LCAT and unesterified cholesterol to discoidal rHDL as described in Chapter 2. The spherical rHDL were isolated by ultracentrifugation and their composition was determined as described.

[a]Determined by nondenaturing gradient gel electrophoresis.

[b]PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; A-1, apolipoprotein A-I.

Fig. 3.2. Hydrolysis of phospholipids in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL by HL. Panel A shows the rate of HL-mediated phospholipid hydrolysis as a function of substrate concentration. Lineweaver-Burk double reciprocal plots of the data in Panel A are shown in Panel B. Varying concentrations of (POPC)rHDL (●), (PLPC)rHDL (▲), (PAPC)rHDL (○) and (PDPC)rHDL (△) (0.1-2.0 mmol/L phospholipid) were incubated, at 37 °C for 3 hr, with BSA (final concentration 20 mg/mL) and a constant amount of HL (24 μL of a preparation that generated 32 nmol NEFA/mL HL/h). The final incubation volume was 120 μL.

A.


0.4
0.2
0
µmol NEFA formed/mL HL/h
0
1.0
2.0
[Phospholipid] (mmol/L)

B.

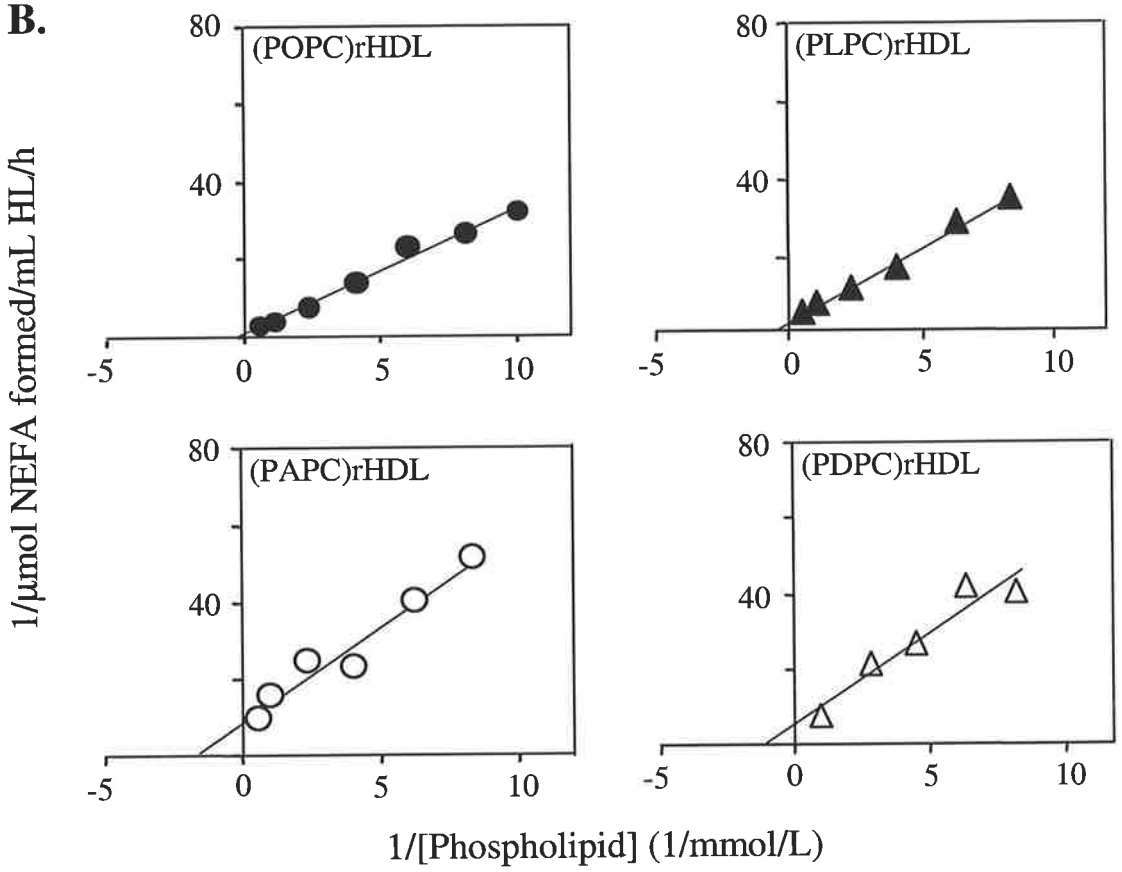

80
40
(POPC)rHDL
(PLPC)rHDL
(PAPC)rHDL
(PDPC)rHDL
-5
0
5
10
1/µmol NEFA formed/mL HL/h
1/[Phospholipid] (1/mmol/L)

Table 3.II. *Kinetic parameters for HL-mediated phospholipid hydrolysis in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.*

Spherical rHDL	V_{max}	$K_m(app)$	r^2	Catalytic efficiency
	μmol NEFA/mL HL/h	*mmol/L PL*		$V_{max}/K_m(app)$
(POPC)rHDL	0.769	2.40	0.978	0.32
(PLPC)rHDL	0.435	1.69	0.993	0.26
(PAPC)rHDL	0.115	0.58	0.952	0.20
(PDPC)rHDL	0.176	0.83	0.942	0.21

The kinetic parameters were calculated from the data in Fig. 3.2, using a Lineweaver-Burk double reciprocal plot of the rate of phospholipid hydrolysis as a function of rHDL concentration.

compared to 1.69 and 2.40 mmol/L phospholipid for (PLPC)rHDL and (POPC)rHDL. The catalytic efficiencies for phospholipid hydrolysis ranged from 0.32 for (POPC)rHDL to 0.20 for (PAPC)rHDL.

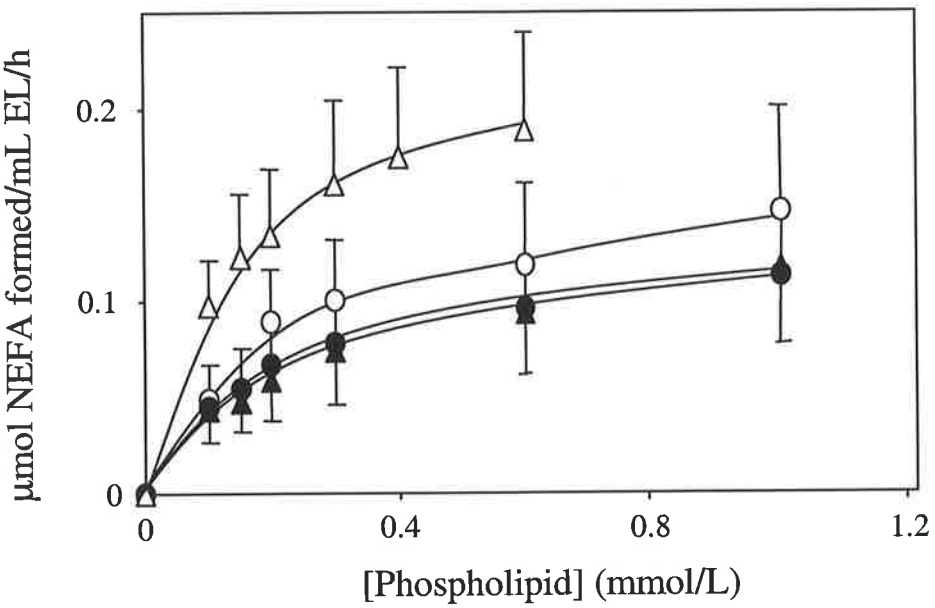
Kinetics of EL-mediated Phospholipid Hydrolysis in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (Fig. 3.3, Table 3.III):

Varying amounts of (POPC)rHDL (closed circles), (PLPC)rHDL (closed triangles), (PAPC)rHDL (open circles) and (PDPC)rHDL (open triangles) (0.01–1.0 mmol/L phospholipid) were incubated at 37 ^{0}C for 0.5 hr with a constant amount of EL. The mass of NEFA generated was measured directly and plotted as a function of rHDL concentration (Fig. 3.3A).

Kinetic parameters were determined from the Lineweaver-Burk double reciprocal plots of the data in Fig. 3.3A (Fig. 3.3B). The V_{max} for (PDPC)rHDL and (PAPC)rHDL were 0.233 and 0.192, respectively, compared with 0.128 and 0.127 μmol NEFA formed/mL EL/h for the (POPC)rHDL and (PLPC)rHDL. The respective $K_{m}(app)$ values for (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL were 0.189, 0.215, 0.309, and 0.139 mmol/L phospholipid. The catalytic efficiency, $V_{max}/K_{m}(app)$, for the phospholipid hydrolysis was greater for (PDPC)rHDL than for any of the other rHDL.

Fig. 3.3. Hydrolysis of phospholipids in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL by EL. Panel A shows the rate of EL-mediated phospholipid hydrolysis as a function of substrate concentration. Lineweaver-Burk double reciprocal plots of the data in Panel A are shown in Panel B. Varying concentrations of (POPC)rHDL (●), (PLPC)rHDL (▲), (PAPC)rHDL (○) and (PDPC)rHDL (△) (0.1-1.0 mmol/L phospholipid) were incubated, at 37 °C for 0.5 hr, with BSA (final concentration 20 mg/mL) and a constant amount of EL (125 μL of a preparation that generated 60 nmol/ml EL/h). The final incubation volume was 250 μL.

A.


µmol NEFA formed/mL EL/h
0.2
0.1
0
0
0.4
0.8
1.2
[Phospholipid] (mmol/L)

B.

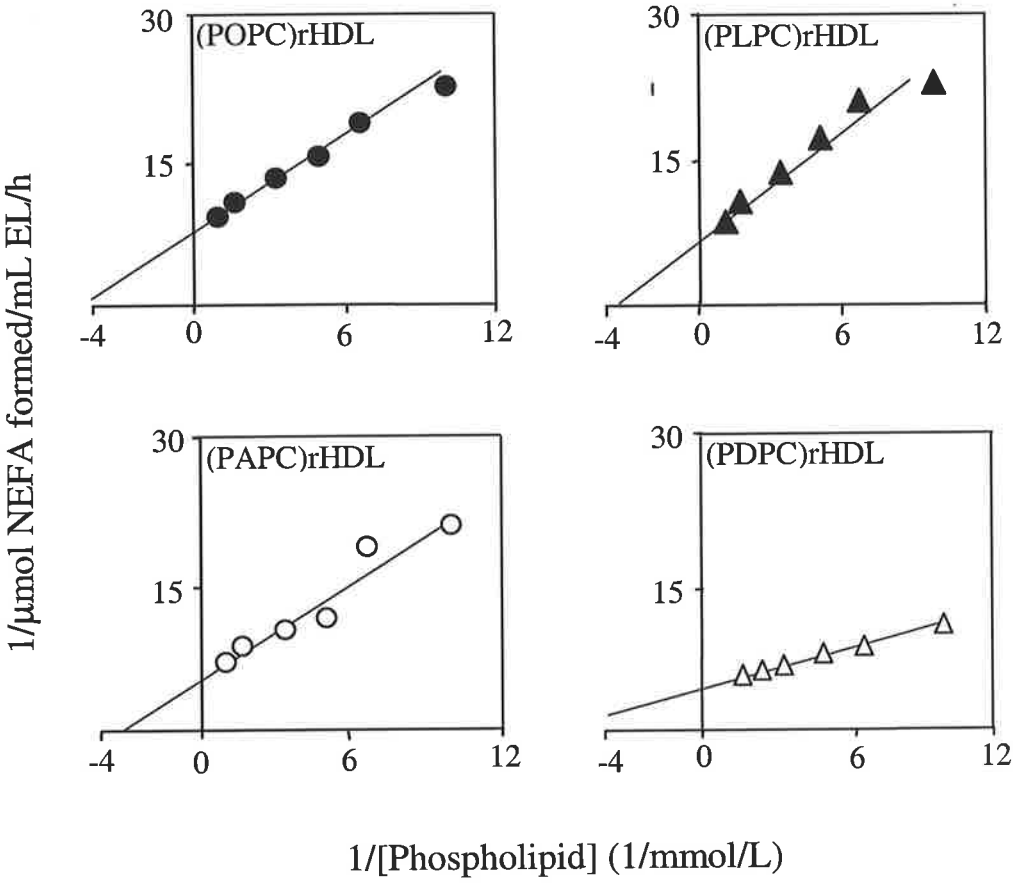

1/µmol NEFA formed/mL EL/h
(POPC)rHDL
(PLPC)rHDL
(PAPC)rHDL
(PDPC)rHDL
30
15
-4
0
6
12
1/[Phospholipid] (1/mmol/L)

Table 3.III. *Kinetic parameters for EL-mediated phospholipid hydrolysis in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.*

Spherical rHDL	V_{max}	$K_m(app)$	r^2	Catalytic efficiency
	μmol NEFA/mL EL/h	*mmol/L PL*		$V_{max}/K_m(app)$
(POPC)rHDL	0.128	0.189	0.991	0.68
(PLPC)rHDL	0.127	0.215	0.958	0.59
(PAPC)rHDL	0.192	0.309	0.926	0.62
(PDPC)rHDL	0.233	0.139	0.996	1.68

The kinetic parameters were calculated from the data in Fig. 3.3, using a Lineweaver-Burk double reciprocal plot of the rate of phospholipid hydrolysis as a function of rHDL concentration.

3.3 DISCUSSION

HL and EL are both members of the triglyceride lipase gene family. Both enzymes hydrolyse HDL phospholipid *sn*-1 acyl ester bonds and decrease HDL levels *in vivo* {Choi *et al.*, 2002; Jin *et al.*, 2002; Jaye *et al.*, 1999; Busch *et al.*, 1994; Barbagallo *et al.*, 1998}.

The main aim of this study was to determine if HL and EL have different substrate specificities for HDL phospholipids. This issue was addressed by using well-defined, homogenous preparations of spherical rHDL that were comparable in size, contained apoA-I as the only apolipoprotein, CE as the only core lipid and either POPC, PLPC, PAPC or PDPC as the sole phospholipid {Rye *et al.*, 2002}.

As the rHDL did not contain triglycerides in their core, only phospholipids were hydrolysed during the incubations. The results showed that HL hydrolyses the phospholipids in (POPC)rHDL and (PLPC)rHDL more rapidly than the phospholipids in (PAPC)rHDL and (PDPC)rHDL (Fig. 3.2). EL hydrolyses the phospholipids in (PAPC)rHDL and (PDPC)rHDL more rapidly than in (POPC)rHDL and (PLPC)rHDL (Fig. 3.3).

Other investigators have also reported that HL hydrolyses the phospholipids in (POPC)rHDL more rapidly than the phospholipids in (PAPC)rHDL {Tansey *et al.*, 1997}. However, the rHDL that were used in those studies were smaller and had a much lower CE/apoA-I molar ratio than the rHDL that were used in the current study. As there is evidence that particle size is an important determinant of HL-mediated phospholipid hydrolysis in HDL, these results do not give an unequivocal indication of

the role of phospholipid composition in HDL interactions {Shirai *et al.*, 1981}. These investigators also only compared phospholipid hydrolysis in (POPC)rHDL and (PAPC)rHDL. PLPC, on the other hand, which is the most abundant phosphatidylcholine species in HDL (and therefore more physiologically relevant) was not studied {Subbaiah and Monshizadegan, 1988}.

The results in this chapter were somewhat unexpected given that the phospholipids in the rHDL preparations all contained palmitic acid in the *sn*-1 position and varied only in the length and unsaturation of their *sn*-2 acyl chains. This observation can be explained in two ways. One possibility is that the rHDL phospholipid *sn*-1 acyl chains access the active sites of HL and EL differently.

It is well recognised that the active sites of most members of the triglyceride lipase gene family are covered by a lid-like structure, which in LPL and HL, and probably EL, consists of two amphipathic helices separated by a 4-residue β-turn {Dugi *et al.*, 1992}. The lid determines the specificities of the enzymes by regulating access of the substrate to the active site {Dugi *et al.*, 1992; Dugi *et al.*, 1995; Kobayashi *et al.*, 1996}. It is also well documented that minor changes in either the lipid binding properties, size, polarity or charge of the lid can have dramatic effects on substrate specificity {Dugi *et al.*, 1992; Dugi *et al.*, 1995; Henderson *et al.*, 1993}.

At neutral pH, the lid of EL contains two negatively and one positively charged residues while, HL has one negatively and five positively charged residues. This difference in net charge may be instrumental in determining why HL and EL hydrolyse different rHDL phospholipids at different rates. When either EL or HL binds to rHDL, their lids

open and partition into the particle surface, thus exposing the catalytic site {van Tilbeurgh *et al.*, 1994}. Given that the phospholipid headgroups tilt away from a negatively charged lipid interface {Scherer and Seelig, 1989}, it follows that the rHDL phospholipid headgroups will tilt further from the particle interface when the lid of EL opens than when the HL lid opens. This may be associated with an enhanced translocation of the phospholipid *sn*-1 acyl chains towards the particle surface. The translocation may enable *sn*-1 acyl chains to access the active site of EL more easily than the active site of HL. Furthermore, as the motion and disorder of phospholipid acyl chains increases with increasing unsaturation {Deckelbaum *et al.*, 1990}, it follows that the palmitic acid in PAPC and PDPC may be able to enter the active site of EL more readily than the palmitic acid in POPC and PLPC.

Another explanation for the different substrate specificities of HL and EL for HDL phospholipids is that HL and EL interact differently with the rHDL surface. This possibility is investigated in Chapter 4 by enriching (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL with triolein and studying the kinetics of triolein hydrolysis in these preparations.

CHAPTER 4

HYDROLYSIS OF TRIGLYCERIDES IN SPHERICAL (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL AND (PDPC)rHDL BY HL AND EL.

4.1 INTRODUCTION

4.2 METHODS

4.3 RESULTS

Physical properties of spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL.

Kinetics of HL-mediated triglyceride hydrolysis in spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL.

Kinetics of EL-mediated triglyceride hydrolysis in spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL.

4.4 DISCUSSION

4.1 INTRODUCTION

HL and EL vary widely in their abilities to hydrolyse triglycerides, with HL having a much higher activity towards HDL triglycerides than EL {McCoy *et al.*, 2002; Rader and Jaye, 2000}. HL also has higher triglyceride lipase activity than phospholipase activity {Wilcox *et al.*, 1991; Simard *et al.*, 1989; McCoy *et al.*, 2002}. On the other hand, while EL is primary considered as a phospholipase, it has low, but detectable triglyceride lipase activity {McCoy *et al.*, 2002}.

The aim of this chapter is to determine if phospholipids regulate the interactions of HL and EL with the surface of rHDL. This is an issue of considerable significance given that HDL phospholipid composition changes according to diet {Sola *et al.*, 1990} and that the ability of HL to hydrolyse HDL triglycerides is associated with the dissociation of lipid-poor apoA-I from the particles {Barrans *et al.*, 1994; Clay *et al.*, 1992}. The lipid-poor or lipid-free apoA-I that is generated during the HL-mediated remodeling of HDL is important in the formation of nascent, discoidal HDL and promoting cellular cholesterol efflux {Clay *et al.*, 1992; Clay *et al.*, 1996; Castro and Fielding, 1988; Fournier *et al.*, 1996}.

To determine if phospholipids regulate the interactions of HL and EL with the rHDL surface, (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL were enriched with [^{3}H]triolein (TO) and the kinetics of TO hydrolysis was determined. This was achieved by measuring the release of [^{3}H]NEFA into the incubation mixture. The results showed that HDL phospholipid composition affects the interaction of EL, but not HL, with the rHDL interface.

4.2 METHODS

Preparation of spherical rHDL

Spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL containing CE as the only core lipid and apoA-I as the sole apolipoprotein constituent, were prepared as described in Chapter 2.

Preparation of POPC/TO, PLPC/TO, PAPC/TO and PDPC/TO microemulsions

Microemulsions, containing triolein (TO) and either POPC, PLPC, PAPC or PDPC and were prepared {Martins *et al.*, 1989}. Each phospholipid (37.5 mg) was mixed with TO (100 mg) and dissolved in chloroform:methanol (2:1 (v/v)). BHT (final concentration 0.12 mmol/L) was also added to the mixtures to inhibit phospholipid oxidation. The mixtures were placed under a stream of N_2 for 2 hrs, then maintained overnight under vacuum. Twelve mL of TBS (pH 7.4) containing 50 μmol/L DETAPAC, 0.006% NaN_3 (w/v) and 10 μmol/L BHT, was added to the lipids and the resulting solutions were sonicated as described {Martins *et al.*, 1989}. The density of the solutions was adjusted to 1.21 g/mL and they were placed into SW41 tubes. The microemulsions were overlayered with density solutions of 1.065, 1.02 and 1.006 g/mL. The microemulsions were then isolated by ultracentrifugation as the fraction of d < 1.21 g/mL. The phospholipid and TO concentrations of each microemulsion was determined.

Labelling of microemulsions with [^{3}H]TO

[^{3}H]TO (28 Ci/mmol) (NEN Life Science Products) (100 μL/mL microemulsion) was dried down under N_2, in a glass test tube, then resuspended in ethanol (200 μL). The [^{3}H]TO in ethanol was added to the POPC/TO, PLPC/TO, PAPC/TO and PDPC/TO microemulsions. The resulting mixtures were incubated, under N_2, at 37 °C for 3 hr.

The specific activity of the microemulsions was determined by adding 5 μL of each preparation to scintillation fluid (10 mL) and counting the samples on a Beckman LS6000 TA liquid scintillation counter. The respective specific activities for the [^{3}H]-POPC/TO, [^{3}H]-PLPC/TO, [^{3}H]-PAPC/TO and [^{3}H]-PDPC/TO microemulsions were $1.9x10^6$, $2.2x10^6$, $2.1x10^6$ and $1.8x10^6$ cpm/mg TO.

Preparation of [^{3}H]TO-labelled spherical rHDL

Spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL were labelled with [^{3}H]TO (28 Ci/mmol) (NEN Life Science Products) as described {Hime *et al.*, 1998}. Briefly, the rHDL (final concentration 0.1 mmol/L CE) were individually incubated at 37 °C, under N_2, for 1 - 3 hr with CETP (final concentration 2.7 units of activity/mL) and the radiolabelled microemulsions (final concentration 4.0 mmol/L TO). To ensure that the phospholipid composition of the rHDL did not change during the incubation, the phospholipids in the microemulsions were matched to those of the rHDL. The resulting [^{3}H]TO-enriched rHDL were isolated by sequential ultracentrifugation in the $1.063 < d < 1.21$ g/mL density range. Enrichment with TO did not affect rHDL size significantly. The [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL were respectively concentrated 7-, 4-, 4.5- and 5-fold before use. Their specific activities were $4.9x10^6$, $2.4x10^6$, $1.6x10^6$, $4.3x10^6$ cpm/mg TO.

Quantitation of HL- and EL-mediated [^{3}H]TO Hydrolysis

TO hydrolysis was quantitated by extracting the rHDL lipids as described by Folch *et al.* (1957) {Folch *et al.*, 1957}. One mL of chloroform:methanol (2:1 (v/v)) was added to each incubation mixture with vortexing, followed by TBS (210 μL). This generated

two phases. The upper, aqueous, phase was removed. Chloroform:methanol:water (3:48:47 (v/v/v)) (500 μL) was added to the organic phase with vortexing and the aqueous phase was again removed. The addition of chloroform:methanol:water was repeated. When the aqueous phase was removed for the final time, methanol (200-300 μL) was added to return the solution to a single phase. The samples were dried down under N_2 at 40 °C. The lipids were taken up into chloroform:methanol (2:1 (v/v)) (50 μL) and spotted onto silica TLC plates. NEFA and TO standards were also applied to the TLC plates. The NEFA were separated from the other lipids by running the plates in *n*-hexane:diethyl ether:acetic acid (70:30:1.6 (v/v/v)). The plates were dried and the lipids were visualised with I_2. The areas corresponding to TO and NEFA were cut out and placed into scintillation fluid (5 mL). The radioactivity of the samples was determined using a Beckman LS 6000TA liquid scintillation counter.

Kinetic parameters, V_{max} and K_m*(app)*, were derived from the line of best fit from linear regression analysis of a Lineweaver-Burk double reciprocal plot of the rate of phospholipid, or triglyceride, hydrolysis *versus* the concentration of rHDL phospholipid or triglyceride. V_{max} was determined as the reciprocal of the *y*-intercept, and K_m*(app)* the product of the V_{max} and the slope. The catalytic efficiencies were determined as V_{max}/K_m*(app)*.

Other techniques

Phospholipid protein, triglyceride, NEFA, total cholesterol and unesterified cholesterol concentrations were quantitated as described in Chapter 2. CE concentrations were determined as the difference between unesterified cholesterol and total cholesterol

concentrations. rHDL particle size was determine by non-denaturing gradient gel (3-35%) electrophoresis.

4.3 RESULTS

Physical Properties of Spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL (Fig. 4.1, Table 4.I) :

The diameters of the [^{3}H]TO-enriched rHDL ranged from 9.5 nm for the [^{3}H]TO-(POPC)rHDL to 10.0 nm for the [^{3}H]TO-(PLPC)rHDL (Fig. 4.1). The [^{3}H]TO-enriched rHDL were slightly larger than the original rHDL that contained only CE in their core (Fig. 3.1, Table 3.II). This is consistent with what has been reported previously {Hime *et al.*, 1998}. The increase in the phospholipid/apoA-I molar ratios of the TO-enriched rHDL, relative to the original rHDL, reflects the spontaneous transfer of phospholipids from the microemulsions to the rHDL {Rye *et al.*, 2002} (Table 4.I). The triglyceride/apoA-I molar ratio was lower for the [^{3}H]TO-(POPC)rHDL than for the other rHDL preparations. This was a consequence of the relatively slow CETP-mediated transfer of TO from the POPC/TO-microemulsions into the (POPC)rHDL {Rye *et al.*, 2002}.

Kinetics of HL-mediated Triglyceride Hydrolysis in [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL (Fig. 4.2, Table 4.II):

In this study, [^{3}H]TO-enriched rHDL (50–600 μmol/L triglyceride) were incubated at 37 °C for 2 hr with a constant amount of HL. HL-mediated triglyceride hydrolysis in [^{3}H]TO-(POPC)rHDL (closed circles), [^{3}H]TO-(PLPC)rHDL (closed triangles), [^{3}H]TO-(PAPC)rHDL (open circles) and [^{3}H]TO-(PDPC)rHDL (open triangles), is shown in Fig. 4.2A. The rate of HL-mediated triglyceride hydrolysis was similar for all the rHDL preparations. Double reciprocal plots of the data in Fig. 4.2A are shown

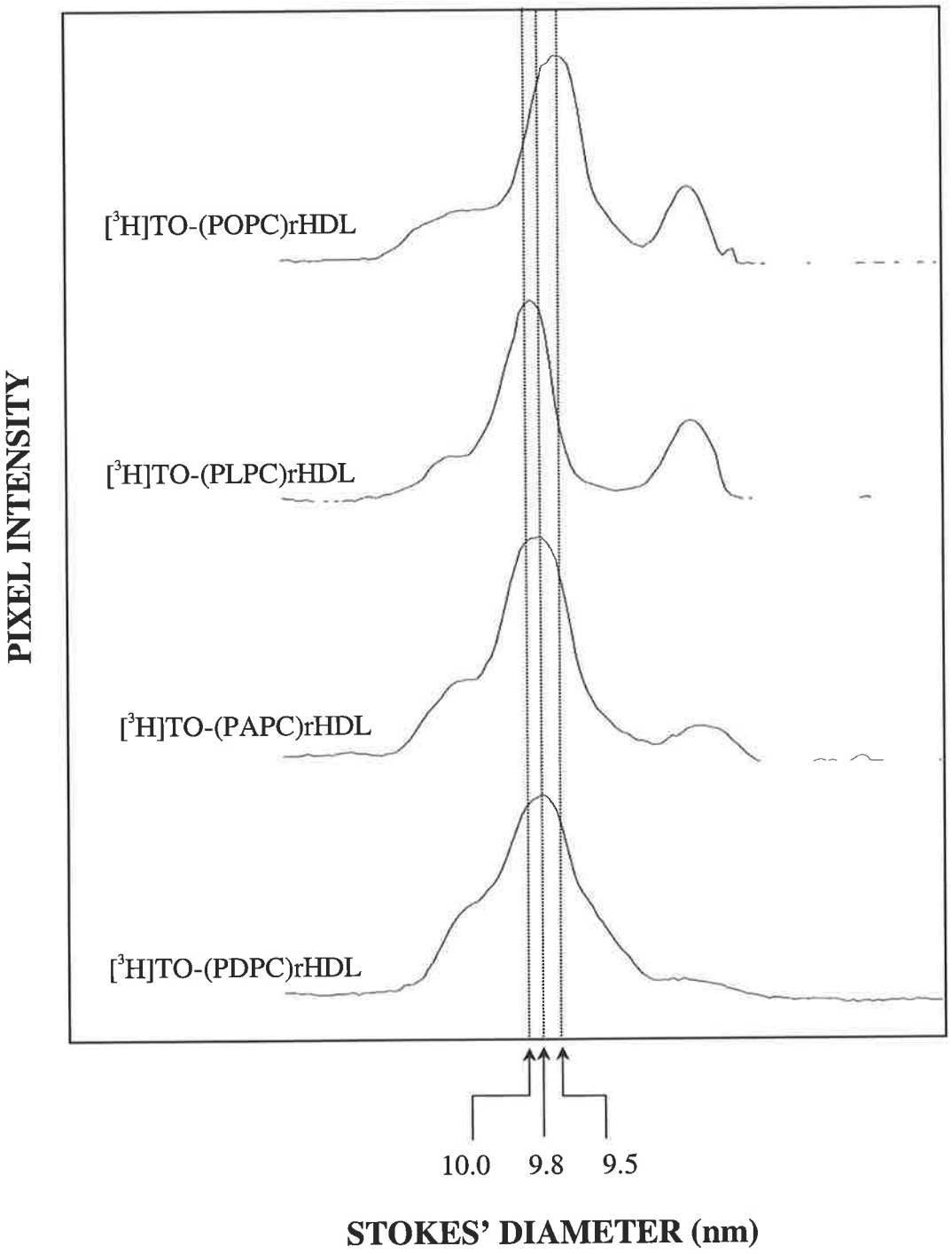


Figure 4.1. Size distribution of triglyceride-enriched spherical rHDL. The spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and[^{3}H]TO-(PDPC)rHDL were subjected to non-denaturing 3-35% polyacrylamide gradient gel electrophoresis and Coomassie Blue staining as described in Chapter 2. Laser densitometric scans of the gels are shown.

Table 4.I. *Physical properties of spherical rHDL.*

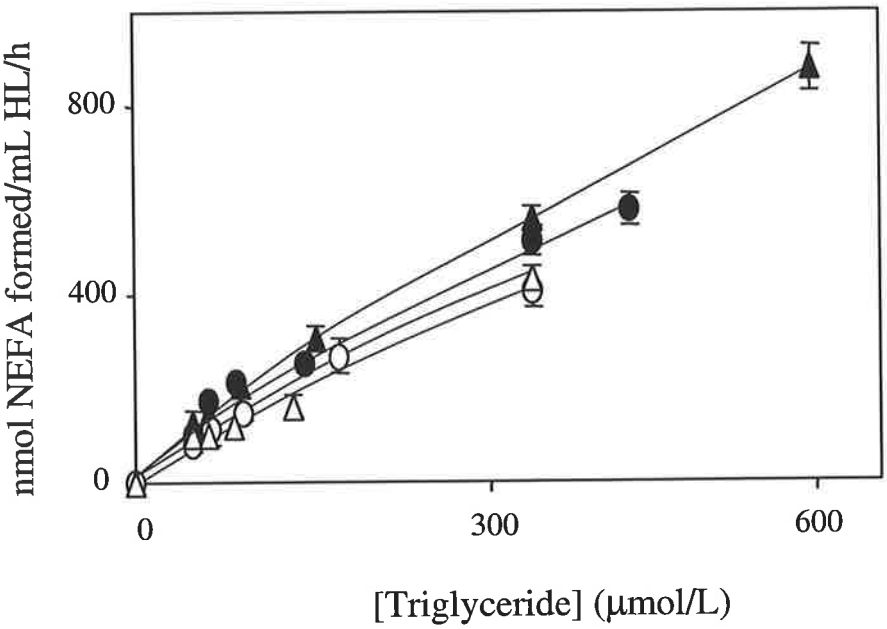
Spherical rHDL	Stokes' Diameter[a]	Stoichiometry[b]				
		PL	UC	CE	TG	A1
	nm	*mol/mol*				
[^{3}H]TO-(POPC)rHDL	9.5	51.7	0.9	19.4	5.8	1.0
[^{3}H]TO-(PLPC)rHDL	10.0	50.8	1.5	16.1	9.3	1.0
[^{3}H]TO-(PAPC)rHDL	9.8	53.1	0.9	16.8	7.2	1.0
[^{3}H]TO-(PDPC)rHDL	9.8	47.3	1.3	14.7	7.0	1.0

Spherical rHDL were prepared by adding LCAT and unesterified cholesterol to discoidal rHDL as described in Chapter 2. The spherical rHDL were isolated by ultracentrifugation and their composition was determined as described. The [^{3}H]TO-rHDL were prepared by incubating the rHDL (final concentration 0.1 mmol/L CE) with [^{3}H]TO-labelled microemulsions (final concentration 4.0 mmol/L TG) and CETP (final activity 2.7 U/mL). The final incubation mixture volumes for [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL was 27.5, 30.6, 28.8 and 23.9 mL, respectively.

[a]Determined by nondenaturing gradient gel electrophoresis.
[b]PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; A-1, apolipoprotein A-I; TG, triglycerides.

Fig. 4.2. Hydrolysis of triolein in [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL by HL. Panel A shows the rate of HL-mediated triglyceride hydrolysis as a function of substrate concentration. Lineweaver-Burk double reciprocal plots of the data in Panel A are shown in Panel B. Varying concentrations of [^{3}H]TO-(POPC)rHDL (●), [^{3}H]TO-(PLPC)rHDL (▲), [^{3}H]TO-(PAPC)rHDL (○) and [^{3}H]TO-(PDPC)rHDL (△) (50-600 μmol/L triglyceride) were incubated, at 37 °C for 2 hr, with BSA (final concentration 20 mg/mL) and a constant amount of HL (5 μL of a preparation that generated 122 nmol NEFA/mL HL/h). The final incubation volume was 50 μL. The values are the means of triplicate data points.

A.


nmol NEFA formed/mL HL/h
800
400
0
0
300
600
[Triglyceride] (μmol/L)

B.

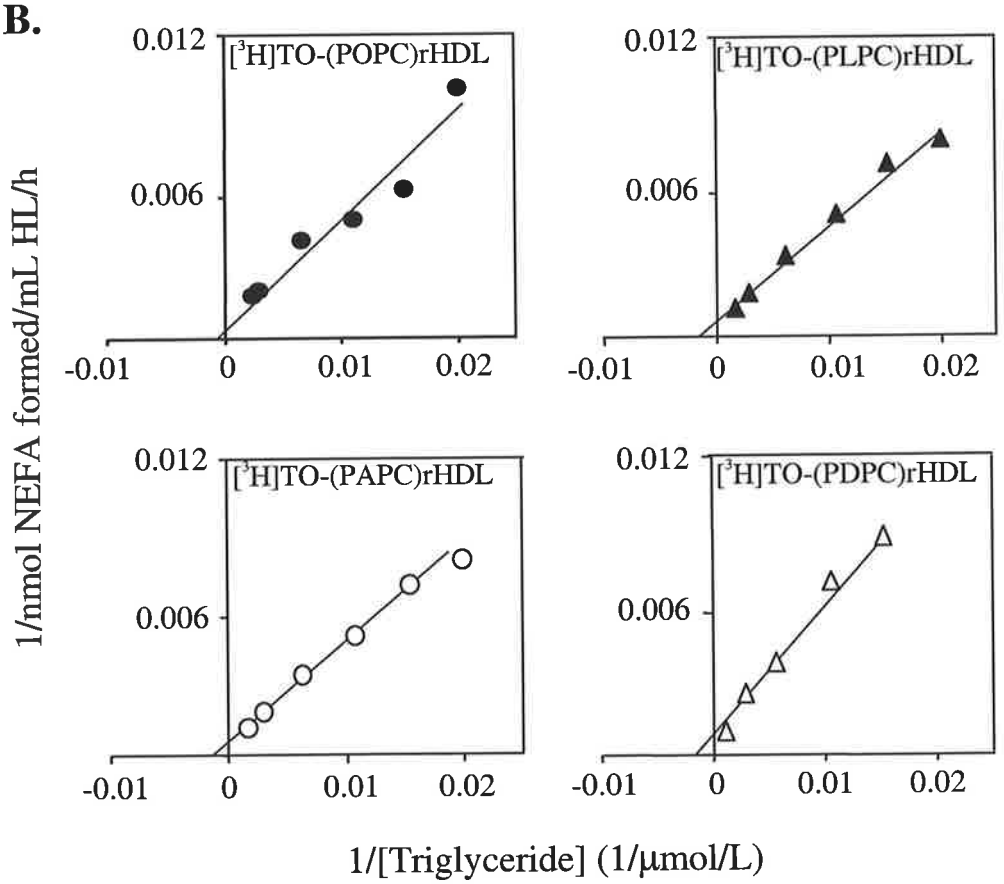

1/nmol NEFA formed/mL HL/h
[³H]TO-(POPC)rHDL
[³H]TO-(PLPC)rHDL
[³H]TO-(PAPC)rHDL
[³H]TO-(PDPC)rHDL
0.012
0.006
-0.01
0
0.01
0.02
1/[Triglyceride] (1/μmol/L)

Table 4.II. *Kinetic parameters for HL-mediated triglyceride hydrolysis in spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL.*

Spherical rHDL	**V_{max}**	**$K_m(app)$**	**r^2**	**Catalytic efficiency**
	nmol NEFA/mL HL/h	*μmol/L TO*		*$V_{max}/K_m(app)$*
[^{3}H]TO-(POPC)rHDL	1000.0	588.4	0.938	1.70
[^{3}H]TO-(PLPC)rHDL	909.0	535.9	0.989	1.70
[^{3}H]TO-(PAPC)rHDL	1000.0	532.3	0.990	1.88
[^{3}H]TO-(PDPC)rHDL	833.3	508.6	0.966	1.64

The kinetic parameters were calculated from the data in Fig. 4.2, using a Lineweaver-Burk double reciprocal plot of the rate of triglyceride hydrolysis as a function of rHDL concentration.

in Fig. 4.2B. The kinetic parameters and catalytic efficiencies were comparable for all of the rHDL (Table 4.II).

Kinetics of EL-mediated Triglyceride Hydrolysis in [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL (Fig. 4.3 and Table 4.III):

For this study [^{3}H]TO-(POPC)rHDL (closed circles), [^{3}H]TO-(PLPC)rHDL (closed triangles), [^{3}H]TO-(PAPC)rHDL (open circles) and [^{3}H]TO-(PDPC)rHDL (open triangles), (6–40 μmol/L triglyceride) were incubated at 37 ^{0}C for 1 hr with a constant amount of EL (Fig. 4.3A). In all cases, the rate of EL-mediated triglyceride hydrolysis was much less than what was observed for HL-mediated triglyceride hydrolysis (Fig. 4.2A).

Kinetic parameters for triglyceride hydrolysis were derived from the double reciprocal plots of the data in Fig, 4.3A. The results are shown in Fig. 4.3B and Table 4.III. The V_{max} for [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL was 32.3 and 16.7 nmol NEFA/mL EL/h respectively, compared to 6.8 and 3.8 nmol NEFA/mL EL/h for [^{3}H]TO-(PLPC)rHDL and [^{3}H]TO-(POPC)rHDL. The $K_m(app)$ for [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL was 41.5 and 20.8 μmol/L triglyceride, compared to 4.2 and 3.9 μmol/L triglyceride for [^{3}H]TO-(PLPC)rHDL and [^{3}H]TO-(POPC)rHDL. The catalytic efficiency of EL-mediated triglyceride hydrolysis ranged from 1.62 for [^{3}H]TO-(PLPC)rHDL to 0.78 for [^{3}H]TO-(PAPC)rHDL.

Fig. 4.3. Hydrolysis of triolein [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL by EL. Panel A shows the rate of EL-mediated triglyceride hydrolysis as a function of substrate concentration. Lineweaver-Burk double reciprocal plots of the data in Panel A are shown in Panel B. Varying concentrations of [^{3}H]TO-(POPC)rHDL (●), [^{3}H]TO-(PLPC)rHDL (▲), [^{3}H]TO-(PAPC)rHDL (○) and [^{3}H]TO-(PDPC)rHDL (△) (6-40 μmol/L triglyceride) were incubated, at 37 °C for 1 hr, with BSA (final concentration 20 mg/mL) and a constant amount of EL (20 μL of a preparation that generated 50 nmol NEFA/mL EL/h). The final incubation volume was 50 μL. The values are the means of triplicate data points.

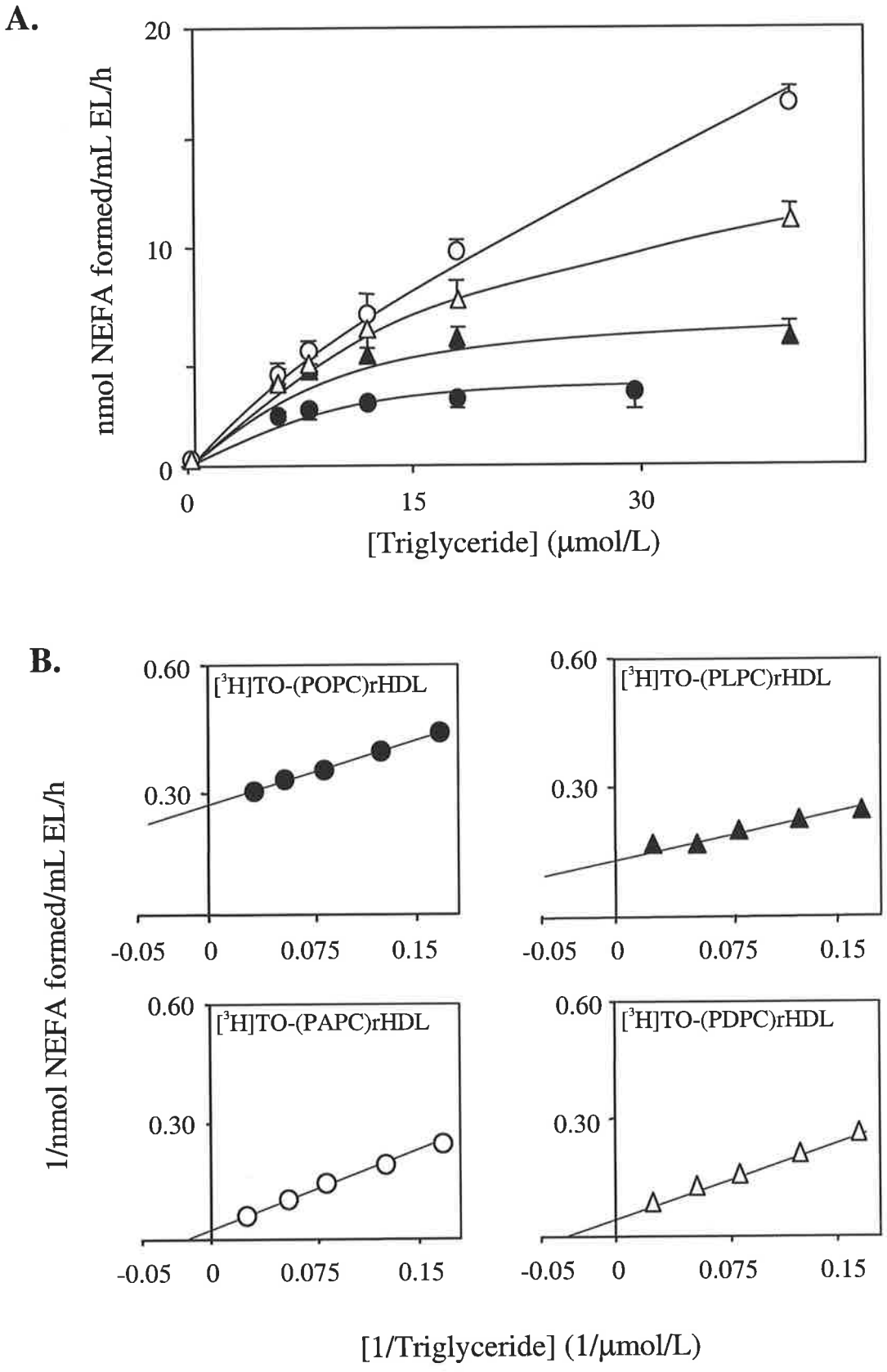

A.
nmol NEFA formed/mL EL/h
20
10
0
0
15
30
[Triglyceride] (μmol/L)
B.
1/nmol NEFA formed/mL EL/h
[³H]TO-(POPC)rHDL
[³H]TO-(PLPC)rHDL
[³H]TO-(PAPC)rHDL
[³H]TO-(PDPC)rHDL
0.60
0.30
-0.05
0
0.075
0.15
[1/Triglyceride] (1/μmol/L)

Table 4.III. *Kinetic parameters for EL-mediated triglyceride hydrolysis in spherical [^{3}H]TO-(POPC)rHDL, [^{3}H]TO-(PLPC)rHDL, [^{3}H]TO-(PAPC)rHDL and [^{3}H]TO-(PDPC)rHDL.*

Spherical rHDL	**V_{max}**	**K_m(*app*)**	**r^2**	**Catalytic efficiency**
	nmol NEFA/mL EL/h	*μmol/L TO*		$V_{max}/K_m(app)$
[^{3}H]TO-(POPC)rHDL	3.8	3.9	0.998	0.97
[^{3}H]TO-(PLPC)rHDL	6.8	4.2	0.964	1.62
[^{3}H]TO-(PAPC)rHDL	32.3	41.5	0.998	0.78
[^{3}H]TO-(PDPC)rHDL	16.7	20.8	0.998	0.80

The kinetic parameters were calculated from the data in Fig. 4.3, using a Lineweaver-Burk double reciprocal plot of the rate of triglyceride hydrolysis as a function of rHDL concentration.

4.4 DISCUSSION

The aim of these studies was to determine how the phospholipid composition of rHDL affects the interactions of HL and EL with the surface of the particles. For these experiments increasing amounts of [^{3}H]TO-enriched rHDL were incubated with a constant amount of either EL or HL. As formation of radiolabelled NEFA was measured in these experiments, the results reflected TO, not phospholipid, hydrolysis. Moreover, as TO was the sole triglyceride constituent in all of the rHDL preparations, differences in TO hydrolysis must reflect differences in the interaction of the Hl and EL with the rHDL surface.

The results of these experiments showed that, while HL-mediated TO hydrolysis was comparable for all of the rHDL preparations, there was a greater than 10-fold variation in the V_{max} for EL-mediated triglyceride hydrolysis (Table 4.III). This result indicates that phospholipids regulate the interaction of EL, but not HL, with the rHDL surface.

The reasons for this difference may reflect differences in the lipid binding domains of HL and EL. The initial contact between a lipolytic enzyme and its substrate occurs at a lipid binding site, which is distinct from its active site {Komaromy *et al.*, 1991}. In the case of LPL, residues 415-438 in the C-terminal region of the protein have been identified as a putative lipid binding domain {Keiper *et al.*, 2001}. As this site mediates substrate recognition, and ultimately changes the conformation surrounding the active site of the enzyme {Keiper *et al.*, 2001}, it follows that variations in the sequence of the lipid binding sites may change the recognition of the enzyme for the substrate, as well as enzyme-substrate interactions. As only 5 out of 24 residues in this region are conserved in HL and EL, it is not surprising that their interactions with lipid-water interfaces differ.

The other important finding to emerge from this study is that EL hydrolyses triglycerides very poorly compared to HL. This result, which is in agreement with what has been reported previously {McCoy *et al.*, 2002}, may be due to the different sizes of the HL and EL lids. In general, the ability of lipolytic enzymes to hydrolyse triglycerides decreases as the number of residues in the lid decreases. For example, human pancreatic lipase (hPL) has a 23 amino acid lid and hydrolyses only triglycerides. HL and LPL have 22 residues in their lids and exhibit both triglyceride lipase and phospholipase activities. EL, which has 19 residues in its lid, has high phospholipase and low triglyceride lipase activity {Jaye *et al.*, 1999}. Phosphatidylserine-phospholipase A_1 with its 11 amino acid lid, has selective phospholipase but no detectable triglyceride lipase activity {Sato *et al.*, 1997}. However, studies on chimeric lipases of hPL, which has high triglyceride lipase activity, and guinea pig pancreatic lipase-related protein 2 (GPLRP2) show that the lid size alone does not regulate triglyceride lipase activity {Carriere *et al.*, 1997}. GPLRP2 which has a lid consisting of only 5 residues, has relatively low triglyceride lipase activity compared to hPL. While, the ability of hPL to hydrolyse triolein was decreased 10-fold in a hPL/GPLRP2-lid chimera, the hPL-lid/GPLRP2 chimera also exhibited lower triglyceride lipase activity compared to unmodified GPLRP2.

The shape and charge of the active sites of the enzymes are also likely to influence HL- and EL-mediated triglyceride hydrolysis. For optimal hydrolysis, the shape of the catalytic site should match that of the acyl chains of the substrate {Pleiss *et al.*, 1998}. Extra space in the catalytic site results in a sub-optimal fit of the substrate and reduces hydrolysis {Pleiss *et al.*, 1998}. For example, site-directed mutagenesis of Val^{209} in the fungal lipase, *Rhizopus delemar*, for the smaller Gly, reduces triglyceride hydrolysis

{Joerger and Haas, 1994}. Conversely, if the active site is too small, access of the substrate is limited by steric hindrance. Thus the replacement of Val^{209} by the larger Trp in *Rhizopus delemar*, markedly decreases the ability of the enzyme to hydrolyse triolein {Joerger and Haas, 1994}. Similarly, altering the charge in the active sites of lipases, such as the substitution of Phe^{112} in *Rhizopus delemar* for a polar Gln essentially abolishes the enzyme's catalytic activity {Klein *et al.*, 1997}. Little is known about the actual shape and charge of the catalytic sites of HL and EL. However, on the basis of the present results, and the above observations, the lower triglyceride lipase activity of EL, compared to HL, suggests that EL has a larger and more charged active site than HL.

The differences in HL- and EL-mediated TO hydrolysis may also be due to variations in the amphipathicity and charge of their lids. The lid of EL extends from residue 234 to 254, while the HL lid spans residues 232 to 255 {Jaye *et al.*, 1999}. As the residues in the HL lid are more polar than in EL, it follows that the α helices in the HL lid are more amphipathic than those in EL {Segrest *et al.*, 1990}. Positively charged residues also increase the affinity of α helices for lipids {Segrest *et al.*, 1990}. The lid of HL has four more positively charged residues than EL. These charged residues are clustered at the polar interface of the α helices in a way that is characteristic of the lipid binding α helices that are found in apolipoproteins {Dugi *et al.*, 1992}. This is also consistent with the lid of HL being more amphipathic than that of EL. Given that more amphipathic α helices are associated with increased TO hydrolysis {Dugi *et al.*, 1992}, it follows that the increased ability of HL to mediate triglyceride hydrolysis may reflect the greater amphipathicity of its lid.

In summary, these experiments provide evidence that the rHDL phospholipid composition affects the interaction of EL, but not HL, with the particle's surface, and therefore the ability of EL to hydrolyse HDL triglycerides.

CHAPTER 5

THE DEVELOPMENT OF A NOVEL SPECTROSCOPIC APPROACH FOR QUANTITATING HL-MEDIATED PHOSPHOLIPID HYDROLYSIS.

5.1 INTRODUCTION

5.2 METHODS

5.3 RESULTS

Physical properties of spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.

Partitioning of NEFA into spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL.

Time course of HL-mediated phospholipid hydrolysis in spherical rHDL as determined by ADIFAB.

Time course of HL-mediated phospholipid hydrolysis in spherical rHDL as determined by NEFA mass assay.

Kinetics of HL-mediated phospholipid hydrolysis in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL as determined by ADIFAB.

5.4 DISCUSSION

5.1 INTRODUCTION

For many years, phospholipids were thought to be important for maintaining the structural integrity of HDL, but not to have any other effects on HDL metabolism. It has recently become apparent that this is not the case and that phospholipids may be important for regulating the cardioprotective properties of HDL. HDL phospholipids influence the ability of HDL to, (i) promote cellular cholesterol efflux {Davidson *et al.*, 1995; Agnani and Marcel, 1993; Fournier *et al.*, 1996; Fournier *et al.*, 1997; Yancey *et al.*, 2000}, and (ii) suppress the cytokine-induced expression of adhesion molecules {Baker *et al.*, 2000}.

Phospholipids may influence these functions directly, by regulating the surface properties of HDL, or indirectly, through their interactions with the apolipoproteins, for example, phospholipid acyl chain saturation can affect the lipid-water interfacial hydration of HDL in ways that alter apolipoprotein conformation {Ho *et al.*, 1994}. Thus, apoA-I is less stable in rHDL that contains phospholipids with long, polyunsaturated acyl chains chain {Jonas *et al.*, 1987; Parks and Gebre, 1997; Huggins *et al.*, 1998}. HDL phospholipid composition also regulates the fluidity and structure of the particle surface in ways that regulate LCAT reactivity {Parks *et al.*, 2000; Mitchell and Litman, 1998}. As one of the main objectives of this thesis was to determine how HDL phospholipids also regulate HL-HDL interactions, it was important to develop a fast and reliable way to monitor phospholipid hydrolysis.

When HDL phospholipid hydrolysis is quantitated by measuring NEFA mass (see Chapter 3), hydrolysis is monitored as a series of time points, rather than in a continuous manner. Measurement of NEFA mass also requires large amounts of

substrate and enzymes. In order to overcome these drawbacks a new spectroscopic approach for monitoring phospholipid hydrolysis has been developed using a fluorescence probe, ADIFAB (AcryloDan-derivatised Intestinal Fatty Acid Binding protein).

ADIFAB is a rat intestinal fatty acid binding protein that is complexed to the polarity-sensitive fluorescent probe Acrylodan {Richieri et al., 1994}. When NEFA binds to ADIFAB the emission wavelength of the probe changes from 432 to 505 nm. The ratio of the wavelengths at 505 and 432 nm is then used to quantitate the amount of NEFA generated during the hydrolysis reaction {Richieri *et al.*, 1992}.

This chapter describes the development of a protocol where ADIFAB is used to monitor HL-mediated phospholipid hydrolysis in rHDL containing either POPC, PLPC, PAPC or PDPC as the sole phospholipid. These rHDL also contained apoA-I as the sole apolipoprotein and CE as the only core lipid. When ADIFAB was used to monitor HL-mediated phospholipid hydrolysis in these preparations of rHDL, the results agreed well with what was obtained using the mass assay (see Chapter 3), with HL hydrolysing the *sn*-1 acyl ester bonds in (POPC)rHDL and (PLPC)rHDL more rapidly than in (PAPC)rHDL and (PDPC)rHDL.

5.2 METHODS

HL-mediated phospholipid hydrolysis in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (Fig. 5.1)

Release of NEFA mass from the rHDL was quantitated spectroscopically using an LS 50 Luminescence Spectrometer (Perkin Elmer, Buckinghamshire, England). As the phospholipids in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL all contain palmitic acid in the *sn*-1 position, and HL has PLA_1 activity {Scagnelli *et al.*, 1991}, palmitic acid was formed in all the incubations. This palmitic acid has several fates. It either remains associated with the rHDL surface, or (ii) partitions from the rHDL surface, into the aqueous phase. The palmitic acid in the aqueous phase either (iii) binds to the walls of the cuvette, or (iv) binds to ADIFAB (Fig. 5.1). Given that one molecule of palmitic acid is released from the rHDL for each phospholipid molecule hydrolysed by HL, the amount of phospholipid hydrolysed can be determined as the sum of the concentration of palmitic acid in these four pools.

Phospholipid hydrolysis (and NEFA formation) is determined by the fluorescence intensity of the incubation mixtures at 432 and 505 nm. When NEFA is not present in the system, the ADIFAB appears as a single peak at wavelength 432 nm. When NEFA binds to ADIFAB, the intensity of the 432 nm peak decreases while that of the 505nm peak increases {Anel *et al.*, 1993}. The concentration of palmitic acid released from the rHDL surface into solution is determined from the following equation {Anel *et al.*, 1993}.

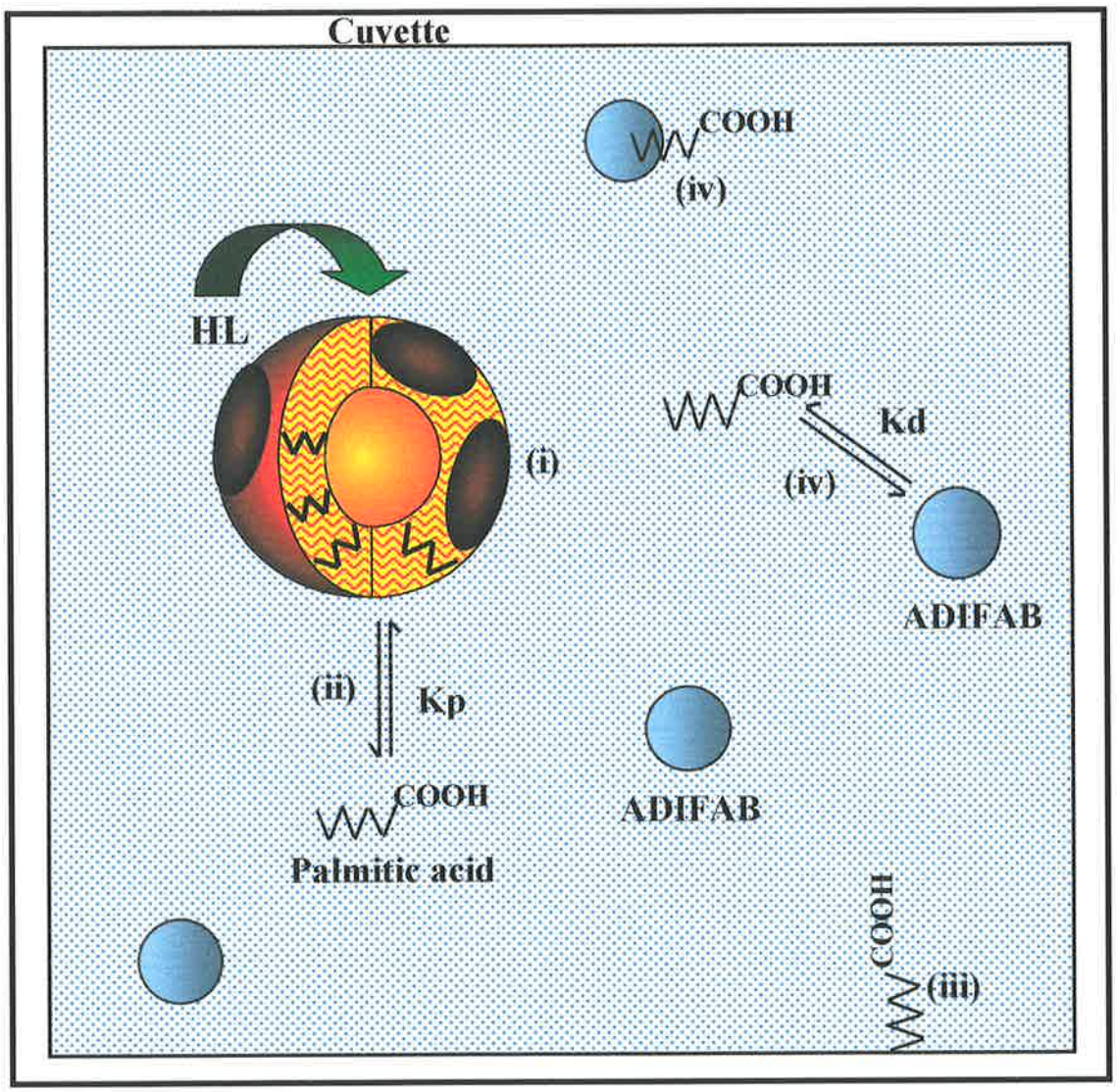


Figure 5.1. A schematic diagram showing the fate of palmitic acid (NEFA) that is generated when HL hydrolyses rHDL phospholipids. As HL hydrolyses rHDL phospholipids, the majority of the palmitic acid (i) partitions into the rHDL surface. The (ii) partition coefficient (Kp) is the ratio of palmitic acid in the rHDL surface and in solution. The palmitic acid that is in solution either, remains in solution, (iii) binds to the cuvette wall, or (iv) binds to ADIFAB. The dissociation constant (Kd) is the equilibrium constant for the binding of NEFA to ADIFAB.

$$[\text{NEFA}] = \frac{K_d Q(R-R_o)}{(R_{max}-R)} \qquad (1)$$

where R and R_o are the ratios of the fluorescence intensities at 505 and 432 nm in the presence of and absence of NEFA, respectively. R_{max} is the ratio of the intensities at 505 and 432 nm when maximal amounts of NEFA are bound to ADIFAB. Q is the ratio of the fluorescence intensities at 432 nm for ADIFAB in the absence of NEFA and when saturated with NEFA. The values for R_{max} and Q are 11.5 and 19.5, respectively {Richieri *et. al.*, 1992}. K_d is the equilibrium constant for the binding of NEFA to ADIFAB. This value varies according to the length and unsaturation of the fatty acid acyl chain. The K_d for the binding of palmitic acid to ADIFAB was determined using the approach described by Richieri *et al.* (1992). Varying amounts of palmitic acid (2.0-72.0 μmol/L) were mixed with ADIFAB (final concentration 0.24 μmol/L) and TBS and preincubated for 10 min. The final volume of the incubation mixtures was 750 μL. The fluorescence intensities at 505 and 432 nm were measured at 3 sec intervals, for 30 sec and the concentration of palmitic acid calculated as described {Richieri *et al.*, 1992}. The log $(Q(R-R_o)/(R_{max}-R))$ was plotted as a function of log [palmitic acid]. The K_d was determined as the intercept on the x-axis. The K_d for the binding of palmitic acid to ADIFAB was 0.32 ± 0.02 μmol/L. This value agrees well with the published value of 0.32 μmol/L for palmitic acid {Richieri *et al.*, 1992}.

The concentration of palmitic acid bound to ADIFAB, [Palmitic $acid_{ADIFAB}$], is determined from equation 2

$$[\text{Palmitic acid}_{ADIFAB}] = \frac{[\text{ADIFAB}] \times 19.5 \times (R-R_o)}{11.5-R+19.5(R-R_o)} \qquad (2)$$

where [ADIFAB] is the final concentration of ADIFAB in the incubation mixtures (0.24 μmol/L), and R and R_o are defined as in equation 1.

Determining the amount of NEFA bound to the cuvette wall

The concentration of NEFA bound to the cuvette walls was determined by adding aliquots of 20 mmol/L palmitic acid/4 mmol/L NaOH/25 μmol/L BHT to TBS to give final concentrations of 1.06, 2.12, 4.82, 9.97, 20.2, 30.4 and 50.6 μmol/L palmitic acid. R was measured at 3 sec intervals over 60 sec. Equation 1 was used to calculate the concentration of palmitic acid in solution at each time point. These values were averaged to give the mean concentration of palmitic acid in solution. The concentration of unbound palmitic acid in solution was calculated by subtracting the mean concentration of palmitic acid in solution from the concentration of palmitic acid that was added to the cuvette. At all concentrations of added palmitic acid, 30% bound to the cuvette walls (data not shown).

Determination of the partitioning of NEFA into the rHDL surface

The partitioning of NEFA into the rHDL surface was determined by adding palmitic acid (1.0–50 μmol/L) to the rHDL. The concentration of palmitic acid in solution was calculated using equation 1 and corrected for binding to the cuvette wall. The concentration of palmitic acid bound to ADIFAB was calculated using equation 2. The concentration of palmitic acid in the rHDL surface was determined by subtracting the sum of the concentration of palmitic acid in solution (equation 1) and palmitic acid bound to ADIFAB (equation 2) from the total NEFA added to the rHDL. This was repeated for each rHDL preparation at a number of different concentrations (10, 20, 50, 70 and 100 μmol/L phospholipid).

Incubations:

TBS (700 μL) was equilibrated at 37 °C for 10 min. A background value for R was determined as the ratio of fluorescence intensities at 432 and 505 nm. ADIFAB (final concentration 0.24 μmol/L) and rHDL (final concentration 0.01–0.10 mmol/L phospholipid) were added to the TBS. After 10 min of equilibration R was measured at 3 sec intervals, for a total of 1 min. The background value for R (obtained with TBS alone) was subtracted from each of these measurements to give $R_o = 0.292 \pm 0.015$ (n=20). This is comparable to what has been reported elsewhere {Richieri *et al.*, 1994; Anel *et al.*, 1993}. HL was added to the incubation mixtures and R was determined at 3 sec intervals over 10 min for the time course experiments, or for 2 min for the kinetic experiments. In all cases, R was calculated at 3 sec intervals and corrected by subtracting the background value of R. The corrected R value was used to calculate the concentration of palmitic acid in solution (equation 1). These concentrations were corrected for binding to the cuvette walls. The concentration of palmitic acid bound to ADIFAB was determined from equation 2. The concentration of palmitic acid in the rHDL surface was determined from the partition coefficients (Table 5.II). These values were used to calculate the total concentration of palmitic acid generated during the incubations. Phospholipid hydrolysis was then determined as a function of time, assuming that each molecule of palmitic acid generated represents the hydrolysis of one phospholipid molecule by HL.

Kinetic Studies:

The initial, linear part of the curves of time *versus* μmol NEFA formed/mL HL was used to calculate the rate of phospholipid hydrolysis. The kinetic parameters, V_{max} and

$K_m(app)$ were derived using linear regression analysis from the line of best fit of a Lineweaver-Burk double reciprocal plot of the rate of phospholipid hydrolysis versus the concentration of rHDL. V_{max} was determined as the reciprocal of the y-intercept. $K_m(app)$ was determined as the product of V_{max} and the slope.

The time course of HL-mediated phospholipid hydrolysis in spherical rHDL quantitated by NEFA Mass Assay

All incubations were carried out under N_2, in stoppered tubes, in a shaking water bath maintained at 37 ^{0}C. Spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (100 μmol/L phospholipid) were individually incubated, at 37 ^{0}C for 3 hr, with BSA (final concentration 20 mg/mL), TBS and a constant amount of HL (10 μL of a preparation that generated 60 nmol/NEFA/mL HL/h). The final volume of the incubation mixtures was 140 μL. When the incubations were complete the tubes were placed immediately on ice. Phospholipid hydrolysis was determined by quantitating NEFA mass, assuming that one molecule of NEFA is produced for each molecule of phospholipid hydrolysed.

Other techniques

An Hitachi 902 automatic analyser was used for all chemical analyses. Phospholipid, protein, NEFA, total cholesterol and unesterified cholesterol concentrations were measured as detailed in Chapter 2. CE concentrations were determined as the difference between unesterified cholesterol and total cholesterol concentrations. rHDL size was determine by 3-35% non-denaturing gradient gel electrophoresis.

5.3 RESULTS

Physical Properties of spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (Table 5.1):

Spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL were prepared as described in Chapter 2. The preparations were comparable in terms of their CE/apoA-I and phospholipid/apoA-1 molar ratios (Table 5.I). Each preparation contained a single, monodisperse population of particles with diameters ranging from 8.9 nm for the (PDPC)rHDL to 10.2 nm for the (PLPC)rHDL.

Partitioning of palmitic acid into the surface of (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (Table 5.II) :

Palmitic acid (final concentration 1.0–50 μmol/L) was added to rHDL (final concentration 10, 20, 50, 70 and 100 μmol/L phospholipid). The difference between the amount of palmitic acid that bound to the cuvette wall and what remained in solution was subtracted from the amount of added palmitic acid. This process was repeated for (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL. The difference represents the amount of palmitic acid that partitioned into the rHDL surface. In general, the partitioning of palmitic acid into the rHDL surface increased as the concentration of rHDL in solution increased. For example, the fraction of palmitic acid that partitioned into the particle surface at 10 μmol/L rHDL phospholipid, ranged from 0.802 for (PDPC)rHDL to 0.840 for (PAPC)rHDL. However, as the concentration of rHDL increased to 100 μmol/L the fraction that partitioned into the rHDL also increased, with values ranging from 0.961 to 0.978 for (PAPC)rHDL and (PDPC)rHDL.

Table 5.I. *Physical properties of spherical rHDL.*

Spherical rHDL	Stokes' Diameter[a]	Stoichiometry[b]			
		PL	UC	CE	A1
	nm	*mol/mol*			
(POPC)rHDL	9.5	26.3	0.9	28.5	1.0
(PLPC)rHDL	10.2	30.4	1.0	30.6	1.0
(PAPC)rHDL	9.5	15.1	0.7	27.6	1.0
(PDPC)rHDL	8.9	24.5	1.7	24.5	1.0

Spherical rHDL were prepared by adding LCAT and unesterified cholesterol to discoidal rHDL as described in Chapter 2. The spherical rHDL were isolated by ultracentrifugation and their composition was determined as described.

[a]Determined by nondenaturing gradient gel electrophoresis.

[b]PL, phospholipid; CE, cholesteryl ester; UC, unesterified cholesterol; A-1, apolipoprotein A-I.

Table 5.II: *The partitioning of palmitic acid into the surface of rHDL.*

rHDL phospholipid concentration	Amounts of Palmitic Acid added	[Palmitic acid in rHDL]/[Palmitic acid in solution]			
		(POPC)rHDL	(PLPC)rHDL	(PAPC)rHDL	(PDPC)rHDL
µmol/L	*µmol/L*				
10	1 - 5	0.807 ± 0.024	0.811 ± 0.053	0.840 ± 0.033	0.802 ± 0.034
20	2 - 10	0.915 ± 0.009	0.888 ± 0.032	0.908 ± 0.017	0.931 ± 0.016
50	5 - 25	0.965 ± 0.015	0.948 ± 0.020	0.968 ± 0.010	0.926 ± 0.035
70	5 - 35	0.964 ± 0.008	0.947 ± 0.011	0.962 ± 0.011	0.918 ± 0.043
100	10 - 50	0.973 ± 0.005	0.962 ± 0.013	0.961 ± 0.006	0.978 ± 0.007

The rHDL preparations (10-100 µmol/L phospholipid), ADIFAB (0.24 µmol/L) and TBS were incubated at 37 ^{0}C for 10 min. The final volume of the incubation mixture was 0.7 mL. R was measured as described in section 5.2. Various concentrations of palmitic acid (10-50% of rHDL phospholipid concentration) were added and R was measured again. The concentration of palmitic acid was calculated as described and compared to the amount that was added initially. The amount of palmitic acid bound to the cuvette was determined as described. The concentration of palmitic acid in the rHDL surface is calculated for each concentration of palmitic acid added. These palmitic acid concentrations that partitioned into the rHDL varied by less than 6% for each rHDL concentration, so were grouped and averaged. This partitioning was expressed as the ratio of palmitic acid in the rHDL to the concentration in solution. Values represent the mean ± S.D. of four determinations.

Time course of HL-mediated phospholipid hydrolysis in spherical rHDL determined with ADIFAB (Figure 5.2):

To ensure that the results that were generated using ADIFAB to measure phospholipid hydrolysis were comparable to what was obtained by measuring generation of NEFA mass, both approaches were used to assess the time dependence of phospholipid hydrolysis for each of the rHDL preparation. The (POPC)rHDL (closed circles), (PLPC)rHDL (closed triangles), (PAPC)rHDL (open circles) and (PDPC)rHDL (open triangles) (final concentration 100 μmol/L phospholipid) were incubated individually, at 37 °C for 10 min in the presence of ADIFAB and HL (Fig. 5.2). At the 10 min time point, HL-mediated phospholipid hydrolysis in (PLPC)rHDL > (POPC)rHDL > (PDPC)rHDL > (PAPC)rHDL. However, in the initial 1 min period after the addition of HL, phospholipid hydrolysis in (POPC)rHDL > (PLPC)rHDL > (PAPC)rHDL > (PDPC)rHDL (Fig 5.2 *inset*).

Time course of HL-mediated phospholipid hydrolysis in spherical rHDL determined by measuring NEFA mass (Figure 5.3):

As the assay for NEFA mass is much less sensitive than the assay using ADIFAB, much longer incubation times were used for this experiment. The (POPC)rHDL (closed circles), (PLPC)rHDL (closed triangles), (PAPC)rHDL (open circles) and (PDPC)rHDL (open triangles) (final concentration 100 μmol/L phospholipid) were each incubated with HL, at 37 °C for 3 hr (Fig. 5.3). In this case, HL-mediated phospholipid hydrolysis in (POPC)rHDL > (PLPC)rHDL > (PAPC)rHDL > (PDPC)rHDL.

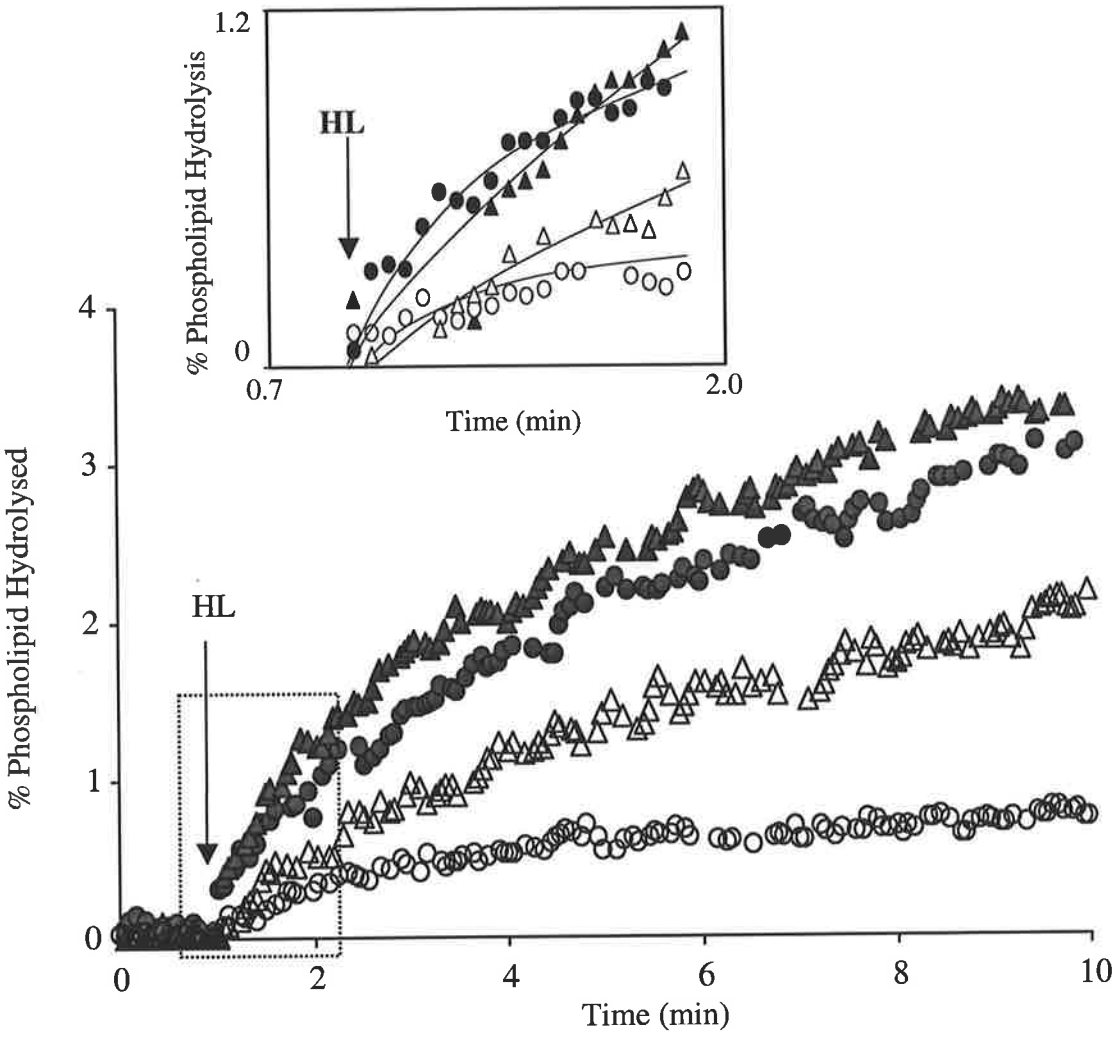


Figure 5.2. Time course for the HL-mediated phospholipid hydrolysis of rHDL determined with ADIFAB. The (POPC)rHDL (●), (PLPC)rHDL (▲), (PAPC)rHDL (○) and (PDPC)rHDL (△) (100 μmol/L phospholipid) were incubated, at 37 °C for 10 min, with ADIFAB (final concentration 0.24 μmol/L) and TBS. The final volume of the incubation mixture was 700 μL. The intensities of wavelengths at 505 and 432 nm were measured for 1 min. before HL (50 μL of a preparation that generated 60 nmol/NEFA/mL HL/h) was added as indicated. The intensities of the wavelengths at 505 and 432 nm were measured for a further 9 min. The inset shows an expanded view of the area of the graph between 0.7 and 2.0 min. These results are representative of three different sets of data.

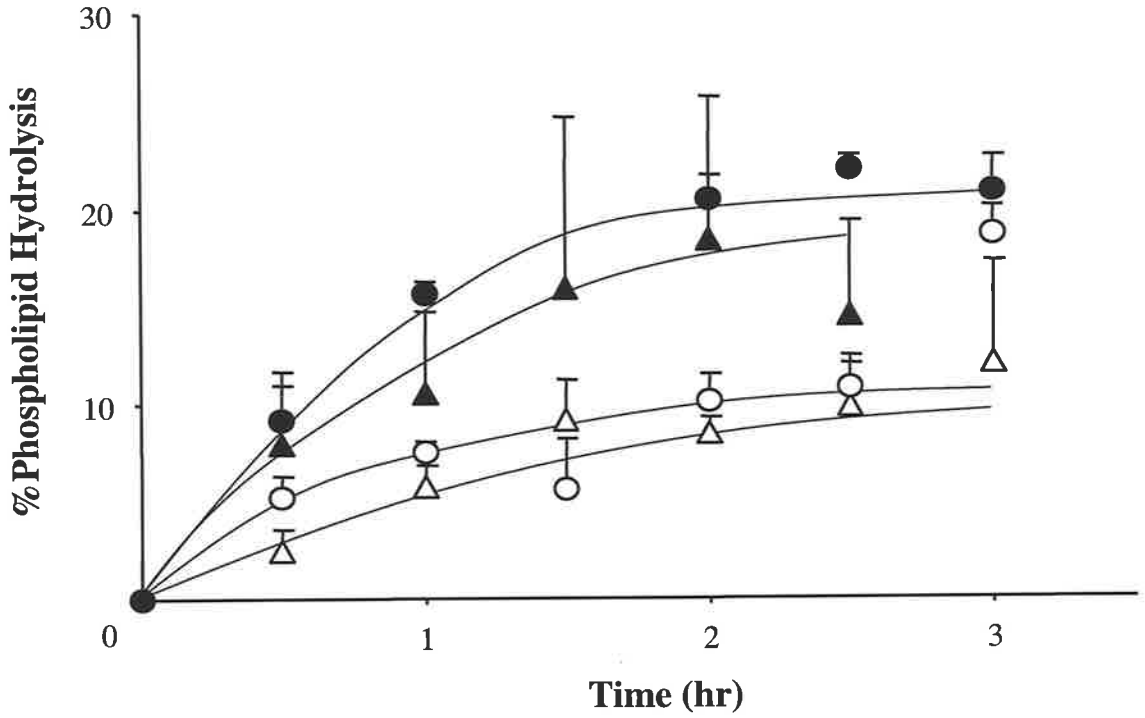


Figure 5.3. Time course for the HL-mediated phospholipid hydrolysis of rHDL using a mass assay to detect NEFA. The (POPC)rHDL (●), (PLPC)rHDL (▲), (PAPC)rHDL (○) and (PDPC)rHDL (△) (100 μmol/L phospholipid) were individually incubated at 37 °C for 3 hr with BSA (20 mg/mL), TBS and a constant amount of HL (10 μL of a preparation that generated 60 nmol/NEFA/mL HL/h). The final incubation volume was 140 μL. NEFA mass was quantitated at 30 min intervals using a mass assay. The values are the means ± SD of triplicate determinations.

Kinetics of HL-mediated phospholipid hydrolysis in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL determined using ADIFAB (Fig. 5.4, 5.5 and Table 5.II):

Aliquots of (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (final concentration 0.01-0.1 mmol/L phospholipid) were each mixed with a fixed amount of HL and ADIFAB (final concentration of 0.24 μmol/L) then incubated at 37 °C for 2 min. Formation of NEFA/mL HL was plotted as a function of time for each concentration of rHDL (Fig. 5.4). In all cases, $r^2 > 0.90$. The slope of the initial linear portion of each curve was determined. These values represented the rate of phospholipid hydrolysis for that particular rHDL phospholipid concentration. The rate of phospholipid hydrolysis was then plotted as a function of substrate concentration (Fig. 5.5). Fig. 5.5 *insets* represent the Lineweaver-Burk double reciprocal plots of the data in Fig 5.5. The kinetic parameters determined from Fig 5.5 *insets* are presented in Table 5.II. The V_{max} was greatest for (POPC)rHDL and (PLPC)rHDL with values of 2.79 and 1.72 μmol NEFA formed/mL HL/h, respectively. The respective maximal rates of hydrolysis for (PAPC)rHDL and (PDPC)rHDL were 1.39 and 0.89 μmol NEFA formed/mL HL/h. The K_{m}*(app)* for (POPC)rHDL and (PLPC)rHDL were 0.043 and 0.019 mmol/L phospholipid, compared to 0.011 and 0.006 mmol/L phospholipid respectively for (PAPC)rHDL and (PDPC)rHDL. The catalytic efficiencies are also shown in Table 5.II.

Figure 5.4. The rate of HL-mediated phospholipid hydrolysis in spherical rHDL determined with ADIFAB. Various concentrations of (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL (final concentration 10-100 μmol/L) were incubated, at 37 °C for 2 min, with ADIFAB (final concentration 0.24 μmol/L), TBS and a constant amount of HL (50 μL of a preparation that generated 60 nmol NEFA/mL HL/h). The final incubation volume was 750 μL. The concentration of NEFA formed/ml HL was plotted as a function of time for each rHDL concentration. The rate of phospholipid hydrolysis in the spherical rHDL was determined from the slopes of the lines. In all cases, $r^2 > 0.90$.

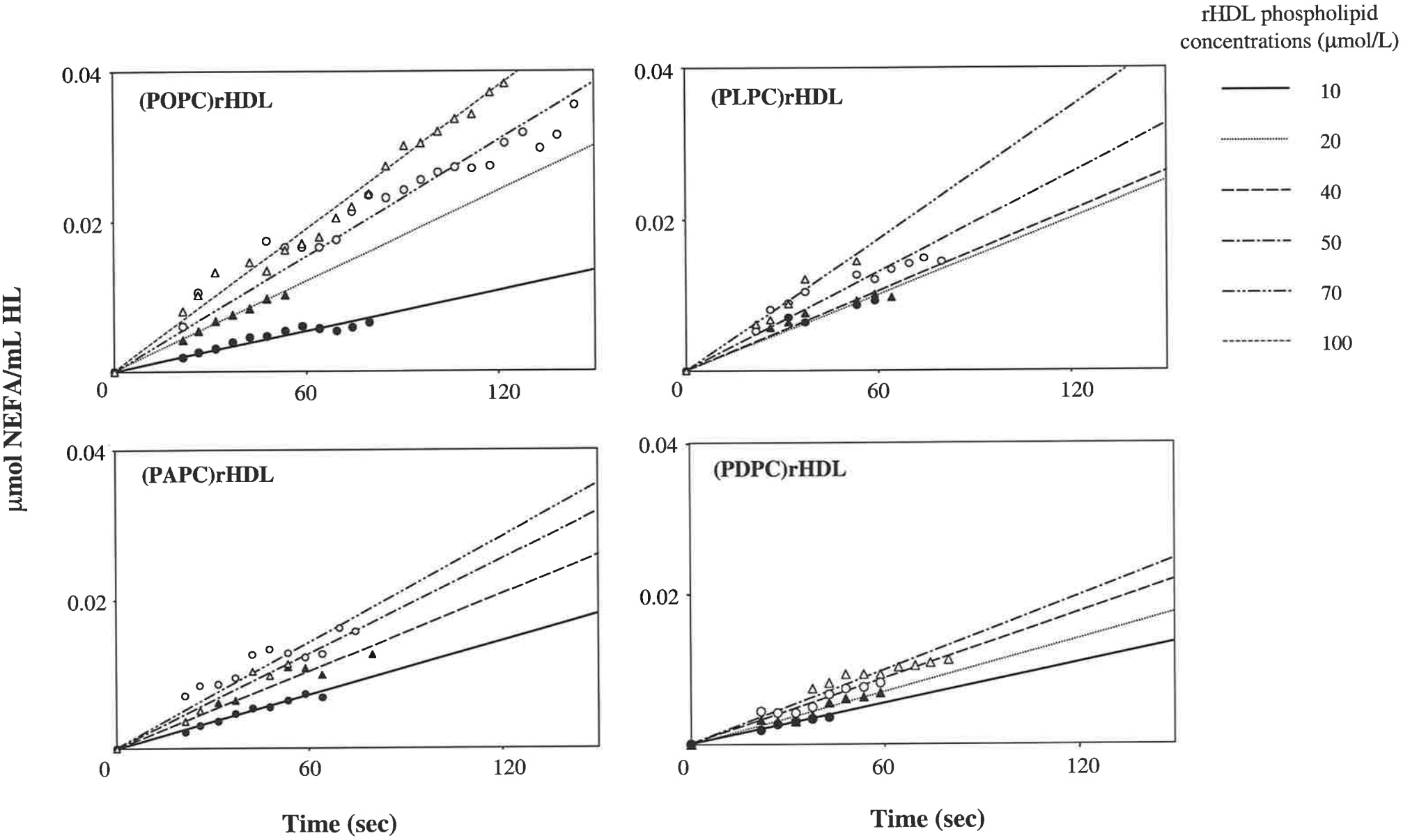

rHDL phospholipid concentrations (µmol/L)
10
20
40
50
70
100
(POPC)rHDL
(PLPC)rHDL
(PAPC)rHDL
(PDPC)rHDL
µmol NEFA/mL HL
Time (sec)
Time (sec)
0.04
0.02
0
60
120

Fig. 5.5. Kinetics of HL-mediated phospholipids hydrolysis in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL determined with ADIFAB. Varying concentrations of (POPC)rHDL (●), (PLPC)rHDL (▲), (PAPC)rHDL (○) and (PDPC)rHDL (△) (final concentration 0.01-0.1 mmol/L phospholipid) were incubated, at 37 °C for 2 min, with ADIFAB (final concentration 0.24 μmol/L) and a constant amount of HL (50 μL of a preparation that generated 60 nmol NEFA/mL HL/h). The final incubation volume was 750 μL. The rate of HL-mediated phospholipid hydrolysis is plotted as a function of substrate concentration. Lineweaver-Burk double reciprocal plots of the data are shown in the insets.

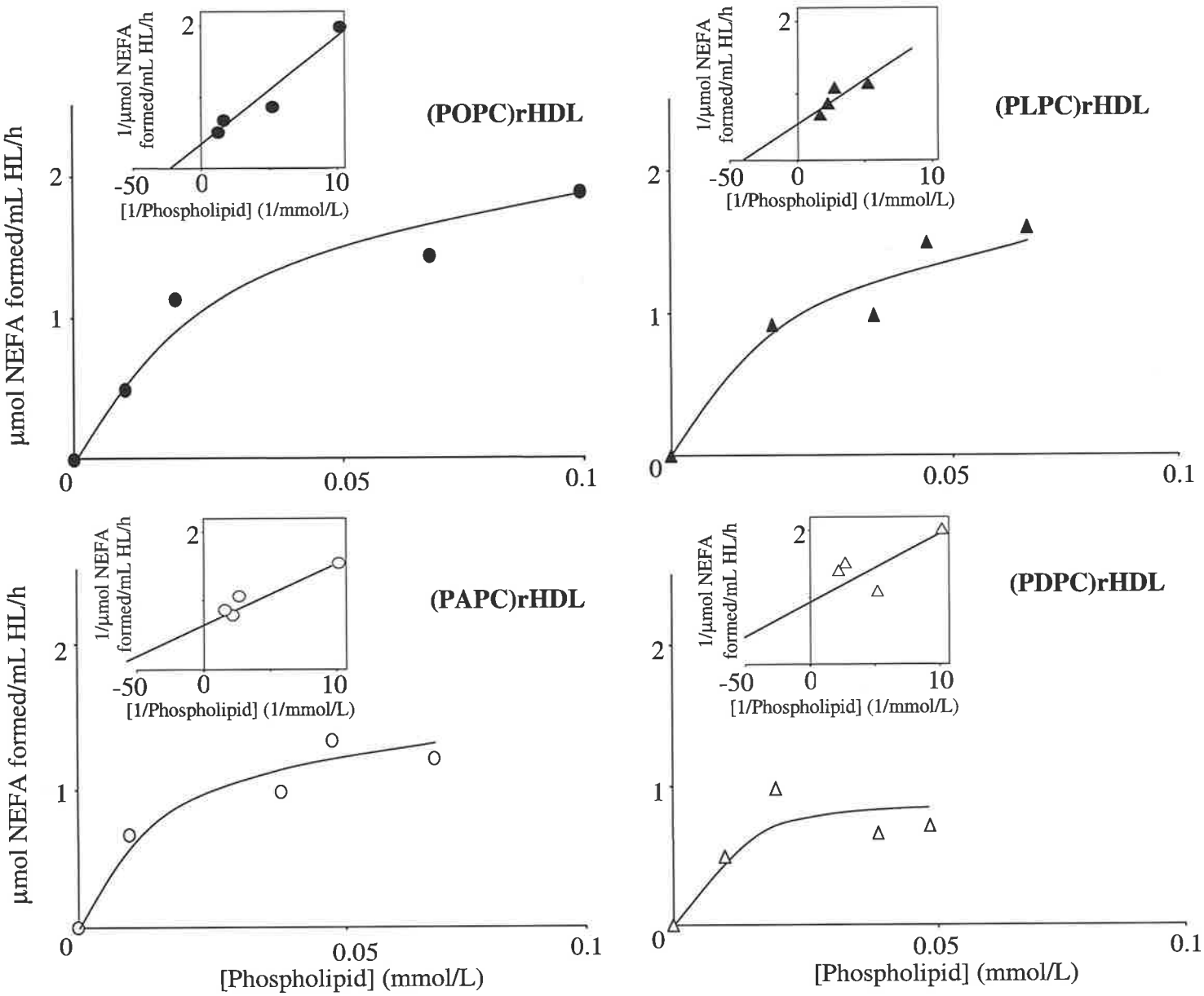

(POPC)rHDL
(PLPC)rHDL
(PAPC)rHDL
(PDPC)rHDL
µmol NEFA formed/mL HL/h
[Phospholipid] (mmol/L)
1/µmol NEFA formed/mL HL/h
[1/Phospholipid] (1/mmol/L)
0
0.05
0.1
1
2
-50
10

Table 5.II. *Kinetic parameters for HL-mediated phospholipid hydrolysis in spherical (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL using ADIFAB.*

Spherical rHDL	V_{max}	$K_m(app)$	r^2	Catalytic efficiency
	μmol NEFA/mL HL/h	*mmol/L PL*		$V_{max}/K_m(app)$
(POPC)rHDL	2.79	0.043	0.94	64.9
(PLPC)rHDL	1.72	0.019	0.70	90.5
(PAPC)rHDL	1.39	0.011	0.92	126.4
(PDPC)rHDL	0.89	0.006	0.62	148.3

The kinetic parameters were calculated from the data in Fig. 5.5, using a Lineweaver-Burk double reciprocal plot of the rate of phospholipid hydrolysis as a function of rHDL concentration.

5.4 DISCUSSION

The previous chapters in this thesis have shown unequivocally that the HL-mediated phospholipid hydrolysis of spherical rHDL is regulated by the phospholipid composition of the particles. In these experiments NEFA formation was determined by using a mass assay. This approach requires large amounts of substrates and long incubation times in order to generate NEFA in amounts sufficient to be detected in the assay.

This chapter describes a new spectroscopic approach for measuring phospholipid hydrolysis in rHDL. The fluorescent probe ADIFAB, which changes its emission wavelength when bound to NEFA, was used to monitor NEFA formation in the incubations. There are many advantages to using ADIFAB. As NEFA formation is measured continuously, true initial rates of hydrolysis are measured. Additionally, ADIFAB does not bind to or modify HDL, and does not associate with lyso-PC, the other product of HL-mediated phospholipid hydrolysis {Richieri et al., 1995}. The sensitivity of this method exceeds that of the mass assay, with ADIFAB detecting NEFA concentrations as low as 1.0 nmol/L, compared to 20 μmol/L for the mass assay. As a result, the incubation times are very short (up to 120 sec) and small quantities of the substrate and enzyme are used.

The results that were obtained using ADIFAB agreed well with the results that were obtained from the mass assay (Chapter 3). In both cases, HL hydrolysed the phospholipids in (POPC)rHDL and (PLPC)rHDL more rapidly than the phospholipids in (PAPC)rHDL and (PDPC)rHDL. The time course for phospholipid hydrolysis that was obtained with ADIFAB (Fig. 5.2) varied slightly from that obtained when the mass assay was used (Fig. 5.3). This may have been due to small amounts of lyso-PC

accumulating in the rHDL and inhibiting the phospholipid hydrolysis. In fact, within the first 30 sec after the addition of HL (Fig. 5.2 *inset*), the times from which the kinetic data is normally obtained, the hierarchy of the curves is consistent with those in Fig. 5.3.

In conclusion, the results in this chapter show that the spectroscopic approach using ADIFAB is a viable alternative for measuring phospholipid hydrolysis. This method has the advantage of requiring shorter incubation times and smaller quantities of substrate than what was necessary when phospholipid hydrolysis was quantitated with the mass assay.

CHAPTER 6

CONCLUDING COMMENTS.

Ever since epidemiological studies first showed an inverse relationship between plasma HDL cholesterol levels and the development of heart disease there has been substantial interest in HDL metabolism and regulation {Miller and Miller, 1975; Stein and Stein, 1999; Gordon, 1990; Barter and Rye, 1996}. The exact reasons why HDL protects against CHD are still not well understood. There are, however, several mechanisms that may be responsible for the cardioprotective effects of HDL: by participating in RCT {Fielding and Fielding, 1995; Barter and Rye, 1996; Rothblat *et al.*, 1999; Oram and Yokoyama, 1996; von Eckardstein *et al.*, 2001}, by acting as an anti-inflammatory agent {Ashby *et al.*, 1998; Cockerill *et al.*, 1995; Baker *et al.*, 1999; Barter *et al.*, 2002}, and through their antioxidant properties {Bowry *et al.*, 1992; Mackness *et al.*, 2000; Banka, 1996}.

Plasma factors that remodel HDL have a major influence on plasma HDL concentrations {Rye *et al.*, 1999}. HDL is the preferred substrate for both HL and EL {Shirai *et al.*, 1981; McCoy *et al.*, 2002}. Several studies have shown that HL activity is inversely related to HDL cholesterol levels and HDL subclass distribution {Shohet *et al.*, 1999; De Oliveira e Silva *et al.*, 1999; Grundy *et al.*, 1999}. Its interaction with HDL generates smaller particles in a process that is accompanied by the formation of lipid-poor apoA-I {Deckelbaum *et al.*, 1986; van Tol *et al.*, 1980; Groot *et al.*, 1981; Barter *et al.*, 1995}. EL also decreases plasma HDL levels {Jaye *et al.*, 1999; Rader and Jaye, 2000}.

The studies described in this thesis investigate how HDL phospholipid composition affects HL- and EL-mediated phospholipid and triglyceride hydrolysis. This was achieved by using well characterised, homogenous preparations of spherical rHDL that

contained either POPC, PLPC, PAPC or PDPC as the only phospholipid constituent. These are the predominant phospholipids in plasma HDL {Subbaiah and Monshizadegan, 1988; Zhang *et al.*, 1998}. PDPC is of particular interest because of its cardioprotective properties {Harker *et al.*, 1993; Siscovick *et al.*, 1995; De Caterina *et al.*, 1994; Conner and Conner, 1997}.

In Chapter 3, the kinetics of HL- and EL-mediated phospholipid hydrolysis in (POPC)rHDL, (PLPC)rHDL, (PAPC)rHDL and (PDPC)rHDL was investigated. The results showed that HL and EL had very different substrate specificities for HDL phospholipids, with HL hydrolysing the phospholipids in (POPC)rHDL and (PLPC)rHDL more rapidly than the phospholipids in (PAPC)rHDL and (PDPC)rHDL. EL, by contrast, hydrolysed the phospholipids in (PAPC)rHDL and (PDPC)rHDL more rapidly than the phospholipids in (POPC)rHDL and (PLPC)rHDL. Given that HL and EL both hydrolysed phospholipid *sn*-1 acyl ester bonds and the phospholipids in the rHDL varied only in the length and unsaturation of their *sn*-2 acyl chains, this result was somewhat unexpected. These findings can be explained either, by phospholipids regulating access of the phospholipid acyl chains to the active sites of the enzymes, or by HL and EL interacting differently with the rHDL surface.

The studies in Chapter 4 addressed the possibility that HL and EL interacted differently with the rHDL surface. This was achieved by studying the kinetics of triglyceride hydrolysis in rHDL that were enriched with [^{3}H]TO. As triolein was the only triglyceride constituent in the rHDL preparations, differences in triglyceride hydrolysis must reflect differences in the interaction of HL and EL with the rHDL surface. The results of these studies showed that HDL phospholipid composition affects the interaction of EL, but not that of HL, with the rHDL surface. Given the structural-

functional information available on the triglyceride lipase family, this result is most likely due to the differences in the lid and lipid binding domains of the enzymes.

Chapter 5 describes the development of a novel spectroscopic technique for quantitating the phospholipase activity of HL. This involved using the fluorescent probe, ADIFAB. The results for phospholipid hydrolysis that were obtained using ADIFAB were consistent with what was obtained when NEFA mass was measured in Chapter 3.

The fact that HL and EL have different substrate specificities for HDL phospholipids is of considerable physiological significance. Several investigators have reported that dietary fat intake can alter HDL phospholipid composition {Myher *et al.*, 1981; O'Brian and Andrews, 1993; Sola *et al.*, 1990; Gillotte *et al.*, 1998}. For example, consumption of a diet high in fish and seafood enriches HDL with PDPC {Siscovick *et al.*, 1995}. The phospholipids in these HDL would be preferentially hydrolysed by EL. On the other hand, a diet high in olive oil or safflower oil respectively increases the POPC and PLPC content of HDL, and generates substrates that are preferred by HL {Sola *et al.*, 1990; Gillotte *et al.*, 1998}. Therefore, the combined expression of both of these lipases allows for efficient hydrolysis of HDL phospholipids, regardless of dietary fat intake.

In conclusion, this thesis shows for the first time that HL and EL have different HDL phospholipid specificities. The results also show that phospholipids affect the interaction of EL, but not HL, with the surface of rHDL. When taken together these findings are consistent with HL and EL both contributing to optimal processing of a wide range of HDL phospholipids *in vitro*. If this is also found to be the case *in vivo*, it will establish complementary roles for both of the enzymes in HDL metabolism.

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