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Lipoprotein Composition Regulates Hepatic Lipase Cell-surface Association and Activity

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Lipoprotein Composition Regulates Hepatic Lipase Cell-Surface Association and Activity

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Abbreviations

ABCA1, adenosine triphosphate binding cassette transporter AI

ABCG1, adenosine triphosphate binding cassette transporter G1

ANOVA, analysis of variance

AP-1, activator protein-1

Apo, apolipoprotein

APOBEC-1, apolipoprotein B messenger ribonucleic acid-editing enzyme, catalytic polypeptide-1

ARP-1, apolipoprotein regulatory protein-1

BSA, bovine serum albumin

cAMP, cyclic adenosine monophosphate

CE, cholesteryl ester

CETP, cholesteryl ester transfer protein

CHO, Chinese hamster ovary

CHO-hHL, Chinese hamster ovary cell line expressing human hepatic lipase

CLA-I, CD36 and LIMPII Analogous-1

CPM, counts per minute

DG, diacylglyceride

DLnPC, dilinoleoyl phosphatidylcholine

DLPC, dilauroyl phosphatidylcholine

DOPC, dioleoyl phosphatidylcholine

DOPE, dioleoyl phosphatidylethanolamine

DOPG, dioleoyl phosphatidylglycerol

DOPI, dioleoyl phosphatidylinositol

DOPS, dioleoyl phosphatidylserine

DPPC, dipalmitoyl phosphatidylcholine

ED_{50}, concentration required to inhibit half of the maximum binding of an antibody

EL, endothelial lipase

ER, endoplasmic reticulum

ERα, estrogen receptor α

ERE, estrogen regulatory element

FBS, fetal bovine serum

FC, free (unesterifed) cholesterol

FH, familial hypercholesterolemia

FXR, farnesoid X receptor

GdnHCl, guanidine hydrochloride

GRE, glucocorticoid regulatory element

HDL, high density lipoprotein

HL, hepatic lipase

HNF, hepatocyte nuclear factor

HRP, horseradish peroxidase

HSPG, heparan sulfate proteoglycan

IDL, intermediate density lipoprotein

IgG, immunoglobulin type G

K_m, Michealis-Menten kinetic constant

LCAT, lecithin:cholesterol acyl transferase

LDL, low density lipoprotein

LDLR, low density lipoprotein-receptor

LpA-I, synthetic high density lipoprotein containing apolipoprotein A-I

LpA-I/A-II, synthetic high density lipoprotein containing apolipoprotein A-I and apolipoprotein A-II

LpA-II, synthetic high density lipoprotein containing apolipoprotein A-II

LPL, lipoprotein lipase

LRP, low-density lipoprotein receptor-related protein

MG, monoacylglyceride

MOPC, monooleoyl phosphatidylcholine

MTP, microsomal triacylglyceride transfer protein

PA, phosphatidic acid

PAPC, palmitoyl arachidonoyl phosphatidylcholine

PBS, phosphate-buffered saline

PC, phosphatidylcholine

PDPC, palmitoyl docosahexanoic acid phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglyceride

PGC-1α, peroxisome proliferation-activated receptor γ coactivator-1α

PI, phosphatidylinositol

PLA_2, phospholipase A_2

PLPC, palmitoyllinoleoyl phospatidylcholine

PLTP, phospholipid transfer protein

POPC, palmitoyloleoyl phosphatidylcholine

PS, phosphatidylserine

RCT, reverse cholesterol transport

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM, standard error mean

SM, sphingomyelin

SNP, single nucleotide polymorphism

SR-BI, scavenger receptor BI

SRE, sterol regulatory element

TG, triacylglyceride

TRE, thyroid regulatory element

USF, upstream stimulatory factor

VHDL, very high density lipoprotein

VLDL, very low density lipoprotein

VLDLR, very low density lipoprotein receptor

$\mathbf{V_{max}}$**,** maximal velocity kinetic rate constant.

Abstract

It is well established that high triacylglyceride (TG) and reduced high density lipoprotein (HDL) levels increase the risk of developing heart disease and atherosclerosis. It has been assumed that the hydrolysis of TG-rich lipoproteins by the enzymes hepatic lipase (HL) and lipoprotein lipase (LPL) is the major determinant of HDL concentrations in the bloodstream. However, data now indicate that HDL is not only a product of TG-rich lipoprotein hydrolysis but also plays an active role in the regulation of TG metabolism by both HL and LPL. Our laboratory has previously shown that HDL regulates the activity of HL by releasing the enzyme from the cell-surface and enhancing its hydrolytic activity. In this study, the factors regulating the ability of HDL to stimulate the cell-surface displacement of HL and control its activity were characterised. Using a Chinese hamster ovary cell line stably overexpressing human HL (CHO-hHL), it has been shown that incubation with HDL or apolipoprotein (apo) A-I alone can displace HL protein and activity as determined by HL immunoblot analysis and enzyme activity measurements. Incubation of different HDL sub-species with CHO-hHL cells showed that the larger and less dense HDL particles were more effective at displacing HL from the surface than the smaller, more dense HDL species. The ability of the different classes of HDL to regulate very low density lipoprotein (VLDL) hydrolysis by HL *in vitro* was also evaluated. The larger, less dense HDL were stimulatory, while the smaller, more dense HDL particles were inhibitory to VLDL hydrolysis by HL.

The hydrolytic activity of HL is well known to be regulated by lipoprotein structure and composition. The effects of the apoA-I and apoA-II content of HDL particles on the structure, hydrolysis and displacement of HL were investigated. Synthetic HDL containing apoA-I (LpA-I)

or apoA-II (LpA-II) and various lipids were generated and used as model HDL particles with well-defined lipid and protein compositions. LpA-I containing apoA-II (LpA-I/A-II) significantly inhibited DG hydrolysis in DG-rich LpA-I particles and phospholipid hydrolysis in TG-rich LpA-I complexes. The effect of HDL apoA-I and apoA-II content on HL-mediated VLDL hydrolysis was also determined. While LpA-I inhibited VLDL hydrolysis, addition of apoA-II showed enhanced inhibition of HL-mediated VLDL lipolysis. The effect of apoA-II on the ability of HDL to displace cell-surface HL was also evaluated. LpA-I particles containing apoA-II (LpA-I/A-II) significantly stimulated the ability of this particle to displace cell-surface HL by approximately 2.3-fold compared to native HDL or LpA-I without apoA-II.

The effects of phospholipid composition on lipoprotein electrostatic properties, HL activity and cell-surface displacement were also investigated. Enrichment of serum or lipoproteins with the anionic lipids oleic acid, phosphatidylinositol (PI), phosphatidylserine (PS) or phosphatidic acid (PA) increased the negative charge of all lipoprotein classes and stimulated HL-mediated lipid hydrolysis. Lipoproteins enriched with anionic lipids showed enhanced TG hydrolysis, while phospholipid hydrolysis was negligible. The electrostatic properties of HDL also had an effect on the hydrolysis of VLDL by HL. Enrichment of HDL with PI significantly stimulated VLDL-TG hydrolysis by HL relative to HDL enriched with the uncharged phosphatidylcholine (PC). HL-lipoprotein interactions were quantified in order to determine whether HDL charge affects the association of HL with HDL and VLDL. Under normal conditions, HL preferentially associates with HDL while only small amounts of HL bind to VLDL. In contrast, PI-enrichment of HDL reduces the binding of HL with HDL and VLDL. The role of lipoprotein charge on the ability of HDL to displace HL from the surface of CHO-hHL cells was also evaluated. Increasing the negative charge of HDL by enriching with PI

significantly inhibited the ability of this lipoprotein to displace cell-surface HL. However, enrichment of HDL with the less charged PC also reduced its ability to displace HL into the media of CHO-hHL cells. These data suggest that HDL charge does not significantly affect the ability of HDL to displace HL from the cell surface.

It now appears that factors affecting HL displacement and HL-lipoprotein association regulate lipid hydrolysis by this enzyme. ApoA-II is well known to enhance the binding of HL to HDL and it is now clear that tighter binding of the enzyme is inhibitory to HL-mediated lipolysis. The tighter binding of apoA-II to HL also appears to enhance its ability to displace cell-surface HL. In contrast, increasing the negative charge of lipoproteins stimulates hydrolysis by reducing the association of HL with lipoproteins. A reduction in the association between HL and HDL may be responsible for the relative inability of negatively charged HDL particles to displace cell-surface HL. Thus, HDL controls the hydrolysis of VLDL by directly affecting the displacement and inter-lipoprotein association of HL. Lipoprotein composition and charge appear to be important regulators of the binding, displacement and activity of HL.

Chapter 1 – Introduction

1.1 – Cardiovascular disease, high density lipoprotein and hepatic lipase

Obesity is a world-wide epidemic affecting 23.1% (5.5 million) of adults in Canada [1-3]. It is a well-known risk factor for diabetes, metabolic syndrome and cardiovascular disease [4-6]. Cardiovascular disease, which causes heart attacks, stroke and atherosclerosis, is responsible for 50% of deaths in developed countries [7]. The risk of developing cardiovascular disease is correlated with elevated triacylglyceride (TG) and low density lipoprotein (LDL) levels as well as reduced plasma high density lipoprotein (HDL) concentrations [8]. TG metabolism is very closely linked to plasma HDL levels and plays a prominent role in the development of obesity, atherosclerosis and diabetes [9,10]. Abnormal TG and lipoprotein metabolism are directly influenced by a number of plasma enzymes including members of the lipase family.

Hepatic lipase (HL) is responsible for the hydrolysis of TG and phospholipids contained within all lipoprotein classes and is a major determinant of plasma TG and HDL levels [8]. In part, the efficient catabolism of TG-rich lipoproteins by members of the lipase family has been shown to be a major determinant of HDL and TG concentrations in the bloodstream [8]. The catalytic regulation of HL and other members of the lipase family is not very well understood and will be the primary focus of this thesis. It has been a long-held view that lipases are hydrolytically active at the vascular endothelium where they are attached via the extracellular proteoglycan matrix to the cell surface [11]. However, an abundance of evidence actually suggests quite the opposite [12-14]. Infusion of the anti-coagulant heparin into the bloodstream has been known for decades to release plasma lipases into the circulation and stimulate TG hydrolysis by over a 1000-fold [15]. In this thesis, I now propose that HDL is not only a product

of TG-rich lipoprotein hydrolysis but that it can actively regulate TG metabolism by affecting HL activity through a cell-surface displacement and catalytic regulatory mechanism. It is now clear that HDL can act much like heparin and release HL from the extracellular matrix and regulate the catalytic activity of this enzyme in the bloodstream. This thesis proposes that a novel cell-surface displacement mechanism induced by HDL is a major regulatory pathway for both HL activity and TG metabolism.

1.2 - Lipid metabolism

The regulation of plasma lipid homeostasis is a crucial physiological process. In particular, cholesterol and TG play very important roles in lipid metabolism. Cholesterol is an essential component of cellular membranes in all tissues whereas most of the energy in the body is stored in the form of TG. Both of these lipids are derived primarily from the diet but can also be synthesized endogenously. Exogenous dietary lipids are metabolized in the intestine and travel through the circulation to the tissues of the body, while endogenous lipids are produced mainly by the liver. Lipid levels are not only controlled through various synthetic pathways but through various catabolic mechanisms. A process called reverse cholesterol transport (RCT) delivers cholesterol from extra-hepatic tissues and cell membranes to the liver for recycling or excretion from the body [16-18]. Regulated control of lipid transport is, therefore, a crucial component of energy metabolism and mammalian physiology.

1.3 – Lipoprotein structure

Plasma lipids such as cholesterol, TG and phospholipid are transported in the plasma by macromolecular lipid-protein complexes called lipoproteins. Macheboeuf was the first to identify and isolate plasma lipoproteins in the 1920s [19] and a structural model of a lipoprotein particle was proposed by Edelstein *et al.* in 1979 [20]. **Figure 1** shows a model depicting the general

structure and composition of a lipoprotein particle. In this model, hydrophobic water-insoluble lipids such as TG and cholesteryl ester (CE) are found within the core of lipoprotein particles and are surrounded by a monolayer of amphipathic phospholipids, unesterified free cholesterol (FC) and protein components called apolipoproteins. Different types of lipoproteins have been characterized and classified based on size, density, composition and function. **Table 1** shows the general properties of the different lipoprotein classes. Chylomicrons and very low density lipoproteins (VLDL) are large particles composed mainly of TG and relatively small amounts of protein. Chylomicrons are synthesized in the intestine and contain apolipoprotein (apo) B48, whereas VLDL are produced by the liver and contain apoB100 as their primary protein component. Both lipoproteins also transport significant amounts of cholesterol, phospholipid and other apolipoproteins. LDL is the major class of lipoprotein responsible for transporting cholesterol in the plasma. These particles contain only one molecule of apoB100 and are believed to originate from the catabolism of VLDL particles. LDL are commonly referred to as the "bad" cholesterol since high levels are a risk factor for cardiovascular disease. HDL, on the other hand, are often referred to as the "good" cholesterol given that high levels of this lipoprotein reduce the risk of heart disease and atherosclerosis [8]. HDL levels in the plasma are partially regulated by the hydrolytic actions of HL and endothelial lipase (EL). HDL are the smallest and most dense class of lipoprotein and are composed mostly of phospholipid and a protein called apoA-I. The function of HDL is to facilitate the removal and excretion of excess cholesterol and lipids from the body. The protein and lipid compositions of each type of lipoprotein class are summarized in **Table 2**.

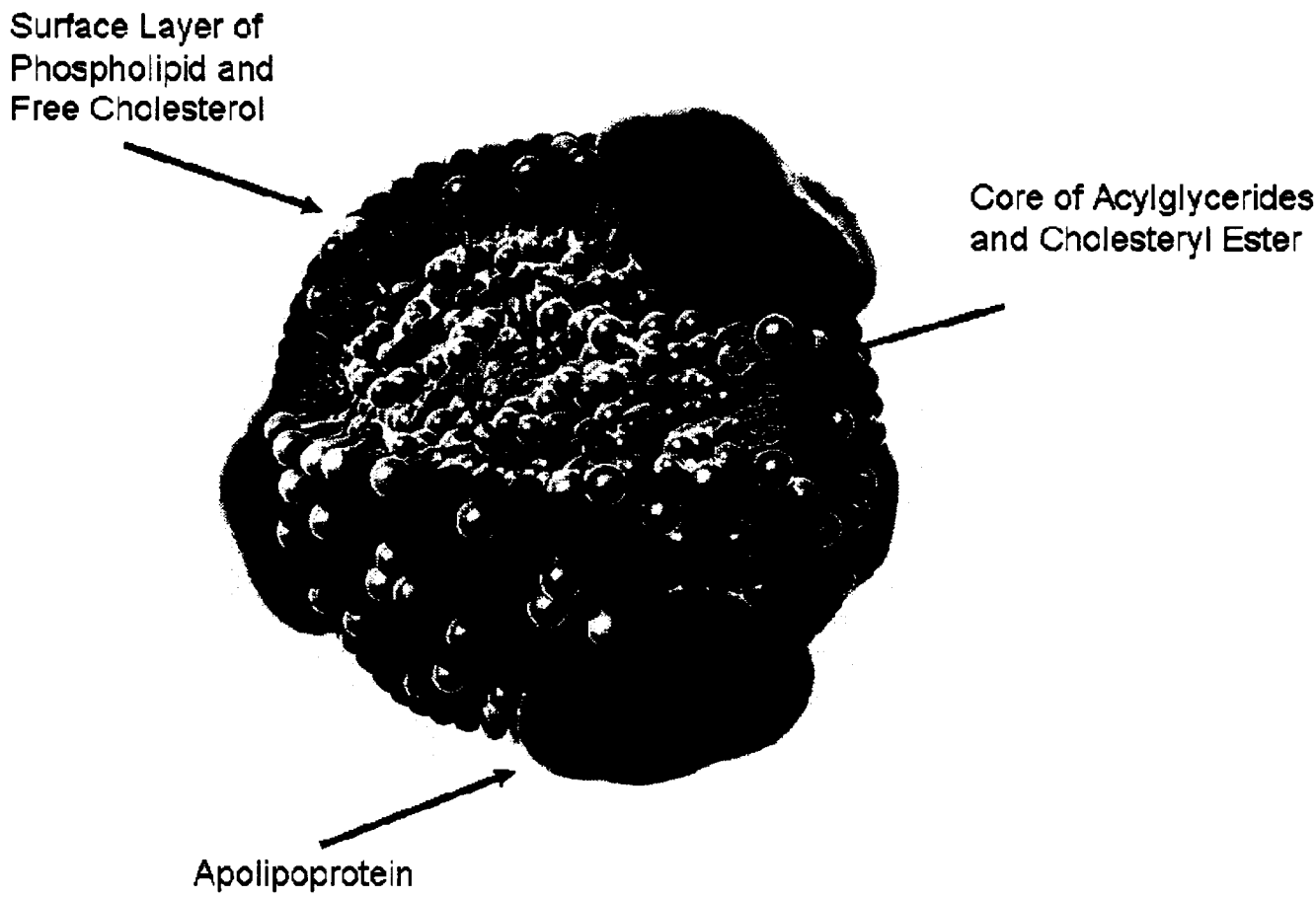


Figure 1. Model illustrating the structure of a lipoprotein particle. Lipoproteins are spherical macromolecular complexes composed of both lipid and protein. Lipoprotein particles contain an outer surface layer composed mainly of amphipathic phospholipids and free cholesterol. The lipoprotein core is composed of hydrophobic acylglycerides and cholesteryl esters. Proteins called apolipoproteins are embedded in the outer surface layer of lipoproteins and are important determinants of lipoprotein class and function. Used with permission from Daniel Sparks, Liponex Inc.

Table 1 – General Properties of the Major Lipoprotein Classes

Class	Density (g/ml)	Diameter (nm)	Molecular Mass (kDa)	Main Apolipoproteins
Chylomicrons	< 0.95	100-1000	400,000	apoB48
VLDL	< 1.006	35-50	10,000-80,000	apoB100, apoE
IDL	1.006-1.019	28-35	5,000-10,000	apoB100, apoE
LDL	1.019-1.063	20-26	2,300	apoB100
HDL	1.063-1.21	7-12	175-360	apoA-I, apoA-II
HDL_2	1.063-1.19	9-12	360	apoA-I, apoA-II
HDL_3	1.19-1.21	7-9	175	apoA-I, apoA-II

VLDL, ver low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein. Adapted from Rye *et al.*, 1999 and Gotto *et al*, 1986 [21,22].

Table 2 – Protein and Lipid Composition of the Major Lipoprotein Classes

	Composition (% w/w)				
	Protein	**Phospholipid**	**Free Cholesterol**	**Cholesteryl Ester**	**Acylglyceride**
Chylomicron	2	5	<1	<1	93
VLDL	10	15	6	14	53
IDL	18	22	7	23	31
LDL	25	21	9	42	4
Preβ-HDL	70	25	5	-	-
HDL_2	43	30	5	20	2
HDL_3	55	25	3	16	1

VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. Adapted from Gotto *et al.*, 1986 [22].

1.4 - Apolipoproteins

Each type of lipoprotein has a characteristic apolipoprotein composition that determines its structure and function. The apolipoproteins can be broadly divided into exchangeable and non-exchangeable groups. The exchangeable apolipoproteins (apoA-I, apoA-II, apoC -I, -II and -III and apoE) can all dissociate to varying degrees from the surface of lipoproteins. This process allows these proteins to be readily exchanged between different types of lipoproteins during their metabolism. In contrast, apoB48 and apoB100 do not exchange between lipoprotein particles. It is believed that the exchangeable apolipoproteins derived from a common ancestral protein through gene duplication because of shared structural and functional features [23]. Most of the exchangeable apolipoproteins have an amphipathic helical structure that readily binds lipid. Each α-helix has a hydrophilic component exposed to the aqueous environment and a hydrophobic face that associates with lipid. The physical properties of these proteins allow them to form monomers in aqueous solution by clustering of their hydrophobic domains. Apolipoproteins also have a high degree of flexibility due to the virtual absence of a defined tertiary structure. This flexibility allows the protein to adjust to the changing composition and size of lipoprotein particles. In addition to contributing to lipoprotein structure, apolipoproteins also function as co-factors for a number of plasma enzymes and as ligands for a range of lipoprotein and lipid receptors. The functional sequences of most apolipoproteins are often charged amino acids that interact with opposite charged residues on enzymes or receptors. The unique characteristics of each apolipoprotein give them distinct roles in lipoprotein structure and metabolism.

Apolipoprotein A-I – The major protein component of HDL is apoA-I, a 243 amino acid apolipoprotein with a molecular mass of 28.8 kDa. The liver and intestine are the main sites of

apoA-I synthesis and studies show that mRNA levels are the primary determinants of the rate of apoA-I secretion [24]. ApoA-I is synthesized as a preproprotein and is cleaved co-translationally in the lumen of the endoplasmic reticulum (ER). Once secreted into the plasma, 6 terminal residues are cleaved from the proprotein to form the mature peptide [25]. The protein contains several repeats of a 22 amino acid long amphipathic α-helical structure that is characteristic of many of the exchangeable apolipoproteins [26]. ApoA-I is intimately involved in RCT and is known for its role in promoting cholesterol efflux from cells and in the formation of HDL. ApoA-I stimulates the plasma enzyme lecithin:cholesterol acyl transferase (LCAT) which esterifies cholesterol and contributes to the synthesis of HDL. Since, apoA-I is the major protein component of HDL, it plays an important role in lipoprotein structure and function. ApoA-I levels have been directly linked to a reduced risk of cardiovascular disease [27,28]. Individuals with low levels of apoA-I (hypoalphalipoproteinemia) are more likely to develop premature cardiovascular disease due to a deficiency of HDL in the plasma. The low levels of apoA-I and HDL seen in these individuals are usually the result of mutations in the apoA-I gene or LCAT deficiency [29]. In contrast, familial hyperalphalipoproteinemia is a disorder that results in elevated plasma HDL levels. These individuals usually have polymorphisms in the promoter region of the apoA-I gene resulting in increased transcription and high HDL concentrations [30]. The physiological function of apoA-I has been very well characterised *in vivo*. Overexpression of apoA-I in transgenic mice increases HDL levels in these animals and protects against the development of cardiovascular disease [31].

Apolipoprotein A-II – ApoA-II is a 77 amino acid protein with a molecular mass of 17.4 kDa. It is synthesized in the liver and found mainly on HDL particles. ApoA-II has a high affinity

for lipid but can also be found in the plasma as a dimer connected by disulphide bridges [32]. ApoA-II is known to displace apoA-I from HDL [33,34] and affects the structure and stability of apoA-I, making it more readily exchangeable [33]. ApoA-II has been shown to regulate the activity of a number of plasma enzymes involved in lipoprotein metabolism. ApoA-II in HDL inhibits the plasma enzymes LCAT [34] and cholesteryl ester transfer protein (CETP) [35,36] as well as the TG-hydrolyzing enzyme lipoprotein lipase (LPL) [37]. Reports on the effect of apoA-II on the lipase HL are inconsistent, some showing an inhibition [38-40] while others indicate an activation of lipolytic activity [41,42]. Overexpression of apoA-II in mice causes atherosclerosis [43] and hypertriglyceridemia [44], although not all studies have suggested an association between apoA-II levels and cardiovascular disease [45].

Apolipoprotein A-IV – ApoA-IV is produced in the liver and intestine and is found on chylomicrons, VLDL and HDL [46]. Once in the plasma, apoA-IV rapidly dissociates from chylomicrons and forms homodimers because of its weak interaction with lipid [47]. The primary function of this protein is believed to involve the synthesis and secretion of TG-rich lipoproteins [48]. Levels of apoA-IV are positively correlated with plasma TG levels [49] and its concentration increases during a postprandial response [50,51]. ApoA-IV has also been shown to promote phospholipid and cholesterol efflux from cell membranes [52] and to play a role in the activation of LCAT and CETP [53,54]. It was also reported that apoA-IV may have anti-atherogenic properties through inhibition of LDL oxidation [55], an important step in the development of atherosclerosis.

Apolipoprotein A-V – ApoA-V is a recently discovered apolipoprotein that appears to be an important regulator of plasma TG levels [56-60]. ApoA-V is synthesized and secreted solely by the liver as a 363 amino acid protein with a molecular mass of 39 kDa [57]. The structure of

apoA-V is typical of other exchangeable apolipoproteins. Once in the circulation it is associated predominantly with large HDL and VLDL particles [57]. ApoA-V knockout mice show a 4-fold increase in plasma TG levels [57]. Overexpression of apoA-V in mice reduces cholesterol and TG in the plasma through inhibition of VLDL synthesis and appears to have no effect on LPL-mediated hydrolysis of VLDL [58,60]. Reports also indicate that apoA-V stimulates the binding and uptake of chylomicrons and VLDL by the liver via interaction with extracellular matrix heparan sulphate proteoglycans (HSPG) [60]. The role of this apolipoprotein in TG metabolism is an active area of investigation.

Apolipoprotein C-I, C-II and C-III - Members of the apoC family are exchangeable apolipoproteins that have similar molecular weights but share no sequence homology. The molecular masses of apoC-I, apoC-II and apoC-III are 6.6, 8.2 and 8.8 kDa, respectively, and these proteins are found primarily on chylomicrons, VLDL and HDL. ApoC-I constitutes around 2% of HDL protein and has been shown to stimulate LCAT activity and HDL formation [61]. The apoC-I content of postprandial TG-rich lipoproteins is also a risk factor for atherosclerosis [62]. ApoC-II is a known co-factor for LPL, stimulating the lipolytic activity of this enzyme and the hydrolysis of TG-rich lipoproteins. Approximately 10-20 apoC-II molecules are present on the surface of each chylomicron or VLDL particle and constitute around 10% of VLDL protein [63]. Inactivating apoC-II mutations or lack of this apolipoprotein on chylomicrons or VLDL result in defective LPL hydrolysis and severe hypertriglyceridemia [64,65]. In contrast, apoC-III is an inhibitor of LPL and HL activity and constitutes approximately 50% of VLDL and 2% of HDL protein [63]. ApoC-III has also been shown to delay TG-rich lipoprotein clearance and uptake by the liver [66].

Apolipoprotein E – ApoE is 299 amino acid glycoprotein with a molecular mass of 34.2 kDa. This apolipoprotein is found on chylomicrons, VLDL, intermediate density lipoproteins (IDL) and HDL and readily exchanges between these lipoproteins. ApoE constitutes around 10-20% of VLDL and 1-2% of HDL protein [63]. The residence time of apoE in the plasma is estimated to be less than one day [67]. ApoE is synthesized primarily in the parenchymal cells of the liver and astrocytes of the brain [68]. Cerebral spinal fluid contains significant amounts of apoE-containing HDL particles [69]. In the brain, it is believed that apoE is responsible for the clearance of lipid during neuron degeneration and delivery of lipid to cell membranes in need of repair [70]. Studies have shown that 20-40% of apoE is also produced in extra-hepatic tissues such as macrophages, spleen, lung, adrenals, ovaries, kidney and muscle [68,69]. The protein is secreted and associates with lipid and lipoproteins via lipid-binding domains at the C-terminus. ApoE plays a central role in cholesterol uptake and is a ligand for lipoprotein receptors such as the LDL-receptor (LDLR), LDL receptor-related protein (LRP) and HSPG [70]. ApoE gene knockout causes severe hypertriglyceridemia and atherosclerosis in animal models due to reduced or delayed removal of atherogenic lipoproteins from the bloodstream [71].

Three isoforms of the protein have been described that differ in the amino acids at positions 112 and 158 of the apoE protein. At these residues, apoE2 contains two cysteines, apoE3 has cysteine and arginine, whereas apoE4 contains two arginines. Alzheimer disease has been linked to specific isoforms of apoE, with up to 80% of Alzheimer patients having at least one copy of the apoE4 allele [72]. The apoE isoforms also preferentially associate with different types of lipoproteins. ApoE2 and apoE3 are found more in HDL while apoE4 associates predominantly with VLDL [70]. Individuals that are homozygous for the apoE2 allele typically

have high lipoprotein TG and cholesterol levels in the plasma because apoE2 does not bind the LDLR very efficiently.

Apolipoprotein B – The human apoB gene is 43 kb long, contains 29 exons and 28 introns and codes for a 4563 amino acid protein [21,73]. It is one of the longest known single chain polypeptide sequences and has a molecular mass of 513 kDa [21]. Two forms of the protein are present in the human body and are products of the same gene. One is the full length apoB100 that is produced almost exclusively in the liver and the other is a truncated form called apoB48 that is produced through an RNA editing mechanism. ApoB48 is synthesized in the intestine where a cytidine deaminase, apoB mRNA-editing enzyme catalytic polypeptide-1 (APOBEC-1), post-transcriptionally de-aminates apoB mRNA at cytidine 6666 to produce a stop codon [74]. ApoB48 represents the N-terminal 48% of the full length protein and has a molecular mass of 264 kDa. ApoB48 is found uniquely on chylomicrons secreted by the intestine whereas apoB100 is the major protein component of VLDL, IDL and LDL. The N-terminus of apoB contains a globular α-helical-rich domain with stabilizing disulphide bonds [75] while the rest of the protein contains two amphipathic β-sheets and additional α-helices responsible for lipid binding [76]. ApoB is a non-exchangeable apolipoprotein because of long stretches of hydrophobic sequences which allow for tight binding to lipid.

Abetalipoproteinemia is a rare genetic condition in which individuals affected have very low or undetectable levels of circulating apoB. As such, plasma cholesterol and TG levels are very low with an absence of chylomicron, VLDL or LDL particles. Abetalipoproteinemia is normally the result of mutations in the microsomal triglyceride transfer protein (MTP) gene that codes for a protein responsible for the intracellular lipidation, assembly and secretion of apoB-

containing lipoproteins. ApoB point mutations or mutations resulting in truncated forms of the protein cause a similar disorder called familial hypobetalipoproteinemia. These individuals have low levels of cholesterol and LDL in the plasma and have a reduced risk of atherosclerosis [77,78].

1.5 - Lipoprotein properties and metabolism

The lipid and protein composition of lipoproteins regulates both their function and metabolic fate in the plasma. Lipoprotein metabolism is complex and involves several plasma enzymes responsible for the synthesis, hydrolysis, conversion and clearance of all lipoprotein classes from the bloodstream. **Figure 2** depicts an overview of the metabolism of lipoproteins by various plasma enzymes.

Chylomicrons – Chylomicrons contain a TG-rich core and are the largest and least dense lipoprotein class. ApoB48 is their main apolipoprotein component, but smaller amounts of apoA-I, apoE and apoCs are also present on these lipoproteins. As indicated in **Table 2**, TG accounts for 93% of chylomicron mass, whereas protein accounts for only 2% of particle weight. Phospholipids are a minor component (< 5%) of chylomicrons and are composed of approximately 80% phosphatidylcholine (PC), as indicated in **Table 3**. The primary function of chylomicrons is to deliver dietary TG from the intestine to the liver and other tissues of the body. However, chylomicrons also transport significant amounts of cholesterol in the bloodstream. While the relative proportion of cholesterol is small compared to TG, it is estimated that these lipoproteins contain 40 times more cholesterol per particle than LDL [79]. Chylomicrons also transport a number of lipid-soluble vitamins and anti-oxidants such as α-tocopherol and β-carotene [80].

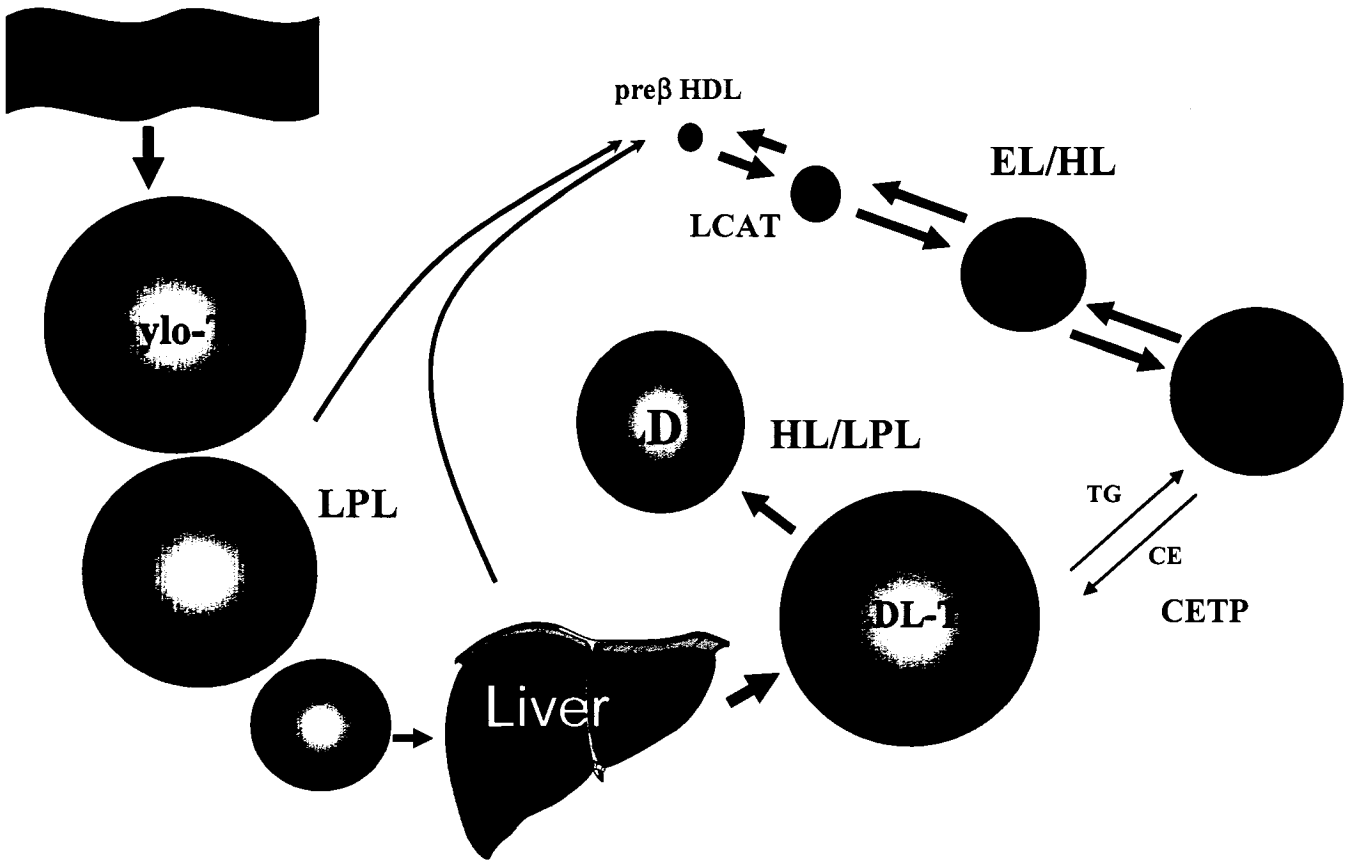


Figure 2. Overview of lipoprotein metabolism. Exogenous dietary lipids are secreted into the lymph in the form of TG-rich chylomicrons. Once in the plasma, chylomicrons are hydrolyzed through the lipolytic activity of the enzyme LPL. Hydrolysis releases free fatty acids that are taken up by cells and used for energy storage and metabolism. Hydrolysis of chylomicrons eventually depletes the core of these particles resulting in the formation of remnants that are rapidly cleared from the circulation by the liver. Endogenous lipids are secreted into the circulation by the liver in the form of TG-rich VLDL particles. VLDL are acted upon in the plasma by the enzymes LPL and HL resulting in the formation of LDL particles. The catabolism of chylomicrons and VLDL results in the release of apolipoprotein and lipid components that are used for the subsequent formation of HDL particles. Cholesterol and other lipids are removed from the tissues of the body by apoA-I to form lipid-poor preβ-HDL. These lipoproteins are acted upon by LCAT to form larger and more heterogenous HDL particles. The larger HDL_2 are then acted upon by HL and EL which hydrolyze these lipoproteins to smaller HDL_3. The enzyme CETP transfers CE from HDL to the TG-rich lipoproteins in exchange for TG. Chylo, chylomicron; TG, triacylglyceride; LPL, lipoprotein lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; HL, hepatic lipase; EL, endothelial lipase; LCAT, lecithin:cholesterol acyl transferase; CETP, cholesteryl ester transfer protein.

Table 3 – Phospholipid Composition of the Major Lipoprotein Classes (% of total phospholipids)

Phospholipid	Chylomicron	VLDL	LDL	HDL_2	HDL_3
PC	78.5	59.7	63.7	73.8	77.1
Lyso PC	4.2	5	2.7	2	4.0
SM	11.7	14.8	25.9	14.5	9.2
PE	5.6	4.6	2.2	3.3	2.5
PI	< 1	3.6	1.6	2.4	2.4
PS	< 1	1.5	0.8	0.9	0.6
PA	< 1	7.6	2.0	2.2	2.0

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid. Adapted from Chapman *et al.*, 1986 [82].

Intestinal lipoprotein assembly occurs through a mechanism that has not yet been fully elucidated. Dietary fat is first emulsified and hydrolyzed in the intestinal lumen and taken up by enterocytes. The lipids are then re-synthesized in the enterocytes and packaged into secreted chylomicrons. ApoB48 and the intracellular enzyme MTP are responsible for chylomicron synthesis and secretion [81]. MTP contributes to TG-rich lipoprotein synthesis by transferring neutral lipids such as TG and CE to apoB in the ER and Golgi apparatus [83]. MTP has been shown to be absolutely required for both chylomicron and VLDL synthesis and secretion and thus plays an essential role in the regulation of plasma lipoprotein concentrations. Newly synthesized chylomicrons are then released into the mesenteric lymph by exocytic secretion. Once in the lymphatic system, the chylomicron particles are released via the thoracic duct into the jugular vein and plasma. The size and composition of chylomicrons depends mostly on the types of dietary lipids ingested [84] and the residence time of chylomicron-TG in the bloodstream is approximately 10 minutes [21]. Chylomicrons can, however, remain in the plasma for up to 10 hours after a meal and may increase plasma TG levels up to 2 mmol/L. The magnitude and duration of this postprandial response have been correlated with cardiovascular disease [85-87].

Once in the plasma, chylomicrons are metabolized by a number of lipases and lipid transfer proteins. LPL and HL hydrolyse the TG core of these particles to produce free fatty acids for storage and energy metabolism, while CETP transfers CE to chylomicrons from HDL in exchange for TG. The metabolism of chylomicrons produces phospholipid, cholesterol and apolipoprotein components that are released into the circulation and can be used as building blocks for the synthesis of other lipoproteins such as HDL. It is well known that HDL levels are correlated with TG-rich lipoprotein hydrolysis due to the fact that chylomicron and VLDL

catabolism result in the release of protein and lipid components that can be used for HDL synthesis [8]. During catabolism, chylomicrons also acquire apoE and apoCs through transfer from HDL. Ultimately, LPL can remove as much as 70-80% of the TG mass of chylomicrons in a short period of time during the postprandial response [21]. As the TG core becomes progressively hydrolysed, the chylomicrons become smaller and eventually form lipoprotein particles called chylomicron remnants. These remnants are rapidly removed from the bloodstream by LRP and the LDLR [88-91]. LRP, a member of the LDLR family, is a large cell-surface protein expressed in the liver with multiple ligand-binding domains that recognize apoE on chylomicrons and VLDL [92]. LRP is the primary receptor involved in remnant clearance from the bloodstream [93]. The LDLR is a 160 kDa glycosylated membrane protein that recognizes apoE on chylomicrons and apoB100 or apoE on VLDL and LDL particles through a negatively charged extracellular ligand-binding domain [94]. Once the receptor and its ligand are internalized, the lipoprotein is degraded in acidic endosomes while the LDLR is recycled back to the cell-surface [95]. Cell-surface expression of the LDLR is regulated by a feedback mechanism that is crucial to the maintenance of cellular cholesterol homeostasis [96]. HSPG, LPL and HL have also been shown to play a role as ligands in receptor-mediated chylomicron remnant clearance from the bloodstream [97-100].

Very low density lipoprotein – VLDL are large TG-rich lipoproteins that contain apoB100 as their primary apolipoprotein component. These lipoproteins also contain smaller amounts of apoA-I, apoCs and apoE, all of which are important for VLDL function and metabolism. The neutral lipid core of these particles contains TG and CE which represent 53% and 14% of VLDL mass, respectively. As indicated in **Table 3**, VLDL phospholipid composition is primarily PC (~ 60%) and sphingomyelin (SM) (~ 15%). VLDL particles are synthesized by

the liver and are responsible for transporting endogenously produced TG and cholesterol through the bloodstream to the tissues of the body. Dietary carbohydrates and fatty acids accumulate in the liver and can be converted and stored as TG. Depending on metabolic requirements, stored hepatic TG and apoB100 are converted into VLDL and secreted. The intracellular synthesis and secretion of VLDL are similar to that of chylomicrons, requiring lipidation of apoB100 by MTP. Once in the plasma, VLDL can acquire apoC, apoE and CE from HDL. VLDL are substrates for various plasma enzymes such as the lipases and lipid transfer proteins. LPL and HL hydrolyse the TG and phospholipid in VLDL whereas CETP exchanges VLDL-TG for LDL- and HDL-CE. VLDL can subsequently be catabolized into IDL and LDL via the lipolytic activity of HL or into lipid-depleted lipoprotein particles called VLDL remnants that are cleared from the bloodstream.

Clearance of VLDL remnants from the circulation occurs primarily via the liver, with small amounts (< 5%) being taken up by extra-hepatic tissues [101]. Several receptors have been shown to mediate clearance of remnants via apoE including the LDLR, LRP and HSPG [102]. Much like for chylomicron clearance, ligands such as LPL and HL enhance interaction of VLDL and VLDL remnants with cell-surface receptors and promote their removal from the plasma [102,103]. The VLDL receptor (VLDLR), found in extra-hepatic tissues such as heart, muscle and adipose tissues also plays an important role in VLDL clearance [104]. This receptor binds apoE-containing lipoproteins and can function more efficiently through interaction with LPL and HSPG [105]. Studies show that delayed clearance of circulating remnant lipoproteins in the postprandial state is a risk factor for hyperlipidemia, diabetes and atherosclerosis [106-109]. Sub-populations of VLDL remnants appear to differ in their atherogenicity. Large, TG-rich, CE-poor VLDL have been shown to be less atherogenic than smaller, CE-rich VLDL [21]. The VLDLR is also expressed in macrophages in atherogenic lesions and promotes the accumulation of

cholesterol and the development of atherosclerosis [110]. The longer residence time of these TG and cholesterol-rich lipoproteins is believed to promote the accumulation of cholesterol in the arterial intima and the development of atherosclerosis.

Intermediate density lipoprotein – The metabolism of VLDL in the circulation results in the formation of a lipoprotein particle with an intermediate density called IDL. IDL contain apoB100 and apoE with almost equal amounts of TG and CE within their core [111]. The function of these particles is uncertain. IDL are transient, short-lived particles found at low concentrations in the bloodstream and are readily hydrolysed by HL, contributing to the formation of LDL particles. In fact, HL deficiency leads to accumulation of IDL *in vivo* [112,113]. IDL levels and delayed clearance from the plasma have been linked to an increased risk of atherosclerosis [114,115].

Low density lipoprotein – LDL are the primary transporters of cholesterol in the plasma. These lipoproteins contain a core of CE and only one surface molecule of apoB100 per particle. LDL are a heterogeneous population of particles that differ in size, density, composition, surface charge and apoB conformation. This lipoprotein class is divided into light (ρ = 1.02-1.03 g/ml), intermediate (ρ = 1.03-1.04 g/ml) and dense (ρ = 1.04-1.06 g/ml) LDL subpopulations [116]. The sizes of light, intermediate and dense LDL are 27.0, 26.6 and 26.0 nm in diameter, respectively [116]. The specific functions of the different LDL sub-species are unknown, however, they appear to differ in their atherogenic potential. In particular, the small, dense LDL are closely linked to the development of cardiovascular disease because they can easily enter the artery wall and are more readily oxidized [117,118].

LDL are produced through the hydrolysis of VLDL and IDL by lipase-mediated hydrolysis. Once formed, LDL deliver cholesterol to the different tissues of the body via receptor-mediated endocytosis. The LDLR, which recognizes apoB100 and apoE, is the primary mediator of the intracellular uptake and clearance of LDL from the plasma. Familial hypercholesterolemia (FH) is a disorder attributed to mutations in the LDLR and results in abnormally high plasma levels of LDL. Over 250 mutations in the LDLR have been described to date, affecting approximately 0.2% of the population [119]. Defects in the LDLR result in an inability of the liver to remove circulating LDL from the plasma and therefore lead to the development of cardiovascular disease [120]. Heterozygous mutations generally result in plasma LDL levels of 8-15 mmol/L, while homozygous mutations result in very high LDL levels of 15-25 mmol/L [120]. LDL have long been known to be correlated with an increased risk of atherosclerosis [121,122]. The proposed mechanism of atherogenicity is believed to be through excessive accumulation and oxidation of LDL in the vessel walls of the cardiovascular system. Once LDL enters the intima of the artery wall, it can become oxidized and very efficiently taken up by macrophages. Uncontrolled lipid accumulation by these macrophages leads to the development of cholesterol-loaded foam cells and the eventual formation of a fibrous plaque and blockage of the blood vessel lumen. Thus, high levels of LDL in the circulation and/or delayed clearance promote lipid uptake, accumulation and oxidation in the intima of the arterial wall [123] and facilitate the progression of atherosclerosis [124,125].

High density lipoprotein – HDL particles contain primarily apoA-I and phospholipid in the outer surface layer with significant amounts of CE in the neutral lipid core. HDL are the smallest and most dense lipoprotein with as much as 40-50% of their total weight due to protein content. The apolipoprotein composition of HDL is approximately 90% apoA-I and apoA-II [21].

However, apoCs, apoE, apoA-IV and apoA-V are also important protein components of HDL particles. As indicated in **Table 2**, HDL lipid composition is approximately 30% phospholipid and almost 20% CE by weight. PC is the most abundant phospholipid in HDL, representing over 70% of the total phospholipid content. In HDL, PC is found mainly as palmitoyllinoleoyl phospatidylcholine (PLnPC) (34.4%), palmitoyloleoyl phospatidylcholine (POPC) (12.9%), palmitoyl arachidonoyl phosphatidylcholine (PAPC) (9.1%) and palmitoyl docosahexanoyl phosphatidylcholine (PDPC) (3.6%) [126]. While acylglycerides represent less than 2% of total HDL weight, they are important determinants of HDL metabolism. TG-rich HDL are cleared more rapidly from the circulation and are much better substrates for hydrolysis by HL. HDL diacylglyceride (DG) content has been reported to be underestimated and may be the major acylglyceride in HDL [127]. HDL also contain small amounts of free fatty acids (between 0.6 - 4.4% of weight), which may play an important role in the electrostatic properties of this lipoprotein class [126,128]. A number of other proteins are associated with HDL including the plasma enzymes CETP, LCAT, phospholipid transfer protein (PLTP) and HL [41,129-131]. The anti-oxidant protein paraoxonase is also transported by HDL in the plasma and confers anti-inflammatory and anti-oxidant properties to this lipoprotein [132,133].

HDL are a very heterogeneous group of particles that are usually divided into HDL_2, HDL_3 and very high density lipoprotein (VHDL) sub-populations. HDL_2 are larger, less dense particles that contain more phospholipid, CE and TG than the other types of HDL and have a higher ratio of apoA-I to apoA-II. In contrast, HDL_3 are smaller and more dense particles with a higher ratio of apoA-II to apoA-I. VHDL are the smallest and most dense HDL sub-population and are isolated within the density range 1.21 g/ml $< \rho <$ 1.25 g/ml. HDL are also classified

according to their apolipoprotein composition. These lipoproteins can be divided into particles containing only apoA-I, those containing apoA-I and apoA-II and, more rarely, those containing only apoA-II [134,135]. Most of the apoA-II, however, is in HDL that contain both apoA-I and apoA-II. Other classification systems are also widely used to define different HDL sub-populations based on size and charge. HDL can been classified into four groups (α, pre-β, pre-α and γ) according to surface charge as determined by agarose gel electrophoresis [136,137]. Using this system, α-migrating HDL represent the HDL_2 and HDL_3 sub-populations whereas the preβ-HDL are small, lipid-poor particles that contain 1-2 molecules of apoA-I with PC, SM and FC [138]. Preα-HDL typically contain only apoA-I and γ-HDL contain only apoE.

The synthesis of HDL is a multi-step process and an important component of the RCT pathway. The formation of HDL begins with the generation of lipid-poor apoA-I through two possible mechanisms. Lipid-poor apoA-I can either be synthesized and secreted by the liver and intestine [139,140] or generated from the remnants of lipoprotein catabolism in the plasma [141,142]. This preβ-HDL is discoidal in shape and contains 1-2 molecules of apoA-I with small amounts of phospholipid and FC. The next step in the formation of HDL involves the accumulation of additional phospholipid and FC and the conversion of these particles into spherical mature HDL by the enzyme LCAT. Preβ-HDL acquire more FC and phospholipid from cell membranes in the extra-vascular space by interacting with the cell-surface transporters ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) [143-145]. These proteins facilitate the diffusion of intracellular and membrane lipids to apoA-I or lipid-poor HDL and are expressed at high levels in macrophages [145]. Ultimately, ABCA1 promotes the removal of cholesterol from extra-hepatic tissues to HDL for delivery to the liver

for excretion into the bile [145]. Knockout of ABCA1 in mice results in reduced levels of HDL and significantly hinders RCT [146]. In humans, Tangier disease, caused by mutations in ABCA1, results in very low HDL levels [147]. While the transfer and accumulation of lipid in increases particle size and plasma levels of preβ-HDL, further maturation requires the activation of LCAT. LCAT esterifies the FC at the surface of preβ-HDL, forming a spherical particle with a core of CE. Thus, this enzyme converts the initially discoidal particles into mature spherical HDL [148,149]. The maturation of HDL by LCAT increases the size and concentration of HDL particles in the bloodstream and is a major determinant of HDL levels. The rate of HDL synthesis is therefore determined by the rate of apoA-I synthesis and secretion into the plasma and by the LCAT-catalyzed production of mature HDL [148,149].

HDL metabolism is regulated by a number of factors that influence the heterogeneity of HDL in the plasma. The transfer and exchange of apolipoproteins between lipoproteins classes contributes significantly to the changing protein composition of HDL. Moreover, many plasma enzymes are involved in the hydrolysis and transfer of HDL lipids. The lipolytic enzymes HL and EL hydrolyse the TG and phospholipid found in HDL and reduce both the size and concentration of these particles in the plasma. Both enzymes convert the larger HDL_2 into small HDL_3 and are directly linked to low HDL levels *in vivo*. HDL structure and composition are also modified by the lipid transfer proteins. CETP transfers the CE contained within the core of HDL particles to chylomicrons and VLDL in exchange for TG, whereas PLTP regulates phospholipid transfer between these lipoproteins. High CETP activity has been indirectly linked to reductions in HDL levels because the TG-enriched HDL particles generated by this enzyme are very good substrates for HL and are rapidly cleared from the plasma [8].

HDL are primarily removed from the circulation by the liver. The CE in HDL can be taken up into cells through various receptor-mediated pathways. The liver and steroidogenic tissues contain an HDL cell-surface receptor called scavenger receptor-B1 (SR-BI) in mice [150], that mediates the uptake of CE from HDL for excretion [151]. The CD36 and LIMPII analogous-1 (CLA-1) receptor, the human version of SR-BI, has not been extensively characterized but is believed to have a similar function [152]. Overexpression of SR-BI prevents the development of atherosclerosis [153] and suggests that the receptor plays an important role in RCT. CETP and LRP have also been shown to mediate the selective uptake of HDL-CE by the liver and may play a role in cholesterol removal and excretion [154,155]. The role of HDL in RCT may explain why levels of this lipoprotein are inversely correlated with cardiovascular disease and the development of atherosclerosis [156]. The risk of developing cardiovascular disease increases by up to 3% for every 1% reduction in HDL-cholesterol [157,158]. Moreover, low HDL levels are often associated with hypertriglyceridemia and postprandial lipemia, further increasing the risk of atherosclerosis [159,160]. Thus, the regulation of HDL synthesis and catabolism are important determinants of human health and disease. It is now clear that HDL and lipoprotein composition have a profound impact on the metabolism of these particles under both normal and dyslipidemic conditions.

1.6 – Lipoprotein charge

Lipoproteins have a net negative charge that is believed to influence their metabolism in the bloodstream. It has been known for decades that patients with hypercholesterolemia have lipoproteins that are abnormally charged [161]. It is not known whether abnormal metabolism is the cause or consequence of the differences observed in lipoprotein charge between healthy and individuals with dyslipidemia. More recently, LDL positive charge has also been linked to the

development of diabetes [162] and many studies have shown that the electrostatic properties of HDL are a major determinant of their metabolism both *in vitro* and *in vivo* [163-165].

The net charge of a lipoprotein particle can be measured by its electrophoretic mobility in an agarose gel, as described by Sparks and Phillips [380]. Net lipoprotein charge is a consequence of the charge density and therefore proportional to the number of exposed electrons and the surface area of the lipoprotein complex. The electrostatic properties of lipoproteins can be defined in terms of net charge (electrons) or as a functional characteristic, surface potential, expressed in voltage (mV). Lipoprotein surface potential is commonly reported as it closely reflects the electrophoretic mobility and charge density on the surface of a the lipoprotein particle.

Lipoprotein charge is determined primarily by acidic amino acids in the apolipoproteins and also by anionic lipid components [126]. Lipid and protein composition influence not only the function of lipoproteins but also determine their physical properties. Apolipoprotein conformation which is influenced by the composition of core neutral lipids as well as free cholesterol is also an important determinant of the charge properties of HDL [126]. Increasing amounts of DG and TG within the core of reconstituted HDL have been shown to contribute to HDL charge and to enhance their clearance from the bloodstream [166,167]. Phosphatidylinositol (PI), phosphatidylethanolamine (PE) and oleic acid are the primary lipids affecting the anionic properties of HDL [126]. It has been estimated that each molecule of PI in HDL contributes 0.4 electrons (–0.3 mV) to a reconstituted HDL particle [126]. In contrast, PC and SM do not affect the charge properties of HDL [126].

The electrostatic properties of lipoproteins have been shown to affect their interaction and metabolism by a number of plasma enzymes including LCAT, CETP and PLTP [168-170]. The

effect of electrostatic properties on phospholipases acting on phospholipid vesicles has also been extensively characterised [171,172]. Many of these studies show that negatively charged vesicle substrates stimulate hydrolytic activity by reducing the binding of the enzyme to the substrate interface. This is consistent with the view that interfacial enzymes such as the lipases must shuttle rapidly between substrates for efficient catalytic activity [173]. The charge properties of lipoproteins must, therefore, have a significant effect on their metabolism by enzymes in the bloodstream.

1.7 – Interfacial enzymes

Enzymes acting at a water-lipid interface have very distinct properties from aqueous-soluble proteins. Most of the important plasma proteins such as CETP, LCAT and the lipases are interfacial enzymes and do not strictly conform to conventional Michaelis-Menten kinetics. This is due to the fact that the enzyme and substrate are not found in the same phase [174]. The surface of a lipoprotein substrate is composed of water and insoluble lipid phases and is an example of a complex interfacial environment. Interfacial enzymes are usually water-soluble and must first associate with and penetrate the lipid interface in a process called adsorption. Enzyme adsorption or penetration is a reversible process and is usually linked to a conformational change in the protein which exposes catalytic or substrate binding sites [174-177]. **Figure 3** depicts an interfacial enzyme binding to the surface of a lipoprotein, resulting in a conformational change in the protein, enabling it to access substrates in the core of the particle. The enzyme can then bind a specific substrate to form the traditional Michaelis-Menten enzyme complex [174]. The following equation is often used to describe the kinetics of interfacial enzyme activation, where E is the enzyme, E^* is the activated enzyme, E^*S is the enzyme-substrate complex, P is the product, and k represents the rate constants for each step [178]:

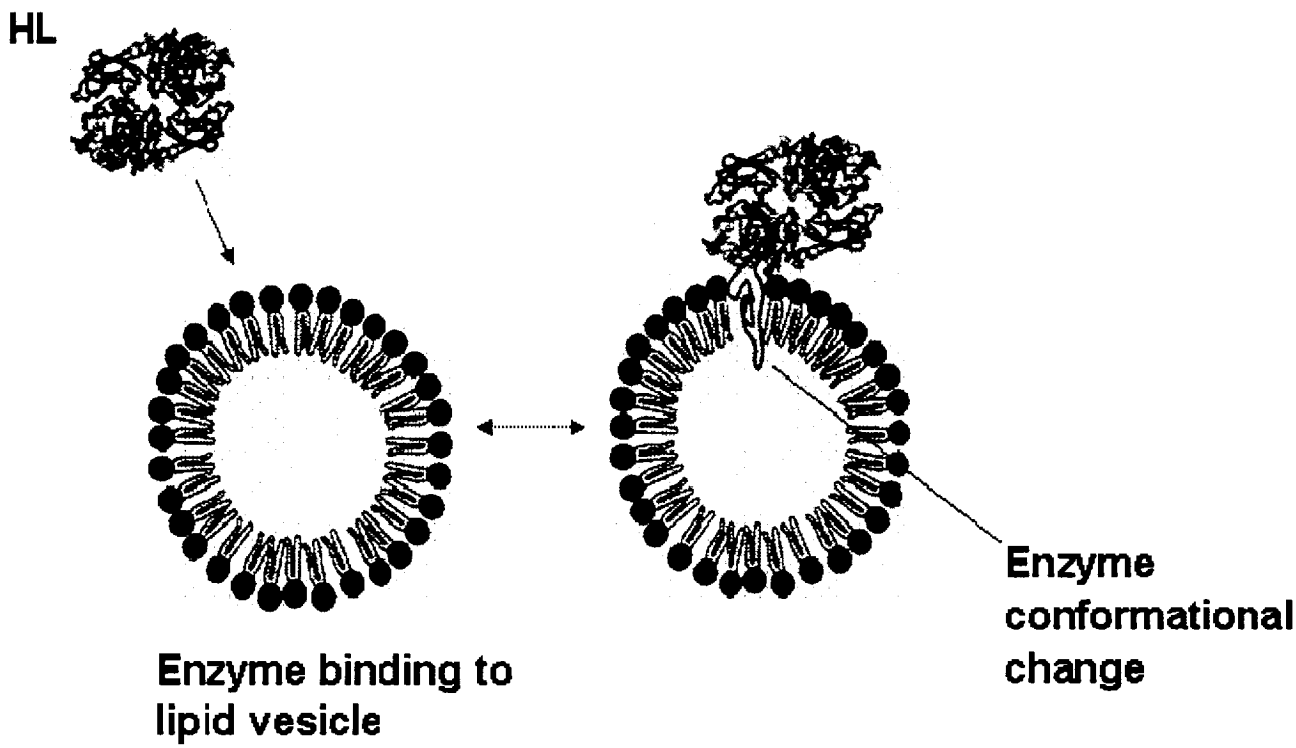
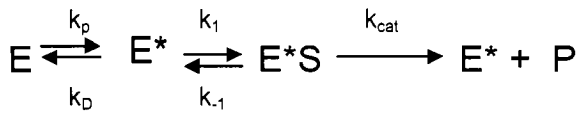


Figure 3. The interfacial binding and activation of HL. The binding of HL to the interface of a lipoprotein particle induces the activation of the enzyme's hydrolytic activity. Interfacial activation is associated with a conformational change that allows the enzyme to access substrates within the hydrophobic core of the lipoprotein. Properties such as electrostatic charge, apolipoprotein composition and lipid packing density of the interface, are believed to affect enzyme interaction with the particle and hydrolytic activity. HL, hepatic lipase.



Jain *et al.* first characterised the properties of enzymes acting at an interface and proposed the scooting and hopping models of interfacial hydrolysis [179]. In the scooting mode, the enzyme binds irreversibly to the interface and is active over the entire surface without dissociating from the larger structure. Thus, all the substrates on a particular vesicle/lipoprotein are available for hydrolysis. In the hopping mode, the enzyme binds but quickly desorbs from the interface after each or every few hydrolytic cycles. In this case, all the substrates on all vesicles/lipoproteins are available for hydrolysis since the enzyme is exchanged between substrates [179]. Weak enzyme binding to the interface is, therefore, associated with efficient hydrolytic activity. Initially, this was believed to be inefficient due to the possibility that the adsorption/desorption process was rate limiting. However, several human lipase kinetic studies confirm the hopping model and show that enhanced enzyme activity is directly linked to increases in V_{max} and apparent K_m values [38,173,180,181]. In contrast, many of the fungal and bacterial lipases are believed to function in the scooting mode of hydrolysis [180]. It would therefore appear that plasma enzymes such as HL and LPL need to rapidly shuttle between substrates to efficiently hydrolyse their lipid substrates. An enzyme fixed at the cell surface seems more unlikely to encounter a substrate. Thus, in the case of HL, displacement from the cell-surface would likely allow the enzyme to move freely between substrates and increase the probability of encountering a substrate for hydrolysis.

Interfacial binding and enzyme activation can be affected by a number of properties including adsorption/desorption, surface pressure, charge and composition. Interfacial enzymes are often regulated by surface pressure which is a measure of the order or packing of the lipid

molecules at the interface. In lipid monolayer studies, HL-mediated hydrolysis of phospholipid and TG has been shown to decrease rapidly with increases in lipid packing or surface pressure [182,183]. Many factors can influence the surface pressure of an interface, including acyl chain composition, apolipoproteins and lipid composition. Increasing surface pressure reduces the ability of enzymes to penetrate the interfacial surface and access hydrophobic substrates [182]. Verger *et al.* showed that pancreatic lipase, a lipid-metabolizing enzyme, cannot penetrate the interfacial surface at high surface pressures, reducing its catalytic efficiency [176]. Apolipoproteins also affect interfacial surface pressure and HL activity. Using PC monolayers, Jackson *et al.* showed that inclusion of apolipoproteins (apoC-II, apoC-III and apoA-I) increased surface pressure and lipid packing, inhibiting the catalytic activity of HL [184]. The mechanism of action for many interfacial enzymes has been fairly well-characterized. Studies with phospholipase A_2 (PLA_2) and pancreatic lipase suggest that vesicles composed of anionic phospholipids are much better substrates for these enzymes and act by affecting enzyme-vesicle electrostatic interaction [185-191]. However, the role of substrate charge properties on human plasma lipase activity and lipoprotein metabolism has not been extensively characterized. Many of the proteins involved in lipoprotein metabolism are interfacial enzymes and regulation of their catalytic activity is an active area of investigation.

1.8 - Plasma enzymes involved in lipoprotein metabolism

Lecithin:cholesterol acyl transferase – LCAT is a 416 amino acid enzyme with a molecular mass of 63 kDa responsible for the synthesis and maturation of HDL particles in the circulation [192]. It is synthesized almost exclusively in the liver and is found associated with HDL in the plasma [193]. The enzymatic reaction catalyzed by LCAT is a transesterification involving the transfer of an acyl group from the *sn*-2 position of PC to the 3-hydroxyl group of

cholesterol to form CE [193]. LCAT primarily acts on newly synthesized lipid-poor apoA-I discoidal HDL and converts FC on the surface of these particles to CE, generating a spherical HDL with a CE-core [193]. These spherical HDL can eventually accumulate more apoA-I and CE, forming larger mature HDL particles. HDL_3 are much better substrates for LCAT than HDL_2 and LCAT activity is significantly stimulated by apoA-I [194] and to a lesser extent by apoA-IV, apoC-I and apoE [53,195,196]. Human LCAT deficiency results in dramatic reductions in HDL lipid and apolipoprotein levels [197] with the presence of primarily discoidal HDL [198]. In contrast, transgenic rabbits and mice overexpressing human LCAT have significantly elevated HDL levels [199,200]. Thus, LCAT mediates the transport of cholesterol from peripheral tissues to the liver and is essential for the generation of HDL in the bloodstream.

Cholesteryl ester transfer protein – CETP transfers CE from HDL to VLDL, IDL and LDL in exchange for TG. The enzyme is a 476 amino acid glycoprotein synthesized in liver, adipose and muscle tissues [201]. CETP is primarily found in the plasma associated with HDL and contains a C-terminal domain with specific hydrophobic residues responsible for lipid transfer [202]. During the postprandial response, the increased presence of TG-rich lipoproteins in the plasma appears to enhance the transfer of TG to HDL [203]. The reaction catalyzed by CETP transfers more CE from HDL than TG is transferred out to other lipoproteins [204]. Thus, CETP depletes the CE core of HDL in exchange for TG, reducing particle size and possibly resulting in the dissociation of apoA-I [205]. Enrichment of HDL with TG enhances its hydrolysis by HL [8], further contributing to the reduction of HDL levels. CETP also affects HDL metabolism by acting as a ligand that mediates the selective uptake of CE from HDL to the liver [154,206]. The role of the ligand function of CETP in RCT as yet to be fully characterised. However, CETP is believed to be atherogenic due to its role in decreasing HDL levels and

enriching apoB-containing lipoproteins with CE [207]. CETP deficiency results in an increase in HDL_2-cholesterol and CE-poor/TG-rich LDL [208,209] and therapeutic CETP inhibitors have been shown to raise HDL levels in humans [210,211].

Phospholipid transfer protein – PLTP is a 476 amino acid protein with a 20% sequence similarity to CETP [212]. PLTP is responsible for the transfer of phospholipids to HDL from other lipoproteins such as VLDL and LDL. The importance of this enzyme in lipoprotein metabolism has yet to be fully determined. However, PLTP activity has been positively correlated with LDL levels as well as diabetes and insulin-resistance [213]. In addition, overexpression of human PLTP in mice resulted in decreased HDL levels and the development of atherosclerosis [214].

Lipases - The mammalian lipase superfamily of proteins includes pancreatic lipase, LPL, EL and HL [215-217]. It is believed that members of the lipase family are all derived from a common ancestral gene which subsequently diverged during evolution [218]. The plasma lipases (HL, LPL and EL) are responsible for the hydrolysis of acylglycerides and phospholipids in the circulation. Lipoprotein and lipid substrate preferences are the primary differences between these lipases. LPL prefers TG in chylomicron and VLDL, HL primarily hydrolyses TG and phospholipid in VLDL, IDL and HDL, while EL is mainly an HDL-specific phospholipase. HL differs from LPL in that it does not require Ca^{2+} or a co-factor (apoC-II) for hydrolysis and is not inhibited at 1 M NaCl or by protamine sulfate [11]. The lipases can associate with negatively charged molecules such as HSPG and are normally bound to the cell surface of the vascular endothelium via the extracellular matrix. Intraveinous injection of heparin causes the release of the lipases into the plasma, resulting in hydrolysis and clearance of lipoproteins from the circulation [219]. Lipases have conserved catalytic sites and a lid domain that regulates substrate

specificity and activity. These enzymes, which normally function as head-to-tail homodimers [220,221], contain a glycine-X-serine-X-glycine motif at the active site, forming part of a conserved aspartate-histidine-serine catalytic triad [222].

Lipoprotein lipase – LPL is a 448 amino acid, 55 kDa protein produced mainly in skeletal, cardiac and adipose tissues [223,224]. The kidneys, adrenal glands, brain and macrophages are also minor sites of protein synthesis [225]. During translation, LPL dimerizes and is glycosylated, both of which are required for secretion and enzymatic activity [226]. The C-terminus of the protein contains TG-rich lipoprotein binding (residues 387-394) and heparin-binding domains composed of positively charged (basic) amino acids (residues 278-282 and 292-300) [227]. Once in the circulation LPL is normally found attached to the extracellular matrix HSPG of the vascular endothelium [228,229]. However, active LPL is also found in the bloodstream associated with lipoproteins and in pre-heparin plasma [230]. Pre-heparin LPL is rapidly removed from the circulation by the liver via LRP or proteoglycans and has a rapid turnover *in vivo* [231]. Unlike the other lipases, LPL activity requires the presence of the protein co-factor, apoC-II, found on chylomicrons, VLDL and HDL. Familial LPL deficiency, resulting from rare homozygous or heterozygous mutations, causes very high chylomicron and plasma TG levels of up to 50-100 mmol/L [232,233]. In addition to its hydrolytic activity, LPL also functions as a cell-surface ligand that facilitates the uptake of chylomicron and VLDL remnants by the liver [234].

LPL has been described as being both pro- and anti-atherogenic. The atherogenic potential of LPL may be mediated through the ligand function of the enzyme, independent of catalytic activity [234,235]. Expression of LPL exclusively in macrophages leads to the development of atherosclerosis in mice by promoting lipid uptake and macrophage foam cell

formation [236]. In contrast, the anti-atherogenic properties of LPL expression are likely due to its role in reducing plasma TG levels. Expression and activity of LPL in heart, muscle and adipose tissues promote a non-atherogenic lipoprotein profile (low TG, low LDL and high HDL) and decreases the risk of atherosclerosis by lowering plasma TG levels [237-239]. Thus, the cell-type and site-specific expression of LPL appear to govern its atherogenic potential.

Endothelial lipase – EL was recently discovered and is now a known member of the TG-lipase family [240,241]. This lipase has conserved catalytic and structural motifs and shows 40-45% identity with both LPL and HL. The enzyme contains four clusters of putative heparin-binding regions and five N-linked glycosylation sites [242]. EL is synthesized in various tissues, however, its production in endothelial cells distinguishes it from the other lipases [240,241]. Much like other members of the lipase family, EL functions as a cell-surface receptor independent of its catalytic activity, facilitating the uptake of lipoproteins and HDL-lipids by the liver [243]. EL displays primarily HDL phospholipase activity and contributes significantly to HDL levels *in vivo* [215,244-246]. An inverse relationship exists between EL expression and plasma HDL-cholesterol concentration [247,248]. EL also hydrolyses TG and has been shown to be involved in the catabolism of apoB-containing lipoproteins [249]. The regulation of EL activity in the plasma is an active area of investigation and initial reports show that apoA-II expression in mice inhibits EL activity and HDL metabolism *in vivo* [250]. Since EL is a very important determinant of HDL levels, it is not surprising that pre- and post-heparin EL plasma concentrations are correlated with atherosclerosis [251].

1.9 - Hepatic lipase: Role in lipoprotein metabolism and disease

HL (EC 3.1.1.3) is a member of the lipase family and plays an important role in lipoprotein metabolism. HL hydrolyzes the acyl ester bonds of TG and phospholipid in all

lipoprotein classes with preferences for VLDL/IDL and TG-rich HDL_2 [39]. HL also functions as a cell-surface ligand, facilitating the uptake of lipoproteins by the liver [252]. The *in vivo* role of HL in lipoprotein metabolism has been well characterized in a number of genetic studies. HL contributes significantly to the regulation of plasma lipid and lipoprotein levels and plays a very important role in the development of atherosclerosis and cardiovascular disease. HL deficiency in humans results in an increase in plasma TG and cholesterol with an accumulation of VLDL, IDL and large HDL_2 [253-255]. In contrast, overexpression of HL in mice and rabbits is atherogenic [256] and causes low HDL and apoB-containing lipoprotein levels even when the enzyme is catalytically inactive [252].

The hepatic lipase gene and its expression

The human HL gene is composed of 8 introns and 9 exons spanning 35 kb, encoding a 1.5 kb mRNA [257-259]. Several transcription factors have been implicated in HL expression. Upstream stimulatory factor (USF), peroxisome proliferation-activated receptor γ coactivator-1α (PGC-1α) and both hepatocyte nuclear factor-1α and -4α (HNF-1α and HNF-4α) are positive regulators of HL expression [260]. In contrast, apolipoprotein regulatory protein-1 (ARP-1), estrogen receptor α (ERα), activator protein-1 (AP-1) and farnesoid X receptor (FXR) negatively regulate HL transcription [260].

A number of single nucleotide polymorphisms (SNPs) in the HL promoter region have been identified that have an impact on HL expression and activity *in vivo*. Four HL promoter SNPs have been characterised including –250G to A [261], –514C to T [262], –710T to C [263] and –763A to G [264]. The –514C to T polymorphism is directly linked to a reduction in HL transcription [265] and has been shown to have an effect on HL activity and lipoprotein

metabolism. In particular, the –514T allele is associated with an almost 40% reduction in HL activity and causing an accumulation of large LDL and HDL_2 [261]. Several regulatory elements have been described in the rat HL promoter, including sterol regulatory element (SRE), estrogen regulatory element (ERE), thyroid regulatory element (TRE) and glucocorticoid regulatory element (GRE) [259]. In addition, the HL promoter is under the control of cyclic adenosine monophosphate (cAMP) and E-box motifs, allowing for regulation of gene expression by glucose and insulin [265]. HL activity is also regulated by several different types of hormones that act through these regulatory elements. It has been known for some time that estrogen decreases post-heparin HL activity [259], whereas androgens, anabolic steroids and glucocorticoids increase HL activity [266-269]. Likewise, insulin, adiponectin and leptin also positively regulate HL transcription or activity [270-274]. Conflicting results on the effect of thyroid hormone have been reported, suggesting either a stimulatory or inhibitory role in the regulation of HL [266,275].

Hepatic lipase synthesis and glycosylation

HL is synthesized primarily in hepatocytes and is localized to hepatic sinusoids, microvilli of parenchymal cells and the sinusoidal endothelium [276,277]. HL is also found in the adrenal glands and ovaries of rats but evidence suggests, due to the lack of mRNA detection, that the enzyme is not synthesized in these tissues but migrates there after synthesis in the liver [278,279]. Recently, HL has been detected in macrophages [280], but the role of the enzyme in these cells has yet to be fully characterised. HL is a 476 amino acid protein with a molecular mass of approximately 65 kDa and shares 50% sequence similarity with LPL [281].

Human HL contains four glycosylation sites at residues 20, 56, 340 and 375 [282,283]. During synthesis, HL is glycosylated with mannose-containing oligosaccharides in the ER and sialic acid-containing oligosaccharides in the Golgi apparatus before rapid secretion of the mature

glycoprotein [284]. Association of HL with calnexin during maturation in the ER is required and enhances the rate of secretion of the protein [285,286]. Proper N-linked glycosylation, particularly at asparagine 56, has been shown to be required for secretion of the protein but not HL activity [282,287]. The mature protein is secreted from the cell as a homodimer and subsequently binds to the cell surface via the proteoglycan matrix. At the cell surface, HL can be rapidly released into the circulation by intraveinous injection of heparin, followed by hepatic uptake and degradation [288].

Protein structure and properties

The structure of the HL protein has been modelled based on the known crystal structure of pancreatic lipase [289,290]. **Figure 4** depicts the predicted structure of HL. Domain exchange studies have contributed significant insight into the functional domains of both HL and LPL. The C-terminus appears to be responsible for lipoprotein and heparin/HSPG binding while the N-terminus contains the active site and controls enzyme kinetic parameters [291,292]. The central domain of HL also contains 10 cysteine residues separated by conserved distances that are important for enzyme conformation and catalytic activity [259].

A number of functional properties are located at the C-terminus of HL, including HSPG/heparin and lipoprotein binding. HL has four heparin binding domains with the BBBXXB or BBXB consensus motifs that bind HSPG [293-296]. The fourth motif at lysine 337 to arginine 443 is not found in the murine HL [15], possibly explaining why the majority of the enzyme is free (not HSPG-bound) in the circulation of these animals. Interaction between the heparin-binding domains of HL and HSPG is believed to occur through an electrostatic interaction favouring a steric fit [295,297,298]. The last 70 amino acids at the C-terminus of HL, and specifically the last 5 residues, are important for binding to the cell-surface [296].

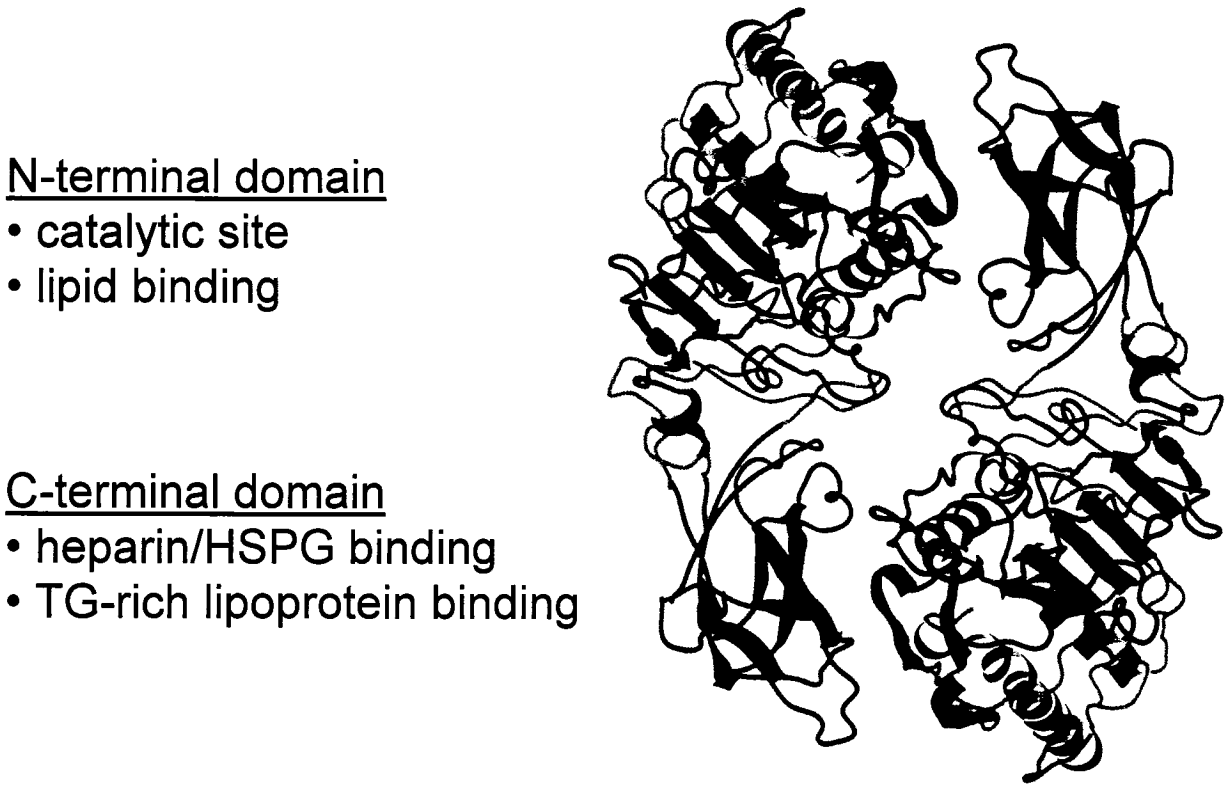


Figure 4. Model of the 3-dimensional structure of HL. HL is a head-to-tail homodimer with functionally important C- and N-terminal domains. The C-terminus of HL contains a heparin/HSPG-binding domain that is critical for the association of the enzyme with the cell-surface extracellular matrix. The C-terminal domain also contains a TG-rich lipoprotein binding domain that facilitates interaction between HL and lipoprotein substrates. The lid domain and catalytic site of HL are located at the N-terminal region of the protein. Adapted from van Tilbeurgh et al. [290,304].

HL dimers bind heparin much more strongly than the monomer and infusion of heparin is known to release HL from cell-surface HSPG and to significantly stimulate HL activity *in vivo* [219].

The N-terminus of HL contains the active site as well as sequences important for substrate binding. Serine 146 is the center of a glycine-X-serine-X-glycine motif that forms a conserved serine-aspartate-histidine catalytic triad common to other lipases [281]. HL is a serine hydrolase enzyme that functions through the formation of an acyl-serine enzyme intermediate [299]. Lipase activity can, therefore, be inhibited by diisopropylfluorophosphate which inhibits enzymes with an active site serine residue [300]. The lipases share a distinctive structure surrounding the active site called an α/β hydrolase fold that brings together the conserved pentapeptide residues of the active site and the serine-aspartate-histidine triad [301,302]. The active serine is located at a turn between a β-strand and an α-helix called the nucleophile elbow [302,303]. The active site is located in a hydrophobic cleft covered by a loop of 23 amino acids called the "lid" domain [306]. The basic structure of the lid domain is similar to a number of bacterial and fungal lipases [306]. Once the enzyme binds to the substrate interface, the lid rotates around a conserved disulfide bond exposing a hydrophobic lipid binding site [306]. In this way, the lid domain regulates access of substrates to the catalytic site. Differences in the lid domain may explain the substrate preferences of HL and LPL. Domain exchange studies showed that an HL chimera with an LPL lid had increased TG and reduced phospholipid hydrolase activity, suggesting that the lid domain confers substrate specificity [306]. *In vivo*, adenovirus-mediated expression of HL and LPL chimeras with exchanged lid domains confirmed that phospholipase activity is characteristic of HL [307].

Hepatic lipase and lipoprotein metabolism

HL has been shown to hydrolyze all classes of lipoproteins [308-312] and is an important plasma enzyme involved in both TG and HDL metabolism. The role of HL in HDL catalysis and remodelling has been well characterised both *in vitro* and *in vivo*. HL preferentially hydrolyzes the larger HDL_2 into the smaller more dense HDL_3, contributing significantly to HDL levels and speciation in plasma [313]. HDL_2 normally contain more TG than the smaller HDL_3 particles and it is well known that TG-enriched HDL are much better substrates for HL [8]. Similarly, studies showed that PC in HDL_2 is hydrolyzed much more readily than HDL_3-PC [314]. Thus, high plasma HL activity reduces HDL_2 and HDL-cholesterol levels and is an important determinant of a healthy plasma lipid and lipoprotein profile [315-319].

Hepatic lipase ligand function

HL also plays a very important role in the uptake and clearance of lipoproteins independently of its enzymatic activity [311]. Enhanced uptake of apoB-containing lipoproteins is mediated by a direct interaction between HL and the apoB protein [320,321] and can regulate lipoprotein clearance by the liver. HL deficiency was shown to hinder the clearance of chylomicrons and injection of HL antibodies into rats and mice caused a reduction in the uptake and removal of chylomicron remnants [322,323]. It is now clear that HL functions as an important ligand for almost every type of lipoprotein class in the plasma. The mechanism of HL-mediated uptake appears to be through a direct interaction with membrane receptors. Studies showed that HL enhanced the uptake of chylomicron remnants, HDL and LDL via LRP- and apoE-mediated pathways [321,324-326]. Likewise, HL enhanced LDL binding and degradation through the LDLR and facilitated CE uptake by the liver via SR-BI [320,324,327]. Thus, HL plays an important role in lipoprotein metabolism in addition to its enzymatic activity and

mediates the clearance of lipid from the bloodstream by enhancing the function of several lipoprotein receptors.

Lipid preferences and substrate properties

Acylglycerides – Monoacylglyceride (MG), DG, TG, phospholipid and CE have all been shown to be substrates of HL *in vitro* [328]. Studies of HL-mediated acylglyceride hydrolysis have resulted in conflicting reports on the substrate preferences of HL. Most studies indicate that TG is a better substrate for HL than either phospholipid or DG [159,329,330]. However, other reports have found that TG and DG are either hydrolyzed to the same extent [331] or that DG and phospholipid are the preferred substrates of HL [38,332]. In fact, some studies even showed that DG is a much better substrate for HL than many phospholipids including phosphatidic acid (PA), PE and PC [333]. The conflicting reports on the acylglyceride preferences of HL are most likely due to the variety of substrate and reaction conditions used (lipid monolayers, vesicles, synthetic or native lipoproteins).

Phospholipids - Phospholipid hydrolysis by HL has been extensively characterised. HL displays some degree of phospholipid head group selectivity with a preference for smaller head groups [328,334]. While PC is the most abundant phospholipid, it is poorly hydrolysed by HL relative to PE and PA which are minor components of lipoproteins [126,313,335-339]. Waite *et al.* also showed that HL hydrolyzes different phospholipids more readily than others PA > PE > PC > phosphatidylserine (PS) [328]. HL appears to have a specific role in PE metabolism. Chylomicron- and HDL-PE are not only cleared from the plasma by HL, but PE-specific hydrolysis was inhibited in the presence of inactivating anti-HL antibodies while PC hydrolysis was completely unaffected [335]. Due to the abundance of PC in lipoproteins, many studies have examined the hydrolysis of this phospholipid by HL. HDL containing POPC, PLPC or dioleoyl

phosphatidylcholine (DOPC) are hydrolyzed more readily than those containing PAPC and dipalmitoyl phosphatidylcholine (DPPC) [183,336,340]. DPPC alone is a poor substrate for HL, however trace amounts in a mixture of phospholipids are readily hydrolyzed [39]. These studies indicate that phospholipid head groups are not the only determinants of HL activity but that factors such as acyl chain composition, interface surface pressure and apolipoprotein composition may also play a role in regulating HL-mediated hydrolysis.

Acyl chain position, degree of saturation and acyl chain length are also factors that influence HL activity [341-344]. The preferred acyl chain position in TG and phospholipid for HL hydrolysis has been extensively characterised. Many studies using phospholipids and acylglycerides as substrates show that the *sn*-1 position is preferentially hydrolysed by HL [328,333]. The degree of fatty acid saturation also regulates HL hydrolysis. Studies show that HL preferentially hydrolyzes unsaturated over saturated lipids [335,337] and that activity increases with the degree of unsaturation of the phospholipid acyl chains [345,346]. Acyl chain length also affects HL-mediated lipid hydrolysis. Phospholipids with shorter acyl chains are hydrolyzed more readily than ones with longer chains and acylglycerides with shorter acyl chains are much better substrates [328,347].

Apolipoprotein regulation of hepatic lipase activity

ApoA-I – ApoA-I is the primary protein component of HDL and would be expected to influence HL-mediated hydrolysis of this lipoprotein. A number of studies have shown that apoA-I is an inhibitor of HL activity [12,40,348,349]. In addition, preβ-HDL particles, which are composed mainly of phospholipid and apoA-I, have also been shown to inhibit HL-mediated lipolysis [350]. This likely explains the hypertriglyceridemia and inhibition of lipase activity seen in patients with nephrotic syndrome [11]. In theses cases, LCAT deficiency causes high preβ-

HDL levels which would inhibit hydrolysis by HL and result in high plasma TG levels. Ramsamy *et al.* have also shown that HDL can inhibit VLDL hydrolysis by HL and that apoA-I is the primary inhibitory component of this lipoprotein [13].

ApoA-II – Reports on the effect of apoA-II on the activity of HL have been inconsistent. ApoA-II has been shown to inhibit as well as activate HL-mediated TG and phospholipid hydrolysis. HDL containing apoA-II were shown to be more readily hydrolysed than HDL containing only apoA-I [39], however, the opposite result was obtained at higher concentrations of HDL [39]. HL hydrolysis of phospholipid and TG was also shown to be higher in apoA-II-containing HDL_2 and HDL_3 [41,42,351,352]. Many studies have examined the effect of apolipoprotein composition on the binding of HL to lipoprotein substrates. It was shown that HL binds HDL containing apoA-II more tightly than HDL containing apoA-I or more than VLDL [41,42] and the enhanced association stimulates hydrolysis. However, this appears inconsistent with the majority of HL/lipoprotein binding studies which show that decreased binding is linked to an enhancement of enzyme activity.

Most studies now show that apoA-II has an inhibitory effect on HL activity. HDL_2-TG hydrolysis by HL was inhibited by apoA-II [40] and HDL containing apoA-II had a 10-fold higher affinity for HL than HDL containing only apoA-I [39]. HDL containing apoA-II are in fact poor substrates for HL [353]. The inhibition by apoA-II has been linked to a higher affinity of HL for apoA-II containing HDL resulting in a decreased apparent K_m [39,354]. Hime *et al.* suggest that the differences in HL inhibition by HDL apoA-II and apoA-I on activity may be due to the differences in charge of the two apolipoproteins [39]. ApoA-II-containing reconstituted HDL are more positively charged than apoA-I reconstituted HDL [355] and, therefore, the net negative charge associated with HL [182] may result in a greater affinity of HDL for the enzyme.

The apolipoprotein composition of HDL can also have an effect on the hydrolysis of other lipoproteins by HL. Co-incubation of apoA-II-containing reconstituted HDL inhibited apoA-I reconstituted HDL hydrolysis by HL, showing that the apolipoprotein composition of one lipoprotein class can have an effect on the hydrolysis of another class [354]. Likewise, HDL_3 inhibited VLDL-TG and total hydrolysis by HL and the inhibition was enhanced in the presence of apoA-II [41].

The effect of apoA-II on HL activity has been characterised in several *in vivo* studies. Evidence suggests that apoA-II overexpression in mice causes inhibition of HL resulting in an increase in HDL-TG and cholesterol [43,356-358]. ApoA-II overexpression inhibits both HL and LPL causing decreased hydrolytic activity and hypertriglyceridemia [356,359]. Boisfer *et al.* also showed that overexpression of apoA-II caused hypertriglyceridemia due to reduced VLDL hydrolysis by HL [37]. Gene knockout studies in mice provide further evidence for an inhibitory role of apoA-II on HL activity. ApoA-II knockout mice have a 70% decrease in HDL-cholesterol levels [356], suggesting that HL activity is high in the absence of apoA-II inhibition. However, two studies showed that overexpression of apoA-II in mice resulted in the formation of small HDL particles, suggesting enhanced hydrolysis of HDL_2 [44,360].

ApoC-I, ApoC-II, ApoC-III - ApoC-I, apoC-II and apoC-III were all shown to inhibit HL activity [182,361-363], however, the effect is believed to be non-specific. The most convincing data showing the effect of apoC-I on HL activity comes from *in vivo* studies. Overexpression of apoC-I in atherosclerosis-prone apoE-knockout mice results in hypertriglyceridemia by the direct inhibition of apoC-I on HL activity [364]. In this case, lack of efficient HL hydrolysis of chylomicrons and VLDL would result in elevated plasma TG levels. HDL from these mice were enriched in apoC-I and also inhibited HL-mediated VLDL hydrolysis *in vitro* as did purified

apoC-I alone [364]. The authors suggest that apoC-I may be inhibitory because of its ability to displace apoE from HDL [361,364].

ApoE - Studies of HL activity using *in vitro* lipid monolayers, phospholipid micelles and native HDL particles show that apoE stimulates phospholipid and TG hydrolysis by HL [338,362,363]. ApoE was also shown to stimulate HL-mediated hydrolysis of VLDL [365]. It has been postulated that the activation of HL activity by apoE occurs at the interface and most likely involves a protein-protein interaction between apoE and HL [338]. Thus, HL may be targeted to larger HDL that are enriched in phospholipid and apoE [338,362,363,366]. HL activity is also regulated by the different apoE isoforms. Studies showed that apoE2 and apoE3 were more efficient at activating HL activity than apoE4 [181,338]. These studies also indicated that an increase in hydrolysis by HL was associated with a reduced affinity for the lipoprotein substrates as seen by an increase in apparent K_m. ApoE may be able to stimulate hydrolysis relative to apoA-I because the surface of apoE is more positively charged and can easily interact with the charged residues in the lipid-binding site of HL [181,367]. In fact, it has been shown for HL and pancreatic lipase that the active site becomes negatively charged during interfacial activation and, therefore, the more positively charged apoE-containing reconstituted HDL may be more readily hydrolysed [181,304]. It is now clear that the apolipoprotein as well as lipid composition of lipoprotein particles is a major determinant of their metabolism and hydrolysis by the plasma lipases.

Regulation of hepatic lipase activity and cell-surface association

The regulation of HL function and activity also occurs through its association with cell-surface HSPG. Traditionally, the lipases were believed to be active at the cell-surface where they interact with lipoprotein substrates and supply fatty acids to local tissues. However, many studies

now suggest that lipase activity is stimulated when released into the plasma. It has been known for some time that release of HL or LPL into the circulation by heparin significantly stimulates the activity of these enzymes [219]. Injection of heparin enhanced HL-mediated hydrolysis of chylomicron-TG in humans without affecting clearance of remnants by the liver [368]. HL activity is present in the blood before and after heparin injection [15,369,370] and mouse models show the majority of the enzyme is active in the plasma and not bound to cell-surface HSPG. In fact, 50% to 80% of mouse HL activity is circulating in the plasma, unlike humans and rats [362,363].

Therefore, a new perspective on the regulation of HL activity by release into the circulation from cell-surface proteoglycans has been proposed. This view suggests that factors regulating the release of HL into the circulation will stimulate its activity and enhance the hydrolysis of lipoprotein substrates (**Figure 5A**). Once released from the cell-surface, the association of HL with different lipoproteins, based on their physical properties, will determine whether its hydrolytic activity is stimulated or inhibited (**Figure 5B**). Evidence supporting this mechanism comes from *in vitro* and *in vivo* studies. Ramsamy *et al.* showed that HSPG-bound HL is less effective than free HL at hydrolysing VLDL, LDL or HDL [12]. It also appears that lipoproteins can regulate the release of HL into the circulation. Studies showed that HDL or purified apoA-I can release cell-surface and HSPG-bound HL and stimulates VLDL hydrolysis [12,13]. This is consistent with data from Brown *et al.* which showed that adenovirus-mediated overexpression of an HSPG-binding deficient human HL in mice increased pre-heparin HL activity and caused reductions in plasma cholesterol and phospholipids and severe hypoalphalipoproteinemia, suggesting a possible increase in lipid hydrolysis due to enhanced amounts of lipase circulating in the bloodstream [14]. The authors of this study suggest that

circulating lipases may play a role in apoB-lipoprotein metabolism and that efficient catalysis may not occur when lipases are bound to cell-surface HSPG [14]. Release of HL into the circulation may also be regulated at the cellular level. Treatment of cultured cell lines with tyrosine kinase inhibitors inhibited the heparin-stimulated release of HL [371]. It appears that heparin release of cell-surface and intracellular HL is stimulated through the activation of Ca^{2+}/calmodulin-dependent protein kinase II and membrane tyrosine kinase activity [371]. Thus, factors that determine the release of HL protein and activity into the circulation may provide a novel mechanism for the regulation of HL lipolysis and lipoprotein metabolism.

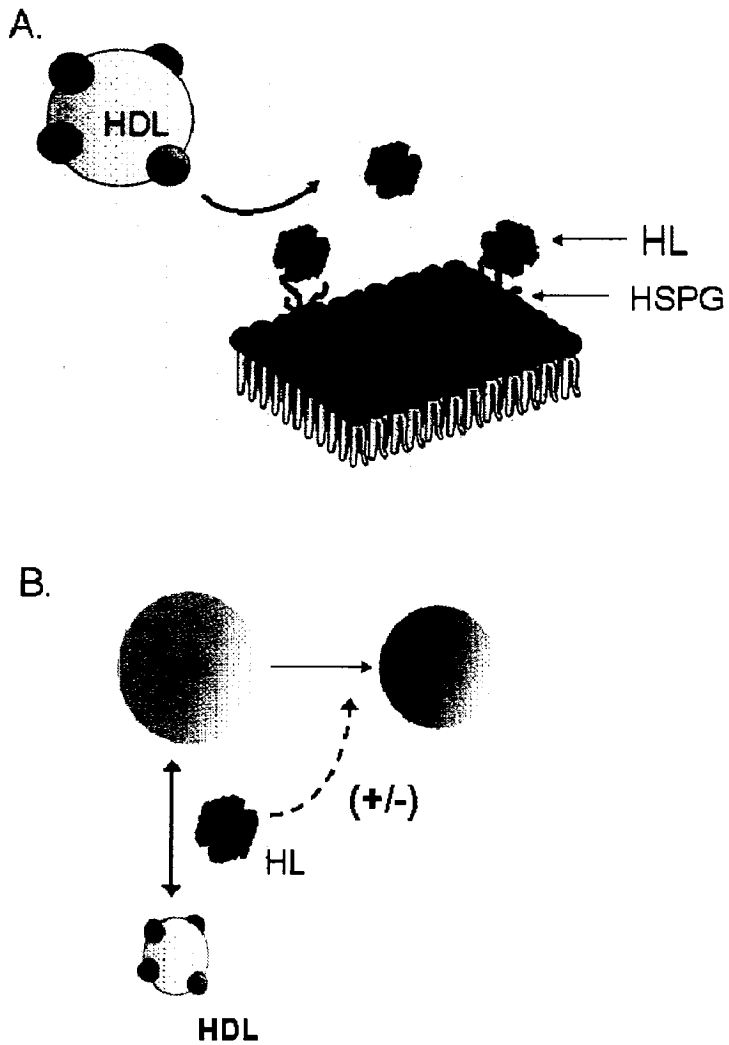


Figure 5. Model illustrating the regulation of HL through cell-surface displacement and lipoprotein association. HL is normally located at the vascular endothelium attached to the cell surface via extracellular matrix HSPG. It has been known for decades that intravenous injection of the anti-coagulant heparin releases the enzyme from the cell surface and stimulates lipase activity in the bloodstream [219]. HDL can act like heparin and displace cell-surface HL and activate lipolysis. This novel mechanism may be an important regulator of HL activity and TG metabolism. HL activity is also regulated by its association with lipoprotein particles in the bloodstream. Tight association of HL with HDL inhibits HL-mediated VLDL hydrolysis whereas a weaker association is stimulatory. HDL, high density lipoprotein; HSPG, heparan sulfate proteoglycan; HL, hepatic lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

Rationale

Cardiovascular disease and atherosclerosis are among the primary causes of mortality in developed countries [7]. The risk of developing cardiovascular disease is linked to elevated TG and low HDL levels [8]. The efficient catabolism of TG-rich lipoproteins by HL has been shown to be a major determinant of HDL concentrations [8]. It is believed that lipases are hydrolytically active at the vascular endothelium bound to the extracellular matrix [372-374]. However, infusion of heparin into the bloodstream has been known for years to release plasma lipases into the circulation and stimulate TG hydrolysis [15]. Data from our laboratory now suggest that HL requires displacement from extracellular matrix HSPG for efficient catalytic activity. It has been shown that HDL and apoA-I can act like heparin and release HL from the cell surface, activating lipoprotein hydrolysis. HDL and apoA-I not only regulate the displacement of HL but also appear to regulate the hydrolysis of VLDL by HL. In order to better understand the mechanisms leading to abnormal plasma lipid metabolism and the development of atherosclerosis, it is essential that the factors regulating lipase interaction with lipoproteins and hydrolysis be determined. Thus, this thesis will characterize the determinants of HDL composition that regulate the cell-surface displacement of HL and the hydrolytic activity of this enzyme. The objectives of this thesis are to evaluate how lipid and protein composition:

- Regulate cell-surface displacement of HL;
- Affect HL-mediated lipid hydrolysis; and
- Control the association and inter-lipoprotein movement of HL with HDL

Many studies have attempted to correlate lipoprotein composition-dependent physical properties to the activation of interfacial enzymes. However, there is still no consensus as to how changes in lipoprotein structure and charge may affect interfacial catalysis by HL. It is well known that interfacial enzymes require rapid shuttling between substrates for efficient catalysis of their substrates [173]. Therefore, I hypothesize that specific components of lipoprotein composition that promote a tighter binding between HL and lipoprotein substrates, such as HDL apoA-II content, will effectively stimulate displacement and inhibit activity of this enzyme. Moreover, factors that decrease association of HL with lipoprotein substrates, such as the anionic properties of the particle, will stimulate lipase activity by promoting inter-lipoprotein shuttling and access to these substrates. It is now clear that lipoprotein composition and electrostatic properties regulate the displacement, association and activity of interfacial lipolytic enzymes.

Chapter 2 – Methods and Materials

Isolation of lipoproteins by sequential density ultracentrifugation. VLDL, LDL and HDL were isolated from fasted human serum by sequential density ultracentrifugation as described within the density ranges $\rho < 1.006$ g/ml, $\rho = 1.006$-1.063 g/ml and $\rho = 1.063$-1.21 g/ml, respectively [375]. HDL_2 and HDL_3 were isolated in a similar manner within the density ranges $\rho = 1.063$-1.19 g/ml and $\rho = 1.19$-1.21 g/ml, respectively. HDL sub-fractions were isolated by KBr-sucrose discontinuous density gradient ultracentrifugation as described [13]. The densities of 12 HDL fractions between $\rho < 1.063$ g/ml and $\rho < 1.27$ g/ml were determined by refractometry. Chylomicrons were obtained by density gradient ultracentrifugation ($\rho < 1.006$ g/ml) from postprandial serum samples of normolipidemic volunteers 4 hours after a lipid-rich meal. All isolated lipoprotein fractions were extensively dialyzed against phosphate buffered saline (PBS) (50 mM sodium phosphate, 150 mM NaCl, pH 7.2). Lipoprotein heterogeneity was determined by agarose gel electrophoresis on Lipogels (Beckman-Coulter, Mississauga, ON) and visualized by neutral lipid staining. Phospholipid and TG concentrations were determined using commercial diagnostic assays from Roche Diagnostics (Laval, PQ). Protein concentration was determined by the Lowry method as modified by Markwell *et al.* [376].

Quantitative measurement of lipoprotein charge. Lipoprotein charge was caculated using particle electrophoretic mobilities after agarose gel electrophoresis as described [380]. Briefly, purified lipoprotein or reconstituted HDL samples were loaded onto preformed Lipogels (Beckman-Coulter), composed of 0.6% agarose, and subjected to electrophoresis at 100 volts for 30 minutes. Lipoprotein bands were fixed with acetic acid and stained with Sudan Black. Electrokinetic theory was used to calculate particle net charge and charge density. Lipoprotein

charge is directly related to electrophoretic mobility and was calculated as described [380]. Surface potential (in mV) was calculated using the following formula surface potential = U x $6\pi n/D$, where U is the electrophoretic mobility, n is the coefficient of viscosity (0.0089 poise) and D is the dielectric constant [380]. Purified apoA-I with a known charge and electrophoretic mobility was used as a standard to calculate the surface potential and charge of each lipoprotein band. Lipoprotein surface potential was calculated from the electrophoretic mobilities of each lipoprotein band as described [380].

Purification of HL and LPL from post-heparin human plasma. Human HL and LPL were purified from post-heparin human plasma by heparin-Sepharose affinity chromatography as previously described [377]. Healthy male volunteers were intravenously injected with 60 units of heparin per kg body mass 10 minutes prior to blood collection. Plasma was subsequently isolated after centrifugation (3,000 rpm for 10 minutes at 4°C) and incubated with a 20% Intralipid®-TG emulsion (100 ml Intralipid® per 400 ml plasma). The intralipid/plasma mixture was incubated with shaking for 15 minutes at 37°C and the lipid cakes were separated by ultracentrifugation at 27,000 rpm for 30 minutes at 8°C. The lipid cakes were then suspended in cold acetone and centrifuged at 16,000 rpm for 15 minutes. The pellet was re-suspended in ether and centrifuged again at 16,000 rpm for 20 minutes. The crude HL/LPL pellet obtained was dried under N_2 gas and solubilized in barbital buffer (5 mM sodium barbital, 0.15 M NaCl, 20% glycerol, pH 7.4). The mixture of crude lipase was then loaded onto a heparin-Sepharose CL-6B chromatography column (Amersham Pharmacia Biotech, Baie d'Urfe, Canada) and washed sequentially with barbital buffer and wash buffer (5 mM sodium barbital, 0.4 M NaCl, 20% glycerol, pH 7.4) to remove unbound lipase and other impurities. HL was then eluted using HL elution buffer (5 mM

sodium barbital, 0.9 M NaCl, 20% glycerol, pH 7.4) followed by the elution of LPL using LPL elution buffer (5 mM sodium barbital, 1.5 M NaCl, 20% glycerol, pH 7.4). HL and LPL were then stored at -80 °C until further use. Lipase activity was quantified into units of enzyme activity (where 1 unit = 1 μmol fatty acid released/hour) using a ^{3}H-TG emulsion assay as described [377]. The specific activities of the purified HL and LPL were determined to be 19,455 units/mg protein and 3,703 units/mg protein, respectively. Immunoblot analyses under denaturing conditions using an HL monoclonal antibody (XHL3-6) obtained from Dr. Andre Bensadoun (Cornell University) and an LPL monoclonal antibody (5D2) obtained from Dr. John Brunzell (University of Washington) showed single bands with apparent molecular masses of 66 kDa and 54 kDa, respectively. In some cases, bovine LPL at 100,000 units/mg protein (Sigma-Aldridge, US) was used in hydrolytic experiments where indicated.

Purification of apoA-I and apoA-II. ApoA-I and apoA-II were isolated from human HDL delipidated in chloroform:methanol as described [378]. ApoA-I was isolated by size exclusion chromatography on a Sephacryl S-200 HR column [32] and apoA-II was purified by anion exchange chromatography on Q-Sepharose [379]. Purified protein samples were lyophilized and stored at –80°C. Prior to use, apoA-I and apoA-II were re-solubilized in 6M guanidine hydrochloride (GdnHCl), 10 mM Tris (pH 7.2) and dialyzed against PBS.

Preparation of reconstituted HDL particles. Reconstituted HDL particles containing apoA-I (LpA-I) or apoA-II (LpA-II) or both apoA-I and apoA-II (LpA-I/A-II) were prepared as described by co-sonication of 3.2 mg of POPC (Avanti Polar Lipids, Alabaster, AL) and 2 mg of apoA-I and/or apoA-II at the molar ratios shown in **Table 4** [167]. In some cases, various amounts of TG (trioleoyl), DG (dioleoyl), FC (Sigma ,St. Louis, MO), ^{3}H-TG (Glycerol tri[9,10(n)-^{3}H]oleate) or ^{14}C-DG (Glycerol di[1-^{14}C]oleate) (Amersham Pharmacia Biotech, Baie

d'Urfe, Canada) were also included as indicated. Reconstituted HDL composed of different types of phospholipids, including DOPC, dioleoyl phosphatidylethanolamine (DOPE) or dioleoyl phosphatidylglycerol (DOPG) were also prepared by co-sonication with apoA-I at molar ratios of 35:1 (lipid:protein).

Determination of reconstituted HDL physical characteristics. The size and homogeneity of the reconstituted HDL particles were determined by non-denaturing gradient gel electrophoresis on 8-25% acrylamide gels and Coomassie blue protein staining. The protein concentration of the HDL particles was determined by the Lowry method as modified by Markwell *et al.* [376]. TG, FC and phospholipid concentrations were determined using commercial enzyme assays (Wako Diagnostics). Particle charge was determined by agarose gel electrophoresis as described [380].

Immunoreactivity of apoA-I in LpA-I/A-II complexes. The effect of apoA-II on the structure of apoA-I in reconstituted HDL was determined by competitive solid phase radio-immunometric assay as described [167,381,382]. Antibodies 2G11 and 4A12 were purchased from Sanofi (Paris, France). A03, A05, A07, A11, A17, A16 and A44 were obtained from the Institute Pasteur (Lille, France). 3D4, 2F1, 4H1 and 5F6 were a gift from Dr. Yves Marcel (University of Ottawa Heart Institute). The 13 different antibodies were used because they recognize defined apoA-I epitopes located between specific residues, as indicated between brackets: 4H1 (2-8), 2F1 (8-82), A16 (14-29,60-82), A05 (25-82), 2G11 (25-96), 3D4 (98-121), A17 (98-121), A11 (98-132), 5F6 (118-141), A03 (135-148), A07 (148-186), A44 (148-186) and 4A12 (173-205). Removawells (Immulon 2, Dynatech Laboratories, MA) were coated with 0.2 μg HDL protein in 100 μl of 15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.02% NaN_3, pH 6.9. The

coated wells were then washed with PBS containing 0.02% NaN_3 and saturated with PBS containing 0.5% gelatin and 0.02% NaN_3. The samples were then incubated for 1 hour at room temperature with mixtures of apoA-I antibodies at predetermined optimal dilutions and serial dilutions of reconstituted HDL complexes in PBS containing 0.1% gelatin. The wells were washed three times with PBS containing 0.05% Tween-20 and incubated for 1 hour with ^{125}I-labelled anti-mouse immunoglobulin type G (IgG) obtain from goat (200,000 cpm/well). After incubation the wells were thoroughly washed and the radioactivity was quantified. Results were graphed as B/B_0, where B and B_0 represent cpm bound in the presence and absence of competitor. The data were used to calculate the particle concentration required for 50% inhibition of the maximal binding (ED_{50}) of the antibody for each reconstituted HDL particle. The immunoreactivity of apoA-I in the various reconstituted HDL particles was determined from the ED_{50} which represents the HDL concentration required to inhibit 50% of the maximal binding of the antibodies to the apoA-I-coated plate.

Quantification of HL cell-surface displacement by HDL. Chinese hamster ovary (CHO) cells overexpressing human HL (CHO-hHL) were obtained from Drs. Robert Brown (University of Ottawa) and Zemin Yao (University of Ottawa). CHO-hHL cells were plated and grown to confluence in Ham's F12 medium containing 10% fetal bovine serum (FBS) and 500 μg/ml geneticin selective antibiotic (G418 sulfate). Prior to incubation with HDL or apoA-I, the cells were grown overnight in medium containing 1% FBS and 500 μg/ml G418. Cell-surface displacement of HL was determined after incubation with 150 μg/ml HDL or apoA-I in FBS-free culture media for various times at 37°C. Following incubation, the media were removed and the amount of HL displaced was quantified by measuring the amount of lipase activity present in the culture media. HL activity was determined in 150 μl media samples using a radioactive ^{3}H-TG

emulsion as described [377]. Where indicated, a portion of the media samples were concentrated by vacuum freeze drying and subsequently solubilized in sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.5% bromophenol blue) for HL quantification by immunoblot analysis. The cell extracts were also solubilized in sample buffer and subjected to 8% polyacrylamide gel electrophoresis and HL immunoblot analysis using the monoclonal human HL antibody as described [13]. Bands representing human HL were quantified by densitometry using Quantity One® software.

HL-mediated hydrolysis of reconstituted HDL particles containing apoA-I or apoA-II. LpA-I and LpA-II HDL particles labelled with ^{3}H-TG or ^{14}C-DG were incubated at the indicated concentrations in a reaction mixture containing 150 µl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% fatty acid-free bovine serum albumin (BSA), 3.33 M NaCl, 5 mM $CaCl_2$), PBS and 214 units of purified HL in a final volume of 500 µl for 3 hours at 37°C. Total lipid hydrolysis was determined by quantifying the amount of fatty acid released during the incubation using a commercial free fatty acid assay (Roche Diagnostics, Laval, Canada). Phospholipid-, TG- and DG-specific hydrolytic rates were then determined subtractively after quantifying the amount of radioactive fatty acids liberated from ^{3}H-TG or ^{14}C-DG after extraction with methanol:chloroform:heptane (1.41:1.25:1) and scintillation counting as previously described [332,377]. After extraction, > 95% of the free fatty acids were present in the aqueous phase, as previously described [332]. V_{max} and apparent K_m values were estimated from double-reciprocal plots of the data in **Figure 11A**.

Effect of reconstituted HDL containing apoA-I or apoA-II on HL-mediated hydrolysis of VLDL. VLDL hydrolysis by HL was characterized in the presence or absence of

native HDL or reconstituted HDL particles as previously described [13]. Incubations were carried out for 30 minutes at 37°C. Each sample contained the lipoprotein substrate (350 μM VLDL), HL (26 units), 75 μl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% fatty acid-free BSA, 3.33 M NaCl, 5 mM $CaCl_2$), PBS and increasing concentrations of HDL or LpA-I/A-II particles as indicated in a final volume of 250 μl. Hydrolysis was determined by quantifying the total amount of fatty acid released during the incubation using a commercial free fatty acid assay. In separate experiments, LPL hydrolysis was determined after a 20 minute incubation at 37°C under similar reaction conditions using 100 μl of purified human LPL and incubation buffer without NaCl.

Preparation of radioactively-labelled (^{3}H-TG and ^{14}C-DPPC) native lipoproteins and serum. Lipoproteins and serum were labelled with ^{3}H-TG or ^{14}C-PC (L-3-Phosphatidylcholine, 1,2-di[1-^{14}C]palmitoyl) obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Canada). First, 50 μg of PC were dried down under N_2 with 0.25 mCi of ^{3}H-TG or 0.01 mCi ^{14}C-PC and re-solubilized in 1 ml of PBS. The mixture was then sonicated for 5 minutes at 90% duty cycle at 15°C and subsequently incubated with 3 ml of serum or isolated lipoprotein (chylomicron, VLDL, LDL or HDL) for 48 hours at 4°C. VLDL, LDL and HDL were re-isolated by sequential density ultracentrifugation as described. It was determined that approximately 85-90% of the radioactive tracer is lipoprotein-incorporated after separation of the lipoproteins by density gradient ultracentrifugation and scintillation counting of the resulting density fractions, as described [13].

Preparation of lipid-enriched lipoproteins and serum. Dioleoyl PC, dioleoyl phosphatidic acid (PA), soy PI, dioleoyl phosphatidylinositol (DOPI) and dioleoyl phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). After ^{3}H-TG

or ^{14}C-PC enrichment, the serum and lipoprotein fractions were enriched with oleic acid (Sigma-Aldrige, St-Louis, MO), PC, PI, PA or PS. First, 3 mg of each lipid were dried under N_2 and solubilized in 1 ml of PBS. In order to facilitate lipoprotein and serum enrichment, 5 μg of PC were added to the oleic acid. The lipid-PBS mixtures were then sonicated for 1 minute at constant duty cycle, incubated at 37°C for 15 minutes and sonicated again at high output and 90% duty cycle for 5 minutes at 15°C. Next, 0.12 μmol oleic acid or 1.2 μmol (except where indicated) phospholipid vesicles were incubated per 1 ml lipoprotein or serum sample for 48 hours at 4°C. After lipid enrichment, lipoprotein charge and homogeneity were determined by agarose gel electrophoresis as described [380]. In order to determine incorporation of phospholipids into serum and isolated lipoproteins, enriched samples containing trace amounts of ^{3}H-PC or ^{3}H-PI (Amersham Pharmacia Biotech, Baie d'Urfé, Canada) were subjected to density gradient ultracentrifugation, as described [13], and the radioactivity associated with each lipoprotein fraction was quantified by scintillation counting. In whole serum, < 3% of the radioactive phospholipid tracers were found in VLDL, 50-60% in LDL and 30-45% were in the HDL fractions. No differences were observed between the lipoprotein enrichment profiles of the ^{3}H-PC and ^{3}H-PI tracers. In similar experiments using isolated lipoproteins subjected to density gradient ultracentrifugation, approximately 85-90% of the radioactive phospholipid tracers were lipoprotein-incorporated.

HL-mediated lipid hydrolysis of lipid-enriched sera and lipoproteins. Each hydrolysis reaction contained the lipoprotein substrate (350 μM TG for VLDL, LDL, HDL or 25 μl serum), 26 units of purified HL (150 units for serum), 150 μl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% fatty acid-free BSA, 3.33 M NaCl, 5 mM $CaCl_2$) and PBS to a final volume of 500 μl. HL-mediated lipid hydrolysis was determined after a 1 hour incubation at 37°C. An aliquot

was removed and total lipid hydrolysis was determined from the amount of fatty acid released during the incubation using a commercial free fatty acid diagnostic assay. TG- or phospholipid-specific hydrolysis were determined from the remaining reaction volume using a methanol:chloroform:heptane (1.41:1.25:1) extraction to separate the fatty acids into an aqueous phase as described [13,244,383]. The ^{3}H- or ^{14}C-labelled fatty acids released were then quantified by scintillation counting. Co-incubations of VLDL and native HDL or reconstituted HDL were performed in a similar manner but contained 350 μM VLDL-TG and 150 μg HDL/LpA-I protein per reaction except where indicated. In separate experiments, LPL hydrolysis was determined after a 20 minute incubation at 37°C under similar reaction conditions containing incubation buffer without NaCl and using 50 μl of bovine LPL (10,000 units/ml) (Sigma-Aldrige, St-Louis, MO) diluted 1:1000 in PBS.

Association of HL with lipoproteins. The association of HL with HDL or VLDL was determined by gel filtration chromatography and immunochemical analysis. Each sample contained 1 mg VLDL-TG, excess purified HL (986 units), 1 mg of either HDL or PI-enriched HDL and PBS to a final volume of 2.5 ml. The mixture was then loaded onto a size exclusion chromatography Superose-6 column (Amersham Pharmacia Biotech, Baie d'Urfé, Canada) and the eluted fractions representing the VLDL and HDL were pooled separately and dialysed against 6 mM NH_4HCO_3. Each sample was then delipidated using a chloroform:methanol (2:1) extraction and re-suspended in 100 μl H_2O and 100 μl of urea sample buffer (28.3 mM NaH_2PO_4, 71.8 mM Na_2HPO_4, 2% SDS, 1% β-mercaptoethanol, 0.5% (w/v) bromophenol blue and 6 M urea). Samples were analyzed on 8% polyacrylamide gels (Invitrogen, Burlington, Canada) under denaturing conditions. Immunochemical analysis was performed using the HL monoclonal

antibody, anti-mouse IgG horseradish peroxidase-linked (HRP) secondary antibody (Amersham Pharmacia Biotech, Baie d'Urfé, Canada) and the SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Co., Rockford, IL) as previously described [13].

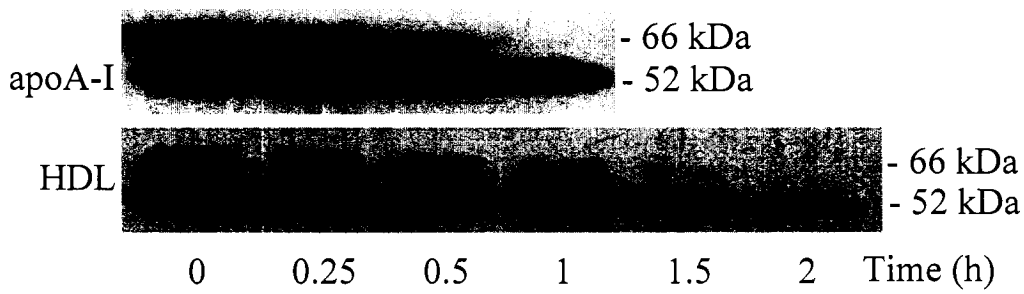
Kinetic analyses. The parameters V_{max} and apparent K_m were used to characterize the kinetics of HL activity. Since HL is an interfacial enzyme, it cannot be analyzed using conventional Michaelis-Menton kinetic models. In the case of HL, K_m is only an apparent value since this constant is both a function of the affinity of the enzyme for its lipid substrate and the binding/dissociation from the lipoprotein interface. While techniques (such as surface plasmon resonance) do exist to measure enzyme-lipoprotein binding, they require the isolation of highly purified protein samples and this has proved problematic in the case of HL. Therefore, conventional kinetic theory has been applied to provide some measure of HL kinetic behaviour. The maximal velocity rate (V_{max}) and the concentration of substrate at half V_{max} (K_m) were calculated from the double reciprocal (Lineweaver-Burke) plots of the the data in **Figure 11**. In addition, nonlinear regression can also be used to calculate these parameters as shown for **Figure 21** and **Table 8**. Nonlineary regression analysis was performed using SigmaStat (Version 9) software and the data was curvefit using the formula for a rectangular hyperbola.

Statistical analyses. Significance of difference between group mean values was calculated by analysis of variance (ANOVA) and Tukey post-test analysis using Instat® GraphPad Software (Version 3.00).

Chapter 3 – Results

HDL and apoA-I regulate the displacement of HL from the surface of CHO-hHL cells. Previous data from our laboratory have shown that apoA-I and HDL can cause the displacement of HL from a synthetic proteoglycan matrix *in vitro*. In order to evaluate the ability of both apoA-I and HDL to displace HL from the surface of a cell-culture model, a stably transfected CHO cell line expressing human HL (obtained from Drs. Brown and Yao) was used. These studies were undertaken in close collaboration with Dr. T. Ramsamy. Incubation of both apoA-I and HDL in the culture media of the CHO-hHL cells caused a gradual displacement of HL from the surface of these cells by 1 and 2 hours, respectively, as determined by immunoblot analysis of the cell extracts (**Figure 6).** Previous reports indicated that the lower HL band on the Western blot represents a smaller, intracellular form of the enzyme that is not glycosylated, whereas the higher band is a larger, glycosylated form of the enzyme located on the cell-surface [13]. The amount of HL was also quantified by measuring the lipase activity released into the culture media. Incubation of the cells with heparin was used as a positive control as it is well-known to displace HL from the extracellular matrix at the cell surface. As expected, heparin caused a rapid release of HL activity into the media of the CHO-hHL cells (**Figure 6**). Consistent with the immunoblot data, incubation of the CHO-hHL cells with HDL also caused the displacement and release of HL activity into the media but to a lesser extent compared to the heparin control (**Figure 6**). Unmodified media alone had almost no effect on the release of HL activity into the media of the CHO-hHL cells (**Figure 6**).

A.



B.

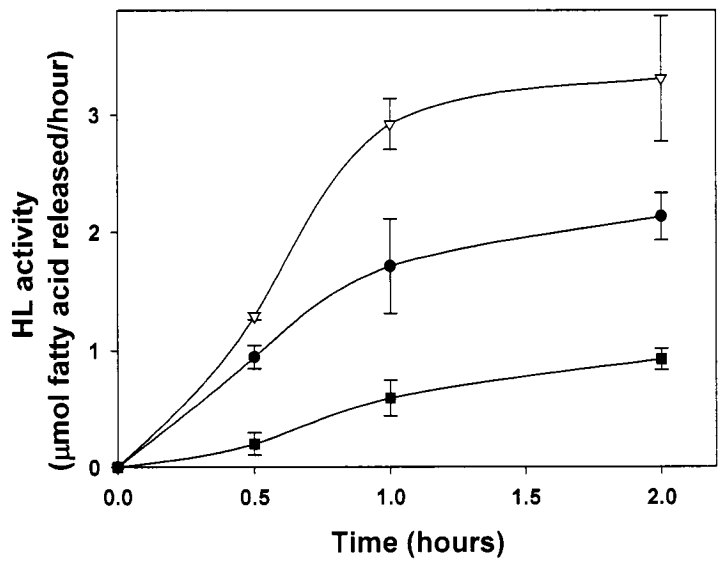


Figure 6. Displacement of HL from the surface of CHO-hHL cells by HDL or apoA-I. CHO-hHL cells were incubated with either apoA-I, HDL or heparin for as much as 2 hours as described. Displacement of cell-surface HL was quantified by immunoblot analysis of the cell extracts or by measuring lipase activity displaced into the cell media. (A) ApoA-I and HDL completely displace cell-surface HL by 1 and 2 hours, respectively. The two bands represent cell-surface (upper) and intracellular (lower) HL, as described [13]. (B) Heparin induced the highest amount of HL activity displaced into the cell culture media followed by HDL. Media alone caused only a small amount of background release of HL into the media. ∇, Heparin; •, HDL; ■, Media; ApoA-I, apolipoprotein A-I; HDL, high density lipoprotein; HL, hepatic lipase. Ramsamy,TA, Boucher,J, Brown,RJ, Yao,Z, and Sparks,DL (2003). HDL regulates the displacement of hepatic lipase from cell surface proteoglycans and the hydrolysis of VLDL triacylglycerol. *J.Lipid Res.*, **44**:733-741.

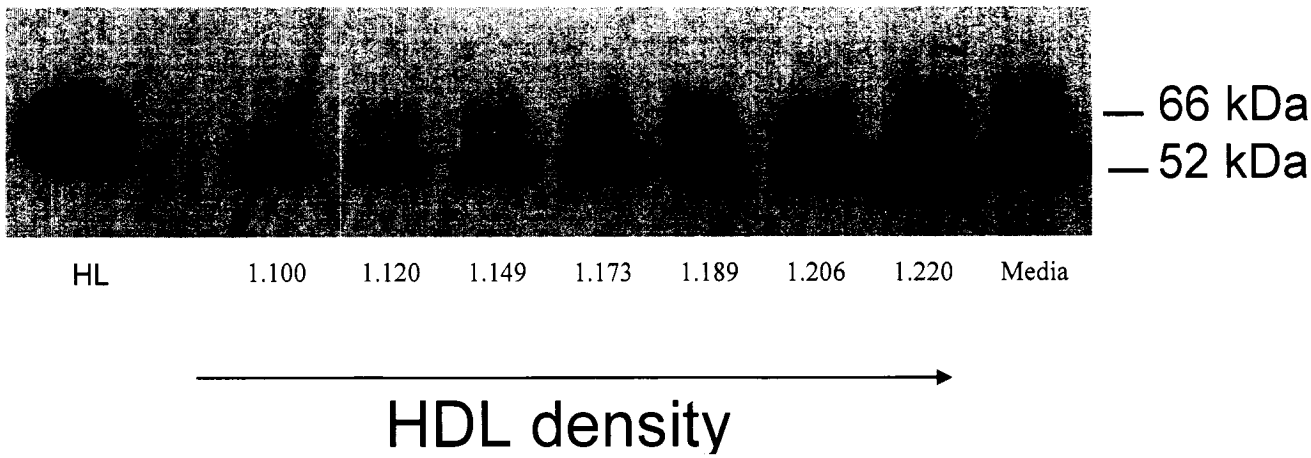


Figure 7. Displacement of HL from the surface of CHO-hHL cells by HDL sub-species. CHO-hHL cells were incubated with 150 μg/ml of various HDL sub-species of increasing densities (from 1.100 to 1.22 g/ml) for 1 hour as described. Displacement of cell-surface HL was quantified by immunoblot analysis of the cell extracts The two bands represent cell-surface (upper) and intracellular (lower) HL, as described [13]. HL, hepatic lipase; HDL, high density lipoprotein. (2003) *J.Lipid Res.*, **44**:733-741.

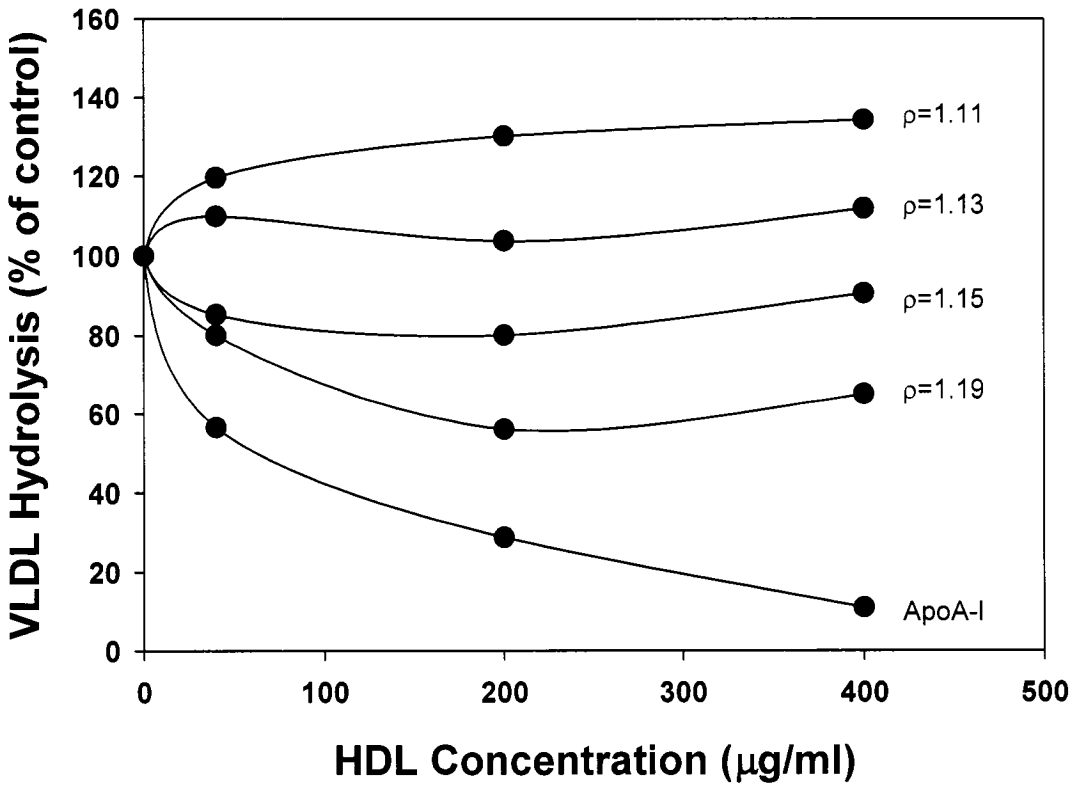


Figure 8. The effect of HDL sub-species on HL-mediated VLDL hydrolysis. VLDL was incubated as described with HL and either apoA-I or increasing concentrations of HDL sub-species isolated at densities ranging from 1.11 g/ml to 1.19 g/ml. Hydrolysis was determined by quantifying the amount of fatty acids released using a commercial assay. Hydrolytic values are the means of triplicate determinations and representative of two separate experiments. VLDL, very low density lipoprotein; HDL, high density lipoprotein; apoA-I, apolipoprotein A-I. (2003) *J.Lipid Res.*, **44**:733-741.

Since HDL in the plasma is a very heterogeneous population of particles, the abilities of the different HDL sub-species to displace cell-surface HL was evaluated. Seven different HDL sub-populations were isolated by density gradient ultracentrifugation and were incubated with CHO-hHL cells in culture media. HL displacement was then determined by immunoblot analysis of the cell extracts. The results showed that the larger and less dense HDL sub-species were more effective at displacing cell surface HL than the smaller and more dense HDL particles (**Figure 7**). Previous data from our laboratory indicated that HDL and apoA-I were inhibitory to HL-mediated VLDL hydrolysis. Thus, the effect of the different HDL sub-species on HL activity was evaluated. **Figure 8** clearly shows that the larger, less dense HDL particles are stimulatory while the smaller, more dense HDL are inhibitory to HL-mediated VLDL hydrolysis with increasing concentrations of HDL.

Effect of apoA-II on the size and charge of the reconstituted HDL particles. Since the different HDL sub-populations have opposing effects on HL activity, the protein and lipid composition of HDL particles were evaluated for their effect on HL displacement and activity. One of the primary differences between the different types of HDL particles is their apoA-I and apoA-II composition. Therefore, reconstituted HDL particles were prepared as described containing various amounts of apoA-I and/or apoA-II. Synthetic HDL were used because of the ability to accurately control the protein and lipid composition of the reconstituted particles. HDL prepared in this manner have been shown to exhibit similar properties and characteristics to native HDL [165,380,384,385]. All HDL complexes were homogeneous in composition and exhibited only one band on non-denaturing gradient gels (**Figure 9**). The sizes of the HDL particles were all determined to be between 7.7-8.7 nm as indicated in **Table 4**.

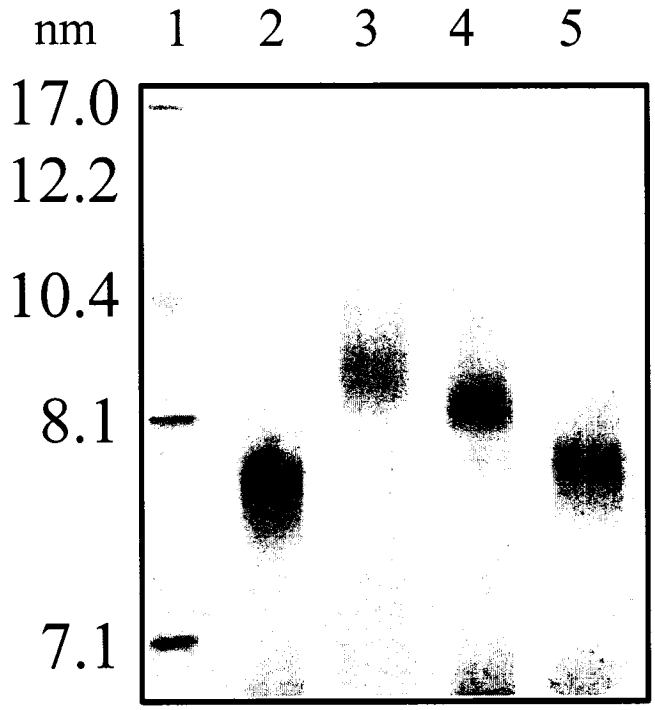


Figure 9. Non-denaturing gradient gel electrophoresis of reconstituted HDL particles. The size and homogeneity of reconstituted HDL particles containing apoA-I and/or apoA-II were determined by non-denaturing gradient gel electrophoresis on pre-cast 8-25% acrylamide gels. Protein bands were visualized by Coomassie Blue R250 staining and particle size (nm) was determined from reference standards. **Lane 1**, molecular weight markers; **lane 2**, LpA-I; **lane 3**, LpA-II; **lane 4**, LpA-I/A-II (1:2); **lane 5**, LpA-I/A-II (1:1). Molecular weight markers are represented by the hydrodynamic diameters provided by the manufacturer. Boucher, J, Ramsamy, TA, Braschi, S, Sahoo, D, Neville, TA and Sparks, DL. (2004) Apolipoprotein A-II regulates HDL stability and affects hepatic lipase association and activity. *J.Lipid Res.*, **45**:849-858.

Table 4 - Effect of Apolipoprotein Composition on the Size and Charge of Reconstituted High Density Lipoprotein Particles

LpA-I Complex[a]	ApoA-I:A-II[a] (mol:mol)	Diameter[b] (nm)	Surface Potential[c] (-mV)
LpA-I	1:0	7.7	8.9
LpA-I,A-II	1:1	7.8	9.0
LpA-I,2A-II	1:2	8.3*	9.1
LpA-II	0:1	8.7*	9.1

[a] LpA-I complexes were prepared by co-sonication as described in methods and materials. Values are representative of 3-5 different preparations of LpA-I complexes. [b] Particle diameters from non-denaturing gradient gel electrophoresis. [c] Charge potential at the particle surface ± 0.2 mV (SEM). * Significance of difference from LpA-I. $p < 0.001$. LpA-I, reconstituted HDL containing apoA-I; LpA-I/A-II, reconstituted HDL containing apoA-I and apoA-II.

The LpA-I/A-II particles with a molar ratio of 1:1 (apoA-I:apoA-II) exhibit a similar size to those containing only apoA-I (LpA-I). However, when greater amounts of apoA-II were included in the LpA-I particles to a molar ratio of 1:2 (apoA-I:apoA-II), the resultant HDL particles appeared approximately 0.6 nm larger in size. Similarly, when particles were prepared with apoA-II alone (LpA-II), they exhibited a size of 8.7 nm, approximately 1 nm larger in diameter than the LpA-I particles. The electrostatic properties of the synthetic HDL particles were determined by agarose gel electrophoresis as described [380] and are indicated in **Table 4**. The surface potentials of the HDL particles were all relatively similar, between -8.9 mV and -9.1 mV. The addition of apoA-II to LpA-I particles did not significantly effect particle charge, increasing the negative surface potential by only -0.2 mV. This is consistent with previous reports [126] and shows that apoA-II is able, to a small extent, to contribute to the electrostatic properties of HDL much like apoA-I.

Effect of apoA-II content on the conformation of apoA-I in LpA-I/A-II particles. The conformation of the apoA-I molecule on the different reconstituted HDL particles was evaluated by competitive solid phase radio-immunometric assay using a series of 13 antibodies specific for various apoA-I conformational epitopes ranging from the N-terminus to the C-terminus of apoA-I [381,386-389]. Competitive binding curves were parallel for all monoclonal antibodies and were used to calculate the ED_{50} values presented in **Figure 10**. The data show that inclusion of apoA-II in LpA-I particles was associated with a decrease in the ED_{50} values for the 2F1, A16, A51, 2G11 antibodies, which recognize epitopes approximately located in the N-terminal domain of apoA-I. The ED_{50} values for the A44 and 4A12 antibodies, which recognize epitopes near the C-terminal domain of apoA-I, were also decreased in the presence of apoA-II.

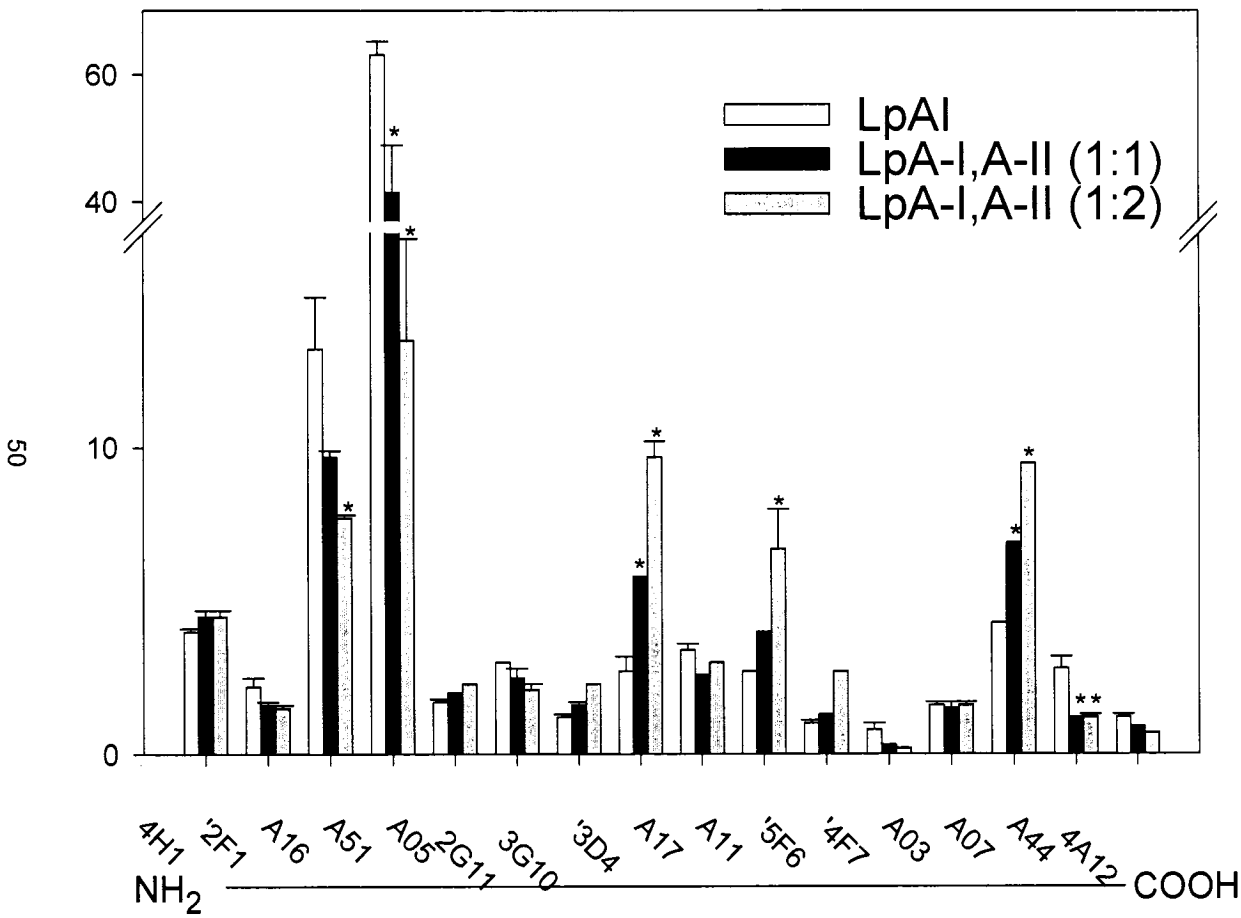


Figure 10. Effect of the apoA-II content on the apoA-I immunoreactivity in LpA-I/A-II complexes. The apoA-I immunoreactivity of the various LpA-I/A-II complexes for each monoclonal antibodies was evaluated by a radio-immunometric assay. ED_{50} were determined as previously described and represent the LpA-I/A-II concentration required to inhibit half of the maximum binding of the monoclonal antibodies. The monoclonal antibodies are roughly positioned on the X-axis as their epitopes appear in the apoA-I sequence and define groups of epitopes localized to the N-terminal (4H1 to 2G11), the central (3G10 to A03) and the C-terminal regions (A07 to 4A12) of the apoA-I molecule. ED_{50} values are expressed in mg/ml and are the mean ± S.D. of triplicate determinations and are representative of two different experiments. *Significance of difference from LpA-I of $p < 0.01$. LpA-I, reconstituted HDL containing apoA-I; LpA-I/A-II, reconstituted HDL containing apoA-I and apoA-II. (2004) *J.Lipid Res.*, **45**:849-858.

In contrast, the presence of apoA-II in LpA-I/A-II particles was associated with an increase in the ED_{50} values for the 3G10, 3D4, A11, 5F6, A07 antibodies, which recognize epitopes in the central to C-terminal region of the apoA-I protein. The data show that the presence of apoA-II on reconstituted HDL significantly affects apoA-I conformation and exposes epitopes located at the N and C-termini of the protein while blocking access of antibodies to epitopes in the central region of the apoA-I molecule.

Effect of apoA-II on HL-mediated hydrolysis of reconstituted HDL particles. Since different types of HDL can either stimulate or inhibit VLDL hydrolysis by HL, the ability of both apoA-I and apoA-II to affect HL-mediated hydrolysis was evaluated. The effect of apoA-II on HL-mediated hydrolysis of phospholipid and acylglycerides in the reconstituted HDL particles was determined. Reconstituted LpA-I/A-II particles were prepared to contain various amounts of DG and TG, as indicated in **Table 5,** and lipid hydrolysis by HL was quantified. **Figure 11** illustrates the effect on HL activity of changing the apoA-I/apoA-II and DG/TG composition of reconstituted HDL particles. Lipid hydrolysis by HL was much higher in DG-rich particles compared to those enriched in TG, suggesting that DG is a preferred substrate of the enzyme. Inclusion of apoA-II in the reconstituted HDL particles significantly reduced hydrolysis of DG-rich HDL particles by HL and to a much lesser extent of TG-rich particles (**Figure 11A**). Consistent with these data, **Table 5** shows that apoA-II reduced the V_{max} values for both the DG- and TG-enriched particles hydrolyzed by HL. ApoA-II also resulted in a reduction of the apparent K_m values (**Table 5**). Enhanced binding of HL to HDL containing apoA-II appears consistent with previous reports by other groups [39].

Table 5 - Effect of Apolipoprotein Composition of Reconstituted High Density Lipoprotein Particles on Hepatic Lipase Activity

LpA-I Complex	DG:TG[a] (molar ratio)	V_{max}[b] (nmol fatty acid released/hour)	Apparent K_m[b] (μM apoA-I)
LpA-I	10:40	94	2049
LpA-I,A-II	10:40	74	990
LpA-I	40:10	160	983
LpA-I,A-II	40:10	102	789

[a] LpA-I were prepared as described, but with the indicated DG and TG ratios. Values are representative of three different preparations of LpA-I/A-II complexes. [b] V_{max} and apparent K_m values, Y- and X-axis intercepts respectively, were calculated from double reciprocal plots. DG, diacylglyceride; TG, triacylglyceride; LpA-I, reconstituted HDL containing apoA-I; LpA-I/A-II, reconstituted HDL containing apoA-I and apoA-II.

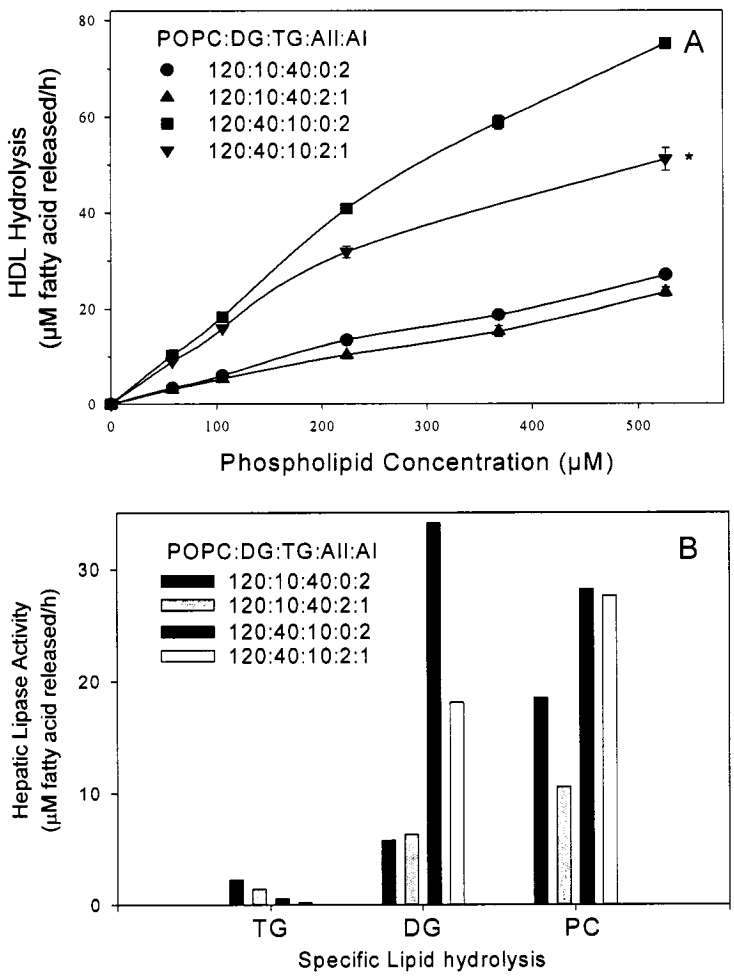


Figure 11. Effect of apolipoprotein A-II content of reconstituted HDL on HL hydrolytic activity. (A) LpA-I and LpA-I/A-II particles (POPC:DG:TG: apoA-II:apoA-I molar ratios) were prepared as described and incubated with purified human HL for 3 hours at 37°C. The reaction rate was linear throughout the incubation. Total lipid hydrolysis was determined by measuring the amount of fatty acid released per hour using a commercially available enzyme kit. Values are the mean ± S.D. of triplicate determinations and are representative of three different preparations of reconstituted HDL particles. **(B)** The differential hydrolysis of PC, TG and DG by HL were determined subtractively by measuring the amount of free fatty acid released from [^{3}H]-TG and [^{14}C]-DG, relative to the total fatty acid release, for each of the reconstituted particles. Values are the average of triplicate determinations and are representative of two different experiments. * Significance of difference from the DG enriched LpA-I. $p < 0.001$. POPC, palmitoyloleoyl phosphatidylcholine; DG, diacylglyceride; TG, triacylglyceride; AI, apoA-I; AII, apoA-II; PC, phosphatidylcholine; HDL, high density lipoprotein. (2004) *J.Lipid Res.*, **45**:849-858.

The TG-, DG- and phospholipid-specific hydrolysis of the reconstituted particles showed that apoA-II selectively inhibits DG hydrolysis and has no effect on phospholipid hydrolysis of DG-rich HDL particles by HL. In addition, apoA-II preferentially inhibited HL-mediated phospholipid hydrolysis in the DG-poor/TG-rich particles (**Figure 11B**). TG hydrolysis, on the other hand, was relatively small in both the DG-rich and TG-rich particles but also appeared to be slightly reduced in the apoA-II containing particles. Overall, apoA-II content of HDL appears to be inhibitory to HL-mediated lipid hydrolysis.

Studies from our laboratory have previously shown that apoA-I inhibits HL-mediated VLDL hydrolysis [12] and that different HDL sub-species can be both inhibitory and stimulatory to HL activity [13]. Experiments were therefore undertaken to evaluate the effect of apoA-II in reconstituted HDL particles on VLDL hydrolysis by HL. As indicated in **Figure 12**, reconstituted HDL particles and native HDL inhibited HL-mediated VLDL hydrolysis as previously reported [13]. Inclusion of apoA-II in the reconstituted HDL further increased the inhibitory effect of the LpA-I particles on HL activity. In contrast, the LpA-II particles had no effect on VLDL hydrolysis by HL.

Characterization of HL activity in phospholipid-enriched serum. In order to assess the effect of changes in lipoprotein composition that may occur during lipoprotein metabolism or a postprandial response, sera samples were enriched with various phospholipids as described. The electrophoretic mobilities of the lipoprotein classes in serum were determined by agarose gel electrophoresis after enrichment with different phospholipids (**Figure 13**). Serum enriched with PI, PA or PS showed increased electrophoretic mobilities for all lipoprotein classes (VLDL, LDL and HDL) compared to PBS or PC-enriched sera. The net negative charge of the phospholipid-enriched lipoproteins was proportional to electrophoretic mobility.

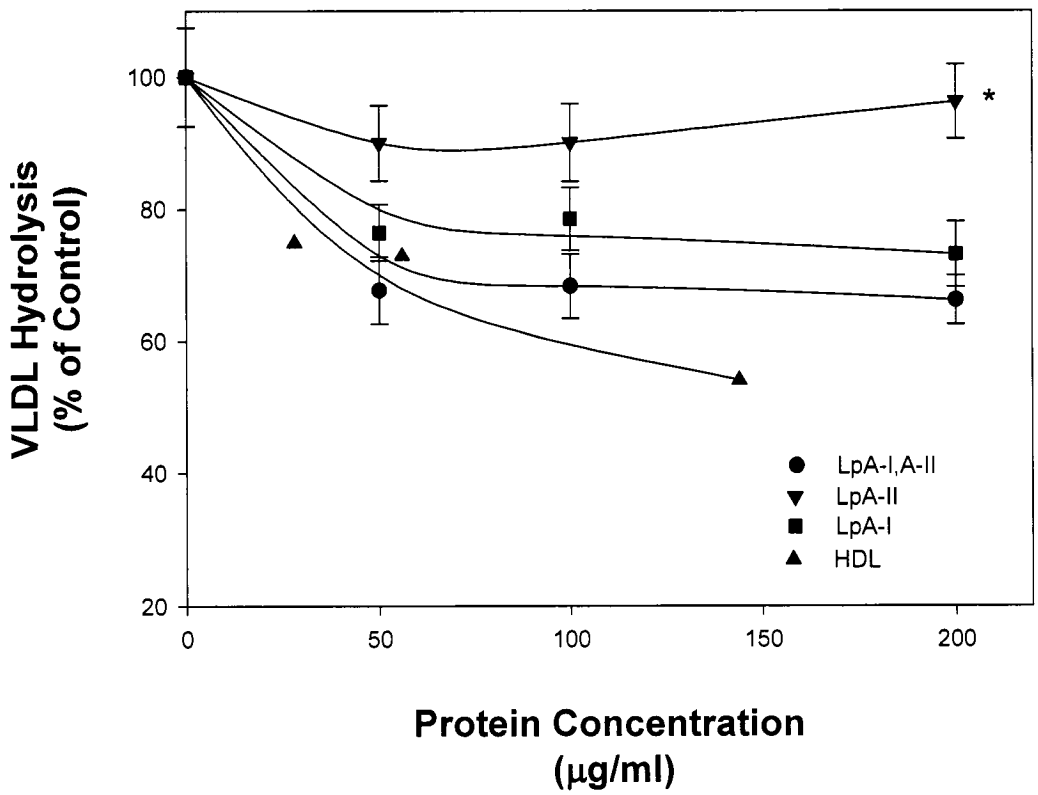


Figure 12. Effect of apolipoprotein content of reconstituted HDL on VLDL-TG hydrolysis by HL. VLDL (350μM TG) was incubated with HL and increasing amounts of either HDL, LpA-I, LpA-I/A-II or LpA-II particles for 30 minutes at 37°C. Hydrolysis was determined by measuring the amount of free fatty acid released using a commercially available enzyme kit. Hydrolytic values are the means ± S.D. of triplicate determinations and are representative of two different experiments. Samples containing no HDL or HDL particles were used as a control. Protein concentration was used as a measure of HDL concentration. * $p < 0.001$ significance of difference from LpA-I. LpA-I, reconstituted HDL containing apoA-I; LpA-II, reconstituted HDL containing apoA-II; LpA-I/A-II, reconstituted HDL containing apoA-I and apoA-II; HDL, high density lipoprotein; VLDL, very low density lipoprotein. (2004) *J.Lipid Res.*, **45**:849-858.

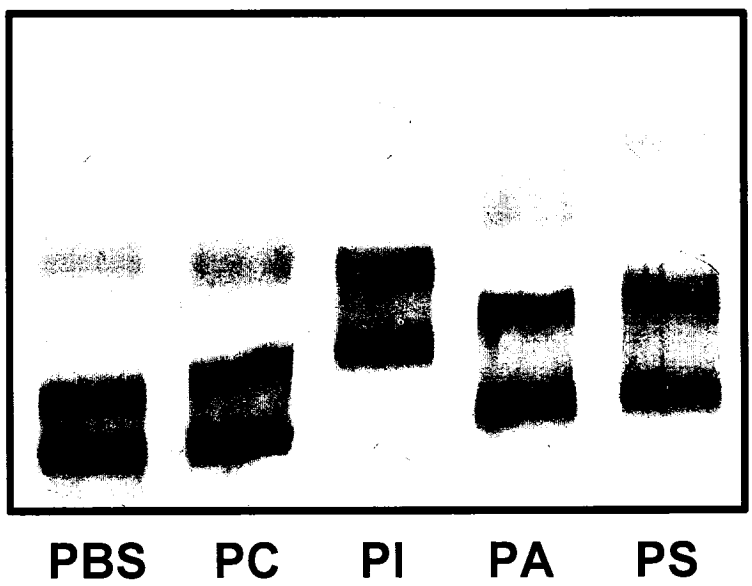


Figure 13. Effect of phospholipid enrichment of serum on lipoprotein electrophoretic mobility. Fasted sera samples were enriched with PBS, PC, PI, PA or PS and subjected to agarose electrophoresis. Lipoproteins bands were visualized by neutral lipid staining and charge was calculated based on electrophoretic mobility as described. Lipoprotein samples move from bottom to top during electrophoresis. Representative of three different preparations of enriched serum samples. PBS, phosphate buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

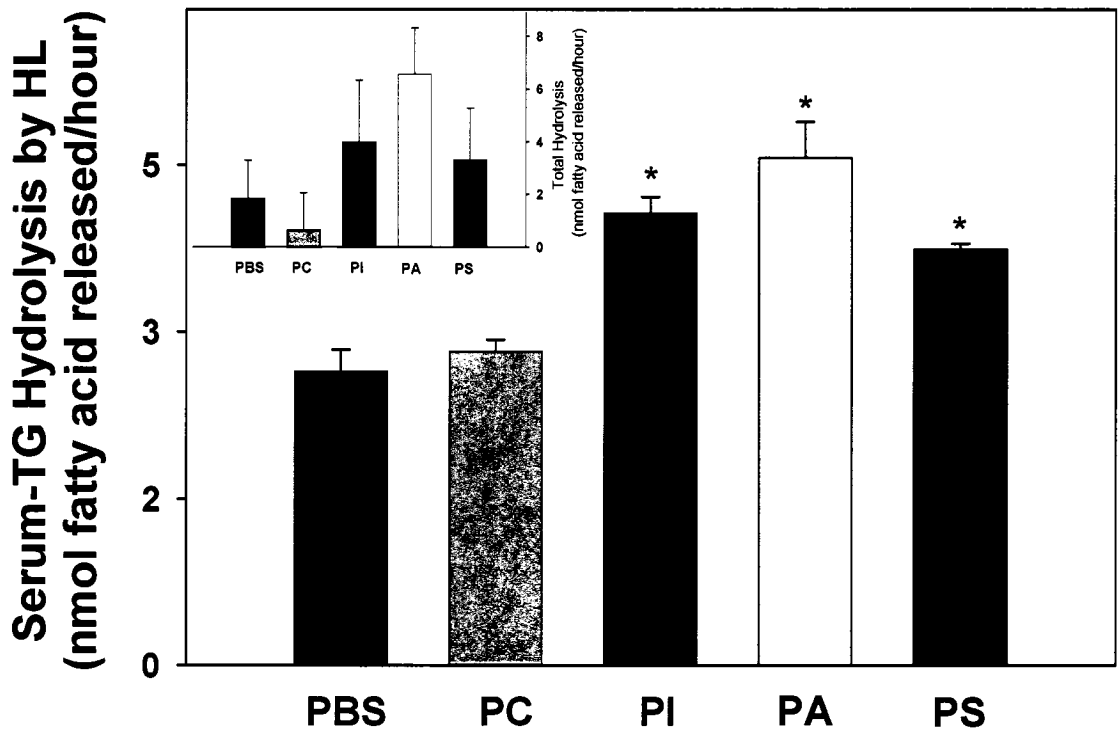


Figure 14. Effect of phospholipid enrichment of serum on total lipid hydrolysis and TG-specific hydrolysis by HL. Fasted serum samples radioactively-labelled with TG were enriched with PC, PI, PA or PS and incubated with HL as described in the Materials and Methods section. Total lipid hydrolysis (**inset**) and TG-specific-hydrolysis were determined after a one hour incubation by measuring the amount of fatty acid released using a commercial assay and by scintillation counting of the labelled fatty acids after extraction. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; TG, triacylglyceride; HL, hepatic lipase. * Indicates a statistical significance of difference from the PC-enriched serum of $p<0.001$.

Table 6 - Effect of Phospholipid Enrichment on Serum, Very Low Density Lipoprotein and High Density Lipoprotein Phospholipid-specific Hydrolysis by Hepatic Lipase

	PBS	PC	PI
Phospholipid Hydrolysis (nmol fatty acid released per hour)[c]			
VLDL[a, d]	0.12	0.45	0.59
HDL[a, d]	0.2	0.56	0.19
Serum[b, d]	N/A	0.46	0.16
Phospholipid Hydrolysis (% of total lipid hydrolysis)			
VLDL[a]	0.005	0.03	0.03
HDL[a]	0.004	0.004	0.01
Serum[b]	N/A	0.2	0.1

[a] Representative values from three independent experiments. [b] Representative values from two independent experiments. [c] Average SEM for hydrolytic values was determined to be +/- 0.22 nmol fatty acid released/hour. [d] Differences in hydrolysis between groups are not statistically significant. VLDL, very low density lipoprotein; HDL, high density lipoprotein; PBS, phosphate buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol.

PI-enrichment of serum caused the largest increase in net negative charge in all lipoprotein classes followed by PS and PA, respectively. PC-enrichment of serum did not result in a significant change in electrophoretic mobility or charge in any lipoprotein class compared to the PBS control serum.

Figure 14 illustrates the rate of lipid hydrolysis by HL in serum enriched with various phospholipids. Enrichment with anionic phospholipids significantly stimulated TG hydrolysis by HL as determined by ^{3}H-TG degradation. Enrichment of serum with PI, PA or PS also stimulated total lipid hydrolysis as determined by total free fatty acid mass measurements (**Figure 14, inset**). Serum enriched with PC did not stimulate either total or TG hydrolysis compared to the PBS control. Comparison of TG hydrolysis and total lipid hydrolysis shows that the majority of the free fatty acids released are generated from the hydrolysis of TG. Phospholipid hydrolysis was determined by measuring ^{14}C-fatty acid release after extraction of ^{14}C-PC-labelled serum as described [244]. No significant difference in ^{14}C-PC hydrolysis between PC- or PI-enriched sera was evident. Phospholipid hydrolysis in both PC- and PI-enriched sera represented only 0.2% and 0.1% of the total lipid hydrolysis by HL, respectively (**Table 6**). In all cases, phospholipid hydrolysis was small when compared to total lipid or TG hydrolysis by HL.

Effect of increasing phospholipid enrichment of serum on lipid hydrolysis by HL. Reports examining the effect of lipoprotein charge on CETP and PLTP have indicated that the activity of these enzymes is inhibited at more extreme lipoprotein negative charge values [168,169]. Therefore, studies were undertaken to determine the effect of increasing amounts of phospholipid-enrichment of serum on HL-mediated TG hydrolysis.

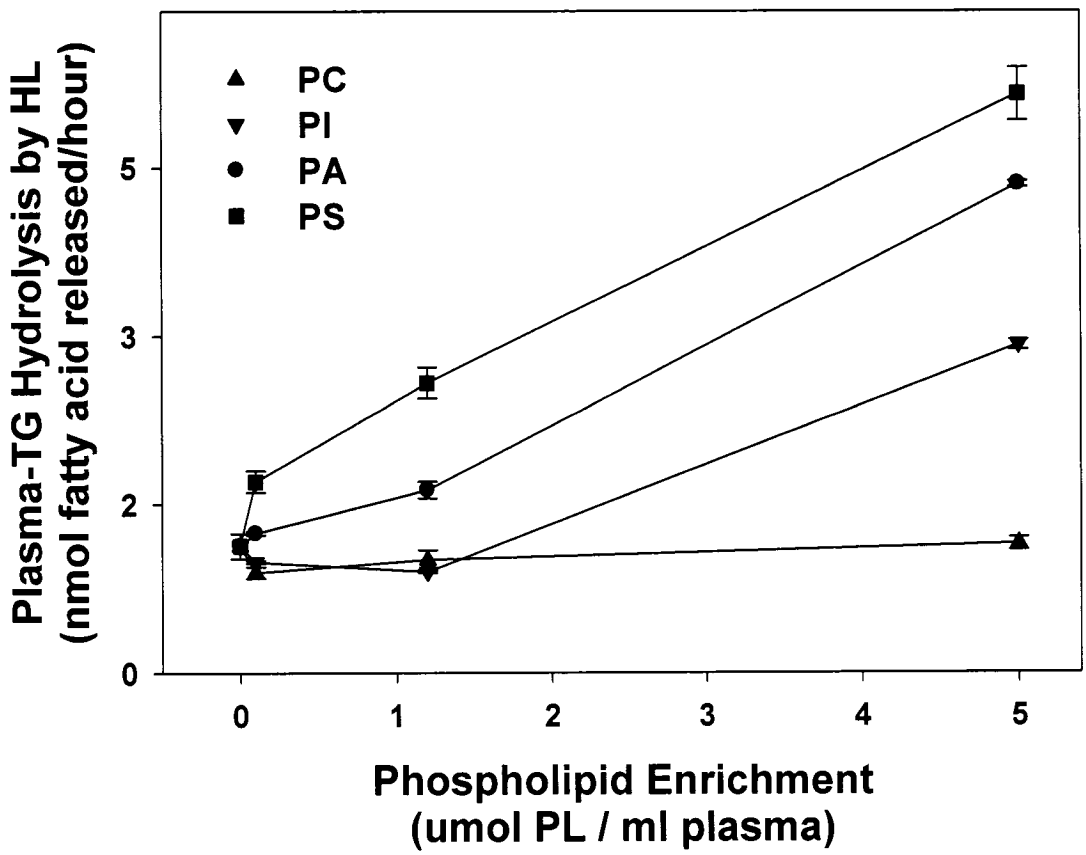


Figure 15. Increasing concentrations of anionic lipids stimulate HL activity in Serum. Fasted serum samples radioactively-labelled with TG were enriched with increasing amounts of PC, PI, PA or PS and incubated with HL as described. TG-specific-hydrolysis was determined after a one hour incubation by measuring the amount of radioactively-labelled fatty acids released. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

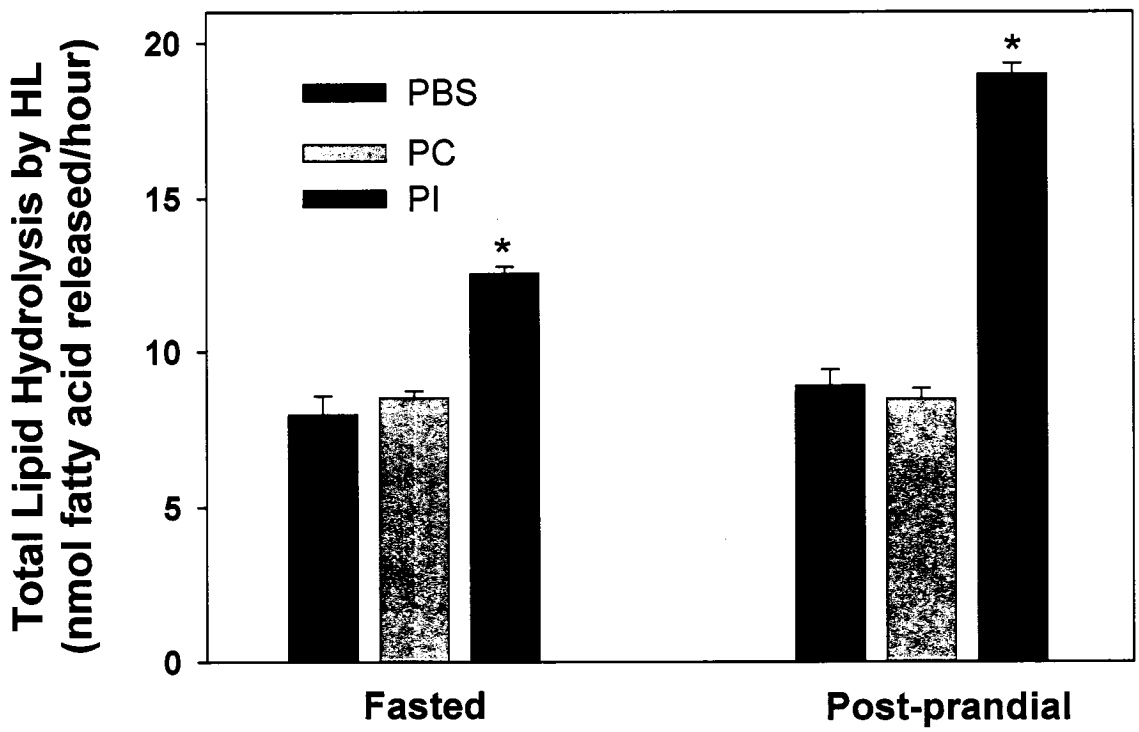


Figure 16. Effect of phospholipid enrichment of serum on total lipid hydrolysis by HL. Fasted and postprandial sera were enriched with PC or PI vesicles as described and incubated with exogenous human HL as described in the Materials and Methods section. Un-enriched serum was used as a control by the addition of an equivalent volume of PBS and enzyme. Hydrolytic rates were determined after a one hour incubation by measuring the amount of fatty acid release using a commercial assay. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; HL, hepatic lipase. * Indicates a statistical significance of difference from the respective PC-enriched serum of $p<0.001$.

Increasing amounts of the anionic phospholipids PI, PA and PS progressively increased the charge of all lipoprotein classes in serum as determined by agarose gel electrophoresis. Increasing amounts of PC had no effect on lipoprotein charge relative to PBS control sera. Likewise, increasing amounts of PI, PA and PS in serum significantly stimulated TG hydrolysis by HL in a linear manner up to 5 μmol phospholipid/ml serum, whereas increasing amounts of PC had no effect on HL activity (**Figure 15**).

Effect of PI-enrichment of fasted and lipemic serum on lipid hydrolysis by HL. One of the primary anionic phospholipids found in the bloodstream is PI and therefore its role in lipoprotein charge and metabolism was evaluated. Studies were performed to determine the effect of PI-enrichment of fasted and lipemic sera on lipoprotein charge and lipid hydrolysis. **Figure 16** shows total lipid hydrolysis by HL in fasted or lipemic (postprandial) sera enriched with PC or PI. Enrichment with PI significantly stimulated total lipid hydrolysis as determined by total fatty acid release. In contrast, serum enriched with PC did not stimulate hydrolysis by HL compared to the PBS control.

Effect of soy PI and DOPI enrichment of serum on lipoprotein charge and lipid hydrolysis by HL. The effect of PI acyl chain composition on lipoprotein charge and HL activity was also evaluated. Sera and VLDL samples were enriched with PC, soy PI or DOPI and lipoprotein charge was evaluated by agarose gel electrophoresis. Both soy PI and DOPI resulted in identical increases in the negative charge of all lipoprotein classes in serum (**Figure 17**) and VLDL compared to PC and PBS. Likewise, sera (**Figure 18A**) and VLDL (**Figure 18B**) enriched with soy PI or DOPI stimulated TG hydrolysis by HL relative to PC and PBS control samples.

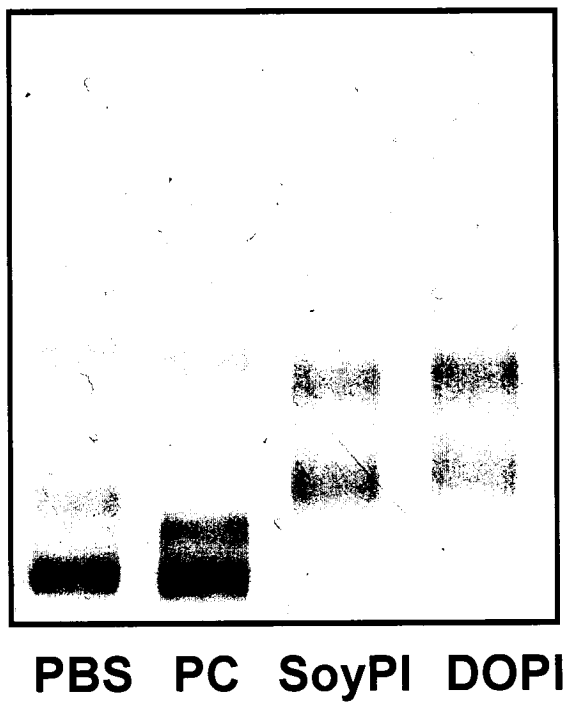


Figure 17. Acyl chain composition of PI and lipoprotein charge. Fasted serum samples were enriched with PC, soy PI, or DOPI and subjected to agarose gel electrophoresis as described. Lipoproteins were visualized by neutral lipid staining and charge was calculated based on electrophoretic mobility as described. Representative of three different preparations of enriched serum samples. PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; DOPI, dioleoyl phosphatidylinositol.

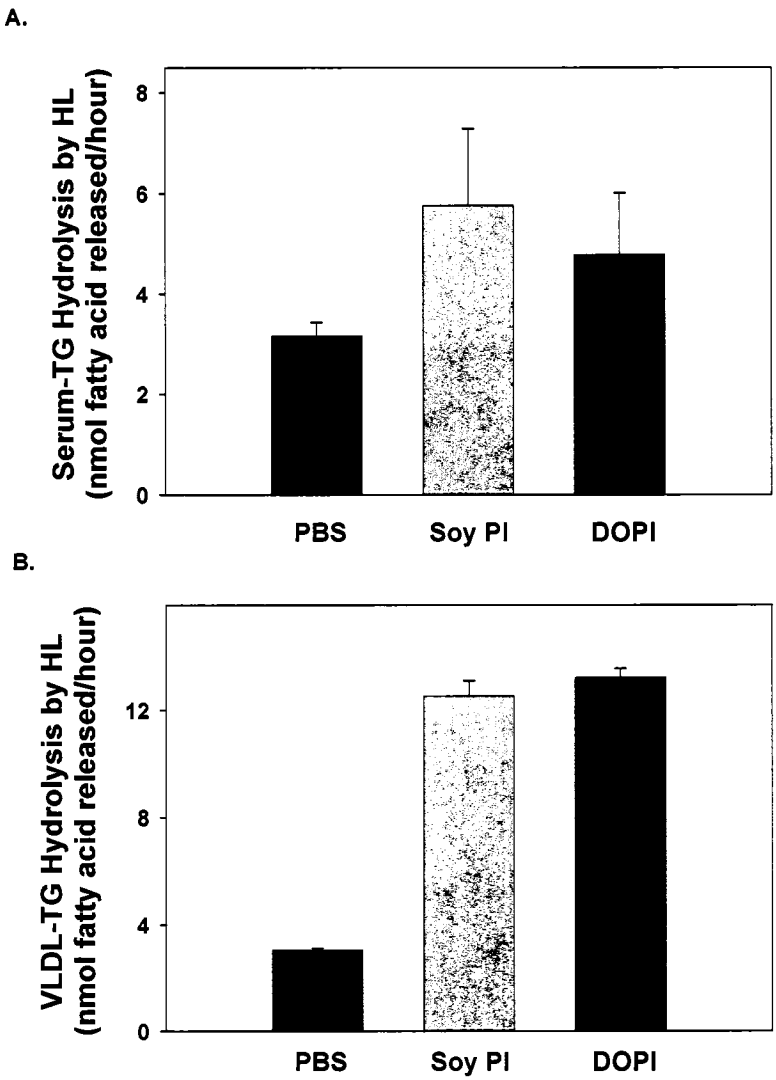
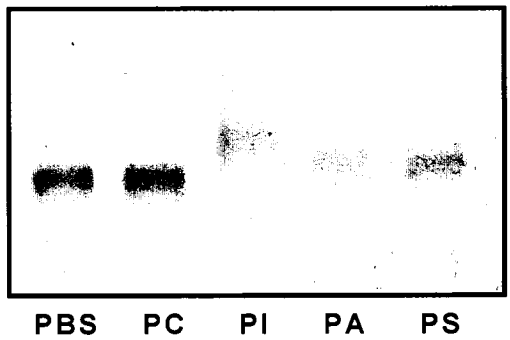
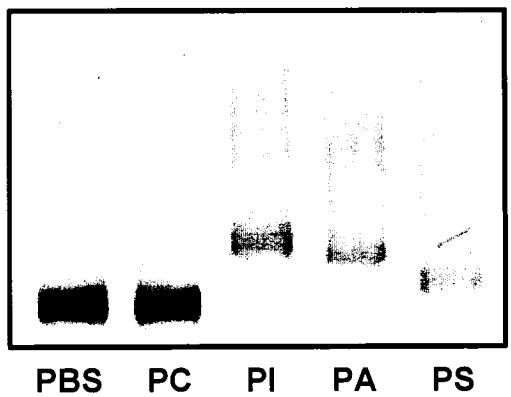


Figure 18. Effect of PI acyl chain composition on HL-mediated hydrolysis of serum and VLDL. Fasted serum (**A**) and VLDL (**B**) samples radioactively-labelled with TG were enriched with soy PI or DOPI and incubated with HL as described. TG-specific-hydrolysis was determined after a one hour incubation by measuring the amount of radioactively-labelled fatty acids released. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. PBS, phosphate-buffered saline; PI, phosphatidylinositol; DOPI, dioleoyl phosphatidylinositol; TG, triacylglyceride; HL, hepatic lipase. * Indicates a statistical significance of difference from the PC-enriched serum of $p<0.001$.

A. HDL



B. LDL



C. VLDL

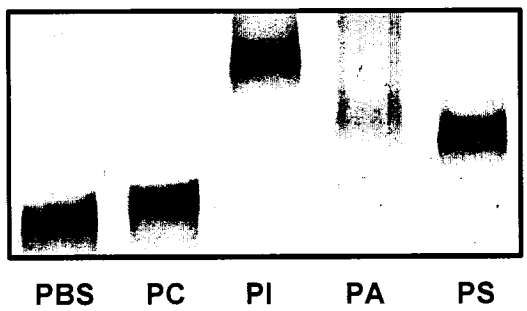


Figure 19. Effect of phospholipid enrichment of lipoproteins on electrophoretic mobility. Isolated HDL (**A**), LDL (**B**) and VLDL (**C**) samples were enriched with PC, PI, PA or PS and subjected to agarose electrophoresis. Lipoproteins bands were visualized by neutral lipid staining and charge was calculated based on electrophoretic mobility as described. Representative of three different preparations of enriched serum samples. PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

Table 7 - Effect of Phospholipid Enrichment on Lipoprotein Electrophoretic Mobility and Charge

Phospholipid	Surface Potential (- mV)		
	VLDL	LDL	HDL
PBS	10.0	5.6	13.9
Phosphatidylcholine (PC)	10.0	5.6	13.9
Phosphatidylinositol (PI)	12.9	8.5	15.4
Phosphatidic acid (PA)	11.9	8.0	14.9
Phosphatidylserine (PS)	11.4	6.6	14.9

[a] Lipoprotein charge calculated from electrophoretic mobility on 0.6% agarose gels as described. Charge potential at the particle surface represents ± 0.2 mV (SEM). Values are representative of three separate experiments. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

No differences were observed in the ability of soy PI or DOPI to change lipoprotein charge or stimulate HL activity.

Characterization of HL-mediated hydrolysis of phospholipid-enriched lipoproteins. The electrophoretic mobilities of isolated VLDL, LDL and HDL enriched with different phospholipids were analyzed by agarose gel electrophoresis (**Figure 19**) and were used to calculate the lipoprotein charge properties indicated in **Table 7**. Incorporation of PI, PA or PS into HDL resulted in an increase in electrophoretic mobility and net negative charge as compared to control or PC-enriched HDL. HDL enriched with PI exhibited the largest change in charge, while PA and PS showed smaller, but similar, changes. In contrast, PBS and PC had no effect on HDL electrophoretic mobility or charge. Much like HDL, enrichment with anionic phospholipids also increases the negative charge of LDL and VLDL. PI, PA and PS enrichment caused an increase in electrophoretic mobility compared to control or PC-enriched LDL or VLDL. Likewise, PI induced the largest change in charge, followed by PA and PS, respectively.

Figure 20A illustrates the rate of TG hydrolysis in HDL enriched with various phospholipids. Enrichment of HDL with the anionic phospholipids PI, PA and PS significantly stimulated HDL-TG hydrolysis by HL. The stimulation of hydrolysis was similar for all three anionic phospholipids, resulting in an approximate 2-fold increase in hydrolysis compared to the PBS control. PC enrichment of HDL had a minor effect on TG hydrolysis by HL compared to the PBS control. Phospholipid-specific hydrolysis was relatively small compared to total lipid hydrolysis and was < 1% of the total lipid hydrolysis by HL (**Table 6**).

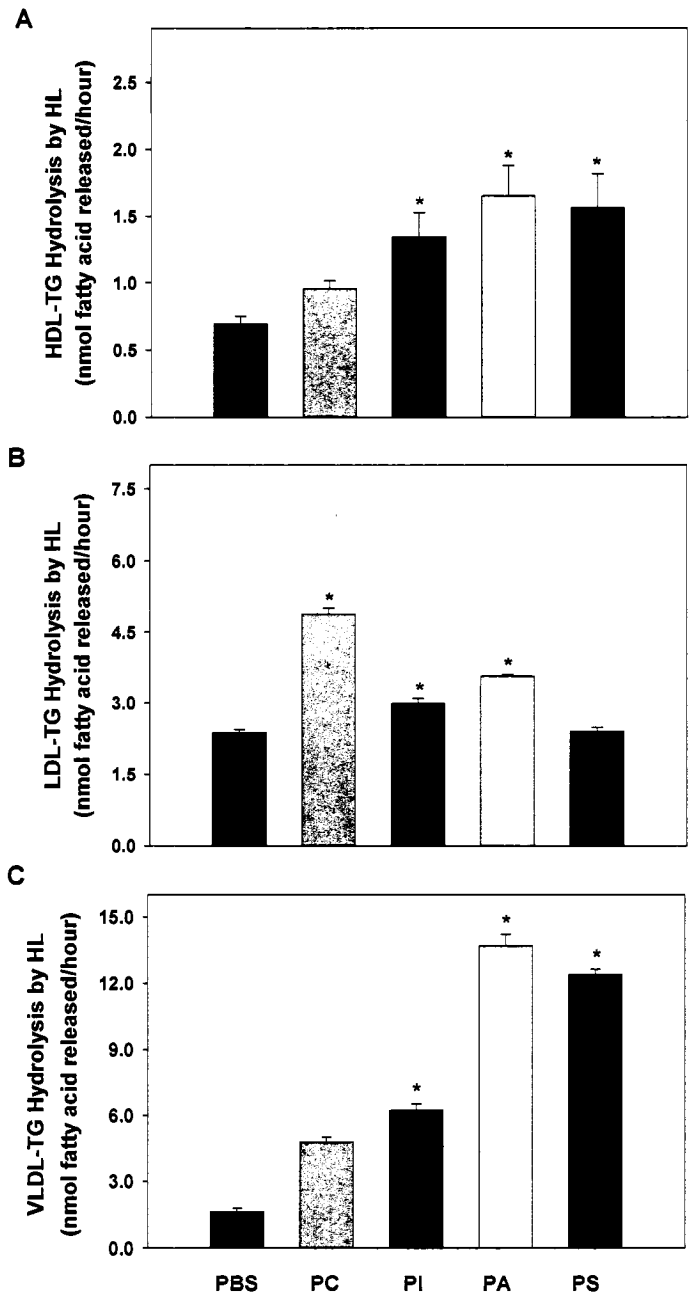


Figure 20. Effect of anionic phospholipid enrichment on lipoprotein-TG hydrolysis by HL. HDL (**A**), LDL (**B**) and VLDL (**C**) samples radioactively-labelled with TG were enriched with PBS, PC, PI, PA or PS and incubated with HL as described. TG-specific-hydrolysis was determined after a one hour incubation by scintillation counting of the labelled fatty acids release. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HL, hepatic lipase. * Indicates a statistical significance of difference from the PC-enriched HDL of $p<0.001$.

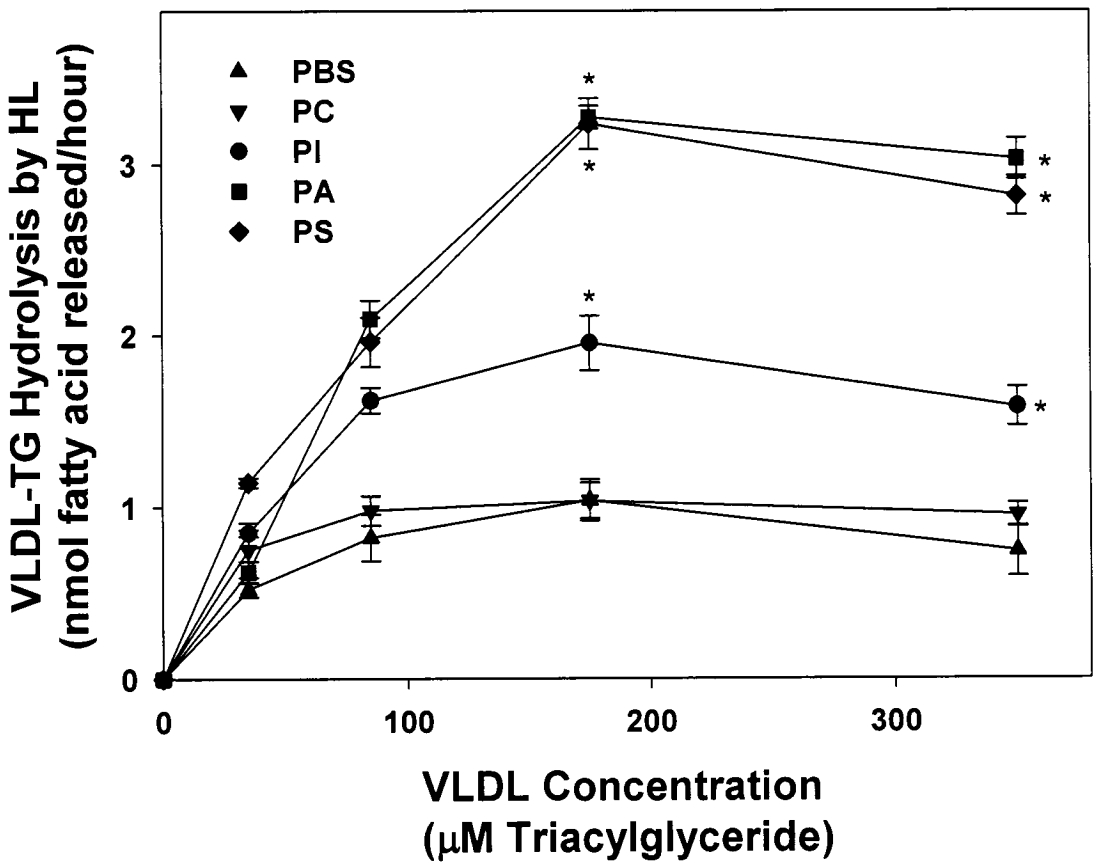


Figure 21. Effect on anionic phospholipid enrichment of VLDL on HL activity. VLDL samples radioactively-labelled with TG were enriched with PBS, PC, PI, PA or PS and incubated at the indicated concentrations with HL as described. TG-specific-hydrolysis was determined after a one hour incubation by scintillation counting of the labelled fatty acids released. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of three different experiments. PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; VLDL, very low density lipoprotein; HL, hepatic lipase, TG, triacylglyceride. * Indicates a statistical significance of difference from the respective PC-enriched VLDL of $p<0.001$.

Figure 20B illustrates the rate of TG-specific hydrolysis by HL of LDL enriched with various phospholipids. Enrichment of LDL with the anionic phospholipids PI and PA significantly stimulated LDL-TG hydrolysis by HL. However, PS did not stimulate LDL-TG hydrolysis by HL, while PC had a significant effect on hydrolysis and stimulated TG hydrolytic rates by almost 2-fold compared to the PBS control. **Figure 20C** illustrates the rate of TG hydrolysis by HL of phospholipid-enriched VLDL. PI, PA and PS significantly stimulated VLDL-TG hydrolysis compared to the PBS control. The stimulation of hydrolysis was highest (almost 6-fold) in the PA and PS-enriched VLDL. PI-enrichment of VLDL stimulated hydrolysis by 3-fold, while PC enrichment of VLDL promoted a 2.6-fold increase in TG hydrolysis by HL. **Table 6** shows that VLDL phospholipid hydrolysis under the described reaction conditions was relatively small, accounting for < 1% of the total lipid hydrolysis by HL.

Figure 21 shows the effect of anionic lipids and increasing concentrations of VLDL on TG hydrolysis by HL. Similar to the previous results, VLDL enriched with the anionic phospholipids PI, PA or PS show significantly higher TG hydrolysis by HL. Kinetic analysis of the data was undertaken to generate the apparent K_m and V_{max} values indicated in **Table 8**. As expected, maximal velocity rates were highest for PI, PA and PS-enriched VLDL, at 1.8, 3.4 and 3.1 nmol fatty acid/hour, respectively. The V_{max} values for PBS and PC-enriched VLDL were reduced and similar at 0.9 and 1.0 nmol fatty acid/hour, respectively. PI, PA and PS also resulted in higher apparent K_m values than either PBS- or PC-enriched VLDL. Previous studies have also observed a stimulation in interfacial enzyme activity associated with parallel increases in both V_{max} and apparent K_m values [38,390].

Table 8 - Effect of Phospholipid Enrichment on the Kinetic Parameters of Very Low Density Lipoprotein-triacylglyceride Hydrolysis by Hepatic Lipase

Phospholipid-enriched VLDL	V_{max}[a] (nmol fatty acid released/hour)	K_m[a] (μM TG)
PBS	1.4	58.5
Phosphatidylcholine (PC)	1.2	19.5
Phosphatidylinositol (PI)	3.3	97.7
Phosphatidic acid (PA)	7.0	198.3
Phosphatidylserine (PS)	3.9	77.2

[a] V_{max} and apparent K_m values were estimated non-linear regression analysis as described. Values are representative of three separate experiments. VLDL, very low density lipoprotein; TG, triacylglyceride.

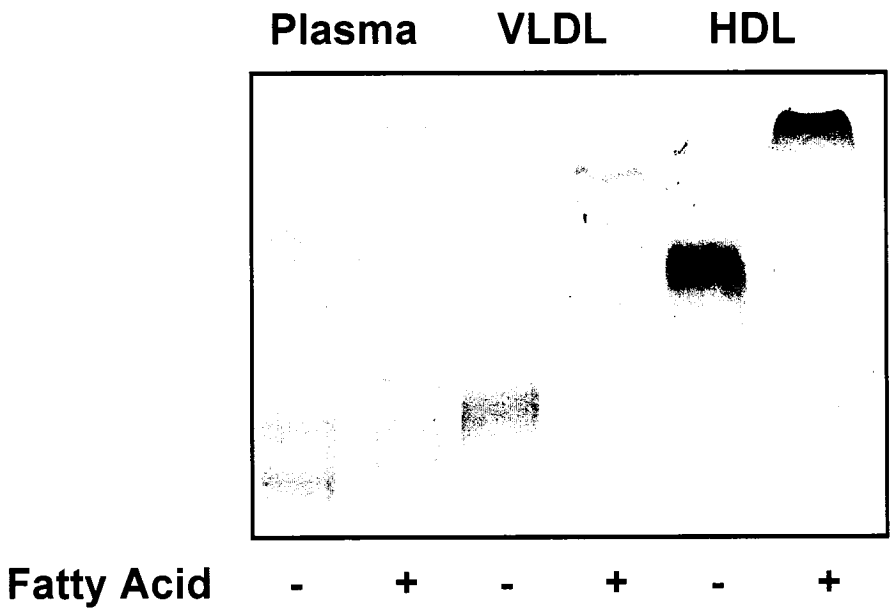


Figure 22. Effect of fatty acid enrichment of serum, VLDL and HDL on lipoprotein charge. Fasted serum, VLDL and HDL samples were enriched with oleic acid and subjected to agarose gel electrophoresis. Lipoproteins bands were visualized by neutral lipid staining and charge was calculated based on electrophoretic mobility as described. Representative of three different preparations of enriched serum samples. VLDL, very low density lipoprotein; HDL, high density lipoprotein.

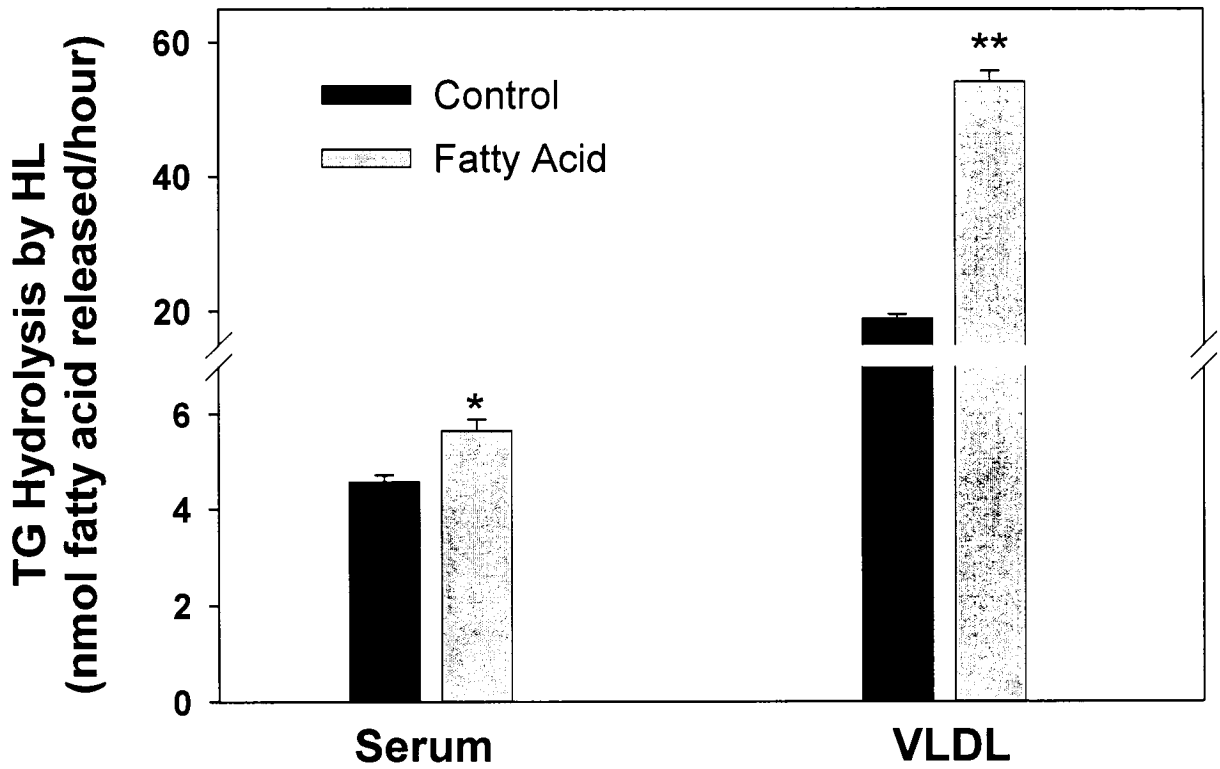


Figure 23. Effect of fatty acid enrichment of serum and VLDL on TG-specific hydrolysis by HL. Serum and VLDL samples radioactively-labelled with TG were enriched with PC (control) or oleic acid and incubated with HL as described. TG-specific-hydrolysis was determined after a one hour incubation by scintillation counting of the labelled fatty acids released. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. TG, triacylglyceride; HL, hepatic lipase; VLDL, very low density lipoprotein. *Indicates a statistical significance of difference from the respective PC-enriched controls of $p<0.05$. **Indicates a statistical significance of difference from the respective PC-enriched controls of $p<0.001$.

Effect of fatty acid enrichment of serum, VLDL and HDL on lipoprotein charge and HL-mediated hydrolysis. Fatty acids released during the postprandial response can remain associated with lipoproteins and contribute to the anionic properties of the particle. Therefore, the effect of fatty acids on lipoprotein charge and HL activity were evaluated. Serum, VLDL and HDL were enriched with oleic acid as described. Much like the anionic phospholipids, oleic acid caused an increase in the negative charge of all lipoprotein classes in serum as well as isolated VLDL and HDL compared to control samples (**Figure 22**). In addition, HL-mediated TG hydrolysis was significantly enhanced in both serum and VLDL enriched with oleic acid compared to control incubations (**Figure 23**).

Effect of lipid enrichment of HDL on VLDL hydrolysis by HL. Additional experiments were undertaken to track the effects of anionic lipids on HL hydrolysis of mixtures of reconstituted or native HDL and VLDL. Reconstituted HDL were prepared containing phospholipids with different head groups but similar acyl chain composition. Reconstituted HDL particles composed of DOPC, DOPE and DOPG were characterized by non-denaturing gradient gel electrophoresis which showed that the particles were homogenous and had diameters between 7-8 nm (**Figure 24**). While the sizes of the reconstituted HDL were similar, the charges of the particles were significantly different. The DOPG particle was the most negatively charged at –25 mV compared to the –10 mV DOPC particle, whereas the DOPE particle had an intermediate charge of –14 mV (**Table 9**). The effect of these different reconstituted HDL particles on VLDL hydrolysis by HL was also evaluated. **Figure 25** shows that the more negatively charged DOPG particles significantly stimulated VLDL hydrolysis by HL in a concentration-dependent manner compared to the more positively charged DOPC particles.

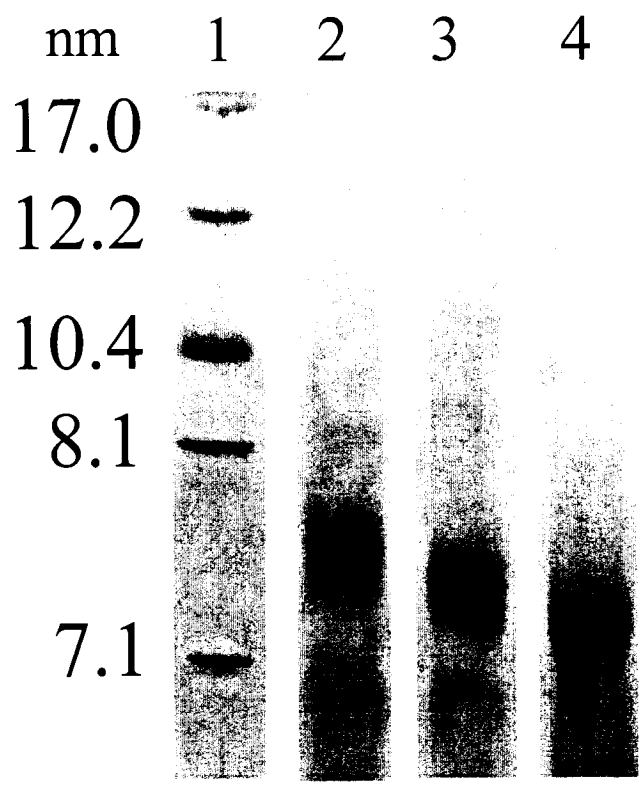


Figure 24. Non-denaturing gradient gel electrophoresis of reconstituted HDL particles containing DOPC, DOPE or DOPG. The size and homogeneity of reconstituted HDL particles were determined by non-denaturing gradient gel electrophoresis on pre-cast 8-25% acrylamide gels. Protein bands were visualized by Coomassie blue staining and particle size (nm) was determined from reference standards. **Lane 1**, size markers; **lane 2**, reconstituted HDL composed of DOPC; **lane 3**, reconstituted HDL composed of DOPE; **lane 4**, reconstituted HDL composed of DOPG.

Table 9 - Effect of Lipid Composition on the Size and Charge of Reconstituted High Density Lipoprotein Particles

LpA-I Complex[a]	Diameter[b] (nm)	Surface Potential[c] (-mV)
DOPC LpA-I	7.6	10
DOPE LpA-I	7.4	14*
DOPG LpA-I	7.2	25*

[a] LpA-I complexes were prepared by co-sonication as described in methods and materials. Values are representative of 3-5 different preparations of LpA-I complexes. [b] Particle diameters from non-denaturing gradient gel electrophoresis. [c] Charge potential at the particle surface ± 0.2 mV (SEM). * Significance of difference from DOPC LpA-I, $p < 0.001$. LpA-I, reconstituted HDL containing apoA-I; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DOPG, dioleoyl phosphatidylglyceride.

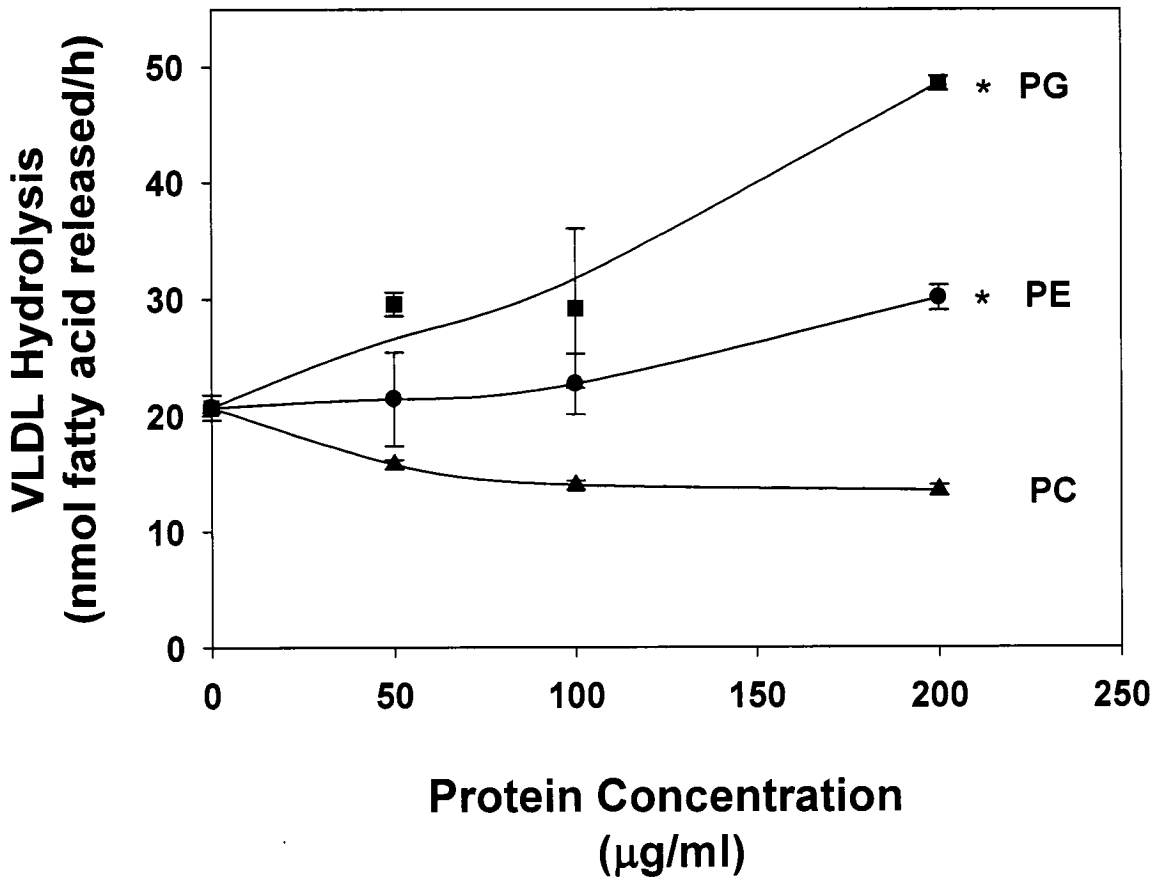


Figure 25. Effect of reconstituted HDL containing DOPC, DOPE or DOPG on VLDL hydrolysis by HL. Reconstituted HDL particles were incubated at the concentrations indicated with purified HL and VLDL for one hour at 37°C. Hydrolysis was determined by quantifying the amount of fatty acids released using a commercial free fatty acid assay as described. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of three different preparations of HDL. VLDL, very low density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. * Indicates a statistical significance of difference from the reconstituted HDL composed of DOPC of $p<0.001$.

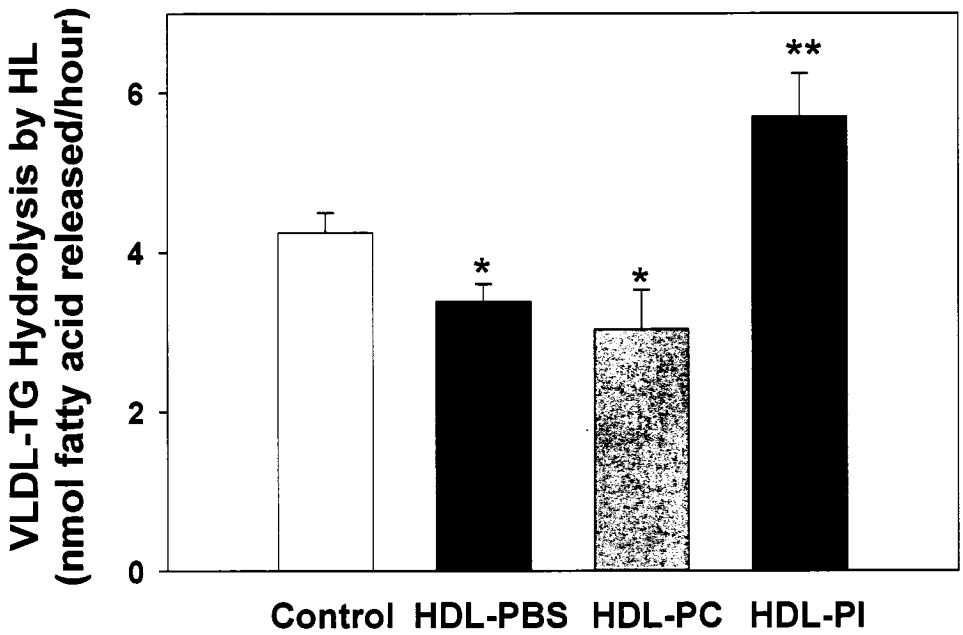


Figure 26. Effect of PI-enriched HDL on VLDL-TG hydrolysis by HL. HDL samples were enriched with PC or PI and incubated with purified HL and VLDL. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of three different preparations of HDL. *Indicates a statistical significance of difference from control (no HDL) of $p<0.001$. **Indicates a statistical significance of difference from the PC- and PI-enriched HDL of $p<0.001$. Values are the mean of triplicate quantification determinations and are representative of three separate experiments. HDL, high density lipoprotein; VLDL, very low density lipoprotein; TG, triacylglyceride; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PC, phosphatidylcholine.

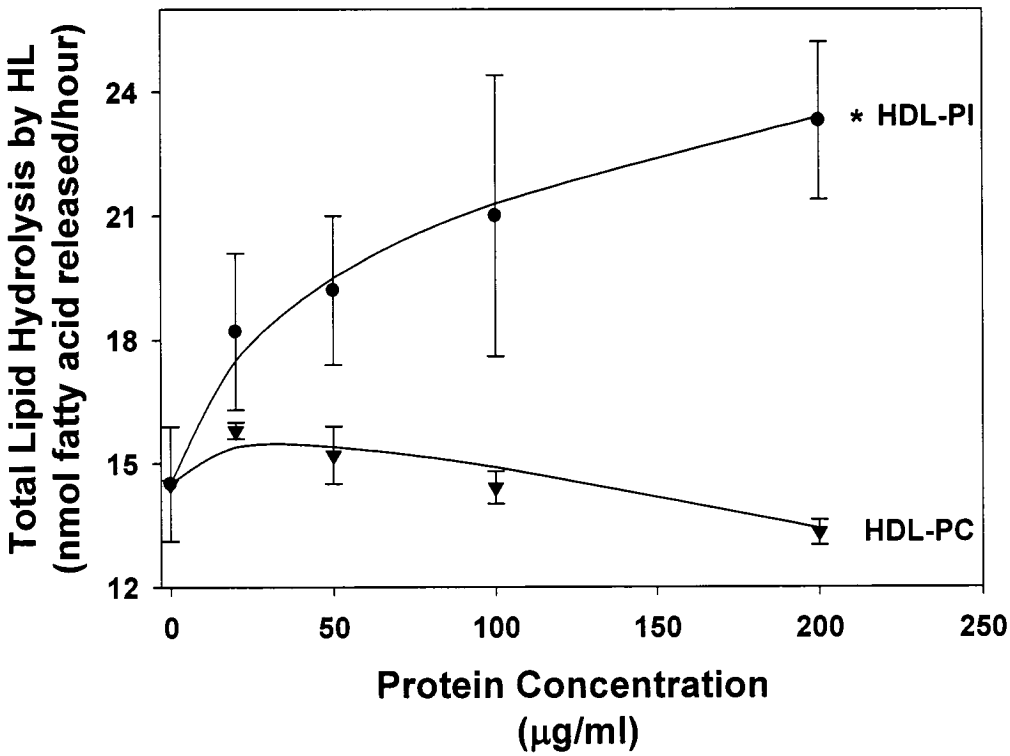


Figure 27. Effect of native HDL containing PI on total lipid hydrolysis by HL. HDL was enriched with PC or PI and incubated with purified HL and VLDL as described. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of three different preparations of HDL. * Indicates a statistical significance of difference from the PC- and PI-enriched HDL of $p<0.001$. Values are the mean of triplicate quantification determinations and are representative of three separate experiments. HL, hepatic lipase; HDL-PI, high density lipoprotein enriched with PI; HDL-PC, high density lipoprotein enriched with PC.

Likewise, the DOPE particles, which had an intermediate negative charge, also significantly stimulated VLDL hydrolysis but to a lesser extent than the DOPG particles. The DOPC reconstituted HDL particles were actually inhibitory to VLDL hydrolysis by HL, consistent with previous data from our laboratory [12]. Similar studies were also undertaken with native HDL enriched with PI or PC. The results in **Figure 26** show that VLDL-TG hydrolysis by HL is significantly stimulated by HDL enriched with PI. Co-incubation of VLDL with PC-enriched or PBS-treated HDL did not stimulate VLDL-TG hydrolysis. Similarly, HDL enriched with PI stimulated VLDL total lipid hydrolysis by HL in a concentration dependent manner compared to PC-enriched HDL (**Figure 27**). In fact, PC-enriched HDL were inhibitory to VLDL total and TG hydrolysis by HL (**Figures 26 and 27**). This is consistent with previous data from our laboratory showing that HDL can be inhibitory to VLDL hydrolysis by HL [12,13,38]. In order to determine if any of the PI in HDL is transferred to VLDL during these co-incubations, control experiments using a ^{3}H-PI tracer in the PI-enriched HDL were undertaken. HDL enriched with ^{3}H-PI were incubated with VLDL under similar reaction conditions then subjected to density gradient ultracentrifugation as described [13] and scintillation counting of the separated HDL and VLDL fractions. These studies indicated that a small amount (~ 8%) of the PI is transferred from HDL to VLDL under these reaction conditions.

Effect of PI-enrichment of HDL on HL association. To determine if PI affects HL association with plasma lipoproteins, either unmodified or PI-enriched HDL were combined with VLDL and HL. Physiological concentrations of VLDL and HDL and saturating amounts of HL were utilized to approximate conditions in the bloodstream.

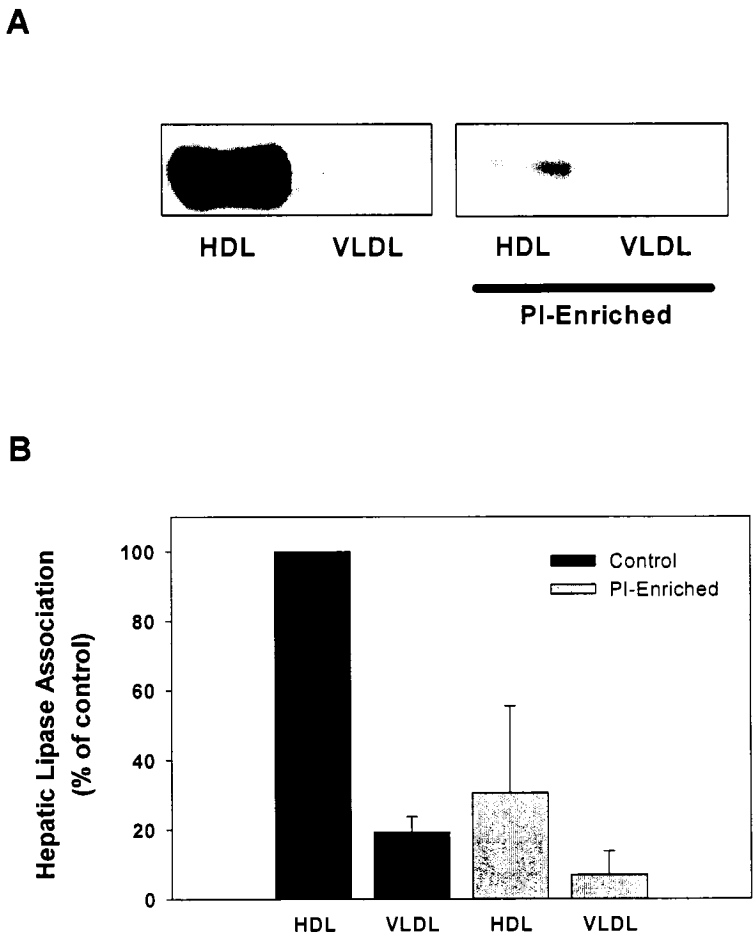


Figure 28. Lipoprotein charge affects HL activity and association. Control HDL or HDL enriched with PI were combined with VLDL and purified HL and then the lipoproteins were separated by size exclusion chromatography on a Superose-6 column. Lipoprotein peak fractions were pooled and probed for HL with an HL monoclonal antibody. **(A)** Immunoblot results are representative of three different experiments. **(B)** Average densitometry analyses of the immunoblot data shown in panel A. Values are the mean ± S.D. of three different experiments. VLDL, very low density lipoprotein; HDL, high density lipoprotein; PI, phosphatidylinositol.

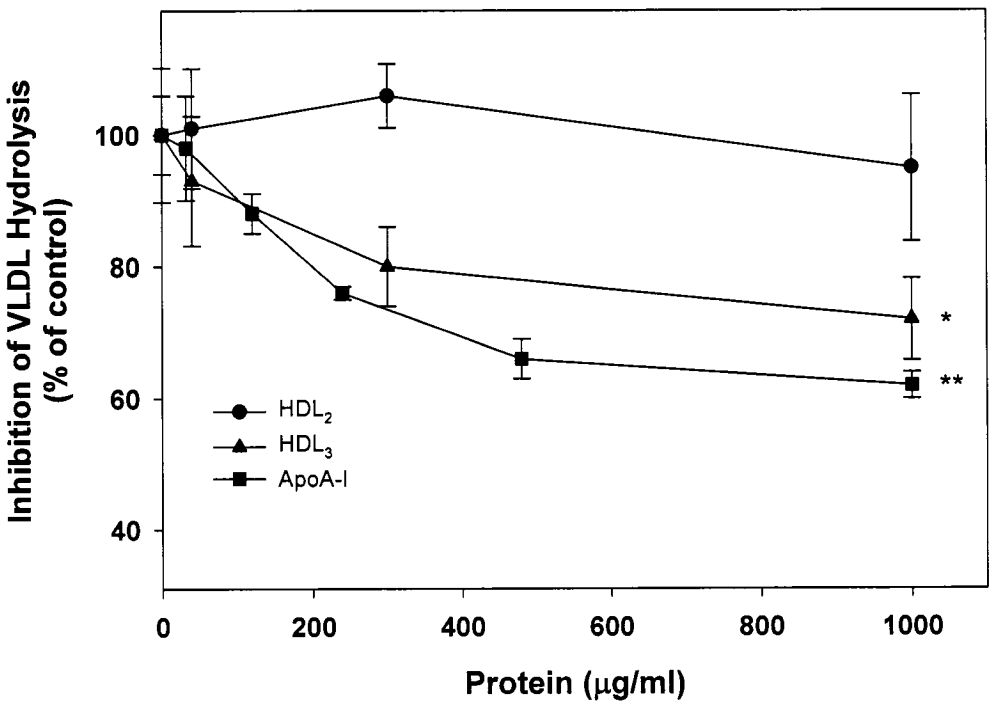


Figure 29. The effect of HDL_2, HDL_3 and apoA-I on VLDL hydrolysis by LPL. HDL_2, HDL_3 or apoA-I were incubated at the indicated concentrations with VLDL and human LPL for 20 minutes at 37°C as described. Total lipid hydrolysis was determined by quantifying the fatty acids released using a commercial free fatty acid assay. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of three separate experiments. VLDL, very low density lipoprotein; HDL_2, high density lipoprotein$_2$; HDL_3, high density lipoprotein$_3$; apoA-I, apolipoprotein A-I. * Indicates a statistical significance of difference from HDL_2 of $p<0.05$, ** Indicates a statistical significance of difference from HDL_2 of $p<0.001$.

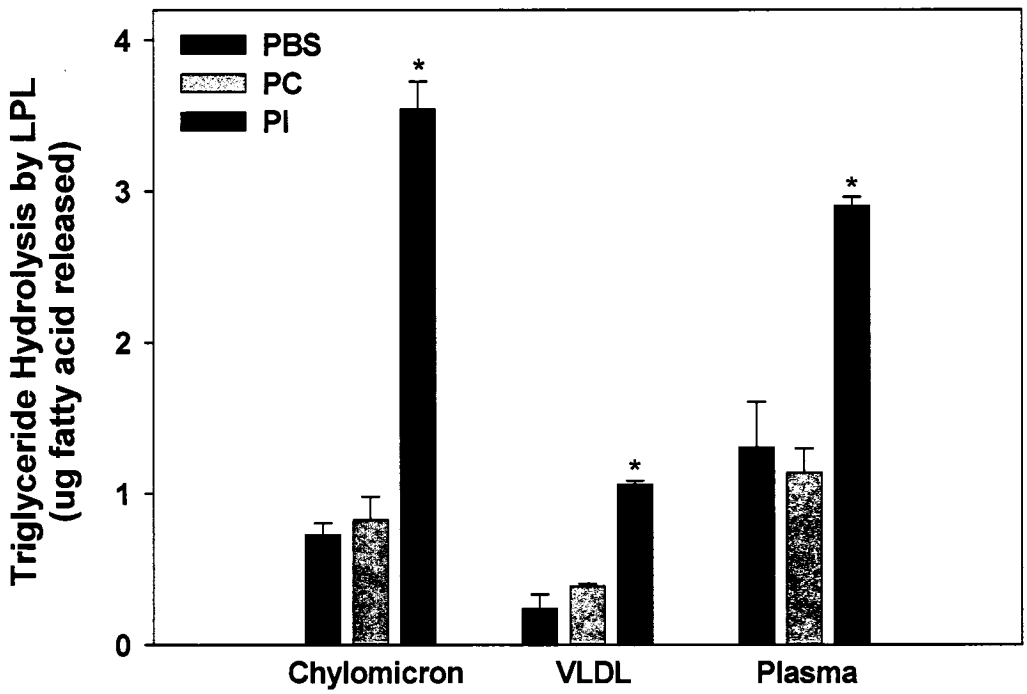


Figure 30. Effect of PI-enrichment on serum, chylomicron and VLDL hydrolysis by LPL. Serum, chylomicron and VLDL samples radioactively-labelled with TG were enriched with PC or PI and incubated with bovine LPL as described. TG-specific hydrolysis was determined after a 20 minute incubation at 37°C by scintillation counting of the radioactively-labelled free fatty acids released. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations and are representative of three different experiments. LPL, lipoprotein lipase; PBS, phosphate buffered saline; PC, phosphatidyl choline; PI, phosphatidyl inositol; VLDL, very low density lipoprotein. * Indicates a statistical significance of difference from the respective PBS-enriched controls of $p<0.001$.

The lipoproteins were incubated at 37°C for 1 hour and then re-isolated and separated from free HL by size exclusion chromatography. Column fractions representing lipoprotein peak profiles were then pooled and probed with a monoclonal HL antibody to track the association of HL with different lipoproteins. Immunochemical (**Figure 28A**) and densitometry (**Figure 28B**) analyses show that, under normal circumstances HL appears to predominantly associate with HDL. Only small amounts of HL were observed associated with the pooled VLDL fractions. However, PI enrichment of HDL caused an almost 70% reduction in HL association with HDL. When VLDL was incubated with PI-enriched HDL, HL association with VLDL relative to the control samples also appears to be slightly reduced but the decrease is not statistically significant.

Effect of HDL, apoA-I and apoA-II on VLDL hydrolysis by human LPL. Previous data from our laboratory showed that the HDL sub-species had different effects on the hydrolysis of VLDL by HL. These studies showed that the larger less dense HDL_2 were stimulatory to VLDL hydrolysis by HL, whereas the smaller HDL_3 and free apoA-I were inhibitory to HL-mediated VLDL hydrolysis. Studies were therefore undertaken to determine whether LPL activity is also regulated by apoA-I and HDL. VLDL were incubated with either apoA-I, apoA-II, HDL_2 or HDL_3 and LPL-mediated VLDL hydrolysis was determined as described. **Figure 29** shows that, much like HL, VLDL hydrolysis by LPL is significantly inhibited by both HDL_3 and free apoA-I. Moreover, HDL_2 appeared to have no effect on VLDL hydrolysis by LPL. In separate experiments, reconstituted HDL composed of apoA-II had no effect on LPL-mediated hydrolysis of VLDL.

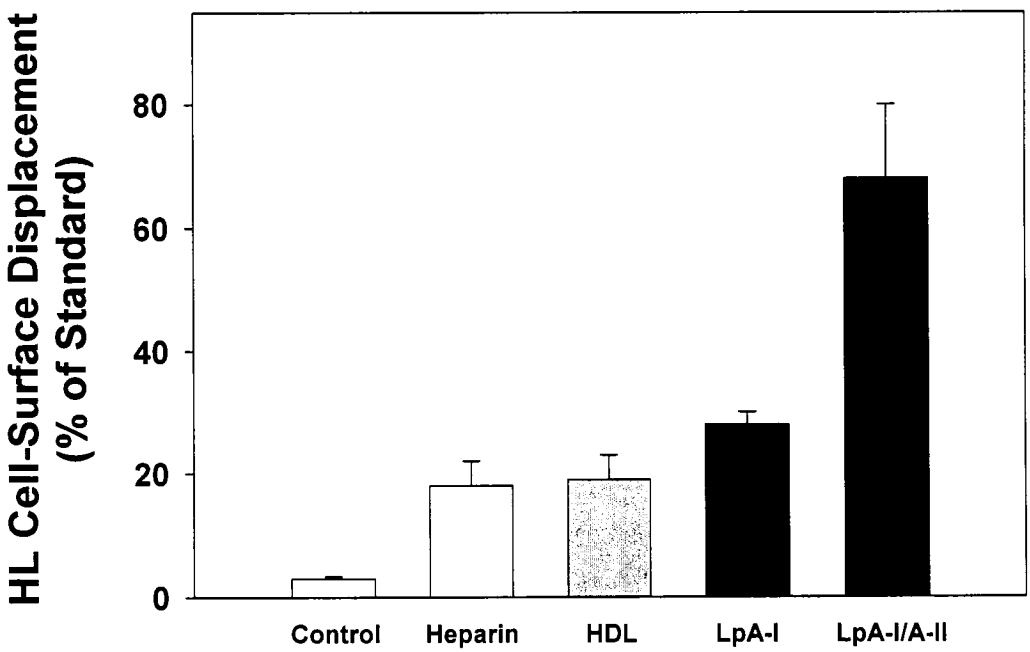


Figure 31. Displacement of HL from the surface of CHO-hHL cells by reconstituted HDL. Native HDL and synthetic HDL particles containing apoA-I (LpA-I) or apoA-I and apoA-II (LpA-I/A-II) were incubated with CHO-hHL cells as described. HL displacement was then determined by quantifying the amount of HL protein released into the media by immunoblot analysis. Bands were quantified by densitometry and compared to a purified HL protein standard control. HDL particles containing both apoA-I and apoA-II were much more efficient at displacing cell-surface HL than either native HDL, LpA-I or heparin. Control incubations using plain media show relatively no displacement of HL as expected. Values are the means of triplicate determinations and are representative of three separate experiments. HL, hepatic lipase; HDL, high density lipoprotein; LpA-I, reconstituted HDL containing apoA-I; LpA-I/A-II, reconstituted HDL containing apoA-I and apoA-II.

Effect of charge on LPL-mediated hydrolysis in serum, chylomicrons and VLDL. Serum, chylomicrons and VLDL were enriched with PI or PC and TG hydrolysis by bovine LPL was determined as described. Consistent with the data for HL activity, LPL-mediated TG hydrolysis was stimulated in serum, chylomicrons and VLDL enriched with the anionic phospholipid PI compared to samples enriched with either PBS or PC (**Figure 30**).

Effect of apoA-II content on HL cell-surface displacement by reconstituted HDL particles. While data from our laboratory showed that apoA-I displaces cell-surface HL, the effect of apoA-II had yet to be determined. Therefore, the effect of apoA-II in synthetic HDL particles on the displacement of HL from the surface of CHO-hHL cells was evaluated (**Figure 31**). The data showed that HDL and heparin displace cell-surface HL into the media relative to control samples. An equal amount of purified HL protein stock used as a loading control for each gel to faciliate gel-to-gel desitometry comparisons. Likewise, LpA-I particles containing only apoA-I and PC displaced HL into the media to a similar extent as native HDL. In contrast, LpA-I/A-II particles significantly stimulated the displacement of cell-surface HL into the media of CHO-hHL cells relative to heparin and HDL.

Effect of HDL charge on HL cell-surface displacement. Since HDL phospholipid composition has a significant impact on HL activity, the effect of HDL charge on its ability to displace cell-surface HL was also evaluated. CHO-hHL cells were incubated as described with native HDL as well as HDL enriched with PC or PI and the amount of HL displaced into the media was quantified by immunoblot analysis. The data show that native HDL displaces HL to a similar extent as heparin (**Figure 32**). In contrast, HDL enriched with either PI or PC were much less able to displace HL from the surface of CHO-hHL cells than either native HDL or heparin (**Figure 32**).

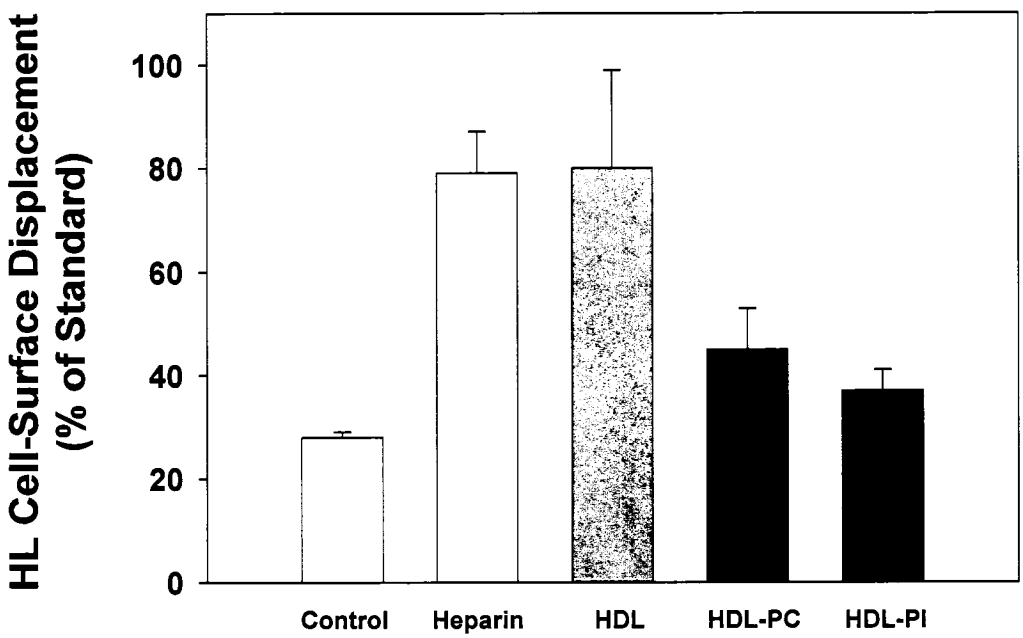


Figure 32. Displacement of HL from the surface of CHO-hHL cells by phospholipid-enriched HDL. Native HDL and HDL enriched with PC or PI were incubated with CHO-hHL cells as described. HL displacement was then determined by quantifying the amount of HL protein released into the media by immunoblot analysis. Bands were quantified by densitometry and compared to a purified HL protein standard control. As expected, heparin and unmodified HDL displaced HL into the media of CHO-hHL cells. In contrast, HDL enriched with PC or PI displaced only a small amount of cell-surface HL compared to the media control. Values are the means of triplicate determinations and are representative of three separate experiments. HL, hepatic lipase; HDL, high density lipoprotein; HDL-PI, high density lipoprotein enriched with PI; HDL-PC, high density lipoprotein enriched with PC.

Chapter 4 – Discussion

4.1 - Introduction

Cardiovascular disease is responsible for 50% of deaths worldwide [7]. The risk of developing cardiovascular disease has been directly linked to elevated TG and low plasma HDL concentrations [8]. Plasma lipases acting on lipoproteins play an important role in initiating the development of this disorder. Moreover, the catabolism of TG-rich lipoproteins by HL and LPL has been shown to be a major determinant of HDL levels in the bloodstream and the development of atherosclerosis [8]. I now propose that HDL is not only a product of TG-rich lipoprotein hydrolysis but that it actively regulates TG metabolism by controlling lipase function. Data from our laboratory and others now suggest that HL is lipolytically inactive when attached to the cell surface and requires displacement or release from the extracellular matrix to stimulate efficient hydrolysis of its substrates in the circulation [12-14]. It is now clear that HDL regulates HL by displacing the enzyme from the cell-surface and affecting its inter-lipoprotein movement in the plasma. In this thesis, I show that HDL composition controls HL activity by regulating the cell-surface displacement of the enzyme and that its interfacial association with lipoprotein substrates is crucial in promoting lipid hydrolysis. Overall, most studies now suggest that specific determinants of lipoprotein composition control interactions between interfacial enzymes and substrates and regulate their metabolism *in vivo*.

4.2 – HDL-apoA-II composition regulates HL activity

In order to understand how lipoprotein composition affects HL activity, a thorough analysis of the roles of HDL apolipoprotein and lipid content on hydrolysis by this enzyme were undertaken. The roles of apoA-I, apoA-II as well as various charged lipids on lipoprotein

structure and their effect on HL activity were determined. In this study, the effect of apoA-II on HL hydrolytic activity was evaluated. The data indicated that apoA-II can significantly modify HL-mediated hydrolysis (**Figures 11 and 12**) . In addition, it appears that the acylglyceride composition of HDL also affects the ability of apoA-II to inhibit HL activity. Lipid hydrolysis of synthetic LpA-I was much higher in DG-enriched particles, consistent with the observation that DG is a preferred substrate of HL [332]. ApoA-II caused a reduction in the lipid hydrolysis of both the DG- and TG-enriched particles; however, the inhibition of HL activity was much greater in the DG-enriched LpA-I/A-II. The addition of apoA-II also resulted in reduced apparent K_m values, indicating that apoA-II increased the affinity of HL for this lipoprotein substrate (**Table 5**). These data suggest that apoA-II is inhibitory to HL-mediated lipid hydrolysis by decreasing the ability of the enzyme to shuttle between substrates. The effect of apoA-II on the remodelling of HDL catalyzed by the interfacially active proteins found in the plasma has been well-characterised. ApoA-II is less efficient than apoA-I at activating LCAT [391] and in the plasma, discoidal HDL containing only apoA-II are very poor substrates for LCAT [392]. Similarly, apoA-II inhibits the lipid transfer activities of both CETP and PLTP [35,393]. The effect of apoA-II on HL is less clear, as there are reports that it can be both stimulatory [41,351,394] and inhibitory [354] to lipid hydrolysis. However, data using apoA-II transgenic or knockout mice crossed to HL-deficient animals clearly showed that apoA-II inhibits HL activity *in vivo* [356,395]. The effect of apoA-II on interfacial enzyme association with lipoproteins appears consistent with recent reports that LPL and EL are also regulated by the apoA-II content of lipoproteins. VLDL hydrolysis by LPL has been shown to be inhibited by the presence of apoA-II on this lipoprotein [37]. *In vivo*, apoA-II significantly inhibits EL activity and delays HDL catabolism [250]. Reconstituted HDL containing only apoA-II were not hydrolysed very

efficiently by EL compared to LpA-I particles [396]. This suggests that apoA-II inhibits the activity of EL by increasing the binding of the enzyme to HDL resulting in decreased V_{max} and apparent K_m values. By uniquely affecting substrate affinity, apoA-II appears to control the interaction of HDL with plasma enzymes and directly regulates lipid metabolism.

Previous studies from our laboratory have shown that HDL and apoA-I are inhibitory to VLDL-TG hydrolysis by HL [12,13]. The ability of one lipoprotein class to affect lipase-mediated hydrolysis of another has been previously reported by a number of groups [13,38,41,354]. It should be noted that while HL can act on most of the plasma lipoproteins, hydrolytic rates are orders of magnitude higher for chylomicrons and VLDL than for HDL or LDL [397,398]. In co-incubations of VLDL and HDL, it has been shown that TG hydrolysis accounts for the bulk of HL and LPL activity [398]. In the present study, the different types of HDL have unique effects on HL hydrolytic activity (**Figure 8**). The larger HDL_2 are stimulatory to VLDL hydrolysis while the denser HDL_3 are inhibitory to both HL and LPL activity. Moreover, reconstituted LpA-I particles are inhibitory to VLDL-TG hydrolysis by HL, while inclusion of apoA-II in the HDL particle (LpA-I/A-II) further increases this inhibitory effect (**Figure 12**). The data now suggest that it is the apoA-I containing HDL that are uniquely inhibitory to HL activity and that apoA-II modifies the structural properties of LpA-I particles. It is likely that the apoA-II content of HDL results in a tighter binding to HL, preventing the enzyme from hydrolyzing VLDL lipids. The greater inhibitory effect of HDL_3 may be partly due to the higher amounts of apoA-II found in this HDL sub-species. Enhanced binding of HL to HDL_3 due to the presence of more apoA-II may directly block the shuttling/association of HL with circulating VLDL and reduce overall hydrolytic rates. This may partially explain why HDL_2 are better substrates for HL than HDL_3 and appears consistent with the work of Mowri *et al.*

showing that HL associates more readily with HDL_3, which contains higher amounts of apoA-II relative to HDL_2 [42].

HDL have also been shown to inhibit HL-mediated chylomicron hydrolysis, possibly by preferential binding of HL to HDL [159,399,400]. These studies showed that an increase in activity was typically associated with an increase in apparent K_m, suggesting that the enzyme is more active when loosely bound to its substrate [340]. *In vitro*, HDL was also shown to inhibit rat and human LPL-mediated hydrolysis of TG-emulsions [401]. The regulation of LPL activity by HDL appears to be due to an apolipoprotein component since inhibition was reduced when the HDL used was made apolipoprotein-poor [401]. It was suggested that an HDL apolipoprotein composition that favored a higher affinity with LPL would reduce the binding and interaction of the enzyme with the TG-emulsion and thus, hydrolysis would be inhibited [401]. It is well-accepted that HDL is related to TG metabolism primarily because it is a product of lipolysis and a substrate for HL and LPL. However, this thesis suggests that HDL can directly modulate TG hydrolysis [12,13,38] and may affect the ability of HL to hydrolyze TG-rich lipoproteins.

4.3 – HDL charge and lipid composition regulate HL activity

The data now show that much like apoA-II, the anionic lipid composition of lipoproteins has a significant effect on particle structure and functional properties. Abnormal lipoprotein charge has long been known to be associated with hyperlipidemia [161]. Lipoproteins from dyslipidemic patients have an abnormal lipid and protein composition associated with changes in electrostatic properties [161]. It is now clear that lipoprotein charge may be a determining factor in the metabolism of lipoproteins in dyslipidemic patients as well as healthy individuals. Lipoprotein composition and charge are not static but change constantly depending on dietary and metabolic factors [8]. Apolipoprotein and lipid exchange between lipoproteins is in a

constant state of flux and not only affect lipoprotein charge but the metabolism of lipoproteins in the circulation [8]. It would be expected that lipoprotein composition would be the most physiologically relevant during a postprandial response when free fatty acids are generated from the hydrolysis of TG-rich lipoproteins. The fatty acids that are produced under these conditions can remain associated with lipoprotein particles and affect their charge properties [126]. Transient changes in phospholipid composition of lipoproteins during the postprandial response and normal metabolism are also known to occur [126]. As a result, phospholipid-dependent changes in lipoprotein composition and charge would also be expected to affect lipid metabolism and clearance in the bloodstream.

In this study, the effect of fatty acid and phospholipid composition on the metabolism of lipoproteins by HL was undertaken. The electrostatic properties of lipoproteins were modified through incorporation of free fatty acids or anionic phospholipids. The data show that changes in lipoprotein charge due to anionic free fatty acids and phospholipids can regulate hydrolysis by HL. Incorporation of oleic acid in serum and VLDL increased the negative charge of all lipoprotein classes and stimulated TG-specific hydrolysis by HL (**Figures 22 and 23**). Stimulation of VLDL hydrolysis by oleic acid was approximately 3-fold, whereas oleic acid partitioning to albumin most likely caused a smaller activation of HL activity in serum. Similarly, changes in lipoprotein negative charge, using the anionic phospholipids PI, PA or PS stimulated the lipolytic activity of HL in serum and in all of the lipoprotein classes studied (**Figures 14, 15 and 19**). Phospholipid hydrolysis by HL under the described reaction conditions was relatively small compared to total and TG-specific hydrolysis (**Table 6**). This is somewhat consistent with reports that TG is hydrolyzed more readily by HL than other lipids [159,329], however, the phospholipid and TG specificity of HL has yielded conflicting results depending on the substrates

and reaction conditions used [331,332]. The effect of HDL phospholipid-enrichment on the hydrolysis of VLDL-TG by HL was also determined. In contrast to the effect of apoA-II, the data show that VLDL hydrolysis by HL is stimulated when co-incubated with HDL made more negatively charged by incorporation of PI, PA, PS, DOPE or DOPG (**Figures 25 and 27**). Consistent with HL studies, LPL activity is also regulated by the charge properties of lipoproteins. The data show that serum, chylomicrons and VLDL enriched with PI display significantly stimulated LPL-mediated TG hydrolysis (**Figure 30**). Until now, the role of interfacial electrostatic properties on the regulation of HL activity has not been well-characterized. Laboda *et al.* showed that TG hydrolysis by HL in monolayers containing SM and cholesterol is reduced by 2-fold when it is made more negatively charged by inclusion of dicetylphosphate in the lipid monolayer [182]. Likewise, inclusion of the non-hydrolysable anionic ether lipids dialkyl-PA or dialkyl–PE stimulated TG hydrolysis by HL relative to monolayers containing the less charged dialkyl-PC [402]. It is now clear that lipoprotein charge plays an important role in the regulation of the lipolytic function of HL.

The ability of different phospholipids to affect lipoprotein charge and HL activity may also be determined by factors such as head group size and acyl chain length. Each of these characteristics is known to have an effect on HL-mediated lipolysis [337,346,403-406]. In this study, differences in acyl chain length do not appear to affect lipoprotein charge or HL activity. All of the phospholipids used contained oleoyl acyl chains except for soy PI which contained a mixture of acyl chain lengths (16:0-18:2). However, studies showed no differences in lipoprotein charge or the stimulation of HL activity using either DOPI or soy PI (**Figure 18**). In contrast, the effect of phospholipid head group size on lipoprotein charge and HL activity may be much more significant. The electrostatic nature of the phospholipid head group is believed to be very

important to the action of some phospholipases [405,406]. A decrease in head group size is most likely associated with an increase in the net negative surface charge of the lipoprotein particle. This observation may also relate the regulation of lipase activity with changes in interfacial hydration that result from the increased negative charge of the lipoprotein. The extent of hydration of this surface layer may play an important role in the catalytic mechanism of HL. The active site serine residues of the plasma lipases are believed to participate in a proton relay mechanism that uses protons from the medium for hydrolysis [222,407]. Interfacial hydration may influence the availability of these protons and control the rate of hydrolysis by HL. In this study, a decrease in phospholipid head group size gave rise to modest differences in lipoprotein charge and a significant stimulation of HL activity. PA and PS caused a smaller change in negative charge than PI but an even greater increase in VLDL hydrolysis. This could suggest that there may be an optimal charge for the stimulation of HL activity. However, when increasing concentrations of anionic phospholipids were used to enrich serum or VLDL, HL activity was stimulated in a linear manner with increasing lipoprotein charge (**Figure 15**). Therefore, the head group of PI must somehow affect lipoprotein electrostatic properties. Cell-surface polyols are known to affect membrane hydration and the carbohydrate group of PI may modify interfacial interactions [346]. This appears to be the case with PI, which causes the largest change in lipoprotein charge but does not stimulate hydrolysis of VLDL or HDL to the same extent as the other anionic phospholipids. Increased incorporation of PI into lipoproteins may also cause a more rigid interface at the particle surface resulting in a reduced ability of HL to access core lipid substrates. It is unclear whether there may be differences in the abilities of different phospholipids, due to head group size or acyl chain composition, to become incorporated into lipoproteins.

The effect of phospholipid enrichment of LDL on HL-mediated hydrolysis does not appear to be affected by lipoprotein charge in the same manner as the other lipoprotein classes (**Figure 20**). PC-enrichment of LDL had no effect on charge but caused the highest stimulation in LDL-TG hydrolysis, whereas PI and PA only mildly stimulated HL activity. On the other hand, PS did not induce stimulation of LDL-TG hydrolysis. Since LDL is not the primary physiological substrate for HL the relevance of this data is unclear. This suggests that phospholipid-enrichment of lipoproteins may have a secondary, non-electrostatic effect on HL activity. LDL is known to have a more rigid surface layer due to a higher concentration of cholesterol and SM [408,409]. Therefore, PC-enrichment of LDL may render the surface layer more fluid and allow HL to penetrate the interface more easily and stimulate hydrolysis.

This is one of the first studies to examine the effect of native lipoprotein charge properties on the activity of human HL. The data appear consistent with reports on other interfacial enzymes found in the plasma. Both CETP and PLTP have been shown to be regulated by lipoprotein electrostatic properties [168,410-413]. Changes in lipoprotein charge, through incorporation of anionic fatty acids or chemical modification of their apolipoprotein components, stimulates CETP activity [168,410,411,414]. Morton *et al.* showed that while the negative charge of LDL stimulated CETP, this stimulation was inhibited at higher lipoprotein electronegativity values [168]. Desrumaux *et al.* also reported that PLTP activity was stimulated by increasing LDL and HDL negative charge up to a maximal value with a subsequent decrease in activity [169]. Likewise, LCAT is also sensitive to the anionic properties of lipoproteins. LCAT activity is almost completely inhibited by anionic lipids both *in vitro* and *in vivo* in rabbits [163]. Previous studies from our laboratory showed that intravenously-administered PI increases lipoprotein and HDL negative charge in New Zealand white rabbits and stimulates RCT [163,164]. In these

studies, PI injection caused a rapid clearance of free cholesterol from the plasma, an increase in CE and FC in VLDL and inhibited both LCAT and CETP activity [163].

It is now clear from studies on members of the lipase family and interfacial plasma enzymes like CETP and LCAT that substrate charge properties play an important physiological role in the metabolism of lipoproteins. It has been shown that anionic lipids such as PI and oleic acid directly affect HL activity. Therefore, plasma levels of these negatively charged lipids may directly impact normal lipoprotein metabolism and the magnitude and duration of a postprandial response. The postprandial lipemic response results in the liberation of fatty acid anions and transient changes in lipoprotein phospholipid composition and charge. PI, one of the main anionic lipids found in the bloodstream, represents approximately 5% of lipoprotein phospholipid content and has been shown to be an important component of chyle and a regulator of lipoprotein secretion [415,416]. Studies have shown that fatty acids play a direct role in regulating lipoprotein charge and lipid metabolism [168,169,411,417,418]. During chylomicron-TG clearance, free fatty acid levels in the plasma increase [419-421] and changes in the phospholipid content of chylomicrons regulates their hydrolysis by HL in the circulation [422]. Under normal physiological conditions, transient changes in lipoprotein charge, either through postprandial metabolic redistribution or *de novo* synthesis of surface lipids, would have an effect on lipoprotein metabolism and interaction with plasma enzymes. Under abnormal metabolic conditions of dyslipidemia, such as hypercholesterolemia or diabetes, lipoprotein charge may more permanently deregulate interaction with plasma lipoproteins and negatively affect their metabolism. Early stage clinical trials show that PI can profoundly affect TG metabolism in humans. Oral doses of PI in normolipidemic individuals over a two week period caused an 18% increase in HDL-cholesterol, decreased TG concentrations by almost 36% and increased plasma

apoA-I levels [423]. It is interesting to speculate that treatment with PI may transiently increase the negative charge of TG-rich lipoproteins such as chylomicrons or VLDL and may ultimately lower plasma TG levels subsequent to prolonged treatment. In this case, increased plasma hydrolysis of TG-rich lipoproteins may also contribute to the increases in HDL levels observed in patients from the same study.

4.4 – Lipase-lipoprotein association regulates lipid hydrolysis

In order to elucidate the mechanism by which HDL charge affects HL activity, the association of HL with different lipoproteins was analyzed by size exclusion chromatography and immunochemical analysis (**Figure 28**). Under normal conditions, most HL was bound to the HDL particles and very little HL was detected on VLDL. This suggests that when circulating in the plasma, HL is predominantly associated with HDL particles. In contrast, PI-enriched HDL showed a significantly reduced ability to bind HL and also resulted in a reduced association of HL with VLDL. A decrease in VLDL binding is most likely due to the small (< 8%) amount of PI transfer to VLDL that was observed. These data are consistent with others showing that in mixtures of VLDL and HDL, most HL can be found associated with the HDL fraction [424]. During these co-incubations of VLDL and HDL with HL, HDL-TG was specifically hydrolysed with only small amounts of phospholipid hydrolysis occurring [398]. This data is similar but opposite to that observed with apoA-II [38,41]. ApoA-II appears to inhibit HL by enhancing its association with HDL, whereas anionic lipids in HDL stimulate VLDL hydrolysis by reducing the association of HL with HDL. Therefore, factors modulating the association of HL with HDL particles should also affect the ability of this enzyme to interact with other available substrates.

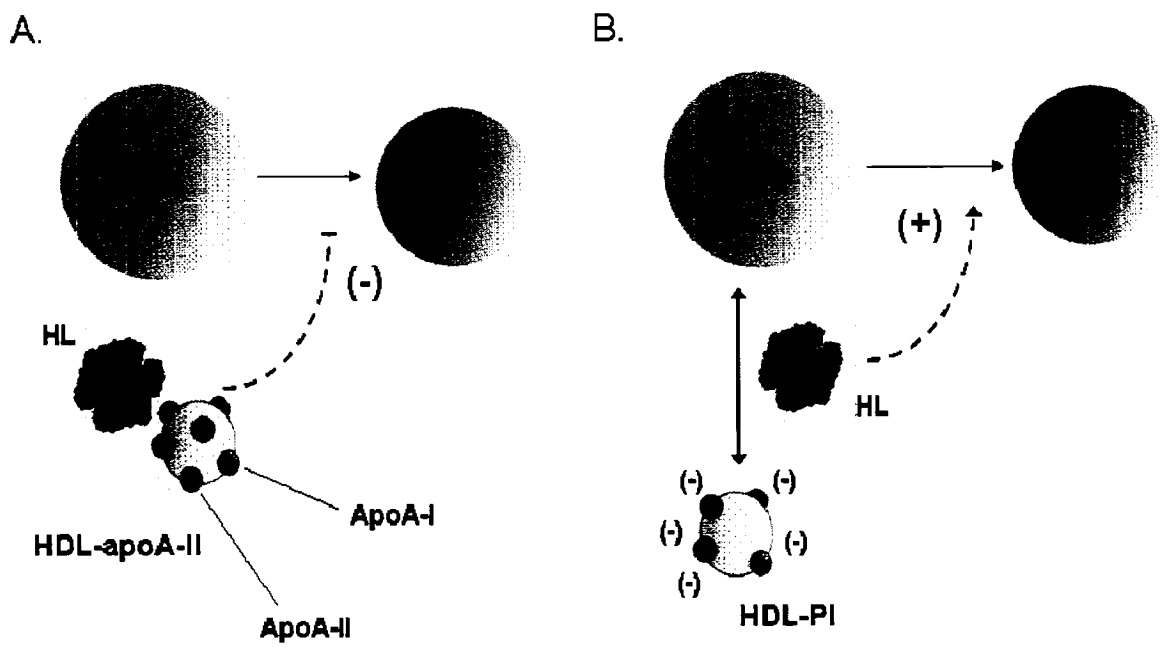


Figure 33. Model depicting effect of apoA-II and PI on HL activity and association. (A) Factors, such as apoA-II, that cause tight binding between HL and HDL cause an inhibition of VLDL hydrolysis by preventing the inter-lipoprotein movement of the enzyme. **(B)** In contrast, increasing the net negative charge on HDL, by enriching with PI, reduces the association of HL with HDL and significantly stimulates the lipid hydrolytic activity of HL. Thus, the inter-lipoprotein movement of interfacial enzymes, such as HL, regulates their hydrolytic activity and lipoprotein metabolism. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HL, hepatic lipase; HDL-apoA-II, high density lipoprotein enriched with apoA-II; HDL-PI, high density lipoprotein enriched with PI; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II.

This study shows that the rate of lipid hydrolysis by HL is governed by the interfacial surface properties of lipoprotein particles. These properties can be affected by the apolipoprotein composition and electrostatic properties of the lipid interface. While different lipids and apolipoproteins have unique effects on the packing and organization of the lipid core of the lipoprotein, they also directly affect the interfacial environment and charge properties of the lipoprotein particle. Factors that prevent the rapid shuttling/hopping of interfacial lipolytic enzymes, such as apoA-II, will inhibit catalytic activity (**Figure 33A**). In contrast, compositional elements that facilitate inter-lipoprotein movement will stimulate the hydrolytic activity of interfacially active enzymes. A decreased binding affinity would be expected to increase the inter-lipoprotein movement of HL (**Figure 33B**). In fact, an increased lipase hopping/shuttling is known to increase the rates of lipid hydrolysis [179]. The data suggest that there is no single component (apolipoprotein or lipid) in HDL that is responsible for the regulation of HL activity, but that all of the constituents of an HDL particle may instead contribute unique structural properties to the complex that directly impact lipoprotein association and activity.

Substrate charge properties are also important regulators of the activity and association of interfacial enzymes from other members of the lipase family [171,172,190,425,426]. Members of the secreted phospholipase A_2 ($sPLA_2$) family have been shown to have a very high affinity for anionic interfaces [180]. These studies showed that tight binding to negatively charged phospholipid vesicles stimulated lipase activity in a typical scooting mode that resulted in the complete hydrolysis of the outer lipid layer [179,427]. Likewise, human $sPLA_2$ is well-known to poorly penetrate neutral vesicles resulting in virtually no hydrolytic activity [428,429]. However, the lipase activity of this enzyme is significantly stimulated on anionic vesicles composed of PG [428,429].

Studies suggest that the anionic properties of the lipid interface not only affect binding of lipase to substrate but also regulate opening of the lid domain and access to the catalytic site [430]. Moreover, the charge properties of the enzyme itself at or near the catalytic site may play an important role in regulating hydrolytic activity [425]. Hydrolysis of the ester bond of the enzyme-substrate acyl intermediate results in the release of a negatively charged free fatty acid molecule [425]. Release of the fatty acid product is crucial to the catalytic process of lipases. Petersen *et al.* propose that the charge properties of the enzyme itself, its products and the substrate interface play critical roles in regulating lipase activity [425]. The authors suggest that once a negatively charged free fatty acid is produced in the active site cleft, the negative charge of the active site itself causes a repulsion that catapults the fatty acid out of the active site and away from the enzyme [425]. Accumulation of fatty acid products is well-known to inhibit lipase activity; therefore, this electrostatic catapult repulsion model may play an important role in lipolytic regulation. It remains to be determined how substrate electrostatic properties affect this electrostatic repulsion model. However, binding of enzyme to a substrate interface is known to induce a conformational change that promotes enzymatic activity. Moreover, a more open active site has been shown to be more negatively charged [425]. Therefore, the extent of the lid domain opening determines the negative charge associated with the active site and may control the extent of lipase activation. The degree of opening of the lid domain, and thus the negative charge of the active site, may be controlled by the charge properties of enzyme substrates themselves.

The charge properties of the interface may also affect the structure of the lipase binding the interfacial surface which may itself regulate binding of the enzyme to its substrates. Opening of the lid after interfacial binding generates an area around the active site that is very hydrophobic [180]. It has been suggested that the lid domain may be the primarily location of

enzyme binding to the lipid interface in some single-domain microbial lipases [180,304,431-433]. However, this may not be the case for lipases closely related to members of the pancreatic lipase family, where other structural features are most likely involved in interfacial binding [180]. The unique β-sandwich C-terminal domain of pancreatic lipase (residues 336-449) is involved in co-factor binding and is required for binding to lipid-water interfaces [433]. In fact, mutagenesis of this same domain in LPL, inhibited binding of the enzyme to lipoprotein substrates [434,435].

In contrast to the effect of lipoprotein charge on the inter-lipoprotein movement of HL, apoA-II promotes the association of HL and HDL. Most reports have concluded that apoA-II is inhibitory to HL activity and that inhibition is related to an increased binding between enzyme and lipoprotein. [354]. In the plasma, apoA-II is predominantly found associated with smaller and less lipid-enriched HDL particles. The more dense HDL_3 sub-species have been shown to contain higher relative amounts of apoA-II than the larger HDL_2 particles (apoA-I:apoA-II ratios 3.7 and 4.8, respectively) [13,32]. This increased content of apoA-II in the more dense HDL sub-species is associated with a reduced ability of these lipoproteins to shed or exchange apoA-I [12,436,437]. The different effects of apoA-I and apoA-II on HL activity may also be due to differences in hydration of the lipoprotein interface caused by these two proteins. The lipid interface on HDL containing apoA-I is believed to be more hydrated [355], suggesting that the hydrophobic catalytic site of HL would preferentially associate with the less hydrated interface of HDL containing apoA-II [39,438]. It has also been suggested that apoA-I and apoA-II may differ in their ability to open the lid domain of HL when the enzyme interacts with the surface of an HDL particle [438]. Studies by several groups have examined the effect of protein and lipid composition on apoA-I conformation in HDL. In this study, apoA-II content directly affects the

conformation of apoA-I in reconstituted HDL particles. An altered conformation induced by apoA-II may have important consequences on the ability of HDL to interact and bind to plasma enzymes. Using a series of thirteen different monoclonal antibodies, it was demonstrated that the addition of apoA-II to reconstituted HDL exposes epitopes at the N-and C-termini of apoA-I, while decreasing expression of epitopes in the central region of the protein (residues 98-186). These data suggest that apoA-II is involved in protein-protein interactions with apoA-I and directly affects its conformation. The ability of apoA-II to alter the conformation of apoA-I may regulate the binding of HL to HDL particles. It is possible that protein-protein interactions between HL and apoA-I on HDL are enhanced through exposure of specific epitopes and/or residues when apoA-II is present on the surface of this lipoprotein (**Figure 10**). Reports from our laboratory have also shown that apoA-II promotes the thermodynamic stability of apoA-I on reconstituted HDL particles [38]. An increase in the stability of HDL most likely renders the lipoprotein particle a poorer substrate for HL and allows the lipase to bind more tightly. The present study now confirms the view that apoA-II generates a more stable HDL complex that binds HL more efficiently and is less susceptible to remodelling by plasma enzymes.

4.5 – HDL composition regulates lipase cell-surface displacement

For decades, it was believed that the plasma lipases were only active when bound to the extracellular matrix of the vascular endothelium. However, an abundant amount of data actually shows quite the opposite [13,14,219]. A new perspective on the regulation of HL activity suggests that release of the enzyme into the circulation is necessary for the efficient hydrolysis of lipoprotein substrates. This is consistent with the view that lipases must be able to easily shuttle between lipoprotein particles for efficient lipid hydrolysis [173]. It now seems likely that the activity of interfacial enzymes would be significantly hindered if the protein was permanently

immobilized on the cell-surface. It has also been known for decades that intravenous infusion of heparin causes a rapid reduction in plasma TG levels [219]. Studies by a number of groups have shown that release of either HL or LPL from HSPG by heparin stimulates the catalytic activity of these enzymes [12,13]. Likewise, overexpression of proteoglycan binding-deficient human HL in mice promoted a hypoalphalipoproteinemia phenotype by stimulating HDL clearance *in vivo*, most likely through increased lipase activity [14]. Ramsamy *et al.* clearly demonstrated that HL was virtually inactive when bound to the surface of cells overexpressing human HL or to purified HSPG and that release of HSPG-bound HL by HDL or apoA-I was critical in stimulating the activity of this enzyme [12,13]. In contrast, VLDL and LDL appeared to have very little or no effect on HL displacement.

The data now show that both HDL and apoA-I can function like heparin and displace HL from the cell-surface and stimulate hydrolysis [13]. Moreover, the different types of HDL differ in their ability to displace this enzyme (**Figure 7**). The larger, less dense HDL_2 are more effective at displacing HL than the smaller and denser HDL_3. HDL not only releases HL protein from the cell surface but an active enzyme that can hydrolyze lipoprotein substrates. In order to further characterize the components of HDL that influence HL displacement, the roles of specific HDL proteins and lipids in this process were evaluated. The main protein components of HDL are apoA-I and apoA-II [134,355]. The data clearly show that apoA-I alone and synthetic HDL containing apoA-I can both displace cell-surface HL. It also appears that apoA-II can modulate the ability of apoA-I to displace HL. The data indicate that apoA-II significantly stimulated the ability of synthetic HDL containing apoA-I to displace HL from the cell surface of CHO-hHL cells (**Figure 31**). Thus, apoA-II plays a very important role in the ability of HDL to regulate HL displacement.

The ability of the different HDL sub-species to uniquely displace HL is likely due to the amount of exchangeable apoA-I on the surface of the larger, less dense HDL_2 particles since HDL is very efficiently displaced by apoA-I alone. It is well known that apoA-I on HDL_2 dissociates more readily than from the smaller HDL_3. This suggests that *in vivo*, high HDL_2 levels would promote the cell-surface displacement of HL and stimulate TG hydrolysis in the plasma. In fact, many studies have shown that delayed postprandial clearance of TG is correlated with low levels of HDL_2 in the bloodstream. Initially, this appears inconsistent with data showing that low HDL_2 levels are correlated with high post-heparin HL activity in the plasma. However, considering the new perspective that HDL can displace HL like heparin, high HDL_2 levels would displace HL more readily and smaller amounts of the enzyme would be liberated by heparin and result in lower post-heparin HL activity. The physiological link between high HDL levels and TG metabolism has been characterized by a number of groups. High HDL levels are often associated with efficient TG catabolism, suggesting that an increase in certain types of HDL may displace more lipase into the circulation and stimulate TG hydrolysis by HL and LPL. Since HDL can act like heparin and stimulate TG hydrolysis releasing HL from the vascular endothelium, this novel regulatory mechanism may help understand the functional regulation of these enzymes.

Preliminary studies on the regulation of LPL show that this enzyme is also regulated in a very similar manner. HDL and apoA-I have been shown to regulate the displacement of HSPG-bound LPL *in vitro* (Ramsamy *et al.*, unpublished observation) and from the cell surface of a CHO cell line overexpressing human LPL (Boucher *et al.*, unpublished observation). While data does show that LPL is active when bound to HSPG *in vitro*, it appears that LPL is even more active when free in solution [439]. Continuous heparin infusion *in vivo* releases a rapid burst of LPL activity and even promotes the secretion of intracellular LPL [440-443]. Studies by de Man

et al. also indicated that binding of LPL to cell-surface HSPG partially inhibited VLDL hydrolysis by this enzyme [444]. This suggests that displacement of lipases from the cell-surface increases enzyme interaction with substrates in the bloodstream and effectively stimulates TG lipolysis. It now appears that HDL plays a very important and novel role in the regulation and displacement of lipase activity and TG metabolism in the plasma.

In contrast to the effect of apoA-I and apoA-II, HDL lipid composition and charge appear to have very different effects on the ability of this lipoprotein to control HL displacement (**Figure 32**). HDL made more negatively charged through incorporation of PI were much less able to stimulate the release of HL from the cell surface. In addition, HDL enriched with the uncharged PC also had a reduced ability to stimulate HL displacement, but to a lesser extent. It appears, therefore, that HDL charge is not a determining factor in the regulation of HL release from the cell surface. The inhibition of HL displacement by phospholipid-enriched HDL may indicate a novel role for phospholipids in this process. In fact, there are some lines of evidence to suggest that negatively charged fatty acids play a role in LPL displacement and the regulation of lipase activity. LPL cell-surface displacement has been shown to occur at physiological concentrations of free fatty acids [417]. Binding of fatty acids to LPL is known to cause a decreased affinity of the lipase for lipid interfaces and heparin [399,445]. Thus, accumulation of fatty acids, which could increase the negative charge of all lipoprotein classes during a postprandial response, would likely decrease lipase-lipoprotein binding as well as cell-surface association of LPL and stimulate lipid hydrolysis [305]. The ability of fatty acids to cause displacement may be specific to this particular lipid or indicate some differences in the regulation of displacement between HL and LPL. As will be discussed in more detail later, the ability of HDL protein and lipid components to affect HL displacement is most likely due to differences in the binding between

HDL and HL. It appears that factors resulting in a tighter binding between HL and lipoproteins, such as apoA-II content, promote the release of HL from the cell-surface. Consequently, factors that reduce the binding between HDL and HL, such as PI-enrichment, inhibit the ability of those HDL particles to stimulate HL displacement and activity. While the control of HL displacement by HDL composition is a major mechanism for the function of this enzyme, lipoprotein composition also regulates lipase activity after the enzyme is released from the cell surface.

It is now clear that interfacial enzymes such as HL must be free to circulate and rapidly exchange between substrates for them to efficiently hydrolyze their lipoprotein substrates. Therefore, displacement of HL from the cell-surface is required for it to function as a lipase. I propose that HDL is not only a product of TG hydrolysis but that it actively regulates its metabolism. The types of HDL in the bloodstream appear to determine the functional characteristics of HL. The large HDL_2 displace HL and stimulate its activity while the smaller HDL particles displace to a much lesser extent. It has been shown that apoA-I is critical in mediating this displacement and that apoA-II stimulates the ability of HDL to release cell-surface HL. In contrast, phospholipid enrichment inhibited the ability of HDL to displace HL. It is now clear that factors promoting the binding between HL and HDL, such as apoA-II, stimulate HL displacement (**Figure 34**).

This new model now indicates that HL is regulated in a two-step mechanism. ApoA-II-rich HDL that bind HL more tightly can release the enzyme from the cell-surface but are inhibitory to HL activity. Moreover, during the postprandial response, lipoproteins can become enriched in anionic lipids such as PI and fatty acids, and are able to interact with the displaced lipases to stimulate hydrolysis. It is unlikely that an increase in certain types of HDL in the plasma will result in the complete release of all HL bound to the vascular endothelium. However,

it is more plausible that transient changes in certain HDL sub-species, such as those enriched in apoA-II, will result in a fractional increase in the amount of HL released into the circulation. While displacement of HDL into the bloodstream is crucial in determining the activity of HL, it is not necessarily the types of HDL that efficiently displace that will stimulate lipase-mediated hydrolysis. Thus, factors that promote HL release from the cell-surface will provide a transient pool of circulating HL that can be stimulated by other types of lipoproteins in the plasma. It is now clear that the types of HDL found in the bloodstream are novel and significant regulators of HL activity as well as lipoprotein and TG metabolism.

4.6 - Future Studies

Lipoprotein composition clearly plays a role in the regulation of lipase displacement, activity and lipase-lipoprotein association. Studies have confirmed that the apoA-I/apoA-II and charged lipid content of HDL have a significant impact on the structure and function of this lipoprotein. It is unknown whether other apolipoproteins and lipids also regulate HL activity and displacement. The apoE- and apoC-content of HDL may have an impact on HDL-mediated displacement of cell-surface HL and hydrolysis. Preliminary data suggests that HDL composition also regulates the displacement and activity of LPL. Since apoC-II is a co-factor of LPL activity, it is interesting to speculate that this apolipoprotein may play a role in the displacement of LPL from the cell-surface. Studies are also currently underway to evaluate the *in vivo* regulation of HL displacement by HDL. Intravenous infusion of HDL and apoA-I into the plasma will be used to determine whether these compounds can stimulate the release of HL protein and activity into the plasma similar to heparin. It is believed that infusion of the different types of HDL into the plasma will also influence TG metabolism and lipase displacement.

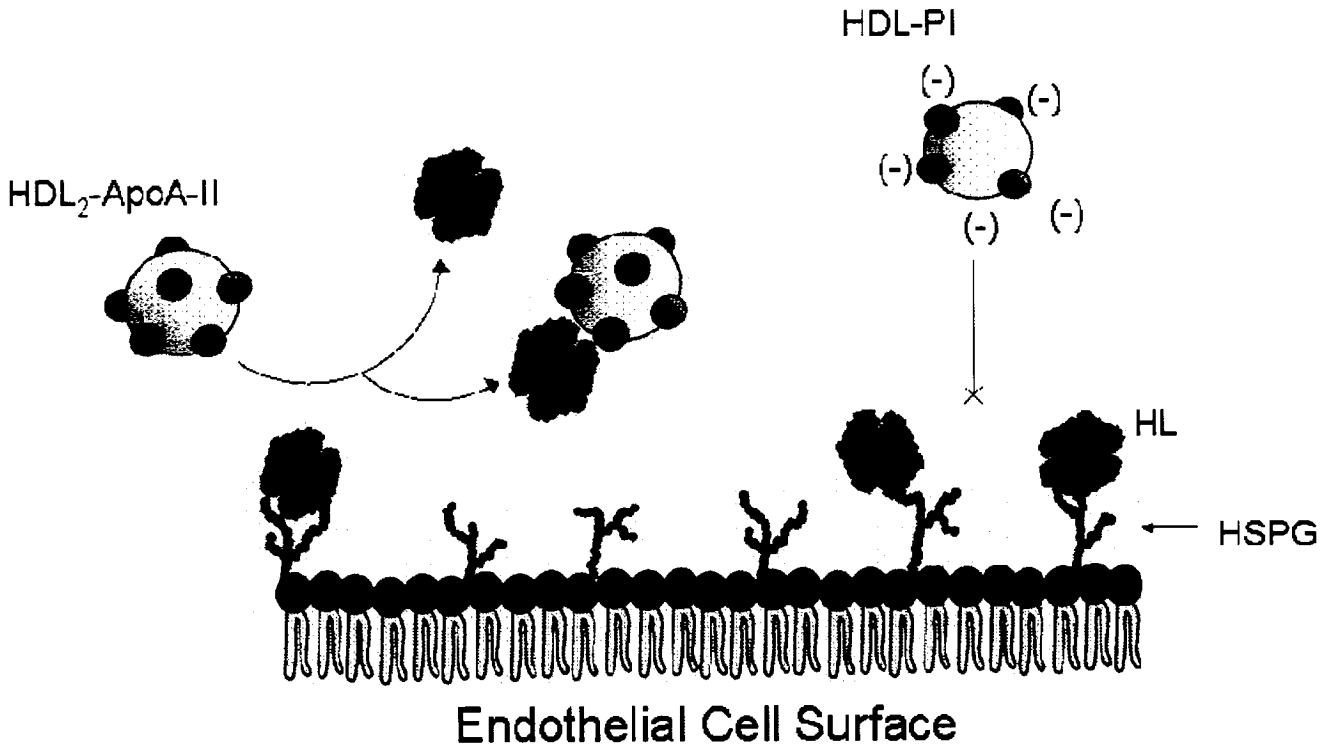


Figure 34. Model illustrating the regulation of HL displacement by HDL composition. HL is normally bound to the vascular endothelial cell surface via HSPG. HL can be displaced by all types of HDL in the plasma and this process is controlled by the protein composition of HDL. Higher amounts of apoA-II on the surface of HDL result in a tighter binding between HDL and HL, stimulating the displacement of the enzyme. Once displaced from the cell surface, the hydrolytic activity of HL is enhanced and results in increased hydrolysis of lipoprotein in the plasma. In contrast, HDL enriched with PI do not displace HL from the cell surface as efficiently. This suggests that lipoprotein charge does not play as important a role in HL displacement as HDL apolipoprotein content. Factors regulating the inter-lipoprotein movement of HL between substrates will then control lipid hydrolysis in the plasma. HDL_2-ApoA-II, high density lipoprotein 2 enriched with apoA-II; HDL-PI, high density lipoprotein enriched with PI; HL, hepatic lipase; HSPG, heparan sulfate proteoglycan.

It has also been demonstrated *in vitro* that the anionic properties of lipoproteins stimulate HL- and LPL-mediated hydrolysis by enhancing inter-lipoprotein shuttling. Further studies are required to determine the exact mechanism of anionic lipid stimulation of lipase activity *in vivo*. Infusion of PI has been shown to have a significant effect on lipid metabolism in rabbits [163] and humans [423]. Future studies examining the specific role of lipases in modulating plasma lipid levels after PI-treatment will significantly contribute to an understanding of lipoprotein metabolism and lipase activity. A comprehensive *in vitro* and *in vivo* analysis of how each of the lipoprotein components affects displacement, inter-lipoprotein movement and activity will enable a more complete understanding of this novel mechanism of lipase regulation.

Contributions of Collaborators

Figures 6, 7, 8 – Collaborators: Jonathan Boucher and Tanya Ramsamy

Figures 31, 32 – Collaborators: Jonathan Boucher and Naghmeh Rouhani

Figure 10 – Collaborators: Jonathan Boucher and Sylvie Braschi

Jonathan G. Boucher – *Curriculum vitae*

EDUCATION

University of Ottawa **2001-Present**
Ottawa, Ontario

Doctor of Philosophy (PhD) in Biochemistry. Program consists of laboratory research at the University of Ottawa Heart Institute in the Lipoprotein and Atherosclerosis Group. Practical lab work is complemented by scientific seminars and biochemistry graduate courses.
PhD supervisor: Daniel L Sparks

University of Ottawa **1998-2001**
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Bachelor of Commerce (B. Com), specialization in marketing. The program consists of courses in finance, accounting, marketing, business administration and management.

McGill University **1994-1998**
Montreal, Quebec

Bachelor of Science (B. Sc) in Microbiology and Immunology. The four-year program consisted of courses related to microbiology, immunology and virology. Theory was complemented with extensive laboratory courses in microbiology, immunology, biochemistry and molecular biology.

RESEARCH EXPERIENCE

Post-Doctoral Fellowship (pending completion of thesis defense) **2007-Present**
Children's Hospital of Eastern Ontario and Ottawa Health Research Institute, Ottawa, Ontario

- Effect of HIV infection and IL-12, IL-23 and IL-27 expression in monocytes.
  Supervisors: Jonathan Angel and Ashok Kumar

Lipoprotein and Atherosclerosis Research Group **2001-2007**
University of Ottawa Heart Institute, Ottawa, Ontario

- PhD Thesis: Lipoprotein composition and charge regulate hepatic lipase activity.
- Techniques: Animal studies, molecular biology, PCR cloning, stable cell line generation, cell culture, adenovirus transfection, immunochemistry, cell biology, lipid biochemistry, use of radioactivity and enzyme kinetics.

Liponex Inc. **Summer 2001/2004**
Ottawa, Ontario

- Performed both collaborative and contract work for a local drug development company.
- Coordinated several animal studies and performed blood lipid analysis, immunochemistry, chromatography and lipid biochemistry.

Teaching Assistant for Molecular Biology Laboratory Course **2002-2004**
University of Ottawa, Ottawa, Ontario

- Instructed 3rd year undergraduate students in various molecular biology and cloning techniques.

PUBLICATIONS

Boucher J, Nguyen T, Sparks DL. Lipoprotein charge regulates hepatic lipase activity. Journal of Lipid Research (Submitted July 2007).

Burgess JW, Sinclair PA, Chretien CM, **Boucher J** and Sparks DL. "Reverse Cholesterol Transport" In Biochemistry of Atherosclerosis (2006). Kaur SC (Ed.), Springer, New York, p3-22.

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Boucher J and Sparks DL. Lipoprotein charge regulates hepatic lipase activity. 7th Annual American Heart Association Conference on Arteriosclerosis, Thrombosis and Vascular Biology. Peer-Reviewed Published Abstract (2006).

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SCHOLARSHIPS AND ACADEMIC AWARDS

- 2005 and 2006 Heart and Stroke Foundation of Canada Doctoral Research Award ($21000/year)
- 2005 and 2006 University of Ottawa Excellence Scholarship ($5000/year)
- 2005 Ontario Graduate Student Scholarship ($15000/year) - Declined
- 2002 and 2003 Heart Institute Research Corporation Tuition Award ($5000.00/year)
- 2002 Graduate Student Departmental Poster Award (1st place, Master's student)
- 1994 Ontario Scholar Award
- 1994 Governor-General Academic Medal

CONFERENCES AND PRESENTATIONS

Boucher J and Sparks DL. Lipoprotein charge regulates hepatic lipase activity - Poster. 7th American Heart Association Conference on Arteriosclerosis, Thrombosis and Vascular Biology, Denver, Colorado (April 2006)

Boucher J and Sparks DL. Phosphatidylinositol regulates hepatic lipase activity – Oral presentation. Canadian Lipoprotein Conference, Montebello, Quebec (October 2005)

Boucher J, Ramsamy TA and Sparks DL. Phosphatidylinositol regulates hepatic lipase and triglyceride metabolism- Poster. 6th American Heart Association Conference on Arteriosclerosis, Thrombosis and Vascular Biology, Washington, DC (April 2005)

Boucher J and Sparks DL. HDL regulates hepatic lipase activity and lipoprotein association – Poster. BioNorth 2005 International Life Sciences and Biotechnology Conference, Ottawa, Ontario (November 2004)

Boucher J and Sparks DL. HDL regulates hepatic lipase activity and lipoprotein association – Poster. Canadian Lipoprotein Conference, Banff, Alberta (October 2004)

Boucher J, Ramsamy TA and Sparks DL. HDL regulates triacylglyceride metabolism – Oral Presentation. Canadian Lipoprotein Conference, Huntsville, Ontario (October 2003)

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