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THE REGULATION OF HEPATIC LIPASE ACTIVITY

by

Tanya A. Ramsamy

A thesis submitted to the School of Graduate Studies in
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Abstract

The regulation of hepatic lipase activity

By Tanya A. Ramsamy

Human hepatic lipase (HL) is a 66-kDa glycoprotein that plays an important role in the metabolism of apoB-containing lipoproteins and high density lipoproteins (HDL). Both the lipid and apolipoprotein composition of lipoproteins have been shown to modulate HL activity. In this study, very low density lipoproteins (VLDL) were found to be the best lipoprotein substrates for HL followed by low density lipoproteins (LDL) and HDL. Only 1% of the fatty acids released from HDL_3 by HL are derived from triglycerides (TG) whereas the remainder of fatty acids produced are from the lipolysis of diglycerides (DG (49%)) and phospholipids (PL (50%)). In order to further study the role of lipoprotein composition on HL activity, HDL and LDL fractions were isolated from subjects with familial combined hyperlipidemia (FCHL) and matched controls and used as substrates for the enzyme. HL-mediated hydrolysis of patient and control LDL and HDL fractions is similar despite elevated serum TG levels in subjects with FCHL. In both patient and control samples, the most buoyant LDL and HDL fractions are better substrates for HL than the corresponding denser fractions when normalized for total protein content. Although differences are observed in the acylglycerol and PL hydrolysis of the two patient groups, these differences are not related to the DG content of the lipoproteins.

The association of HL with pure heparan sulfate proteoglycans (HSPG) has little effect on hydrolysis of HDL particles, but significantly inhibits (> 80%) the hydrolysis of LDL and VLDL. Lipolytic inhibition is associated with a differential ability of the lipoproteins to remove HL from the HSPG. LDL and VLDL are unable to displace HL,

while HDL readily displaces the enzyme from the HSPG. This is consistent with the view that HSPG-bound HL is inactive. HDL also displaces HL from the surface of the hepatoma cell line, HepG2, and Chinese Hamster Ovary (CHO) cells stably overexpressing human HL. Purified apolipoprotein (apo) A-I is more efficient than HDL at liberating HL from both pure and cell surface HSPG. Furthermore, different HDL fractions vary in their abilities to displace the enzyme from the cell surface. The buoyant HDL_2 has a greater capacity to remove HL from the cell surface and intracellular compartments when compared to the smaller denser HDL particles.

Displacement of HL by apoA-I does not enhance the hydrolysis of VLDL. This somewhat paradoxical finding appears to result from the direct inhibition of HL by apoA-I, as both apoA-I and HDL are able to inhibit VLDL-lipid hydrolysis. The different HDL subspecies also uniquely affect the activity of the enzyme. A detailed evaluation of different HDL fractions shows that HDL_2 stimulates HL-mediated hydrolysis of VLDL-TG, while the smaller HDL_3 is inhibitory. Inhibition of VLDL hydrolysis appears to result from a decreased interlipoprotein shuttling of HL between VLDL and the smaller more dense HDL particles. These findings suggest that high HDL_2 levels are positively related to efficient TG hydrolysis by their ability to enhance the liberation of HL into the plasma compartment and by a direct stimulation of VLDL-TG hydrolysis.

Taken together, these results demonstrate that the lipid and apolipoprotein composition of lipoproteins, hence lipoprotein structure, in addition to interlipoprotein interactions are central to the regulation of HL activity.

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Abbreviations

[^{3}H]-TG: tri-[9,10-^{3}H(N)]-olein

ABCA1: ATP-binding cassette transporter type A1

ANOVA: analysis of variance

Apo: apolipoprotein

bLPL: bovine lipoprotein lipase

CAD: coronary artery disease

CE: esterified cholesterol

CETP: cholesteryl ester transfer protein

CHO: Chinese Hamster Ovary

CHO-hHL: human HL-transfected Chinese Hamster Ovary

CVD: cardiovascular disease

DG: diglyceride

DPPC: L-3-phosphatidyl[N-*methyl*-^{3}H]choline,1,2-dipalmitoyl

ECM: extracellular matrix

EGF: epidermal growth factor

EL: endothelial lipase

EMEM: Eagle's Minimal Essential Medium

ER: endoplasmic reticulum

FAF-BSA: essentially fatty acid-free bovine serum albumin

FBS: fetal bovine serum

FC: free cholesterol

FCHL: familial combined hyperlipidemia

FH: familial hypercholesterolemia

G418: Geneticin® Selective Antibiotic G418 sulfate

GGE: gradient gel electrophoresis

HDL: high density lipoproteins

HDL-C: HDL cholesterol

HL: hepatic lipase

hLPL: human lipoprotein lipase

HPTLC: high performance thin layer chromatography

HRP: horseradish peroxidase

HSPG: heparan sulfate proteoglycans

IDDM: insulin-dependent diabetes mellitus

IDL: intermediate density lipoproteins

K_m: Michaelis-Menten constant

LCAT: lecithin:cholesterol acyltransferase

LDL: low density lipoproteins

LDLr: low density lipoprotein receptor

LIPC: human hepatic lipase gene

Lp2A-I: reconstituted HDL particle containing 2 molecules of apoA-I per particle

LPL: lipoprotein lipase

LRP: low density lipoprotein receptor-related protein

MG: monoglyceride

mAb: monoclonal antibody

MTP: microsomal triglyceride transfer protein

NIDDM: non-insulin-dependent diabetes mellitus

NO•: nitric oxide

oxLDL: oxidized LDL

PC: phosphatidylcholine

PBS: phosphate buffered saline

PE: phosphatidylethanolamine

PI: phosphatidylinositol

PL: phospholipid

PLTP: phospholipid transfer protein

POPC: 1-palmitoyl-2-oleoyl-phosphatidylcholine

RAP: receptor-associated protein

RCT: reverse cholesterol transport

rHDL: reconstituted HDL

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMC: smooth muscle cell

SR-A: scavenger receptor A

SR-B1: scavenger receptor B1

TG: triglyceride

TLC: thin layer chromatography

U: units

VLDL: very low density lipoproteins

V_{max}: maximum velocity

Chapter 1 – Introduction

1.1 Atherosclerosis: A disease of inflammation and lipid accumulation

1.1.1 Cardiovascular disease

Cardiovascular disease (CVD), primarily heart disease and stroke, is the leading cause of death in North America and Europe among both men and women regardless of race and ethnic background (1). It accounts for one third of global deaths and approximately 40% of deaths in Canada (2, 3). CVD encompasses a number of dysfunctional conditions of the heart, arteries and veins but the most common forms of this disease are coronary artery disease (CAD) and stroke.

CAD accounts for 54% of all CVD deaths in Canada (3) and 20% of all deaths worldwide (2, 3). CAD is a disease in which the coronary arteries become narrowed or blocked by a gradual build-up of fat within the artery wall, a process known as atherosclerosis (discussed below). When blood flow to the heart muscle is significantly reduced and the heart muscle does not receive enough oxygenated blood to meet its needs, severe life threatening conditions such as arrhythmias, angina pectoris or myocardial infarctions (heart attacks) may occur.

1.1.2 Overview of atherosclerosis

Atherosclerosis comes from the Greek words athero, meaning gruel, and sclerosis, meaning hardness. The gradual build up of fatty deposits on the inside walls cause a narrowing of the arteries that can eventually restrict blood flow. It is one of several types of arteriosclerosis, a term applied to the general thickening and hardening of artery walls. Atherosclerosis is a disease process that, until recently, was considered an inevitable part of

aging. With new advances in research, this view is rapidly changing and it has become clear that atherosclerosis is a modifiable disease that can respond positively to therapeutic interventions and lifestyle modifications.

Atherosclerosis often begins in infancy and progresses throughout life at various rates. Risk factors associated with atherosclerosis and CAD are both genetic and environmental. Some of the factors with a strong genetic component thought to contribute to the onset and the rate of development of atherosclerosis include elevated levels of low density lipoproteins (LDL) (discussed in section 1.2.5) (4), decreased high density lipoproteins (HDL) levels (discussed in sections 1.2.8 and 1.2.9) (5), gender (6), obesity (4), diabetes (4), family history (7) and hypertension (4, 8). Environmental factors that contribute to CAD and atherosclerosis include a diet high in saturated fats, tobacco use and a sedentary lifestyle (4).

1.1.3 The endothelium and blood flow hemodynamics

The pathogenesis of atherosclerosis is an extremely complex process and is believed to begin with injury to the endothelium (9), the innermost layer of the artery exposed to the bloodstream. The endothelium is a monolayer of endothelial cells bound together by tight junctions, which allows it to function as a permeable barrier between the blood and the underlying tissues of the artery. However, the endothelium is not simply a passive surface that acts as a barrier but instead, senses, integrates and responds quickly and sensitively to mechanical forces created by blood flow hemodynamics and the cardiac cycle (10). By doing so, the endothelium plays a central role in maintaining vasodilation, inhibiting platelet aggregation and smooth muscle cell (SMC) proliferation through the release of effector molecules.

Shear stress, one of the three main forces acting upon the endothelium, is the dragging frictional force created by blood flow (10). It is of particular importance because it stimulates the release of molecules that regulate thrombosis, inflammation, vascular tone, and cell morphology (11). Both the nature and the magnitude of the shear stress play an important role in determining whether the endothelium will be atheroprotective or will promote the formation of atherosclerotic lesions.

The type of shear stress is determined by the blood flow patterns throughout the vasculature generated by the cardiac cycle. In linear areas of the vasculature (not at branched points), the endothelial cells experience pulsatile shear stress with fluctuations in magnitude (10). Endothelial cells exposed to this type of shear stress reorient themselves so that their longitudinal axes are parallel to the flow of blood (12, 13). This decreases the effective resistance and reduces the shear stress (14), which promotes endothelial cell survival (15) and the release of molecules that inhibit coagulation (nitric oxide (NO•), prostacyclin, tissue plasminogen activator), the migration of leucocytes (NO•) and SMC proliferation (NO• and transforming growth factor-ß) (10).

At areas of abrupt curvature or bifurcations (branch points), the laminar flow of the blood is disrupted (10). In these susceptible areas, the blood flow is slower and changes direction with the cardiac cycle. Consequently, the endothelial cells experience oscillatory flow with flow reversal and low mean shear stress (16). As a result, these cells do not reorient themselves and are exposed to a high shear gradient (11, 17). There is a strong correlation between this altered blood flow and endothelial cell dysfunction (16). Low shear stress and flow reversal promotes apoptosis (programmed cell death) (18, 19), inhibits the release of factors that prevent coagulation (NO• and prostaglandins) and increases the release of factors that promote the migration of leucocytes (monocyte chemoattractant protein-1 and

vascular cell adhesion molecule-1) (20, 21) and SMC proliferation (angiotensin II, platelet derived growth factor and endothelin-1) (10, 22). In addition, the endothelial cells at these areas have increased permeability to LDL (discussed in sections 1.2.5 and 1.2.7) (23-25). Therefore, areas of bifurcations are the preferred sites for lesion formation (26, 27).

1.1.4 The progression of the atherosclerotic lesion

A large artery consists of three layers. The intima is the innermost layer (see figure 1.1.1). It is bound by a monolayer of endothelial cells on the luminal side (discussed above) and the internal elastic lamina on the peripheral side. The internal elastic lamina is composed of a sheet of elastic fibres, which limits the migration of cells from the media (see below). The intima itself is a very thin region composed of loose connective tissue components, primarily proteoglycans and collagen. The middle layer is called the media and consists primarily of SMC. It is separated from the outermost layer, called the adventitia, by the external elastic lamina. The adventitia is a layer of loose and dense connective tissues, primarily collagen and elastic fibers, which are interspersed with fibroblasts and SMC (28).

The atherosclerotic lesions are known to progress through three main stages; the fatty streak, the fibrous plaque and the complex lesion. The fatty streak is the earliest morphologically identifiable lesion (9) often present in infancy (29). Although not clinically significant, it is the precursor to more damaging and potentially life-threatening lesions. These fatty streaks are characterized by the accumulation of lipid-laden macrophages (foam cells) within the intima that resemble yellowish dots 1-2 mm in diameter just barely elevated above the surface of the surrounding intima (28). When the integrity of the endothelium has been compromised, LDL transport and retention into the subendothelium, via interactions with matrix proteoglycans (30), is increased. Within the intima, LDL undergoes a number of

modifications, including oxidation, which contributes to the inflammatory response and the formation of foam cells (discussed in section 1.2.7). This oxidized LDL (oxLDL), in combination with hemodynamic forces, stimulates endothelial cells to release pro-inflammatory molecules (adhesion molecules and growth factors), which trigger the recruitment of leucocytes (monocytes and lymphocytes) to the site of injury. The association of leucocytes to the endothelial cells is mediated by adhesion molecules (31-36). The leucocytes migrate into the subendothelial space of the arterial wall under the influence of

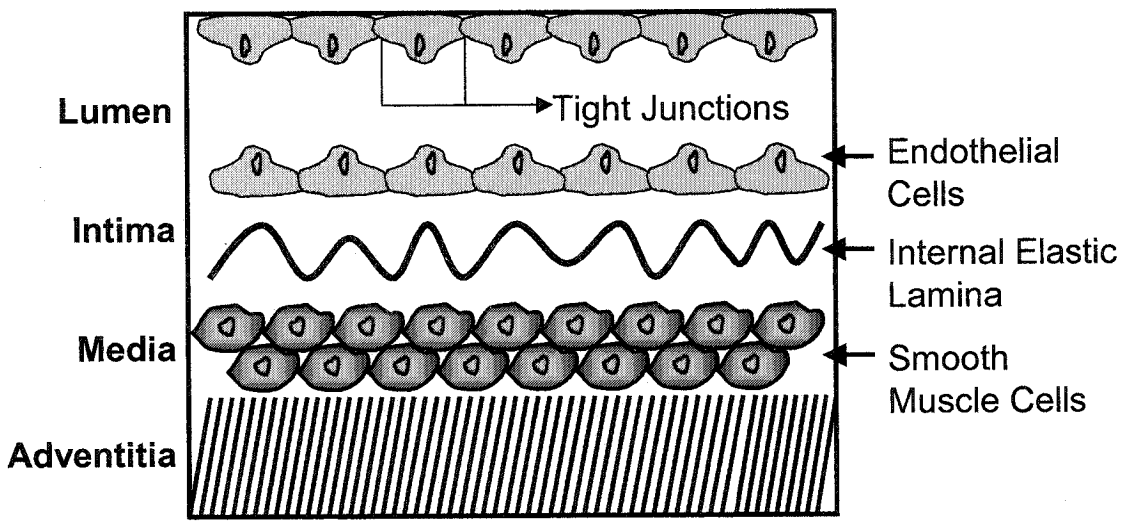

Figure 1.1.1 Structure of a healthy artery

Endothelial cells, bound together by tight junctions, form a monolayer that separates the lumen of the artery from the intima layer and functions as a permeable barrier. The outer most layer, the intima, is primarily composed of proteoglycans and collagen, which acts as a basement membrane for the endothelial cells. The internal elastic lamina, composed of a sheet of elastic fibres, separates the intima from the media thereby limiting the migration of cells from the SMC rich media. The adventia, the outermost layer of the artery, consists of connective tissue with some fibroblasts and SMC.

chemoattractant chemokines (macrophage chemoattractant protein-1) (37, 38) and lymphocyte chemoattractants (39). Once within the intima, macrophage-colony stimulating factor stimulates the proliferation and the differentiation of monocytes into macrophages (40). These monocyte-derived macrophages accumulate large amounts of lipids via their scavenger receptors (discussed in section 1.2.7), which preferentially bind modified forms of lipoproteins, such as oxLDL, eventually becoming foam cells, a hallmark of fatty streaks.

The progression of fatty streaks eventually gives rise to fibrous plaques, the lesions representative of atherosclerosis, which are white in appearance and protrude into the vascular lumen (28). They contain a core of extracellular lipids derived from necrotic foam cells and a fibrous cap comprised of SMC and extracellular matrix (ECM) originating from these cells (see below) (41). The transition from fatty streaks to fibrous plaques is characterized by the migration of SMC from the media into the intima followed by SMC proliferation and the production of connective tissue matrix, which is rich in collagen, elastic fibers and proteoglycans (42). This process, which is affected by both genetic and environmental factors (reviewed in 41), is mediated by various cytokines and growth factors produced by endothelial cells, macrophages and SMC themselves (41). All of these elements contribute to the characteristic thickening of the intima. In the well advanced fibrous lesions, the accumulated SMC, foam cells and ECM within the intima form a fibrous cap of extracellular lipid and necrotic debris on the luminal side of the core (43, 44). Arteries affected by atherosclerosis lose their elasticity, and as the lesions grow, the arteries narrow.

The growing fibrous plaque can develop into a complex lesion upon calcification (hardening of the tissue by calcium deposits), haemorrhage (the escape of blood from the vessel), ulceration (sloughing of inflammatory necrotic tissue) or increased cellular necrosis

(28). During the formation of the complex plaque, SMC continue to proliferate and produce ECM components, which act to further occlude the artery.

1.1.5 Plaque rupture and thrombosis

The transition from the chronic to the acute phase of atherosclerosis is dictated by the strength of the fibrous cap rather than the degree of occlusion of the artery (41, 45). Vulnerable plaques have an increased number of inflammatory cells (macrophages and lymphocytes (T cells)) that contribute to the formation of the thin cap (46, 47). The balance of components that stimulate the production of ECM components (mainly collagen) from SMC and those that either inhibit their production or degrade them determines the tensile strength of the fibrous cap (41).

Once the fibrous cap has ruptured, blood coagulation factors come into contact with tissue factors released from the lipid core (41) and leads to a cascade of events that can result in a thrombus (blood clot), which can have dire consequences. Briefly, tissue factor within the lipid core form a complex with factor VIIa, the active form of factor VII, which preexists in the plasma at low concentrations (48). This factor VIIa-tissue factor complex initiates a cascade of events leading to the activation of factor X and the subsequent conversion of prothrombin to thrombin (reviewed in 49). Thrombin can convert fibrinogen to fibrin, the key step in blood clotting, in addition to activating factor XIII. Factor XIII, the fibrin-stabilizing factor, is responsible for crosslinking fibrin and forming the clot. The endothelial cells, which can normally secrete anticoagulants, such as antithrombin and heparin, to prevent blot clotting are dysfunctional or absent in the complex lesion and the production of anticoagulants is therefore impeded (50). The thrombus produced can occlude the blood vessel or form an embolism. If either happens and blood supply to the heart muscle is

blocked, a myocardial infarction occurs but if it occludes a blood vessel supplying brain, it causes a stroke. The events that lead to plaque formation and rupture are complex, yet it is clear that lipoproteins play a key role in the atherosclerotic process. Hence, much research has focused on gaining a greater understanding of lipoprotein metabolism and its relationship to atherosclerosis (discussed in section 1.2).

1.2 Lipoprotein metabolism

1.2.1 General properties of lipoproteins

Lipoproteins are intimately involved in the development of CAD. As mentioned in section 1.1 and discussed further in section 1.2.7, LDL plays a crucial role in the initial stages and progression of atherosclerosis. HDL, on the other hand, is often referred to as the anti-atherogenic lipoprotein, in part, because of its antioxidant properties (51, 52) and its ability to remove excess cholesterol from the macrophages thereby inhibiting their transformation to foam cells within the intima (discussed in section 1.2.10) (41). It therefore follows that the respective levels of both LDL and HDL are crucial in determining the progression of the disease.

Plasma lipoproteins, for the most part, are spherical macromolecular complexes of lipids and proteins that are responsible for the transport of insoluble lipids. In the circulation, they undergo a number of modifications that alter their composition, structure and functions. Once internalized by cells, lipoproteins and/or their lipids are metabolized and their components are used for energy, membrane biogenesis and the synthesis of lipid derived bioregulators. Lipoprotein lipids include phospholipid (PL), free cholesterol (FC), esterified cholesterol (CE), mono-, di- and triglycerides (MG, DG, and TG), lipophilic hormones and vitamins. The chemical composition of different classes of lipoproteins shows considerable variation in the percentage of surface and core lipids (see table 1.1). In addition, each has a unique complement of amphipathic proteins known as apolipoproteins (or apoproteins) that function to stabilize the lipoprotein (see table 1.2 and discussed in section 1.2.2). Lipoproteins increase the solubility of these lipids by sequestering them in a lipophilic environment created by the surrounding apolipoproteins (53). This is reflected by the

Table 1.1: Chemical composition of human lipoproteins

Lipoprotein Class	Surface Components (% of dry mass)			Core Lipids (% of dry mass)	
	Apolipoprotein	FC	PL	TG	CE
Chylomicrons	2	2	7	86	3
VLDL	8	7	18	55	12
IDL	19	9	19	23	29
LDL	22	8	22	6	42
HDL_2	40	5	33	5	17
HDL_3	55	4	35	3	13
Pre-β-HDL	90	5	5	0	0

Adapted from 53.

general properties of all lipoproteins that contain a neutral lipid core composed of TG and CE surrounded by a monolayer of PL stabilized by the interactions of the apolipoproteins on the surface (see figure 1.2.1) (53).

Lipoproteins are grouped on the basis of their hydrated densities, which reflects their lipid to protein ratio where the size is inversely related to density (see table 1.2) (53). Chylomicrons have the lowest density followed by the very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and LDL. HDL is the most dense lipoprotein class and can further be divided into of a number of subclasses, the most common ones being HDL_2 and HDL_3. Each class of lipoproteins is characterized by and has a preferential association with specific apolipoproteins (see table 1.2). Lipoproteins can also

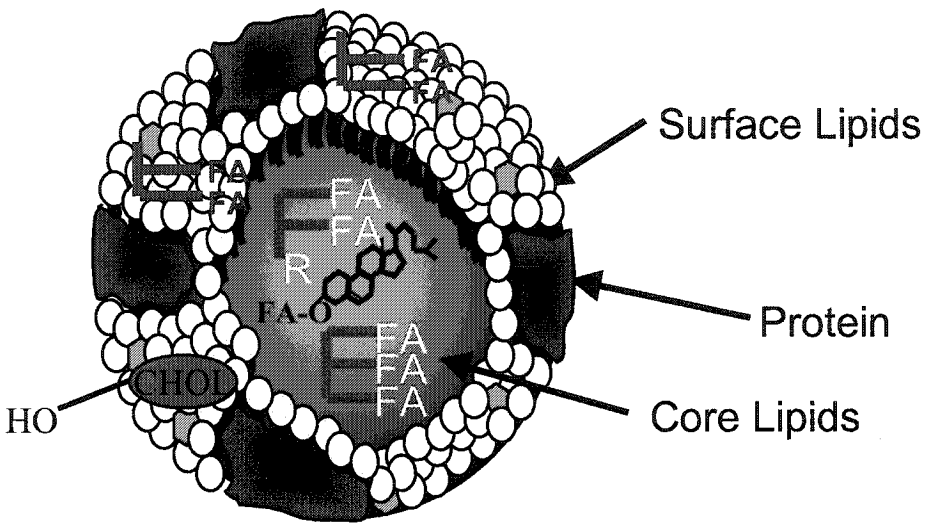

Figure 1.2.1 Generic structural model of a plasma lipoprotein

Lipoproteins consist of a core of neutral lipids, primarily TG, CE and DG surrounded by a PL monolayer, FC, DG and apolipoproteins. The amphipathic apolipoproteins function to stabilize the lipoprotein.

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be separated on the basis of their electrophoretic mobilities, which reflects the overall surface charge of the lipoproteins (53).

1.2.2 The apolipoproteins

The apolipoprotein component of the various lipoproteins can be broadly divided into two groups, the exchangeable (apolipoproteins A-I, -II, -IV, C-I, -II, -III and E) and the non-exchangeable apolipoproteins (apolipoproteins B-100 and -48). The genomic similarities of most of the exchangeable apolipoproteins suggest that they were derived from a common ancestral gene (54). Furthermore, the structures of most of the exchangeable apolipoproteins are similar and consist primarily of repeated amphipathic alpha-helices (multiple repeats of 11 or 22 amino acids), a concept first proposed by Segrest *et al.* (55), which contributes to

Table 1.2: Physical properties of human lipoproteins

Lipoprotein Class	Density (g/ml)	Molecular Weight (kDa)	Electrophoretic Mobility	Diameter (nm)	Protein Associated
Chylomicrons	> 0.93	50-100x10^3	Remains at origin	75-1200	A-I, A-II, B-48, C-I, C-II, C-III, E
VLDL	0.93-1.006	10-80x10^3	pre-β migration	30-80	A-I, B-100, C-I, C-II, C-III, E
IDL	1.006-1.019	5-10x10^3	slow pre-β migration	25-35	B-100, C-I, C-II, C-III, E
LDL	1.019-1.063	2.3x10^3	β migration	18-25	B-100
HDL_2	1.063-1.125	360	α migration	9-12	A-I, A-II, A-IV, E C-I, C-II, C-III
HDL_3	1.125-1.21	175	α migration	5-9	A-I, A-II, A-IV, E C-I, C-II, C-III
Pre-β-HDL	1.28	67	pre-β migration	~5	A-I

Adapted from 53.

the detergent-like properties of these proteins (53). The non-polar residues on one side of the helices penetrate the surface of the lipoprotein and interact with the fatty acyl chains of PL whereas the polar residues on the opposite side associate with and are exposed to the PL head groups and the aqueous environment (56, 57). This distribution of polar and non-polar residues enables the exchangeable apolipoproteins to associate with the surface of the lipoproteins and yet at the same time also allows for their transfer between lipoproteins (53).

One of the best characterized exchangeable apolipoproteins is apolipoprotein (apo) A-I, the major protein constituent of HDL. The gene for apoA-I is located on chromosome 11 (58, 59) and is part of a gene cluster that includes apoA-IV and apoC-III (60). The gene

encodes for a 267 amino acid preproapoA-I, which is cotranslationally cleaved to give rise to the secreted form of the protein, a 249 amino acid proapoA-I that is in turn processed in the circulation producing the mature 243 amino acid apoA-I (reviewed in 54). Although apoA-I is secreted by the intestine bound to chylomicrons or by the liver bound to VLDL, a significant proportion of the apoA-I is secreted in its lipid-free form or as a lipid-poor nascent HDL (pre-ß-HDL) particle (discussed in section 1.2.9) (61).

In addition to its structural role, apoA-I is an essential co-factor for lecithin:cholesterol acyltransferase (LCAT) (62), an enzyme required for HDL maturation. LCAT, which is predominantly synthesized by the liver (63), circulates in the plasma associated with HDL (64). It functions to esterify cellular FC to form CE by catalyzing the transfer of a fatty acid from the sn-2 position of lecithin to the 3-ß-hydroxyl group of FC (62, 65). This LCAT mediated production of CE, which is located at the center of the lipoprotein core, is believed to be responsible for the maturation of the discoidal pre-β-HDL to a spherical HDL (discussed in section 1.2.9). Although the actual mechanism of LCAT activation by apoA-I remains unknown, *in vivo* studies have implicated several domains of the protein (66, 67).

Another important role of apoA-I is to promote facilitated diffusional and receptor mediated (active) cholesterol efflux from cell membranes. Scavenger receptor B type 1 (SR-B1) binds HDL and selectively delivers cholesterol to the liver and steroidogenic tissues (68, 69). Binding of SR-B1 to apoA-I, a known ligand (70), is believed to involve both the C- and N-terminal domains of apoA-I (71). The interaction of apoA-I with another important protein, the ATP-binding cassette transporter type A1 (ABCA1), appears critical for cholesterol flux. This interaction results in the apoA-I-mediated removal of cholesterol and PL from peripheral tissues and the liver (72). Genetic defects in ABCA1 were recently

identified as being responsible for Tangiers disease (reviewed in 78) (73-77) and familial HDL deficiency syndrome (73, 79). In patients with Tangiers disease, the inability to mobilize these lipids to apoA-I prevents the maturation of HDL (80). Consequently, the nascent HDL is rapidly cleared from the plasma, which gives rise to extremely low levels of HDL.

ApoA-II is the second most abundant protein in HDL (20% of total HDL protein content) and, like apoA-I, it is synthesized in the liver and the intestine as a preproprotein, which is cleaved cotranslationally (81). The mature 77 amino acid apoA-II, unlike apoA-I, exists primarily as a homodimer linked by a disulphide bond (82). Although a structural component of HDL, the physiological role of this apolipoprotein is less certain than apoA-I although it has been shown to modulate LCAT (83-86), cholesteryl ester transfer protein (CETP) (87, 88), lipoprotein lipase (LPL) (89) and hepatic lipase (HL) activities (discussed in section 1.3.8).

ApoA-IV is a 376 amino acid protein thought to be loosely associated with HDL and chylomicrons (90). Although it is predominantly found unassociated with lipoproteins in the 1.21g/ml infranate upon centrifugation, apoA-IV has been shown to redistribute itself readily between the lipid-bound and the lipid-free fractions (91). Like apoA-I, apoA-IV has been shown to be a lipoprotein antioxidant (92), participate in reverse cholesterol transport (RCT) (93, 94) as well as activate LCAT (95). In addition, apoA-IV has also been shown to be a post-prandial satiety signal (96, 97).

The apoCs are a group of apolipoproteins synthesized in the liver and to a lesser extent by the intestine (98, 99) and are found on chylomicrons, VLDL and HDL (90). Named as such because of their similarity in size rather than homology (53), the apoCs do have some functional features in common. All of the apoCs have been shown to modulate

LCAT activity (100, 101). In addition, appear to inhibit the uptake of VLDL by the liver (102-104). Although some believe this to be due to their ability to displace apoE from the VLDL surface (105, 106) it has been suggested that the apoCs can obscure the apoE on VLDL thereby inhibiting the binding of VLDL to remnant receptors and preventing their premature clearance (81).

Chylomicrons and VLDL, which are largely devoid of apoC-II, acquire this apolipoprotein from circulating HDL. It is on these lipoproteins (chylomicrons and VLDL) that it exerts its main role as an activator of LPL activity (107, 108). The importance of apoC-II is illustrated in familial apoC-II deficiency in which LPL activity is absent. These patients have severe hypertriglyceridemia and impaired clearance of chylomicrons and VLDL (109). Although these individuals do not develop premature atherosclerosis, they present with severe pancreatitis (110). ApoC-III, on the other hand, appears to have an antagonistic function and has been shown to inhibit both LPL and HL activities (discussed in section 1.3.8) (109).

The *apoE* gene is 3.6 Kb in length and like apoC-I and II, it is located on chromosome 19. The mature 299 amino acid protein, which is primarily secreted by the liver, contains two domains. The N-terminal domain is responsible for its interaction with the low density lipoprotein receptor (LDLr) (discussed in section 1.2.6) and heparan sulfate proteoglycans (HSPG) (discussed in section 1.2.11) via ionic interactions whereas the C-terminal domain contains the lipid-binding region (111, 112). Several apoE isoforms exist which arise from mutations within a single ancestral gene. These have been designated as apoE2 (Cys-Cys), apoE3 (Cys-Arg) and apoE4 (Arg-Arg) (113). The apoE3 isoform is considered the wild-type allele. The apoE2 and apoE4 isoforms differ from apoE3 by a

single amino acid substitution at two different sites (residues 112 and 158) and can cause various lipoprotein abnormalities (discussed below) (114).

ApoE is a protein constituent of most classes of lipoproteins including chylomicrons, VLDL, LDL and some subclasses of HDL. The major role for this protein is to mediate the interaction of these lipoproteins, especially VLDL and chylomicron remnants, with various receptors such as the LDLr and LDL receptor-related protein (LRP) (also known as the chylomicron remnant or apoE receptor) (115). The replacement of Arg for Cys at residue 158 of the apoE3 isoform, which gives rise to the apoE2 isoform, can have detrimental consequences on lipoprotein metabolism since it has been shown to indirectly disrupt the binding of the protein to the LDLr (114, 116, 117). Homozygote carriers of apoE2 have LDLr binding defective apoE and a small population of these subjects (< 5%) develops type III hyperlipoproteinemia. Patients with type III hyperlipoproteinemia are characterized by hypertriglyceridemia and hypercholesterolemia due to the accumulation of chylomicron remnants and VLDL remnants. ApoE4, which has an Arg residue at position 112 instead of Cys, also results in profound changes to the structure of the molecule. As a result, apoE4 binds preferentially to VLDL instead of HDL (118, 119) and carriers of this isoform have increased prevalence of hypercholesterolemia.

Unlike the apoAs, Cs and E whose amphipathic nature is derived predominantly from their alpha helical structure, apoB-48 and -100 contain significantly less alpha helical content (43%) and substantial amounts of beta sheets, beta turns and random coils (81). ApoB-100 and -48 derive their lipophilicity in a large part from long hydrophobic sequences interspersed with hydrophilic ones. This allows the hydrophobic portion of the protein to bury itself and interact strongly with the core lipids while the hydrophilic regions interact with the surface lipids and the aqueous environment (81, 120). Although on the whole

amphipathic, apoB has a high average hydrophobicity, which makes it unique and contributes to the non-exchangeable nature of this apolipoprotein.

The apoBs are the largest of the apolipoproteins. Human apoB-100, which is associated with VLDL and LDL, is a 4536 amino acid protein synthesized in the liver and has a molecular mass of 549 kDa. ApoB-48, which, in humans, is found solely in chylomicrons, is synthesized exclusively in the intestine and has a molecular mass of 264 kDa. Unlike the apoAs, which are evolutionarily related proteins derived from different genes, apoB-48 and -100 are the products of a single gene (apoB-48 represents the N-terminal 48% of apoB-100). The synthesis of apoB-48 from the common apoB gene is not a result of alternate splicing. Instead, this editing process requires the presence of APOBEC-1, a cytidine deaminase only present in the intestine (121, 122), which converts the C nucleotide at position 6666 to U thereby introducing a stop codon (123).

Each VLDL and LDL particle contains one molecule of apoB-100 per particle (124, 125). Similarly, chylomicron remnants, and by inference chylomicrons, also have one molecule of apoB-48 per particle (126). A number of studies have shown that in order to accommodate greater amounts of lipid, the conformation of apoB-100 in VLDL and apoB-48 in chylomicrons differ from that observed for apoB-100 in LDL (127-129). The "ribbon and bow" model for apoB-100 describes the protein forming a kinked ring-like structure around the circumference of the LDL particle (130, 131) with a redundant loop in the C-terminal of the protein representing the bow. By "crossing over" the LDLr ligand domain, the bow is thought to play a regulatory role in the binding of apoB-100 to the LDLr (132). Interestingly, the binding of chylomicrons and VLDL to the LDLr occurs via apoE and not apoB. The binding domain for the LDLr is found in the portion of apoB-100 that is absent in apoB-48 (133) and as such, apoB-48 does not interact with the LDLr. This is supported by

the observation that there is an accumulation of chylomicron remnants in apoE-deficient patients (134). Similarly, VLDL is not a ligand for the LDLr (135, 136) suggesting that conformational changes within the apoB-100, induced by the remodeling of the lipoprotein, are required for the interaction of apoB-100 with this receptor.

By virtue of the fact that apoBs are the primary protein constituents of chylomicrons, VLDL and LDL, it is not surprising that mutations within these proteins can have grave consequences. Hypobetalipoproteinemia is a genetic disorder that primarily results from the premature truncation of apoB (-100 and in some cases -48 as well) and the subsequent decreased production or increased catabolism of apoB-100 and -48 (137). A number of mutations, many of which result from substitutions or deletions that cause frameshifts and introduce a premature stop codon in the gene, have been identified (138). These patients have hematological, gastrointestinal, neuromuscular and ophthalmic symptoms similar or identical to those observed for abetalipoproteinemia (discussed in section 1.2.4) depending on the mutation(s) involved (137). Point mutations within the apoB-100 ligand-binding domain have also been observed (R3500Q, R3500W and R3531C) (139-143). The resulting disorder, which has been termed familial ligand-defective apoB, is a common cause of hypercholesterolemia. It is characterized by the accumulation of LDL in the plasma due to the decreased affinity of the apoB-100 for the LDLr and the premature development of atherosclerosis (137).

1.2.3 Chylomicron synthesis and metabolism

ApoB-48 (discussed in section 1.2.2) is the main protein constituent of chylomicrons, which are responsible for the transport of dietary lipids from the intestine into the circulation. These dietary lipids consist primarily of TG and also PL, FC and CE, which are converted

into oil-water emulsions of coarse droplets within the stomach. In the duodenum, they are solubilized by bile salts to form smaller oil droplets. The hydrolytic activities of pancreatic lipase (produces MG and fatty acids from TG) and pancreatic acyl esterase (converts CE to FC) convert the oil globules to mixed micelles. This process is essential for efficient fat absorption in the intestine. Due to their small size, the micelles interact efficiently with the microvilli in the jejunum at which point the fatty acids, MG, PL and cholesterol are taken up by the enterocyte. Although fatty acids and PL are absorbed readily, dietary cholesterol absorption is incomplete; only about 30-60% enters the body. This is due to the fact that in addition to the bile salts present, the detergent properties of PL and fatty acids are essential for optimal fat absorption. In the enterocyte, the resynthesized TG and re-esterified cholesterol are complexed with PL and apolipoproteins, primarily apoB-48 (discussed in section 1.2.2), to form chylomicrons, which are secreted into the lymphatic system (81).

Chylomicrons are large TG-rich lipoproteins and the major carrier of dietary lipids, which contain only 1-2% protein by mass (144). They are secreted into the lymphatic system and enter the blood circulation through the thoracic duct where they come into contact with other lipoproteins. The secreted chylomicrons contain apoB-48, apoA-I and apoA-IV (145, 146) and acquire the apoCs and apoE (discussed in section 1.2.2) through their interactions with HDL in the circulation (discussed in section 1.2.9) (reviewed in 145 and 147). ApoC-II on the chylomicrons activates LPL (discussed in sections 1.2.2 and 1.3.1) (148) found on the vascular endothelium of skeletal muscle tissue, adipose tissue and cardiac muscle. This enzyme hydrolyzes the TG within the chylomicron core, thereby releasing fatty acids and MG, which can be used as a source of energy by various tissues. As the chylomicrons are hydrolyzed and the size of the lipoprotein is reduced, the relative amounts of cholesterol and protein increase. This favours the net transfer of PL, cholesterol and

apoCs to HDL (146). Both apoB-48 and apoE (discussed in section 1.2.2) remain associated with this newly formed TG-poor cholesterol-rich chylomicron remnant. The removal of the apoCs exposes the receptor-binding domain of apoE, which binds the LRP and the LDLr, in order for these remnants to be cleared by the liver (discussed in section 1.2.6).

1.2.4 Synthesis and secretion of VLDL

VLDL are TG-rich lipoproteins produced by the liver which are substantially smaller than chylomicrons but are thought to undergo a similar metabolism in the circulation. They transport endogenously synthesized lipids, primarily TG (60%) and some CE, in their neutral lipid core and have a surface monolayer composed of PL, FC, and apolipoproteins; mostly apoB-100 (40%), apoCs and apoE (see tables 1.1 and 1.2).

The assembly and secretion of apoB-100-containing lipoproteins has garnered significant interest over recent years primarily because of the belief that their overproduction is responsible for a large portion of the hyperlipidemias observed in patients (149-154). ApoB is believed to associate with the endoplasmic reticulum (ER) membrane co-translationally or early in the post-translational period (155-160). Although synthesized constitutively, not all the apoB is secreted in association with lipoproteins. In fact, a significant proportion is degraded intracellularly. The first study to suggest this was by Borchardt and Davis (161) using primary rat hepatocytes as a model system. Subsequent work supports this hypothesis (162, 163) and has further shown that the lipid availability (TG, PL, CE and FC) is a key determinant in the secretion of apoB-containing lipoproteins (see below) (153, 156, 164-171).

In the absence of lipids, apoB translocation into the ER lumen is slowed considerably (172). Since apoB is constitutively expressed, there is often an abundance of apoB which

complexes to heat shock protein 70 in the cytoplasm (173) leading to its ubiquitination and ultimately proteasomal degradation of the nascent protein (174). On the other hand, when there is an abundance of lipids, apoB is targeted for assembly and secretion rather than degradation (175). Although a complete understanding of the regulation of VLDL assembly and secretion has yet to be established, various studies indicate that both the amount of apoB synthesis and the availability of the lipids are two key factors in determining the rate of particle secretion.

The initial lipidation of apoB occurs during its translation and translocation across the ER membrane (176-178). Microsomal triglyceride transfer protein (MTP), which transiently binds apoB (179, 180), transfers TG to the nascent particles (181) in the ER lumen (182). Although the steps involved in the maturation of the nascent particle to the mature VLDL are still unknown, two possible models have been proposed. In the "two step" model, the partially lipidated VLDL is released into the lumen and fuses with TG droplets, possibly supplied and mediated by MTP, to form VLDL. Alternately, in the "concerted assembly" model, apoB lipidation, possibly via MTP, occurs during translocation into the ER lumen until a VLDL-sized particle is formed (183). Although the exact role of MTP in the assembly of apoB-containing lipoproteins is not known, the importance of this protein in this process is exemplified in abetalipoproteinemia. Abetalipoproteinemia, a disease in which MTP is defective (184, 185), is characterized by the absence of chylomicrons, VLDL and LDL within the circulation and the accumulation of dietary fats within cells of the intestine and liver. Patients afflicted by this disease have fat malabsorption, hematological abnormalities and degeneration of the nervous system (81).

Not only does TG availability play a role in determining the number of VLDL particles secreted, it is also partially responsible for determining the size of the lipoproteins

produced (186, 187). When the availability of TG is high, as is the case in obesity (188), diabetes mellitus (189) and a diet rich in simple carbohydrates (186, 190), the liver secretes large TG-enriched lipoproteins. Interestingly, these TG-rich lipoproteins are characteristic of the type of VLDL secreted by patients with familial hypertriglyceridemia (191, 192). On the other hand, small VLDL or IDL/LDL-like particles are secreted when the availability of TG is reduced (193, 194) or when the secretion of apoB is increased without a concomitant increase in TG synthesis, as is the case in familial combined hyperlipidemia (FCHL) (151, 191, 192) and hyperapobetalipoproteinemia (152).

1.2.5 VLDL catabolism and LDL metabolism

VLDL secreted from hepatocytes contain only one molecule of apoB-100. Although these lipoproteins may also contain apoC-I, -C-II, -C-III and -E, VLDL acquires the majority of these apolipoproteins in the circulation. Within the circulation, two key enzymes, LPL and HL (discussed in section 1.3) hydrolyze the TG core to yield a smaller particle. As the lipoprotein size is reduced, excess PL, cholesterol and apolipoproteins are delivered to HDL (195). Some of the IDL is catabolized in the liver and the remainder is further acted upon by HL and converted to LDL. Concurrently, plasma lipid transfer proteins (discussed below) transfer TG, CE and PL between the apoB-containing lipoproteins and HDL (figure 1.2.2).

The plasma lipid transfer proteins include CETP and phospholipid transfer protein (PLTP). PLTP promotes the net transfer of phosphatidylcholine (PC) from VLDL to HDL and enhances the removal of surface components from chylomicrons, VLDL and their remnants during lipolysis (53, 196). CETP, on the other hand, is associated with the HDL pool (197) where it transfers CE from HDL to the apoB-containing lipoproteins, primarily the TG-rich lipoproteins (chylomicrons and VLDL), in exchange for TG. Since the liver

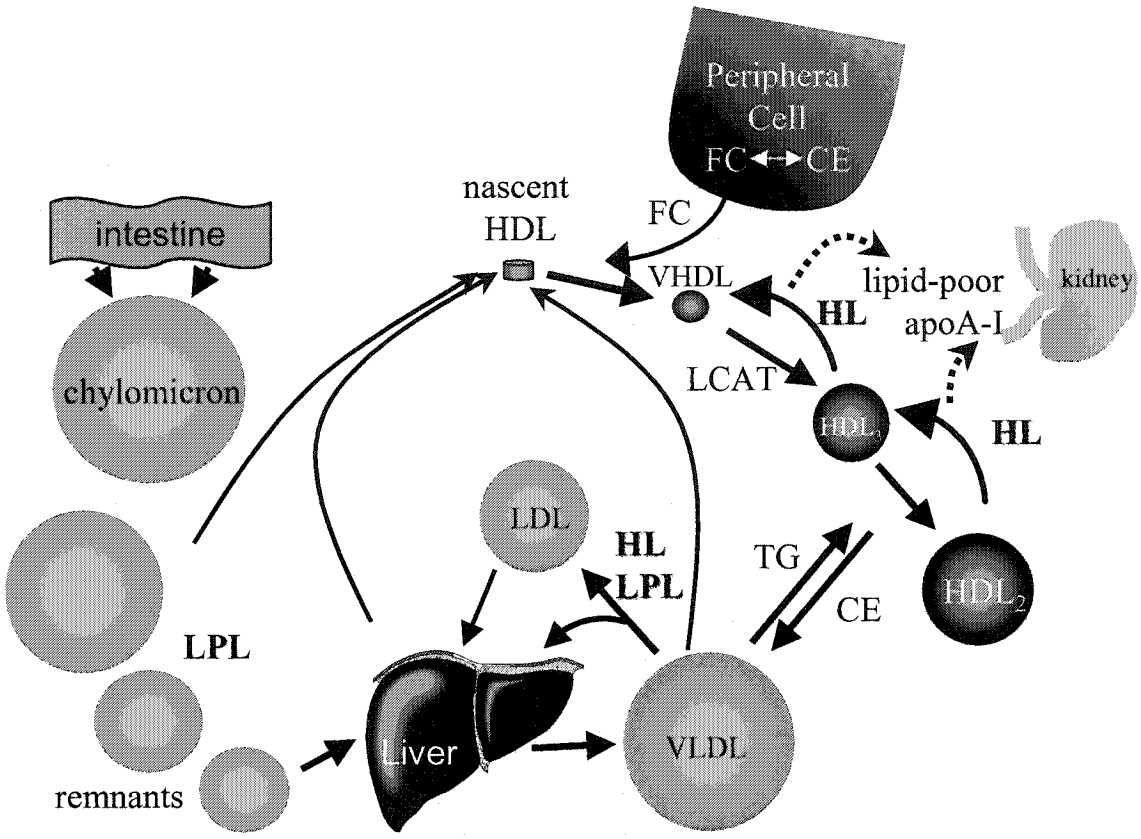

Figure 1.2.2 Reverse cholesterol transport and lipoprotein metabolism

Chylomicrons and VLDL secreted from the intestine and the liver, respectively, are hydrolyzed by the lipolytic activities of LPL and HL. The remnants lipoproteins produced can be taken up by the liver or, in the case of the VLDL remnants, can further be hydrolyzed by HL to form LDL. ApoA-I, secreted by the liver or shed as a byproduct of chylomicron and VLDL lipolysis as a nascent HDL (or pre-β-HDL) is the recipient of FC and PL effluxed from peripheral cells. The accumulation of FC and PL with nascent HDL favours its interactions with LCAT, which convert FC to CE thereby promoting the formation of a neutral lipid core and as such, the formation of larger spherical HDL particles. These, in turn, can exchange CE for VLDL-TG, a process mediated by CETP. The larger HDL particles produced are good substrates for HL, which hydrolyzes them to form smaller denser particles.

clears only part of the CE transferred (via remnant particles), the remainder enters the atherogenic LDL pool. Although this suggests an atherogenic role for CETP, its role in this process has been the subject of much debate.

The factors which determine the fate of IDL (clearance versus conversion to LDL) are not known although some evidence suggests that the larger VLDL are predestined for clearance while the smaller VLDL are targeted for conversion to LDL (190, 193, 194, 198, 199). Some studies suggest that the fate of IDL, either for direct clearance or conversion to LDL, is dependent on the number of apoE molecules on the surface of large VLDL. The more apoE, the greater the chance IDL are removed from the circulation prior to their conversion to LDL (discussed in section 1.2.6) (115, 200, 201). Yet, others have suggested that TG-rich VLDL becomes CE-enriched via the action of CETP. Consequently, the CE-enriched remnant lipoproteins are not good substrates for LPL and HL and are therefore not hydrolyzed to form LDL but instead are targeted for clearance (149, 202). However, it may simply depend on particle circulation time. The larger VLDL, which require more time to be fully hydrolyzed to LDL than the smaller VLDL, have a greater probability of interacting with the receptors targeting clearance (discussed in section 1.2.6) (149, 203).

LDL is not derived solely from the metabolism of IDL but can also be produced and secreted by the liver (193, 204). The composition of the LDL, independent of its origin, is influenced by the composition and number of VLDL and is dependent on the actions of HL and CETP. In the presence of TG-rich VLDL, CETP enhances the transfer of VLDL-TG to LDL in exchange for CE (202, 205, 206). The resulting TG-rich LDL is a good substrate for HL, which hydrolyzes it to generate small dense LDL (207, 208). The small dense LDL are believed to be cleared very slowly because of a poor affinity for the LDLr (209). Consequently, due to their longer residence time in the circulation, they are prone to

oxidative modification (210, 211) and as such, are more atherogenic (discussed in section 1.2.7). LDL is primarily removed from the plasma via the classical LDL receptor pathway (60-80%) (212, 213) whereas the remainder of the LDL is cleared via other receptors (LRP and scavenger) and possibly by non-receptor mediated mechanisms (discussed in section 1.2.6).

1.2.6 Remnant receptors

The LDLr, first discovered by Goldstein and Brown in 1974 (214), is a highly conserved protein amongst species and is comprised of a number of domains. The ligand-binding domain, which interacts with apoE and apoB-100, consists of seven cysteine-rich repeats. This region is adjacent to an epidermal growth factor- (EGF) precursor homology domain (215, 216), which contains three cysteine-rich growth factor repeats. This domain is required for the dissociation of the receptor-ligand complex in the endosome (217). The next domain contains a number of attachment sites for O-linked carbohydrate chains (218, 219) however, the function of this domain has yet to be determined. The protein is anchored to the cell surface by a membrane-spanning domain. The cytoplasmic domain contains a signal that localizes the receptor within the clathrin coated pits (220) and another motif (NPYX) to target the receptor to the sinusoidal surface of polarized hepatocytes (221).

The primary role of the LDLr is to provide the cells with an exogenous supply of cholesterol. The LDLr within clathrin coated pits (222) binds LDL for subsequent internalization (223). Once internalized, the endocytotic vesicles fuse to form endosomes. The acidic internal environment of the endosome enhances the release of the LDLr-LDL complex allowing the receptor to be recycled back to the cell surface in order to initiate another cycle of endocytosis (224). The internalized LDL, on the other hand, is delivered to

lysosomes where it is degraded to its various components (225, 226). The cholesterol released in this fashion can be used for membrane synthesis and/or to regulate intracellular cholesterol levels within narrow limits (227) via the suppression of HMG-CoA reductase activity (228), stimulation of acyl-coenzyme A: cholesterol acyltransferase activity (229) and down-regulation of LDLr synthesis (230).

The importance of the LDLr is most clearly demonstrated in individuals that do not have functional receptors, a disease known as familial hypercholesterolemia (FH). The mutations responsible for FH are numerous and can be divided into five classes, which are based on their phenotypic effects on the LDLr. Class 1 mutations interfere with protein synthesis (null alleles). Class 2 mutations prevent the transport of the receptor from the ER to the Golgi apparatus (transport-defective alleles). Class 3 mutations do not bind LDL efficiently (binding-defective alleles). Class 4 mutations do not internalize LDL due to their inability to cluster in the clathrin coated pits (internalization-defective alleles). Class 5 mutations are unable to release the internalized LDL in the endosome and can therefore not be recycled back to the cell surface (recycling defective alleles). Regardless of the mutation responsible for the disorder, the rate of LDL clearance from the plasma of these patients is reduced and instead, the LDL accumulates in scavenger cells producing atheromas and premature atherosclerosis.

Another important receptor in LDL metabolism is LRP. It belongs to the LDLr super-gene family and is structurally similar to the LDLr although significantly larger. The receptor is found in most tissues and cell types although it is primarily expressed in the liver, brain and placenta (231-233). LRP is a massive protein, consisting of 4525 amino acids and has four ligand-binding domains that are composed of cysteine-rich repeats (234). Each of these domains is separated by an EGF-precursor homology domain that is required for the

acid-dependent release of ligands in the endosomes (217) similar to its function in the LDLr. However, instead of the sugar-rich domain found in the LDLr, LRP has six additional EGF repeats, which link the molecule to the membrane-spanning domain and the cytoplasmic tail.

LRP, which has a high affinity for apoE yet does not bind apoB, is partially responsible for the clearance of chylomicron remnants, VLDL remnants and apoE-containing IDL (235, 236). This clearance can be inhibited by the presence of apoCs, which compete effectively with apoE for binding to VLDL thus depleting the surface of the lipoprotein of its ligand (106). Furthermore, LRP is proposed to play a role in the cellular uptake of HDL in conjunction with HL and apoE (237). In addition to its roles in lipoprotein metabolism, LRP has been shown to bind and internalize a number of biologically diverse ligands including alpha-2-macroglobulin-protease complexes (238, 239), plasminogen activator-inhibitor complexes (240, 241) vitellogenin (242) and *Pseudomonas* exotoxin A (243).

1.2.7 LDL modification, scavenger receptors and atherosclerosis

LDL is the major carrier of cholesterol and normally accounts for two-thirds of total plasma cholesterol. Elevated plasma LDL levels and decreased LDL size are epidemiologically and clinically known risk factors for atherosclerosis (244-250). The chronic presence of these lipoproteins in the circulation and their proximity to the arterial wall renders them particularly susceptible to chemical modification such as oxidation, aggregation and glycation.

Under normal circumstances, LDL is not taken up by macrophages. However, upon chemical modifications, it is readily internalized. Unlike most cells, which tightly regulate fluctuations in intracellular cholesterol levels via feedback mechanisms, the uptake of modified forms of LDL by scavenger receptors is not regulated by cholesterol.

Consequently, these macrophages become lipid-engorged, which leads to the formation of foam cells, the initial step of atherosclerosis (discussed in section 1.1.4).

Although there are a number of different scavenger receptors on macrophages, scavenger receptor class A (SR-A) and B are thought to be the two main classes of receptors involved in foam cell formation. Two forms of SR-A, which are produced by the alternative splicing of the same gene, are referred to as SR-AI and SR-AII. Although slightly different structurally, both these receptors are located predominantly on macrophages (251-253) where they can bind and internalize glycated and oxLDL (254-256). As well, SR-A has been shown to be highly expressed in atherosclerotic lesions (257, 258). CD36, a class B scavenger receptor found on macrophages, monocytes and platelets (259-261) is also involved in the high affinity binding and uptake of oxLDL (262-264).

Overexpression of SR-A in both cell culture and animal models gives rise to the formation of foam cells in the presence of modified LDL (265, 266). These observations are supported by studies utilizing SR-A knockout mice in atherosclerosis susceptible mouse strains. SR-A deficiency, in an apoE-deficient or LDL receptor-deficient background, significantly reduced the lesion severity and the development of atherosclerosis (267, 268). Similar results were obtained when CD36-deficient mice were crossed on an apoE-deficient background (269). In the apoE-deficient backgrounds, the decrease in the lesion size was related to the reduced binding and uptake of oxLDL by these receptors (267, 269). As such, these observations offer a potentially crucial role for the scavenger receptors in the development of atherosclerosis. On the other hand, a study by De Winther *et al.* observed the development of more severe atherosclerotic lesions in SR-A knockout mice bred on an APOE3Leiden transgenic background (these mice carry a dominant variant of the human apoE gene), which suggests an anti-atherogenic role for SR-A in these mice (270). In

support of this, Thiery *et al.* observed that rabbits with a low susceptibility to atherosclerosis have elevated SR-A expression levels compared with levels in rabbits that have a high susceptibility to atherosclerosis (271). These somewhat conflicting findings illustrate that more research is necessary in order to obtain a clear understanding of the role of SR-A in atherosclerosis.

In addition to its direct role in the formation of foam cells, modified LDL, more specifically, oxLDL, can contribute to the formation of atherosclerosis in a number of other ways. These modified lipoproteins act as chemoattractants for monocytes (272), mitogens for macrophages and SMC (273), in addition to stimulating the release of molecules that increase the adherence and penetrance of monocytes (274) and the differentiation of monocytes into macrophages (40, 275). As well, oxLDL inhibits the motility of macrophages, which prevents their movement out of the arterial wall (276) and inhibits the release of NO• from the arterial endothelium thereby preventing vasodilation (277). These are just some of the examples that demonstrate, in addition to its direct role in foam cell formation, that the oxidative modification of LDL can elicit a number of events that are important in the development of atherosclerosis.

1.2.8 General properties of HDL

HDL are a heterogeneous group of lipoproteins that span the density range 1.063-1.21 g/ml. They are composed of an apolar core of CE and TG that are surrounded and stabilized by apolipoproteins (predominantly apoA-I and apoA-II) and amphipathic lipid molecules, PL and FC. HDL is primarily divided into HDL_2 (density 1.063-1.125 g/ml) and HDL_3 (density 1.125-1.21 g/ml) (see table 1.1), which separate into additional distinct subpopulations (HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c}) upon ultracentrifugation and

differ not only in density, but also in size, charge, apolipoprotein, lipid composition and function. In addition to HDL_2 and HDL_3, there exist lighter and denser HDL particles, HDL_1 and HDL_4 respectively, although these make up only a small portion of the total HDL.

HDL are often defined in terms of density, although another important distinction is their apolipoprotein composition (278). As such, HDL can be divided into apoA-I-only, apoA-I and apoA-II (apoA-I/-II) containing HDL and apoE-enriched HDL. The apoA-I-only HDL contain on average 4 molecules of apoA-I per particle and are predominantly found in the HDL_2 density range whereas the apoA-I/II particles contain two molecules of each apolipoprotein and are found primarily in the HDL_3 density range. ApoE-enrich HDL, which contain apoE as their predominant apolipoprotein, are less dense than HDL_2 and comprise primarily the HDL_1 subfraction.

The plasma levels of HDL are usually based on HDL cholesterol (HDL-C) levels and can vary widely from person to person, as do the levels of HDL_2 and HDL_3, which also vary in a predictable manner. HDL_3 is the main HDL subclass found when HDL-C levels are low (< 40-45 mg/dl). However, increases in HDL-C levels are often due to the presence of increased levels of HDL_2 while HDL_3 levels remain constant (279). Therefore, typically, when the HDL-C levels are high, HDL_2 outnumber HDL_3.

1.2.9 Origin and metabolism of HDL

The major HDL apolipoproteins (apoA-I and apoA-II) are synthesized primarily in the liver and the intestine and can be secreted bound to lipoproteins (chylomicrons or VLDL) (discussed in sections 1.2.3 and 1.2.4) or as lipid-poor nascent HDL (pre-ß-HDL) particles (280). In addition to being secreted directly from the liver and the intestine, nascent HDL are generated as remnants of VLDL lipolysis (206). Nascent HDL particles account for 5-

15% of total serum apoA-I. They are thought to be discoidal in shape and composed of apolipoproteins with small amounts of lipids, primarily PC, which is responsible for the bilayer structure of the disk, and FC (281-283). They have a hydrated density that is greater than that of HDL_3 and are isolated at a density > 1.21 g/ml upon centrifugation. These lipoproteins may have a crucial role in RCT (see below) and are readily converted to HDL_3 by the actions of LCAT when FC is supplied (discussed in section 1.2.2).

Traditionally, it has been believed that the maturation of HDL involves the efflux of FC and PL from the peripheral cells to nascent HDL down a concentration gradient via passive diffusion or mediated by ABCA1. This FC is esterified by LCAT to form CE, which shifts the equilibrium towards further transfer of FC from cells to the HDL molecules. The movement of the hydrophobic CE into the HDL core contributes to the formation of its spherical shape, which is followed by the interlipoprotein transfer of HDL-CE to apoB-containing lipoproteins in exchange for TG in a process mediated by CETP (discussed in section 1.2.5). As the nascent HDL molecule gains a lipid core and becomes spherical in shape, it loses some of its apolipoproteins (apoC and apoE) to VLDL and chylomicrons, which are in turn replaced with other apolipoproteins (apoA-I, apoA-II and apoA-IV) necessary for its stability and its function in RCT (see below). The larger, TG-enriched HDL are hydrolyzed by HL (discussed in section 1.3.7) to regenerate the smaller, CE-depleted nascent HDL which can initiate further rounds of cellular cholesterol efflux. However, recent studies have offered a new perspective on the role of HDL in RCT. The work of Stamler *et al.* (284) has shown that the intravenous administration of phosphatidylinositol (PI) liposomes into rabbits increased the net negative charge of HDL and almost completely inhibited LCAT activity while concomitantly stimulating RCT by promoting the clearance of FC from the circulation. More recently, the work of Burgess *et al.* (285) corroborated these

findings and further showed the PI enrichment of HDL, which promoted the movement of cholesterol from the plasma to the feces, appears to involve membrane transporters and cellular signaling systems. These studies therefore suggest that the role of LCAT is to determine whether cholesterol is to be stored in the plasma or excreted from the body and, as first proposed by Schwartz *et al.* (286), that HDL is the primary source of biliary cholesterol.

HDL maturation, as described above, is thought by many to play a key role in RCT, a process whereby cholesterol from the peripheral tissues is transferred to HDL for return to the liver for excretion (287). In this pathway, HDL-CE obtained from peripheral cells can be delivered to the liver via three pathways: remnant uptake, selective uptake and HDL uptake. Remnant uptake, described in detail in sections 1.2.5 and 1.2.6, involves the clearance of chylomicron remnants and IDL, which are enriched in CE, primarily by LDLr and LRP. Selective uptake of HDL-CE is mediated by SR-B1 (288), which is predominantly expressed in steroidogenic tissues and the liver (289-291). HDL particle uptake, on the other hand, is believed to be mediated by apoE in conjunction with HSPG and possibly LRP and HL (discussed in section 1.3.4) (237).

1.2.10 HDL and atherosclerosis

HDL levels are inversely related to the risk of developing atherosclerosis, and low levels of HDL-C are a predictor of coronary disease (292). As such, HDL is often referred to as an anti-atherogenic lipoprotein. The role of HDL in preventing atherosclerosis has yet to be fully elucidated, however, HDL has been shown to inhibit a number of steps in the atherogenic process. The most common anti-atherogenic functions of HDL are its role in RCT and as an antioxidant. Normally, cell cholesterol content is tightly regulated and maintained at constant levels. Under specific conditions, such as when macrophages are

exposed to modified or oxidized lipoproteins, they accumulate cholesterol uncontrollably. This can lead to the formation of fatty lesions and early signs of atherosclerosis (discussed in section 1.1.4). Cholesterol efflux from these cells to nascent HDL, which appears to be promoted by apoE (293, 294) and possibly mediated by ABCA1, is thought to slow down, reverse and possibly prevent this process (295).

HDL also negates a number of other pro-atherogenic effects of oxLDL (discussed in section 1.2.7). For example, HDL has been shown to prevent the migration of monocytes into the subendothelial space (296) as well as their adhesion to the endothelial cells overlaying the atherosclerotic lesion (297). Furthermore, HDL has the ability to inhibit the retention (298) and aggregation (299, 300) of LDL in the arterial wall and the oxidation of LDL by preventing its interaction with oxidized lipids (301), hydroperoxides (302) and transitions metals (303).

1.2.11 Proteoglycans

Proteoglycans are glycoproteins that fulfill a diverse range of biological functions. They influence cell growth (including tumor growth and invasion), function as biological filters, play a role in lipid metabolism and thrombosis and mediate the interactions of the cell surface with enzymes such as LPL and HL (discussed in section 1.3) (304-306). These macromolecules, which are synthesized by all cells and are present in all tissues, are located throughout the ECM, within intracellular structures (secretory storage granules), associated to structures within the ECM (basement membrane) or the plasma membrane of cells (307). They are composed of two main components: a protein core and sulfated polysaccharide side chains known as glycosaminoglycans. The protein core anchors the molecule to the cell surface and covalently binds the glycosaminoglycans via O-glycosidic linkages to serine

residues (307). Since one type of glycosaminoglycan tends to predominate on a protein core, proteoglycans have been divided into groups based on this distinction. The four main types of proteoglycans are chondroitin sulfate proteoglycans, dermatan sulfate proteoglycans, keratan sulfate proteoglycans and HSPG.

HSPG are present in most tissues and can be located within the cell as well as on the pericellular and extracellular matrices (308). The assignment of the term HSPG to define a proteoglycan containing a heparan sulfate glycosaminoglycan is rather broad since heparan sulfate can bind to a variety of different protein cores including agrin and perlecan in the ECM (309) and syndecan and glypican on the cell surface (310). The carbohydrate backbone of HSPG (the heparan sulfate portion) consists of alternating D-glucuronic acid and D-glucosamine units. In heparan sulfate, the D-glucosamine unit is primarily N-sulfated and N-acetylated with few N-unsubstituted units (311, 312). The number and location of the N-sulfated and N-acetylated units on a specific core protein is believed to contribute to its interaction with various protein ligands and, therefore, the function of the HSPG (313). The interaction of the heparan sulfate portion of the HSPG with protein ligands occurs via electrostatic interactions between the negatively charged sulfate and carboxyl groups of the heparan sulfate and positively charged amino acid residues in the protein (312).

The role of HSPG in lipoprotein metabolism has received significant interest in recent years due to its involvement in atherosclerosis and in the cellular uptake and turnover of lipoproteins. The interaction of HSPG with lipoproteins appears to be determined, in part, by the apolipoprotein content of the lipoprotein since both apoB and apoE have been shown to bind with high affinity to heparan sulfate (314). As important, however, is the surface charge of the LDL, which can either enhance or decrease the binding affinity of the lipoprotein for the heparan sulfate (315). In addition to binding lipoproteins, HSPG also

bind LPL and HL (discussed in sections 1.3.3 and 1.3.4). In fact, HSPG are thought to play a role in the HL-mediated clearance of lipoproteins, either by bridging the HL/lipoprotein complexes to cell surface receptors or by directly mediating the uptake of the complex (discussed in detail in section 1.3.4) (237,316).

1.3 Hepatic lipase

1.3.1 Hepatic lipase and lipoprotein lipase

As alluded to earlier, HL is very closely related to LPL. In humans, LPL is a 448 amino acid, 55 kDa glycoprotein that is also anchored to cell surfaces via HSPG. Unlike HL, whose synthesis is believed by most to occur exclusively in the liver (discussed in section 1.3.2), LPL is synthesized in a variety of tissues but primarily in the adipose and muscle (317). The localization of the two enzymes also differs. HL is located on the liver with lesser amounts associated with steroidogenic tissues (adrenals and ovaries), while LPL has a broader distribution and is found in abundance at the vascular endothelium of extrahepatic tissues.

HL and LPL are structurally closely related enzymes and members of the same gene family, which also includes pancreatic lipase and the recently discovered endothelial lipase (EL) (318-320). They both contain a N- and a C-terminal domain that are independently folded (discussed in section 1.3.3) (321). The N-terminal domains contain a conserved active site (321, 322) enclosed by a loop (lid) domain, the latter of which differs significantly between the proteins and has been shown to confer the substrate specificities of the enzymes (322-325). The interaction with apoC-II, which is required for LPL activity, occurs within the N-terminal domain of LPL (326). Although the primary structures of the C-terminal domains are much less conserved (327), the predicted overall tertiary structures are similar (322) as are their functions. The C-terminal domains of both enzymes contain regions involved in heparin- (326, 328-331), lipid- (332-334) and receptor-binding (332, 335) (discussed in section 1.3.3).

The functions of HL and LPL are, not surprisingly, similar and complementary in nature. These lipolytic enzymes have important roles in lipoprotein clearance. HL and LPL, in addition to their lipolytic activities, have been shown to mediate the interaction of lipoproteins with cell surface HSPG (336, 337) as well as with specific receptors such as the LDLr, LRP and SR-BI in order to enhance the clearance of various lipoproteins (discussed in detail in section 1.3.4) (237, 336, 338-347). As lipolytic enzymes, LPL is primarily involved in the conversion of chylomicrons and VLDL to chylomicron remnants and IDL (348), respectively, whereas HL is responsible for a portion of VLDL to IDL conversion, essentially all of the conversion to LDL (349) and some of the HDL hydrolysis (discussed in detail in section 1.3.5) (350). Although HL has previously been thought to be solely responsible for HDL phospholipid hydrolysis, EL, has now also been shown to be an HDL phospholipase (351-357). HL and LPL also differ in their substrate preferences. Although both enzymes have the ability to hydrolyze glycerides (TG, DG and MG) and PL, LPL appears to preferentially hydrolyze TG whereas HL, depending on the lipoprotein substrate, can hydrolyze glycerides and PL equally well (discussed in detail in section 1.3.7) (358-360).

1.3.2 Synthesis, secretion and localization

In humans, HL is a 66 kDa, 476 amino acid protein that is anchored by HSPG to cell surfaces. It is widely distributed on the sublumenal ECM component of endothelial cells, the microvillar surfaces of hepatocytes in the space of Disse, interhepatocyte spaces, as well as on the lumenal surfaces of the hepatic sinusoidal endothelium (361, 362). HL is postulated to function both as a cell-surface ligand for lipoprotein uptake and as a lipolytic enzyme (237, 338, 339, 341, 344, 363-365). Enzymatically, HL hydrolyses the sn-1 fatty acyl ester bonds of sn-3 PL as well as the sn-1 (sn-3) ester bonds of mono-, di- and triglycerides found

in all classes of lipoproteins thereby contributing to the clearance of TG from the blood stream as well as the conversion of VLDL to LDL and HDL_2 to HDL_3 (discussed in section 1.3.5) (360, 366-371).

HL is synthesized in the ER and acquires N-linked oligosaccharides co-translationally at all four N-linked glycosylation sites (amino acids 20, 56, 340 and 375) (372, 373). Subsequently, HL oligosaccharides undergo a series of trimming steps, which involve the removal of the three terminal glucose residues, a process catalyzed by ER glucosidases I and II in the rough ER (374). The trimming of glucose is required for the interaction of HL with the chaperone proteins calnexin and calreticulin, which act as a quality control system by preventing poorly folded proteins from leaving the ER (375, 376). This is followed by the Golgi-specific maturation of the oligosaccharides and secretion of the mature protein (377).

Studies in rat primary hepatocytes suggest that the addition and initial trimming of the N-linked oligosaccharides in the ER is crucial for the secretion of an active rat HL (378-381) whereas latter steps in N-linked oligosaccharide processing (Golgi-specific maturation) affect neither the secretion nor the activity of the enzyme (379). Using human HL-transfected Chinese Hamster Ovary (CHO) cells, Boedeker *et al.* showed that HL secretion (mass and activity) is inhibited in the presence of tunicamycin, a glycosylation inhibitor. However, they further showed that active HL could be secreted in the absence of processing by ER glucosidase enzymes, although at reduced levels (40% of control) (382). Verhoeven *et al.*, in contrast, showed that treatment of HepG2 cells expressing endogenous human HL with an ER glucosidase inhibitor completely abolished its activity and secretion (383). Whether processing of HL N-linked oligosaccharides within the ER is required for secretion and activity of the enzyme is unclear as it appears to depend on the source of the enzyme (rat

versus human) and the cell culture model used (CHO versus HepG2). Nonetheless, the evidence thus far suggests that N-linked glycosylation and ER processing of the oligosaccharides are crucial for its interaction with chaperone proteins and efficient secretion of a functional enzyme.

The synthesis and secretion of HL occurs primarily in the liver parenchymal cells (384) although HL protein and mRNA have been detected in steroidogenic organs of rats and humans (385). Verhoeven *et al.* found that the mRNA in the adrenal tissues of rats differs from that of the liver since the adrenal tissue produces a truncated version of the mature mRNA that is translated into a 40-45 kDa protein, which remains intracellular (386-388). However, the evidence for this local synthesis is still controversial and the function of the intracellular enzyme is unknown. Most, however, believe that HL is synthesized exclusively in the liver and is transported to these tissues via the circulation where it subsequently binds to tissue-specific sites (389, 390). The role of HL in these tissues has yet to be determined but it is thought to promote lipoprotein cholesterol delivery needed for steroidogenesis.

1.3.3 Structural motifs

HL is a member of a gene family that includes pancreatic lipase, LPL and EL (318-320, 391). These enzymes show high sequence homology to one another (392) suggesting that certain regions of these lipases are highly conserved. Initial analyses of these lipases suggested that the commonly shared Gly-X-Ser-X-Gly pentapeptide sequence (X = any amino acid) was the likely active site since this is its function in serine proteases (393). Amino acid sequence homology to serine proteases and thioesterases subsequently identified Ser147 of rat HL (Ser145 of human HL) as part of a highly conserved element. Davis *et al.*

confirmed this when the catalytic activity of rat HL was abolished by site directed mutagenesis of this conserved serine residue (394).

The crystal structure of pancreatic lipase (395) has contributed significant insights into the three dimensional structure and mechanism of action of this enzyme. Consequently, computer generated models of LPL and HL, based on the crystal structure of pancreatic lipase, have provided similar information about these enzymes. Since the crystal structure of pancreatic lipase was solved in 1990 subsequent studies involving the use of domain-exchange strategies, truncation analysis and site-directed mutagenesis, have provided additional information regarding the functional domains of HL and LPL. These strategies have since confirmed that the N-terminal domain is catalytic in nature and contains the active site (394, 396). In addition, the N-terminal contains the lid domain (324) as well as a C-II binding domain in the case of LPL (326). The C-terminal domain, on the other hand, contains regions involved in heparin- (discussed below) (326, 328-331), lipid- (332-334) and receptor-binding (335, 336, 397). Although each domain appears to have distinct functions, their roles often overlap. Both domains have been implicated in the regulation of substrate specificity (324-326), the activation of LPL by apoC-II (326, 328) and the formation of homodimers (334).

The association of proteins with HSPG involves electrostatic interactions between conserved basic amino acid residues of a protein and the negatively charged sulfate groups of the HSPG (reviewed in 398). A number of consensus sequences for these heparin-binding domains have been proposed and include (X)BBBXXB(X) and (X)BBXB(X) where B is a basic residue (399). Margalit *et al.* (400) found additional consensus sequences and showed that the spatial orientation of basic residues is important for protein-HSPG interactions. These consensus sequences have been found in a number of heparin binding proteins

involved in lipoprotein metabolism including apoB-100, apoE, LPL and HL (330, 399, 401, 402).

Although the heparin-binding sequences of LPL have been extensively studied (322, 403-407), much less is known about the heparin-binding domains of HL. Early chimeric studies by Davis *et al.* (using human LPL and rat HL) (326) and Dichek *et al.* (using human LPL and human HL) (408) implicated the C-terminal domain of HL in heparin-binding. Similarly, Hill *et al.* found that the final 60 C-terminal residues (415-476 human HL and 389-448 human LPL) determined the relative heparin affinity of human HL and LPL (328). Recently, using chimeras created from mouse and human HL, Brown *et al.* (331) found that heparin-binding was determined within the C-terminal 70 amino acid residues of human HL of which the last 5 residues play an important role. This has been corroborated by the finding that the protein sequences of mouse HL, which is primarily found free in circulation, diverge significantly at the extreme C-terminus of human HL and lack the sequence homologous to cluster 4 of human HL (residues 337-443) (402, 409).

1.3.4 Ligand function

Although HL and LPL are lipolytic enzymes (discussed in section 1.3.5), an additional role for these enzymes in cellular lipoprotein metabolism has been suggested. It was first proposed some thirty years ago that LPL might play a role in lipoprotein clearance by a remnant receptor (410). A number of studies have since then provided significant *in vitro* and *in vivo* evidence supporting a role for LPL and HL in mediating the interaction of lipoproteins with cell surface HSPG (336, 337, 341, 344) and specific receptors (LDLr, LRP and SR-BI) (237, 336, 338, 339, 341-343) in order to enhance the clearance of all classes of lipoproteins (237, 338-341, 344-347). Although different lines of evidence have suggested

independent roles for HSPG and various receptors in the HL-mediated clearance of lipoproteins, these are not mutually exclusive and often involve complementary pathways.

HSPG are thought to play a role in the HL-mediated clearance of lipoproteins, either by bridging the HL/lipoprotein complex to cell surface receptors or by directly mediating the uptake of the complex (316). Ji *et al.* suggested that the treatment of human HL-transfected rat hepatoma cells with either heparinase (removes the sulfated glycosaminoglycan chains from HSPG) or cholate (inhibits proteoglycan sulfation) inhibited the HL-mediated binding and uptake of ß-VLDL, chylomicron remnants and HDL (237, 344). Similar results were obtained with primary rat hepatocytes (346). In addition, HL-mediated lipoprotein uptake was prevented by proteoglycan-deficient CHO cells (341) further substantiating the view that binding to HSPG is a necessary step. The collaborative roles of HL and HSPG in remnant clearance have been nearly impossible to prove *in vivo* because of the multitude of other factors that come into play (LRP, LDLr). Nonetheless, Ji *et al.* have shown that intravenous heparinase treatment of mice inhibited remnant lipoprotein clearance (337) whereas the overexpression of catalytically inactive human HL in mice decreased plasma levels of apoB-containing lipoproteins (363) suggesting an independent role for HSPG and HL.

In addition to cell surface proteoglycans, various receptors, including the LDLr, LRP and SR-BI, are thought to participate in HL-mediated uptake of lipoproteins and their lipids. LRP has been shown to bind and enhance the clearance of ß-VLDL and chylomicron remnants *in vivo* (411, 412). Evidence for the role of HL in this LRP-mediated pathway has come from a number of *in vitro* cell culture experiments. Kounnas *et al.* showed that the internalization and degradation of HL, which binds LRP, is partially inhibited in LRP-deficient fibroblasts and in HepG2 cells treated with an anti-LRP IgG or receptor-associated protein (RAP), a known LRP antagonist (336). Similarly, it was shown that the HL-

mediated HDL binding and uptake was also partially inhibited by RAP (237) further suggesting the existence of HL-mediated LRP-dependent and -independent pathways. These findings are also supported by the *in vivo* observation that RAP significantly reduced, but did not completely inhibit, HL-mediated removal of chylomicron remnants by the liver (340). In contrast, Amar *et al.* found that adenovirus expression of native or catalytically inactive HL in apoE-deficient mice resulted in the selective uptake of CE from remnant lipoproteins suggesting a role for HL that is independent of lipolysis, apoE and therefore LRP (413). CHO cells incubated with HL in the presence of an active site inhibitor, Orlistat, resulted in a 3-fold increase in ß-VLDL and chylomicrons clearance (341) which is similar to the results obtained by Ji *et al.* using HL-transfected rat hepatoma cells (237). Together, these studies suggest that HL contributes to lipoprotein uptake independent of its catalytic function and that interactions between HL and LRP have significant contributions to lipoprotein clearance.

Although Krapp *et al.* concluded that the LDLr was not important for HL-mediated clearance of human chylomicrons and rabbit ß-VLDL in human fibroblasts (341), others have since suggested a definitive role for this receptor in the HL-mediated uptake of lipoproteins. CHO cells transfected with either a glycosylated phosphatidylinositol-anchored HL (339) or rat HL (338) had an increase in LDL binding and degradation that could be inhibited with an anti-LDLr antibody. *In vivo* observations of DeFaria *et al.* (340) support this claim by showing that chylomicron clearance from the plasma and its uptake by the liver is inhibited by the administration of an anti-LDLr antibody to mice.

Wang *et al.* first suggested a role for SR-BI in the HL-mediated uptake of lipoproteins after observing that, despite increased adrenal SR-BI expression, female HL knock-out mice had decreased adrenal cholesterol stores (342). Similarly, Lambert *et al.*

(343) showed that embryonic kidney 293 cells transfected with SR-BI and HL increased HDL cell association and CE selective uptake compared to cells transfected with either HL or SR-BI alone. This implied that HL might be required for SR-BI-mediated selective uptake of cholesterol by the adrenals and that there is a link between HL levels and SR-BI expression.

1.3.5 Hepatic lipase activity

As discussed (see section 1.2.5), LDL is produced by the combined actions of LPL and HL through the lipolytic hydrolysis of VLDL and IDL and the transfer/exchange of lipids and apolipoproteins with HDL (414, 415). Reports from numerous laboratories have shown that both LPL and HL play important roles in the metabolism of apoB-containing lipoproteins (349, 365, 416-418). While LPL is primarily involved in the conversion of VLDL into IDL (348), HL appears responsible for a portion of VLDL to IDL conversion and essentially all of the conversion to LDL (349, 419).

In humans, HL activity is inversely correlated with LDL size and buoyancy, which is a reflection of IDL catabolism (420, 421). This correlation is corroborated by the finding that overexpression of human HL in transgenic rabbits is associated with a marked reduction in IDL levels and a concomitant increase in LDL levels (422). Similarly, the inhibition of HL activity *in vivo* by infusion of anti-HL antibodies into rats or cynomolgus monkeys gives rise to a significant increase in plasma IDL levels (423-425). In one kinetic investigation of VLDL metabolism in a HL-deficient patient, the authors identified a significant impairment in the conversion of small VLDL to IDL (50% reduction) and IDL to LDL (90% reduction) (349). What little LDL this patient had was abnormal in composition and significantly enriched with TG, an observation seen previously in other HL-deficient patients as well as in

animal models (423, 426). Taken together, these *in vivo* findings support an important role for HL in the conversion of VLDL to LDL.

HL is the only lipolytic enzyme that has a prominent role in the metabolism of both apoB-containing lipoproteins (as discussed above) and HDL. The assimilation of TG-rich lipoprotein-derived surface material, such as PL, cholesterol and apolipoproteins, by HDL promotes the formation of HDL_2 from HDL_3 (427). Concurrently, HL hydrolyses the TG and PL transferred to HDL by CETP and PLTP respectively, leading to the conversion of the larger HDL_2 into smaller HDL_3 (350). The hydrolysis of HDL by HL lowers the HDL-C levels (428) and generates smaller, less stable particles (429, 430) that can either be cleared from the circulation or re-enter the HDL pool for subsequent rounds of HDL maturation (discussed in section 1.2.9). This explains the inverse relationship between HL activity and HDL-C observed in both normal and hypertriglyceridemic subjects (431). In addition to its catalytic role in HDL remodelling, HL appears to play a significant role in the clearance of HDL-CE by the liver (432, 433).

Patients with HL deficiency and animal models in which the enzyme has been inactivated have been extensively studied and have provided significant insights into the role of HL in HDL metabolism. In HL-deficient patients, there is redistribution of the HDL species resulting in a greater proportion of the less dense TG-enriched HDL_2 subfraction (349, 426, 431, 434). Rabbits, which naturally have very low enzymatically active HL, also possess a greater proportion of large TG-enriched HDL (422, 435). Similarly, the inhibition of HL activity *in vivo* by the intravenous administration of antibodies against HL or the targeted inactivation of the gene prevents the conversion of the buoyant HDL to the dense HDL suggesting an obligatory role for HL in this process (436, 437).

In addition to models of HL deficiency, significant information has been obtained from the overexpression of HL. The expression of human HL in various murine backgrounds results in decreased HDL particle size, HDL-C levels and leads to the formation of a more homogenous HDL population (438-440). Similarly, Fan *et al.* (422) found that overexpression of HL in transgenic rabbits led to the near absence of large HDL particles as well as a marked reduction of plasma HDL levels. Likewise, using an identical model, Kee *et al.* observed reduced native and reconstituted HDL particle sizes in addition to increased rates of apoA-I catabolism (441) further supporting a role for HL in HDL metabolism.

1.3.6 Regulation of hepatic lipase expression

HL activity is regulated in a number of ways including its expression level and through a direct modulation of its activity. The expression of HL is determined by numerous factors including hormones, genetic factors, diet and lifestyle. In addition, there is significant evidence that the activity of HL can be modulated by apolipoproteins and non-substrate lipids as well as via its interaction with the cell surface.

The rat HL promoter has been shown to have a number of putative regulatory elements suggesting that the gene may be sensitive to many factors including various hormones, cholesterol, cAMP and glucose (442, 443). The hormones that have been shown to play a role in regulating HL expression include steroid hormones (sex hormones), peptide hormones (insulin and leptin), and amine hormones (thyroid hormones and catecholamines). The role of the steroid sex hormones in regulating HL activity has long been suspected. It is widely known that premenopausal women, have lower HL activity than postmenopausal women (444) and men (445) and this difference is often attributed to the regulation of HL by estrogen. It was found that estrogen treatment or castration was associated with decreased

HL activity and mRNA levels (446-448) while increased HL activity and mRNA levels were observed upon testosterone treatment or following ovariectomies (446, 447, 449-452). In addition to the effects that naturally occurring steroid sex hormones have on HL expression, a number of groups have shown that treating patients with synthetic anabolic steroids, such as the androgen stanozolol, upregulates HL expression (453-455).

The role of the peptide hormone insulin in regulating HL expression and activity is still inconclusive but clinical studies suggest that there is a direct relationship between HL activity and insulin levels. Subjects with insulin-dependent diabetes mellitus (IDDM) have decreased HL activity (456) and treatment of these patients with intraperitoneal insulin therapy increased HL activity (457, 458). In contrast, subjects with non-insulin-dependent diabetes mellitus (NIDDM) have elevated levels of HL activity (459). These results suggest a direct and positive correlation between insulin levels and HL expression and activity. This observation is supported by the studies of Emmison *et al.* (460) who found that insulin treatment of rat-cultured hepatocytes resulted in an increase in HL activity. However, a number of other studies have found that treatment of NIDDM patients with insulin therapy decreased HL activity (461-463). These diametrically opposing results were explained by Rivellese *et al.* who suggested that the subcutaneous administration of insulin may induce a relative hepatic hypoinsulinization, which helps explain the reduction in HL found in type 1 and 2 diabetic patients receiving insulin therapy (462, 464).

Leptin, a 16-kDa circulating protein synthesized primarily by white adipose tissue, is another important peptide hormone that has been shown to influence the regulation of HL activity and expression. Ob/ob mice, which have genetic defects in the leptin signalling pathway, have a marked decrease in HL mRNA levels (465). It was found that leptin treatment of these mice causes a large up-regulation (5.5-fold) of HL mRNA (465). In

contrast, adrenaline (a catecholamine) has the opposite effect of leptin. It has been shown, in rat hepatocytes, to decrease protein secretion but not mRNA levels (466, 467), an effect that was obliterated by treatment with the protein synthesis inhibitor cycloheximide (466). The inhibition of HL maturation and increase in intracellular degradation induced by catecholamines are evoked by changes in Ca^{2+} homeostasis and appear to involve the activation of the alpha-1-adrenoceptor subtype B (468).

Hypo- and hyperthyroidism in humans is associated with decreased and increased HL activity, respectively. In a human clinical study comparing overt hypothyroidism, subclinical hypothyroidism, euthyroidism and hyperthyroidism, Valdemarsson *et al.* (469) found an inverse relationship between the levels of thyroid hormones and HL activity, an observation supported by others (469-472). Hormone replacement treatment of hypothyroism normalized the levels of HL activity, again suggesting a role for thyroid hormones in the regulation of HL activity (469, 471-473). In the hypothyroid rat, decreased HL activity was associated with decreased HL mRNA (474, 475). Although this suggests that thyroid hormones regulate HL activity via a thyroid response element, Kihara *et al.* (476) found that thyroid hormone treatment of HepG2 cells increased HL activity but was not associated with changes in mRNA levels, rate of transcription nor translation. This view is supported by Hoogerbrugge *et al.* (474) and Neve *et al.* (475) who found that treatment of hypothyroid rats with growth hormones normalized HL mRNA levels. This may suggest that the decrease in HL activity observed in hypothyroidism is due, in part, to a concomitant growth hormone deficiency and that growth hormones rather than thyroid hormones may regulate HL mRNA levels. Neve *et al.* go on to postulate that although the growth hormone normalized the HL mRNA levels, the thyroid hormones still may play a role in the translation of the HL mRNA.

In addition to hormonal regulation, HL expression is also determined by genetic factors. The human hepatic lipase gene (*LIPC*), located on chromosome 15q21 (392), consists of nine exons and is over 30 kb in size (477, 478). A number of polymorphisms have been found within the *LIPC* gene or its promoter region; some of which contribute to the variability of HL activity seen within different populations. Of all the HL polymorphisms identified to date, the most studied is the C-514T polymorphism (C to T substitution at position –514 with respect to the transcription start site) of the *LIPC* gene. This polymorphism is associated with decreased HL activity, and accounts for 20-30% of the variance in HL activity in both men and women of various ethnic backgrounds (479-484). This polymorphism actually refers to four polymorphisms (G-250A, C-514T, T-710C and A-763G) identified in the 5' flanking region of the *LIPC* gene, which are in complete linkage disequilibrium and therefore identified as a single allele. A high frequency of these polymorphisms are found among Caucasian (454, 479-482, 485), African American (454, 482-484), Japanese (482, 486, 487) and Turkish men (454).

In addition to the roles of hormones and genetic factors in regulating HL expression and activity, there is also evidence to suggest that HL is regulated by cholesterol. In cultured HepG2 cells, both Ragab *et al.* (488) and Busch *et al.* (489) found that HL mRNA levels were controlled by cholesterol levels. Whereas Ragab *et al.* observed that there was an inverse relationship between the cell cholesterol content and the levels of HL mRNA, Busch *et al.* (489) had earlier found that treatment with an inhibitor of cholesterol biosynthesis (Mevinolin) upregulated HL mRNA levels. These observations were supported by *in vivo* studies that illustrated rats fed a diet supplemented with cholesterol had decreases in HL activity consistent with the lower mRNA levels found (490, 491).

1.3.7 Substrate specificity

As described in detail above, there is a significant amount of research that implicates HL in the metabolism of HDL and the apoB-containing lipoproteins. Independent of the lipoprotein class, the activity of HL is determined in part by the nature of the lipids and the apolipoprotein components. Although HL is often referred to as hepatic triglyceride lipase, the substrate specificity of the enzyme is broader. Collet *et al.* found that traditional methods of measuring TG content misrepresented and actually overestimated the HDL-TG content by neglecting to consider contributions of DG and MG. Using gas-liquid chromatography, they found significant amounts of DG in HDL and noted that the distribution of the various DGs (different acyl chains), either located on the surface or in the core, appeared to depend on the fatty acyl chain length (492). Coffill *et al.* subsequently found that HL preferentially hydrolyzed DG over TG in HDL (360). These results further suggested that the DG content in HDL regulates HL-mediated hydrolysis of both PL and TG possibly by changing the structural organization of the surface lipids and therefore the structural properties of HDL. This, in turn, affects the ability of HL to interact with the interfacial surface of the lipoprotein and act as an acylglyceride lipase. As such, in normolipidemic HDL, HL was found to act primarily as a surface lipid lipase, hydrolyzing PL and DG (360).

HL not only shows a preference in terms of the acylglyceride content (MG, DG and TG), it also shows a preference in terms of the fatty acid chain length of the acylglyceride. Using TG or MG of various chain length as substrates, HL was shown to be more active on the shorter acyl chain length species (493, 494). Similarly, Deckelbaum *et al.* observed that HL hydrolyzed medium chain TG twice as fast as the long chain TG, an observation attributed to the greater mobility of the medium chain TG in the PL monolayer, although the affinity of HL for long chain TG was much higher (495).

In addition to its role in hydrolyzing TG and DG, HL also has phospholipase activity (437). While HL has been shown to mainly hydrolyze TG in VLDL and IDL (496) other studies have shown that the enzyme has a preference for PL in HDL particles (360, 497). Although lipid analysis suggests that phosphatidylethanolamine (PE) is actually a minor component of HDL (498, 499), both monolayer and intact lipoprotein studies have shown that PE is the preferred PL substrate for HL (497, 498, 500). These and other studies have given rise to the view that the phospholipase activity of the enzyme is conferred, in part, by the nature of the PL head group (497, 501). Mutagenic studies support this view and further suggest that the lid domain of HL may be able to accommodate the polar head group of the PL molecule and account for the enzymes selectivity for different PL (324). Similarly, chimeric studies in which HL and LPL lid domains were exchanged suggested that the surface loop dictates the substrate specificity *in vitro* (324, 502). Studies by Kobayashi *et al.* (325) confirmed this observation *in vivo* by using adenovirus vectors to express native (HL and LPL) and chimeric enzymes (in which the lid domains had been swapped) in HL-deficient mice. When compared to mice expressing human HL, the mice expressing HL with the LPL lid had reduced PL hydrolysis thereby confirming the importance of the lid domain for efficient phospholipase activity.

The phospholipase activity of HL also appears to be sensitive to fatty acid chain length, the degree of unsaturation and the nature of the PL head group (as briefly mentioned above). Different PL head groups have dramatic effects on the rate of HL hydrolysis. Kinetic analyses have shown that of the different LpA-I particles, phosphatidylglycerol is the preferred substrate followed by PE, phosphatidylserine and PC (Sparks *et al.*, unpublished observations). These observations are supported by a number of other studies using various

systems (493, 503-508) and appears to be related to the interfacial hydration of the HDL particles (509-511).

Variations in the degree of unsaturation of the PL also affect the phospholipase activity of HL. HL-mediated hydrolysis increases as the degree of acyl chain unsaturation increases in the PL. This has been shown using lipid monolayers (503) and reconstituted (rHDL) particles (493) and may be related to the packing order of the lipids in the lipoprotein. Ho *et al.* (509) observed that increasing the degree of unsaturation introduces packing defects into the lipid bilayer and causes the lipid matrix to have less order and stability, which may have an impact on HL activity.

PL acyl chain length also appears to influence HL activity. Sparks *et al.* (unpublished observations) have shown that PL with shorter acyl chains promote a greater hydrolytic rate by HL. These observations are in accordance with others who have seen a similar relationship between HL activity and the acyl chain length of TG (494). The longer saturated acyl chain length would also be expected to increase the order of the lipid layer and may, therefore, have the same effect as that observed when acyl chain unsaturation is decreased.

1.3.8 Regulation of hepatic lipase lipolytic activity (co-factors and inhibitors)

Unlike LPL, which requires apoC-II for activation (107), HL does not appear to have an absolute requirement for an apolipoprotein co-factor. Despite this, a number of studies have suggested that various purified apolipoproteins, including apoAI/II, apoCI/II/III and apoE and non-substrate lipids as well as factors in serum stimulate or inhibit the activity of this enzyme. HL activity is slightly stimulated by the addition of serum at low concentrations and progressively inhibited at higher concentrations (512, 513). Although

VLDL and HDL isolated by ultracentrifugation were shown to inhibit HL activity (512, 514) these lipoproteins did not fully account for the inhibitory effect observed with serum. Kubo *et al.* (515) found that the combination of the ρ = 1.21 g/ml bottom fraction and HDL mimicked the effects observed with whole serum although the inhibitory component of the bottom fraction was not identified.

In addition to the inhibitory effect of normal plasma on HL activity, uremic serum was found to inhibit LPL (516) and HL (517) activities to a greater extent than normal serum. In both cases, the unidentified inhibitor was predominantly found in the lipoprotein-free fraction ($\rho > 1.225$ g/ml). Cheung *et al.* (518) found that the inhibitor in the lipoprotein-free fraction was an apoA-I containing particle of pre-ß-electrophoretic mobility and minimal lipid content (pre-ß-HDL). In addition, they showed that the greater inhibition of lipase activity in the uremic serum was due to an increased concentration of this particle in the non-lipoprotein fractions as well as to increased inhibition by uremic lipoproteins (518). The elevated levels of pre-ß-HDL in chronic renal failure has previously been observed (519, 520) and appears to be a direct result of renal impairment (519) a notion which is supported by the fact that the catabolism of pre-ß-HDL takes place predominantly in the kidney (521-523).

A number of studies have also explored the effects of purified apolipoproteins on lipolysis by HL. Although ApoA-I, A-II, C-I, C-II, C-III and E have all been reported to inhibit HL-mediated hydrolysis of TG and PL in lipoproteins, emulsions and monolayers (512, 524-526), apoA-II (527-529) and apoE (500, 530) have also been reported to stimulate TG hydrolysis by HL. The discrepancies observed may be due to the choice of system employed and the fact that HL is greatly affected by the physiochemical state of the substrate.

Using a well-controlled lipid monolayer technique where the physiochemical state of the substrates (the surface pressure and composition/concentration) have been carefully controlled and maintained throughout the reaction, Laboda *et al.* (526) showed that at a constant surface pressure of 24 mN/m, the hydrolysis of TG by HL was inhibited by apoA-I, ApoC-I and apoC-III and to a lesser degree by apoA-II. Similarly, Thuren *et al.* (500) showed that most of the apolipoproteins studied (apoA-I/II, C-I/II/III), except for apoE, inhibited TG hydrolysis at a surface pressure of 18.5 mN/m and all inhibited PL hydrolysis at a surface pressure of 25 mN/m. However, Thuren *et al.* also showed that at a lower surface pressure (10 mN/m), apoE was the only apolipoprotein to stimulate both HL-mediated TG and PL hydrolysis. The other apolipoproteins studied, with the exception of apoA-I (no effect), inhibited TG hydrolysis and slightly stimulated PL hydrolysis (apoC-I had no effect and apoA-II inhibited). At the higher surface pressures, HL can not penetrate the phospholipid monolayer containing the various apolipoproteins (500), which explains the inhibition of TG and PL hydrolysis by HL observed at the higher surface pressure used by both Thuren *et al.* and Laboda *et al.* The true inhibitory/stimulatory capability of the apolipoproteins thus depends on the surface pressure of the HDL, which is unknown but estimated to range between 15 and 25 mN/m.

The inhibitory effects of apoA-II on HL activity observed are in agreement with the study of Zhong *et al.* but contrast the results obtained by Mowri *et al.* (528, 529). The study of Zhong *et al.* (87) showed the inhibition of HL activity by HDL in an emulsion-based assay was dependent upon the apoA-I/apoA-II ratio in HDL. In contrast, Mowri *et al.* found that HDL_2 (528) and, to a lesser extent, HDL_3 (529) containing both apoA-I and apoA-II were better substrates for HL than HDL containing only apoA-I. The addition of purified apoA-II to HDL containing only apoA-I stimulated TG and PL hydrolysis to the levels observed with

the native HDL containing apoA-I and apoA-II. The hydrolytic difference observed between the two types of HDL particles (apoA-I versus apoA-I and apoA-II) was attributed, by Mowri *et al.*, to the enhanced interaction of HL with HDL containing apoA-I and apoA-II implying that apoA-II may enhance the interaction of all HDL fractions for HL (528, 529). In contrast, the work of Hime *et al.*, showed a reduced HL affinity for apoA-I enriched HDL, relative to apoA-II containing particles and that the apoA-II HDL were substantially poorer substrates for HL than apoA-I HDL suggesting that apoA-II may function as an inhibitor of HL (531).

The differences reported suggest that the inhibitory and stimulatory capabilities of the apolipoproteins depend on differences in assay conditions. Although using a monolayer system allows control over interfacial properties, it is known that important differences exist between various monolayer systems and native or reconstituted lipoproteins. While the surface pressure of these artificial systems can be controlled, the surface pressure of the HDL is still not known. Furthermore, substrate preferences exist between monolayers and lipoproteins and specifically, it is known that PC is not hydrolyzed by HL in these monolayers but is an important substrate for HL in HDL (500, 528, 532).

ApoA-IV was found to have no effect on TG hydrolysis by HL in an emulsion, but stimulated PE and inhibited PC hydrolysis in a model membrane system. Furthermore, apoA-IV was found to stimulate PC and PE but inhibit TG hydrolysis when HDL_2 and VLDL were used as substrates (533). Therefore, as with other apolipoproteins, studies with apoA-IV show varying results dependent on the type of model system used.

In addition to the effect of apolipoproteins on HL-mediated lipolysis, non-substrate lipids have also been shown to influence HL activity. In a monolayer system, PC was not a substrate for HL (500). However, the addition of up to 90 mol % of PC stimulated the

hydrolysis of TG without any adverse effects on the binding of HL to the lipid interface. Nonhydrolyzable ether lipids were used to determine the effect of altering the sn-3 position on HL activation. It was found that dialkyl-PE and -phosphatidic acid were the best activators (15-fold) and dialkyl-PC and -glycerol were poor activators (5-fold) (534) of HL suggesting that HL activation may be influenced by lipid packing or possibly by the direct activation of HL by the binding of activator lipid molecules. Tansey *et al.* (535) showed that unesterified cholesterol had no effect on HL activity whereas the addition of cholesterol has been shown to stimulate (526) and inhibit (534) HL-mediated TG and PL hydrolysis in monolayers. The effect of cholesterol on hydrolysis by HL depended on the mol % of cholesterol present, which caused alterations in the organization of the lipid film.

HSPG, which are present in most tissues and are located within the cell as well as on the extracellular matrices (308), anchors HL to cell surfaces. A number of studies have suggested that interactions with HSPG may affect the catalytic activity of lipolytic enzymes. Waite *et al.* (366) identified changes in the substrate specificity of HL after it was released from the liver by heparin. In addition, studies with LPL have suggested that the binding of this enzyme to HSPG may inhibit its catalytic activity (536, 537).

It is generally believed that HSPG association of lipases assists the lipolysis of the TG-rich lipoproteins (348, 538). Previous work suggests that lipolysis is initiated when lipoproteins interact with cell associated LPL and therefore, factors that promote HSPG association are thought to also promote lipolysis (367). Clark *et al.* showed that apoE could stimulate TG hydrolysis by heparin-bound LPL and proposed that the apolipoprotein may promote lipolysis by anchoring the TG-rich particles to cell surface HSPG (537). However, this and other investigations have also shown that association with heparin may have significant inhibitory effects on lipolysis (539). Studies, by both Posner *et al.* (536) and

Clark *et al.* (537) looked at the effect of immobilizing LPL onto heparin sepharose and found significant reductions in the reactivity of the heparin-bound enzyme. More recently, de Man *et al.* demonstrated that the binding of LPL to HSPG also significantly inhibited the lipolysis of VLDL (540). Saxena *et al.* have reported similar findings and directly showed that LPL on the surface of cultured endothelial cells was less active than LPL in solution (541). Their study also revealed that cell surface LPL activity could be reduced by the addition of chylomicrons, VLDL or fatty acid-free bovine serum albumin (FAF-BSA). The authors concluded that this reduction might have been due to enzyme displacement by free fatty acids. However, their simple tracking of activity complicates the interpretation of these studies, which could be a result of either displacement or inhibition. None of these studies carefully monitored enzyme association and activity.

1.3.9 Hepatic lipase and atherosclerosis

HL plays a role in the metabolism of pro- as well as anti-atherogenic lipoproteins (discussed in sections 1.2.5 and 1.2.9) (542, 543). Studies have shown that post-heparin plasma HL activity is inversely related to HDL-C levels (350, 544, 545) and LDL particle size (416, 421, 482, 546). In addition, increased HL activity is associated with the more atherogenic LDL subclass pattern B known as the small dense LDL (421). Despite these pro-atherogenic outcomes of HL activity, HL has been shown to stimulate HDL cholesterol ester uptake, a function thought to be anti-atherogenic.

The pro-atherogenic role of HL is supported by a number of clinical observations in which elevated levels of HL activity are observed in conditions associated with an increased atherosclerotic risk. For example, it has been shown that premenopausal women are partly protected from heart disease by having higher HDL levels than either men or postmenopausal

women and that these elevated HDL levels are due in part to a lower HL activity (547). In addition, a sedentary lifestyle (548, 549) smoking (550, 551), and visceral obesity (552-554), which are positively correlated with heart disease, have all been associated with elevated levels of HL activity that is reduced upon lifestyle modifications.

Clinical observations of primary and secondary hyperlipidemias have produced inconclusive data with regards to the relationship between increased HL activity and the risk of developing CAD. Familial combined hyperlipidemic patients (555) and type 2 diabetics (459), who have been shown to be at a higher risk for CAD, have significantly elevated HL activities. In these patients, HL appears to contribute to the atherogenic lipoprotein phenotype (combination of small dense LDL particles, decreased plasma HDL-C, and increased TG levels) (556-558). Furthermore, the presence of premature cardiovascular disease in at least some HL-deficient patients (420, 426, 559) also suggests an anti-atherogenic role for HL. Similarly, Zambon *et al.* (560) found strong evidence to suggest that the regression of coronary atherosclerosis observed is partly due to the effect of lipid-lowering therapy on HL-mediated improvement in LDL buoyancy. In contrast, a significant inverse correlation was found between HL activity and the extent of CAD in men undergoing elective coronary angiography (561). Although familial hypercholesterolemic patients, whom are known to be at a higher atherosclerotic risk, have been reported to have an increase HL activity (562), Dugi *et al.* found an inverse relationship between HL activity and the extent of coronary calcification in these patients (563).

A variety of animal models together with *in vitro* studies have been used to try to resolve whether HL plays a pro- or anti-atherogenic role in lipoprotein metabolism. Overexpressing apoC-I in apoE-null mice inhibited HL activity and resulted in increased atherosclerosis (564). Similar results were obtained by overexpressing apoA-II in mice (565,

566). In addition, increased expression of HL in mice led to a 42% reduction of aortic cholesterol content compared to control mice (439). All of these observations suggest that the presence of HL attenuates atherosclerosis whereas a number of other studies suggest the opposite. Breeding apoE-deficient mice on a HL-deficient background was found to reduce the susceptibility to atherosclerosis (567). In a study of cholesterol-fed HL transgenic rabbits, Taylor *et al.* found that although both control and transgenic rabbits developed lesions, the lesions of the HL transgenic rabbits were significantly thicker than those in the control rabbits (568). In addition, Gonzalez-Navarro *et al.* (569) found that both mouse and human macrophages synthesize HL raising the possibility that HL may have a direct role in the pathogenesis of atherosclerosis. Aviram *et al.* showed that degradation of TG-rich LDL from an HL-deficient patient by macrophages was only 50% that of normal LDL (570). They further showed that normal LDL, after TG-depletion by incubation with HL, were degraded at twice the rate of native LDL by macrophages and arterial SMC. These results suggest that lipase-modification of LDL can cause significant cholesterol accumulation in these cells thereby contributing to the formation of foam cells and the development of atherosclerosis (570).

Clearly, the role of HL in atherosclerosis is far from being completely understood. Both *in vitro* and *in vivo* studies have provided evidence for both a pro- as well as an anti-atherogenic role of HL. Although still controversial, the atherogenic nature of the enzyme appears to be determined by a balance of factors that regulate the activity of the enzyme.

1.4 Rationale and objectives

The complexity of the atherosclerosis process and the identified role of the various lipoprotein classes in this disease has led to an extensive amount of research into these lipid-protein complexes. As discussed, the plasma concentrations of HDL and LDL (and the various subclasses) are negative and positive predictors, respectively, of the development of atherosclerosis. The concentration of these two lipoprotein classes is determined to a large extent by the activity of HL. Consequently, understanding the mechanism of action of this enzyme and what regulates its activity in the plasma is central to attaining a clearer understanding of the complexities of lipoprotein metabolism and the atherosclerotic process.

The objective of this research consists of two parts: 1) to further define the lipoprotein and lipid substrate preferences of HL and 2) to determine the effects of HDL and apoA-I on the displacement of HL from HSPG and on HL-mediated VLDL hydrolysis.

HL activity is directly influenced by the apolipoprotein and lipid composition of the lipoproteins (discussed in detail in section 1.3.7). In order to better understand how differences in lipoprotein composition affect HL activity, LDL and HDL fractions isolated from subjects with FCHL and matched controls were used as substrates for the enzyme.

It has been known for over five decades that displacement of lipolytic enzymes from cell surface binding sites with heparin results in rapid hydrolysis of TG-rich lipoproteins in lipemic serum (571, 572). Nonetheless, there is a prevailing notion that both HL and LPL are catalytically active when bound to cell surface proteoglycans and that this association may indeed enhance the lipolysis of TG (reviewed in 348). This common view is in fact counter-intuitive to the interfacial catalytic models proposed for lipases, which have shown a clear requirement for enzyme hopping or shuttling between substrates for optimal hydrolysis

(573, 574). Therefore, both *in vitro* and cell culture systems were designed to determine the ability of lipoproteins to displace HL from proteoglycans and how this affects the activity of the enzyme.

The experiments detailed within provide new insights into the regulation of HL (and possibly LPL) activity. The model proposed suggests that the dissociation of HL from HSPG is necessary for catalytic activity and dependent on HDL composition. In addition, evidence is provided that further suggests a direct role for HDL and apoA-I in regulating the activity of the dissociated enzyme.

Chapter 2 – Experimental procedures

2.1 Materials

Free fatty acid diagnostic kits were purchased from Roche Diagnostics (Laval, PQ). Anti-mouse IgG, horseradish peroxidase- (HRP) linked whole antibody (isolated from sheep), broad range molecular weight markers, ^{125}I, L-3-phosphatidyl-[N-*methyl*-[^{3}H]choline,1,2-dipalmitoyl ([^{3}H]-DPPC) and di[1-^{14}C]oleoyl phosphatidylcholine were obtained from Amersham Biosciences (Baie d'Urfé, PQ). The SuperSignal West Pico and West Dura chemiluminescent substrates were purchased from Pierce Chemical Co. (Rockford, IL). Novex polyacrylamide gels, Ham's F12 medium, Geneticin® Selective Antibiotic (G418), Eagle's Minimal Essential Medium (EMEM), L-glutamine and penicillin/streptomycin were purchased from Invitrogen (Burlington, ON). Cytochalasin B, fetal bovine serum (FBS), triolein, heparin, essentially FAF-BSA, HSPG, phospholipase C (Type I from *C. perfringens*) and bovine LPL (bLPL) (3300 units/mg where 1 unit releases 1 nano mol of p-nitrophenol per minute using p-nitrophenol butyrate as a substrate) were purchased from Sigma Chemical Co. (St-Louis, MO). 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Tri[9,10-^{3}H(N)]olein ([^{3}H]-TG) was purchased from Dupont Canada Inc. (Mississauga, ON). The anti-HL monoclonal antibody (mAb) XHL3-6, the anti-LPL mAb 1D2, the anti-apoA-I mAbs (5F6 and 4H1) and the anti-apoB-100 mAb (1D1 and 4G3) were obtained from Drs. Bensadoun, Brunzell, Marcel and Milne, respectively. All other reagents were of analytical grade.

2.2 Methods

2.2.1 Human hepatic lipase and lipoprotein lipase purification

HL and LPL were purified from post-heparin human plasma by heparin affinity chromatography as described by Ehnholm *et al.* (575). Normolipidemic subjects were injected with 60 units (U) of heparin per kg of body weight prior to collecting one unit of post-heparin blood. The blood was aliquoted into cold centrifuge tubes (50 ml) and spun at 3000 rpm for 10 minutes at 4°C in a Sorval high-speed centrifuge. The top plasma layer was collected and a 20% TG emulsion (Intralipid 20%, Baxter Corp. Toronto, ON) was added (100 ml of liposyn per 400 ml of plasma). The plasma/liposyn mixture was incubated for 15 minutes at 37°C in a shaking incubator, the lipid cakes harvested centrifugally (27 000 rpm for 35 minutes in an ultracentrifuge or at 20 000 rpm for 1 hour in the Sorvall high-speed centrifuge at 8°C) and the procedure repeated. Following the second extraction, the lipid cakes were resuspended in cold acetone and spun at 16000 rpm for 15 minutes at 4°C in the Sorvall high-speed centrifuge (ss34 rotor) twice. The pellet was then resuspended twice in ether and centrifuged at 16 000 rpm for 20 minutes at 4°C. The ether was decanted and the remaining pellet dried using a gentle stream of N_2. The crude HL/LPL was then solubilized overnight in a 0.15 M NaCl, 20% glycerol, 5 mM sodium barbital solution pH 7.4.

The resuspended, aqueous solution of crude HL/LPL was loaded onto a Heparin Sepharose CL-6B column at a flow rate of 0.75 ml/minute. The column was washed with a 0.15 M NaCl, 20% glycerol, 5 mM sodium barbital solution pH 7.4 followed by a wash with a 0.4 M NaCl, 20% glycerol, 5 mM sodium barbital solution pH 7.4 until baseline was reached. The NaCl concentration was increased to 0.9 M to elute the bound HL and 1.5 M to

elute the bound LPL. Fractions containing HL and LPL activities were pooled separately and frozen at –80°C until use.

HL and LPL activities were characterized using a [^{3}H]-TG emulsion and quantified into units of enzyme activity (where 1 U = 1 μmol fatty acid hydrolyzed/hour). The specific activities of the isolated HL and LPL were determined to be 19,455 U/mg of protein and 3703 U/mg of protein, respectively. The isolated HL was further characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE) and immunoblotting using the anti-HL mAb. A single band with an apparent molecular mass of 66 kDa was present.

2.2.2 Isolation of lipoproteins by sequential ultracentrifugation

Plasma samples from fasting, normolipidemic subjects were collected and VLDL, LDL and HDL were isolated by sequential ultracentrifugation within the density ranges $\rho < 1.006$ g/ml, $\rho = 1.019$-1.063 g/ml and $\rho = 1.063$-1.21 g/ml, respectively, using a previously published method (576). The protein concentrations of the different lipoprotein fractions were determined by the Lowry method as modified by Markwell *et al.* (577). PL, cholesterol (FC and total cholesterol) and TG contents were determined enzymatically using commercially available diagnostic kits (Roche Diagnostics, Laval, PQ).

2.2.3 Isolation of lipoproteins by discontinuous gradient ultracentrifugation

Plasma samples from fasting, normolipidemic and FCHL subjects were collected and VLDL, LDL and HDL subfractions were isolated by KBr discontinuous gradient ultracentrifugation as previously described (578, 579). Briefly, for every 3 ml of plasma, 1 g of KBr and 50 mg of sucrose were added. Using the underlay method, 12.33 ml of a 1 mM EDTA containing 0.02% NaN_3 was added to an open top centrifugation tube followed by

10 ml of 1.080 g/ml density solution, 6.67 ml of a 1.210 g/ml density solution and finally 10 ml of the plasma KBr mix. The plasma was centrifuged at 27500 rpm for 22 hours at 8°C in the SW28 rotor in a Beckman ultracentrifuge. Following centrifugation, 15-2 ml fractions were removed and dialyzed against PBS pH 7.2 no EDTA or NaN_3. The protein concentrations of the lipoprotein fractions were determined as described (see above). PL, TG, total cholesterol and FC contents were determined enzymatically using diagnostic kits (Roche Diagnostics, Laval, PQ). Lipoprotein charge was determined by Lipogels®. The size and homogeneity of the VLDL/LDL and HDL fractions were estimated by non-denaturing gradient gel electrophoresis (GGE) on precast 4% Tris-glycine gels (Novex) and 8-25% acrylamide gels (Pharmacia Phastgel), respectively.

2.2.4 Isolation of HDL fractions by discontinuous gradient ultracentrifugation

Plasma samples from fasting, normolipidemic subjects were collected and HDL subfractions were isolated by KBr discontinuous gradient ultracentrifugation following HDL (ρ = 1.063-1.21 g/ml) isolation by sequential ultracentrifugation (described above). Briefly, 50 mg of sucrose was added to the isolated HDL. Using the underlay method, 12.33 ml of a 1 mM EDTA containing 0.02% NaN_3 was added to an open top centrifugation tube followed by 10 ml of 1.080 g/ml density solution, 6.67 ml of a 1.210 g/ml density solution and finally 10 ml of HDL/KBr/sucrose mix. The HDL was centrifuged at 27500 rpm for 22 hours at 8°C in the SW28 rotor in a Beckman ultracentrifuge. Following centrifugation, the upper 14 ml were discarded and 8-2 ml fractions were obtained and dialyzed against PBS pH 7.2 no EDTA or NaN_3. The protein concentrations of the various HDL fractions were determined as described (see above). Lipoprotein charge was determined by Lipogels®.

The size and homogeneity of the HDL fractions were estimated by non-denaturing GGE on 8-25% acrylamide gels (Pharmacia Phastgel).

2.2.5 Preparation of [^{14}C]-DG

[^{14}C]-DG was isolated after a 4 hour incubation at 37°C of 5 μCi di[1-^{14}C]oleoyl phosphatidylcholine, 1 ml of 0.2 U/ml of phospholipase C in phosphate buffer (pH 7.3), 1 ml of 1% $CaCl_2$ and 1 ml of diethyl ether. The reaction was stopped with 5 drops of 0.1 M HCl and the [^{14}C]-DG extracted with 5 ml of chloroform-methanol (1:1, v/v). After centrifugation for 15 minutes at 2200 rpm, the lower phase was removed and dried under N_2. Preparative thin layer chromatography (TLC) using Silica Gel 60 plates and a solution of chloroform-acetone-methanol-acetic acid–water (60:80:20:20:10, v/v) was used to separate the [^{14}C]-DG from its substrate.

2.2.6 [^{3}H]-TG and [^{14}C]-DG labeling of HDL

Incorporation of [^{3}H]-TG and [^{14}C]-DG into HDL was accomplished by incubating HDL with POPC vesicles containing the labeled lipids (580, 581). PL, TG and DG vesicles were prepared by drying 50 μg of POPC, 100 000 cpm of [^{3}H]-TG and 50 000 cpm of [^{14}C]-DG under N_2, adding 200 μl of 10 mM phosphate buffered saline (PBS), pH 7.2, and vortexing for 1 minute. Vesicles were combined with HDL_2 or HDL_3 (2 mg of protein) and incubated with gentle shaking for 8 hours at 37°C. Greater than 95% of the radioactive lipids were incorporated into the HDL particles after reisolation. HDL lipid mass values were determined as described below and the lipid specific activities were calculated.

2.2.7 Quantification of acylated glycerols in HDL

HDL lipids (per 1 mg protein) in the presence of internal standards of [^{3}H]-TG and [^{14}C]-DG (15000 cpm each) were extracted by the method of Bligh and Dyer after the addition of 12 µl of formic acid per ml of aqueous phase (492). The organic phase was removed, evaporated to dryness, and resolubilized in 50 µl of chloroform. The glycerol containing neutral lipids were separated using Silica 60 plates and a solvent system of hexane-diethyl ether-acetic acid (105:45:1.5, v/v). The following Rf values were obtained: MG = 0.01, 1,2-DG = 0.12, 1,3-DG = 0.14 and TG = 0.53. Spots corresponding to DG and TG were isolated and analyzed for glycerol content using the Boehringer Mannheim TG kit.

2.2.8 Hepatic lipase activity assay using [^{3}H]-TG and [^{14}C]-DG labeled HDL

Double-labeled HDL was characterized as a substrate for HL using a standard assay system. Each enzyme assay contained the lipoprotein substrate, 214 U of purified HL, 3 mg of FAF-BSA, 1.5 mM $CaCl_2$, and 1 M NaCl in a 0.15 M Tris buffer (final volume = 500 µl). Incubations were carried out for 3 hours at 37°C and were terminated by placing the tubes on ice. The total amount of fatty acids release during the incubation was determined using an enzyme kit. The PL, TG and DG hydrolytic rates were determined by subtraction after quantifying the radioactive fatty acids liberated from [^{3}H]-TG and [^{14}C]-DG during the incubation with HL using a liquid-liquid partitioning system (575). Under these conditions, > 95% of the radioactive fatty acids were recovered in the supernatant aqueous phase. Subtracting the TG and DG hydrolytic values from the total hydrolysis gave the PL hydrolytic rate.

2.2.9 [^{3}H]-DPPC labeling of lipoproteins

[^{3}H]-DPPC was incorporated into the various lipoprotein fractions following incubations with [^{3}H]-DPPC vesicles. Vesicles were prepared by drying 250 μCi of [^{3}H]-DPPC under N_2, adding 1 ml of PBS, pH 7.2, and sonicating 3 times for 30 seconds with increasing output. The [^{3}H]-DPPC micelles (175000 cpm per 0.15 mg of LDL protein or 0.35 mg of HDL protein) were incubated with the various control and FCHL lipoprotein fractions isolated by discontinuous gradient ultracentrifugation overnight at 4°C. Using tricarboxylic acid precipitation, greater than 98% of the radioactive lipids were shown to be incorporated into the lipoprotein fractions.

2.2.10 Hepatic lipase activity assay using [^{3}H]-DPPC labeled lipoproteins

[^{3}H]-DPPC labeled lipoprotein fractions were characterized as substrates for purified HL using a standard assay system. Each enzyme assay contained the lipoprotein substrate, 26 U of purified HL, 150 μl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% FAF-BSA, 5 mM $CaCl_2$) and PBS (final volume 500 μl). Incubations were carried out for 3 hours at 37°C and terminated at 4°C. The total amount of fatty acids released during the incubation was determined using a free fatty acid diagnostic kit. The phospholipid hydrolytic rates were determined indirectly. Briefly, following the 3 hour incubation with HL, an aliquot of the incubation mixture containing the lipoprotein was extracted using the acidic Bligh and Dyer procedure. Briefly, ddH_2O was added to the mixture to a final volume of 1 ml followed by 2 ml of methanol, 1 ml of chloroform and 12 μl of formic acid. Following a 30 minute incubation at 24°C, an additional 1 ml of ddH_2O, 1 ml of chloroform and 12 μl of formic acid were added with vortexing. The samples were subsequently centrifuged for 20 minutes at 3000 rpm. The lower solvent phase containing the radioactive substrates and products,

[^{3}H]-DPPC and [^{3}H]-monopalmitoyl PC respectively, was removed and dried under N_2. TLC, using Silica Gel 60 plates and a solvent system of chloroform-methanol-acidic acid-formic acid-ddH_2O (70:30:12:4:2, v/v) was used to separate the [^{3}H]-monopalmitoyl PC from the unreacted substrate. The radioactivity associated with the [^{3}H]-monopalmitoyl PC and [^{3}H]-DPPC was determined by scintillation counting. The ratio of PL to lyso-PL to sphingomyelin was calculated for each fraction by high performance thin layer chromatography (HPTLC) using the TLC solvent system. The HPTLC plates were scanned and analyzed by densitometry using the BioRad, Quantity One ® (version 4.1) software. Using this method, the amount of PL in every fraction was determined and compared to the amount of PL hydrolyzed by HL. The acylglyceride (MG, DG and TG) hydrolytic rates were then calculated by subtracting the PL hydrolytic rate from the total hydrolytic rates.

2.2.11 Binding of hepatic lipase and bovine lipoprotein lipase to proteoglycans

The binding of human HL and bLPL to HSPG was investigated in assays performed in 96 well Removawell plates. Removawells were incubated with 5 μg pure HSPG for 2 hours at room temperature, washed 3 times with PBS and pre-incubated with PBS containing 1% FAF-BSA overnight at 4°C. The Removawells were again washed 3 times with PBS and incubated at room temperature for 2 hours with HL (120 U) or for 1 hour with bLPL (11 U) in PBS (final volume 125 μl). After incubation, Removawells were washed once with PBS to remove any unbound HL or bLPL.

2.2.12 Substrate specificity of bound versus free hepatic lipase and bovine lipoprotein lipase

Native lipoproteins were characterized as substrates for purified HL and bLPL (Sigma) bound to HSPG (as described above) or free in solution. Each Removawell

contained the lipoprotein substrate, 26 U of purified HL or 2.9 U of bLPL, 75 µl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% FAF-BSA, 5 mM $CaCl_2$) and PBS (final volume 250 µl) in the presence or absence of HSPG. Incubations were carried out for either 30 minutes (inhibition studies) or 3 hours at 37°C and terminated by placing the plate on ice. The total amount of fatty acids released during the incubation was determined with a free fatty acid diagnostic kit.

2.2.13 Hepatic lipase and bovine lipoprotein lipase binding to proteoglycans

HSPG-bound HL and bLPL were incubated for 3 hours and 1 hour, respectively, with the lipoprotein substrates and then Removawells were rinsed with PBS. 60 µl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.5% (w/v) bromophenol blue) was then added to the Removawells to elute the HSPG-bound HL or bLPL and the mixture was incubated at 37°C for 30 minutes. Samples were electrophoresed on 12% acrylamide gels, under denaturing conditions and transferred to a nitrocellulose membrane. The membrane to be probed for HL was cut at the 35 kDa marker and the upper portion of the nitrocellulose was blocked overnight at 4°C in blocking solution A (PBS containing 1% BSA and 0.2% Tween-20). The membrane to be probed for bLPL was blocked overnight at 4°C in blocking solution C (PBS containing 1% BSA and 0.5% Tween-20). After a 2 hour incubation at room temperature with the anti-HL mAb (3:5000) in blocking solution A containing 0.02% NaN_3 or with the anti-LPL mAb (1:5000) in blocking solution C containing 0.02% NaN_3, the membranes were rinsed 3 times and washed 4 times for 15 minutes each with PBS containing 0.2% Tween-20 (PBS-0.2%Tween) for HL and containing 0.5% Tween-20 (PBS-0.5%Tween) for bLPL. Sheep anti-mouse IgG, HRP-linked whole antibody was used as a secondary antibody and diluted (1:5000) in blocking solution A for

HL and in blocking solution C for bLPL. After a 1 hour incubation, the membranes were rinsed 3 times and washed 4 times 15 minutes with the appropriate PBS-Tween wash solution. 10 minutes incubations with the Pierce Super Signal West Pico Chemiluminescent Substrate were used to visualize the HL and bLPL. The membranes were exposed to film for various times and the apparent molecular mass of HL and bLPL were determined using broad range molecular weight markers as a reference. The film was subsequently scanned and the percentage of HL that remained bound to the HSPG under the various conditions was determined by densitometry using the BioRad, Quantity One ® (version 4.1) software.

2.2.14 ApoA-I binding to proteoglycans

After the samples were electrophoresed, transferred and the nitrocellulose membrane cut, the lower portion of the nitrocellulose was blocked overnight at 4°C in blocking solution B (PBS containing 5% skim milk and 0.2% Tween-20). After a 1 hour incubation at room temperature with the anti-apoA-I mAbs 5F6 and 4H1 (1:5000 dilution each) in blocking solution B containing 0.02% NaN_3, the membranes were rinsed and washed 3 times for 10 minutes with PBS-0.2%Tween. Sheep anti-mouse IgG, HRP-linked whole antibody was used as a secondary antibody and diluted (1:5000) in blocking solution B. After a 1 hour incubation, the membranes were washed 3 times with PBS-0.2%Tween. The apoA-I that bound to the HSPG was visualized, scanned and analyzed as described for HL. Similar protocols were used to evaluate the binding of apoB (mAb 1D1) and apoE (mAb 1D7) to HSPG.

2.2.15 Characterization of hepatic lipase in CHO-hHL and HepG2 cells

CHO cells stably transfected with human hepatic lipase (CHO-hHL) were plated in Ham's F12 medium containing 10% FBS and 500 μg/ml G418 (plating medium). Following

attachment, the medium was changed to Ham's F12 medium containing 1% FBS and 500 μg/ml G418 (complete medium). HepG2 cells were plated and grown to confluence in EMEM containing 10% FBS, 2% L-glutamine and 1% penicillin/streptomycin (complete medium). Once confluent, the cells were washed and incubated with their respective fresh medium (in the absence of FBS) containing cytochalasin B at a final concentration of 10 μg/ml for 1 hour at 37°C. Plates were then placed on ice and cells were subjected to repeat aspiration of the medium to remove the cell monolayer. This process was repeated with three washes in PBS. The cells and wash buffer were centrifuged at 1300 rpm for 10 minutes at 8°C in the Sorvall RT 6000D low speed centrifuge. The supernatant was removed and the pellet was solubilized in 60 μl of SDS sample buffer. The ECM, which remained on the plate, was incubated with 60 μl of SDS sample buffer overnight at 24°C with shaking. The solubilized pellet and the ECM were subjected to electrophoresis on an 8% polyacrylamide gel under denaturing conditions, transferred to a nitrocellulose membrane and blocked overnight at 4°C in blocking solution (PBS containing 1% BSA and 0.2% Tween-20). The membrane was incubated for 2 hours at room temperature with the anti-HL mAb in blocking solution containing 0.02% NaN_3, and following washes in PBS-0.2%Tween, a sheep anti-mouse IgG HRP-linked whole antibody was used as the secondary antibody. After a 1 hour incubation, the membranes were washed in PBS-0.2%Tween and visualized by chemiluminescence following a 10 minutes incubation with the Pierce Super Signal West Pico substrate or a 5 minute incubation with a 1:5 dilution of the Pierce Super Signal West Dura chemiluminescent substrate. The membranes were exposed to film for various times and the apparent molecular mass the of cell-derived HL were determined using broad range molecular weight markers as a reference. The film was subsequently scanned

and the percentage of HL that was associated with the cells or the ECM under the various conditions was determined by densitometry using the BioRad Quantity One ® (version 4.1) software.

2.2.16 Hepatic lipase binding to CHO-hHL and HepG2 cells

CHO-hHL and HepG2 cells were grown to confluence in complete medium as described above. After an overnight incubation with serum-free media, the cells were washed and incubated with fresh serum-free medium ± apoAI or HDL at 37°C for the various amounts of time as indicated in the figure legend. Following removal of the medium, the cells were washed with PBS and solubilized with 60 µl of SDS sample buffer overnight at room temperature with shaking. The cell lysates were electrophoresed, transferred to a nitrocellulose membrane and the HL content was analyzed by Western blot analysis as detailed above. The membranes were exposed to film for various times and the percentage of HL that was associated with the cells under the various conditions was determined by densitometry with the Quantity One ® (version 4.1) software.

2.2.17 Hepatic lipase activity in CHO-hHL and HepG2 cell medium

CHO-hHL and HepG2 cells were treated as described in section 2.2.16 except that they were incubated with either heparin or HDL for the indicated times. Following this, 150 µl of the medium were removed from the cells and the HL activity was determined using a [^{3}H]-TG emulsion as previously described (575). Briefly, each test tube contained 200 µl substrate emulsion, 150 µl cell media and 150 µl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% FAF-BSA, 3.33 M NaCl and 5 mM $CaCl_2$). Incubations were carried out for 1 hour for CHO-hHL cell medium and 3 hours for HepG2 cell medium at 37°C. The reactions were

terminated by the addition of 3 ml methanol:chloroform:heptane (1.41:1.25:1, v/v) and 750 µl of 0.14 M K_2CO_3/H_3BO_3 buffer pH 10.5. The samples were vortexed and the phases separated with centrifugation at 2200 rpm for 15 minutes in a Sorvall RT 6000D low speed centrifuge. 1 ml of the aqueous phase was removed to scintillation vials and the amount of [^{3}H]-fatty acids released during the incubation was determined.

2.2.18 Determination of apoA-I binding to VLDL

Removawells were coated with an anti-apoB mAb (1D1) (at a predetermined dilution) overnight at 4°C, washed with PBS and saturated with 0.5% gelatin in PBS. Serial dilutions of VLDL in the presence or absence of HDL or apoA-I (in PBS with 0.5% gelatin), that had been incubated with or without HL for 3 hours at 37°C, were added to 1D1-coated Removawells for 2 hours at room temperature. After 3 washes (PBS-0.05% Tween), the Removawells were incubated for 1 hour with ^{125}I-labeled anti-apoA-I mAbs (4H1 and 5F6) in PBS with 0.5% gelatin (approximately 200 000 cpm/well). Removawells were washed 3 times with PBS-0.05%Tween and the amount of radioactivity was measured.

2.2.19 Purification of apoA-I and preparation of spherical LpA-I complexes

ApoA-I was isolated by size exclusion chromatography on a Sephacryl S-200 HR column (280). Prior to use, the apoA-I was resolubilized in 6 M guanidine HCl, 10 mM Tris, pH 7.2, and dialyzed extensively against PBS pH 7.2. Reconstituted LpA-I complexes were prepared by co-sonicating POPC and apoA-I (molar ratios indicated in figure 3.3.7) as previously described (580). Briefly, specific amounts of lipids in chloroform were dried under N_2 in a 12 x 75 mm test tube. 800 µl of PBS were added and the lipid-PBS mixture was successively sonicated under N_2 for 1 minute at constant output, incubated at 37°C for 30 minutes and sonicated again for 5 minutes at 95% duty cycle. ApoA-I (2 mg of a 1.4 mg

protein/ml PBS solution) was added to the lipid mixture and co-sonicated for 4 x 1 minute at 90% duty cycle with 1 minute cooling periods between sonications.

2.2.20 Total hepatic lipase activity assay

VLDL hydrolysis by HL, in the absence of HSPG, was characterized in the presence or absence of HDL fractions of various densities or rHDL particles. Each test tube contained the lipoprotein substrate (350 μM VLDL), purified HL (26 U), 75 μl incubation buffer (0.33 M Tris-HCl pH 8.3, 1% FAF-BSA, 5 mM $CaCl_2$), PBS (to a final volume of 250 μl) and increasing concentrations of HDL or rHDL particles as indicated in figures 3.3.5 and 3.3.7, respectively. Incubations were carried out for 30 minutes and the reactions terminated by placing the samples on ice. The total amount of fatty acids released during the incubation was determined using a free fatty acid diagnostic kit.

2.2.21 Statistical analysis

All statistical analyses were performed using GraphPad InStat ® software (version 3.00).

Due to the exploratory nature of the patient studies described in section 3.1 combined with the small sample size, complex analysis of variance (ANOVA) was not used. Instead, the significance of difference between two means was calculated where indicated using a two-tailed Student's t-test for unpaired data. A value of $p < 0.05$ was considered statistically significant and a value of $p < 0.1$ was considered a trend.

For the results described in sections 3.2 to 3.4, a one-way ANOVA was performed in order to determine the significance of difference between multiple group means. If the p value was found to be less than 0.05, post-test analyses were performed using the Tukey-Kramer Multiple Comparisons Test (compares all pairs of samples), the Bonferroni Multiple

Comparisons Test (compares selected pairs of samples) or the Dunnett Multiple Comparisons Test (compares all samples to a control). $p < 0.05$ was regarded as statistically significant and $p < 0.001$ was considered highly statistically significant.

Chapter 3 – Results

3.1 Hepatic lipase substrate specificity and patient studies

3.1.1 Lipoprotein hydrolysis by hepatic lipase

In order to determine HL lipoprotein substrate preference, Removawells were pre-incubated with 1% FAF-BSA, washed and then incubated with the various lipoproteins (VLDL, LDL or HDL) and 26 U of HL for 3 hours at 37°C. Extended incubation times

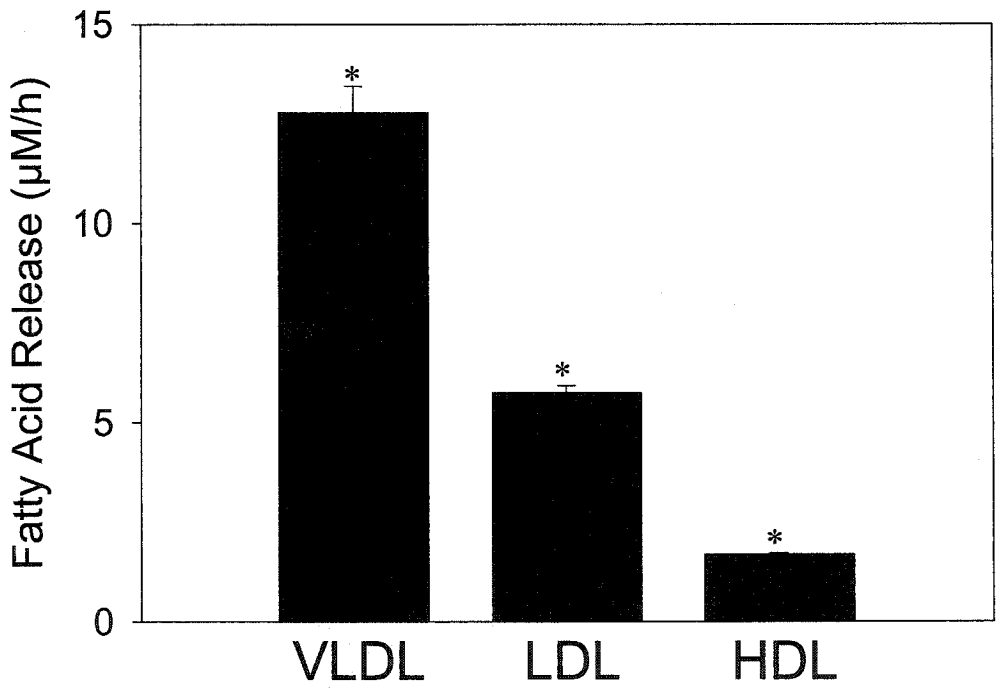

Figure 3.1.1 VLDL, LDL and HDL hydrolysis by HL

Removawells, pre-incubated with 1% FAF-BSA in PBS overnight at 4°C, were incubated with 26 U of HL and VLDL, LDL or HDL isolated by ultracentrifugation normalized for TG (350 µM) for 3 hours at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of two independent experiments. A one-way ANOVA was performed and the results were considered highly significant ($p < 0.0001$). Post-test analysis using the Tukey-Kramer Multiple Comparisons Test was performed comparing all samples to each other for statistical significance (*$p < 0.001$).

(3 hours) and high substrate concentrations (350 μM TG) were used to promote sufficient hydrolysis to allow for comparison of hydrolytic rates of the different substrates. Figure 3.1.1 shows that when compared on the basis of TG content, VLDL (13 μM/h) is the preferred lipoprotein substrate for HL followed by LDL (6 μM/h) and HDL (2 μM/h).

3.1.2 Phospholipase, diglyceride and triglyceride lipase activities of hepatic lipase

HDL subfractions isolated from normolipidemic subjects by sequential ultracentrifugation were characterized as substrates for HL. HDL_3 was incubated with [^{3}H]-TG/[^{14}C]-DG/POPC vesicles to incorporate both [^{3}H]-TG and [^{14}C]-DG into the lipoprotein. The amount of DG and TG in the HDL_3 was determined by preparative TLC and estimated to be 2.2:1, DG:TG. HL-mediated lipid hydrolysis of the HDL_3 showed that only 1% of the fatty acids released were derived from TG whereas the remainder of fatty acids were derived

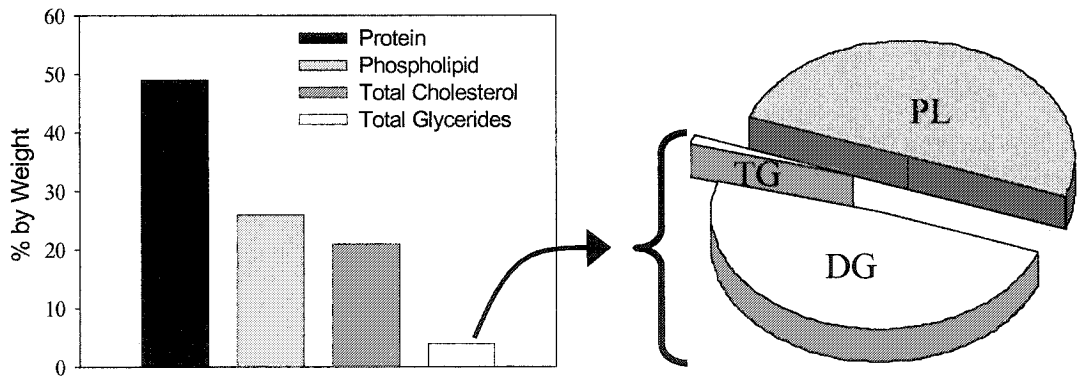

Figure 3.1.2 Phospholipase versus acylglyceride lipase activities of HL

Normolipidemic HDL_3 was labelled with [^{3}H]-TG and [^{14}C]-DG and incubated with HL for 3 hours. HDL_3 protein, total cholesterol, phospholipid and total glyceride composition (% by weight) are shown (left bar graph) relative to the percentage of fatty acids released from TG, DG and PL by HL (right pie chart).

from DG and PL (figure 3.1.2 right pie chart). Although DG represents only about 1% of the HDL_3 lipids, DG hydrolysis represents 49% of total lipid hydrolysis. Similar results were obtained with HDL_2 with 5% and 30% of the fatty acids released from TG and DG, respectively (data not shown). These results indicate that although DG is only a small component of HDL it is a preferred substrate for HL.

3.1.3 Hydrolysis of lipoproteins isolated from control subjects and patients with FCHL by hepatic lipase

In order to determine whether lipoproteins derived from subjects with FCHL were better substrates for HL than those isolated from normolipidemic subjects and if a relationship existed between HL-mediated hydrolysis and DG content in these subjects, six patients and six controls matched for age and gender were recruited from the Cliniques des Maladies Lipidiques by Dr. Godet (Quebec City, Quebec). Plasma samples obtained from each subject were sent to the Ottawa Hospital Clinical Laboratory for lipid analysis. The results summarized in table 3.1 show that within our small sample group, subjects with FCHL had elevated levels of total cholesterol and TG as well as decreased HDL-C levels. LDL-C, on the other hand, did not differ significantly between the two sample groups (table 3.1). These values are consistent with the diagnosis of FCHL, which is based on the occurrence of either increased plasma concentrations of cholesterol, TG or both within a family (582, 583).

From the plasma samples obtained, six LDL and HDL fractions, ranging in density from 1.019 to 1.25 g/ml, were isolated by discontinuous density gradient ultracentrifugation (described in section 2.2.3). VLDL fractions were excluded from the study since insufficient amounts of VLDL were obtained from control subjects to allow for comparison.

Table 3.1: Clinical and biochemical characteristics of study subjects

	Control	Patient
Sample size	6	6
Age (years)	41 ± 5	41 ± 6
Body Mass Index (kg/m^2)	27.30 ± 1.21	28.01 ± 1.73
Total Cholesterol (mmol/l)	4.92 ± 0.83	6.03 ± 1.18[b]
TG (mmol/l)	1.07 ± 0.58	2.55 ± 0.78[a]
HDL-C (mmol/l)	1.29 ± 0.24	0.915 ± 0.11[a]
LDL-C (mmol/l)	3.08 ± 0.79	3.97 ± 0.99

Data are representative of mean ± S.D. of six patients and controls. Clinical and biochemical characteristics of patient samples were compared to the corresponding control samples for statistical significance using the Student's t-test ($^{a}p < 0.05$ or $^{b}p < 0.1$). Values with no symbols are not statistically significant ($p > 0.1$).

The isolated lipoprotein fractions were radioactively labelled with [^{3}H]-DPPC and the association of the [^{3}H]-DPPC with the various lipoprotein fractions was determined to be > 98% by tricarboxylic acid precipitation (data not shown). HL (26 U) was incubated with either LDL (0.15 mg/ml protein) or HDL (0.35 mg/ml protein) fractions for 3 hours at 37°C and total free fatty acid release was determined. Constant protein concentrations were used as the basis for comparison in the enzyme assays since they approximate a constant particle number. Figure 3.1.3 shows that, when compared on this basis, HL-mediated hydrolysis of patient and control LDL and HDL is similar despite the fact that subjects with FCHL have

elevated serum TG levels (table 3.1). Furthermore, in both the patient and the control groups, the most buoyant lipoproteins of each class of lipoprotein (fraction 1 of HDL and LDL) are better substrates for HL than the denser lipoproteins (fraction 6). As the density of the LDL increases (from fraction 1 to 6), there is a 3 to 4-fold decrease in hydrolysis.

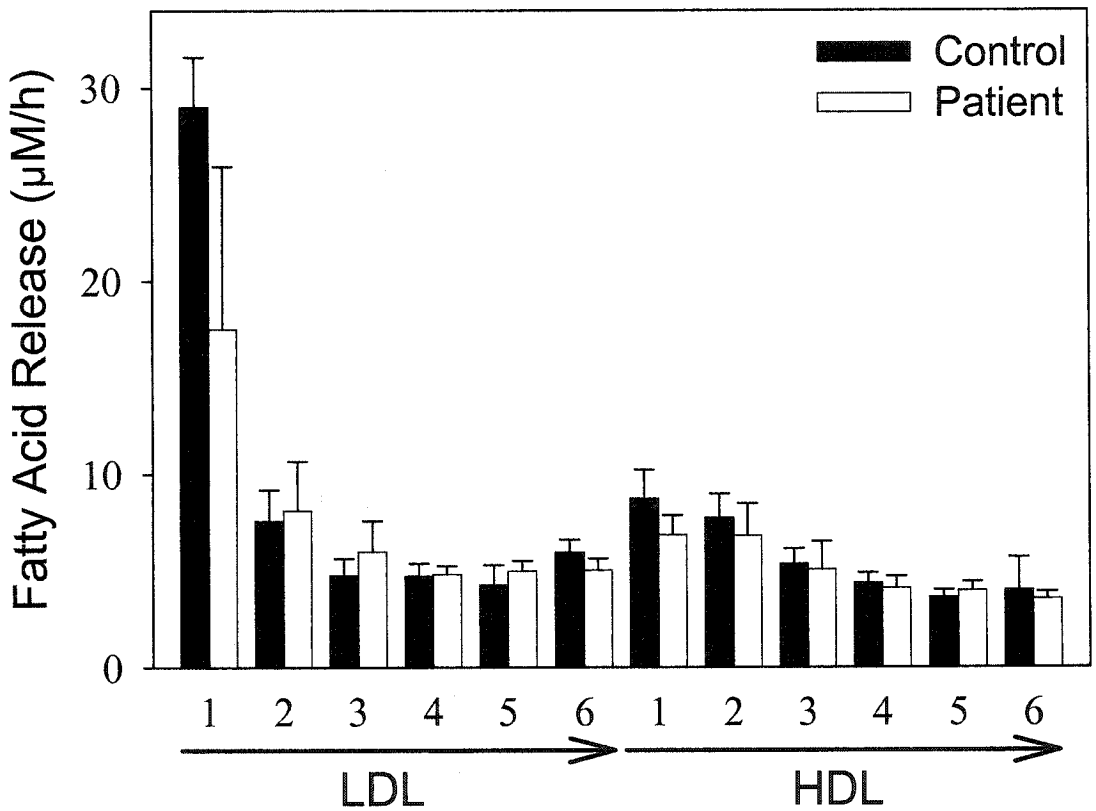

Figure 3.1.3 Hydrolysis of control and FCHL LDL and HDL fractions by HL

Lipoprotein fractions were isolated from normolipidemic and FCHL subjects by discontinuous density gradient ultracentrifugation. Purified HL was incubated with LDL (0.15 mg/ml protein) or HDL (0.350 mg/ml protein) fractions for 3 hours at 37°C. Total fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of six patients or controls each determined in triplicate. Differences between control and patient samples were determined, using the Student's t-test, not to be statistically significant ($p > 0.1$).

Similarly, there is a 2-fold decrease in HL-mediated hydrolysis of HDL as the density of the HDL increases (from fraction 1 to 6).

3.1.4 Phospholipid and acylglyceride hydrolysis of LDL and HDL fractions isolated from control subjects and patients with FCHL by hepatic lipase

Experiments were also undertaken to determine the contribution of PL and acylglyceride hydrolysis within each fraction and whether the contribution of each differed in the patient and control groups. Following a 3 hour incubation with HL, an aliquot of the incubation mixture was extracted and TLC was performed to separate the substrate ([^{3}H]-DPPC) from the product ([^{3}H]-monopalmitoyl PC). In order to convert the ratio of product to reactant into a PL hydrolytic rate, this ratio first had to be related to the absolute amount of PL in the incubation mixture. In order to determine the amount of PL present, the PL:lyso-PL:sphingomyelin ratio was first determined by HPTLC. The PL mass was then determined by multiplying the percentage of PL by the total choline lipids (determined with an enzyme kit). The PL hydrolysis by HL was calculated from the conversion of [^{3}H]-DPPC to [^{3}H]-monopalmitoyl PC. The remaining hydrolysis attributed to acylglyceride (DG and TG) was determined by subtracting the PL hydrolysis from total hydrolysis (enzymatically determined using a total free fatty acid kit). The left panel of figure 3.1.4 shows that in the control group, HL acts primarily as an acylglyceride lipase with the most buoyant LDL and HDL fraction (fraction 1) but hydrolyses PL and acylglycerides equally well as the density of the LDL (fractions 2 to 5) and HDL (fraction 2 and 3) increase. However, with the most dense LDL fraction (ρ = 1.095 g/ml) and the three most dense HDL fractions (ρ = 1.173-1.238 g/ml), HL acts predominantly as an acylglyceride lipase ($^a p < 0.05$). A similar trend is observed with the patient LDL fractions (figure 3.1.4 right panel), however, only the most

buoyant LDL fraction (ρ = 1.019 g/ml) was found to have significantly more acylglyceride hydrolysis compared to PL hydrolysis ($^{a}p < 0.05$). Contrary to that observed with the control HDL fractions, there was more acylglyceride than PL hydrolysis for all HDL fractions ($^{a}p < 0.05$ or $^{b}p < 0.1$). When the PL and acylglyceride hydrolysis of the patient and control LDL and HDL fractions are compared, acylglyceride hydrolysis was not significantly

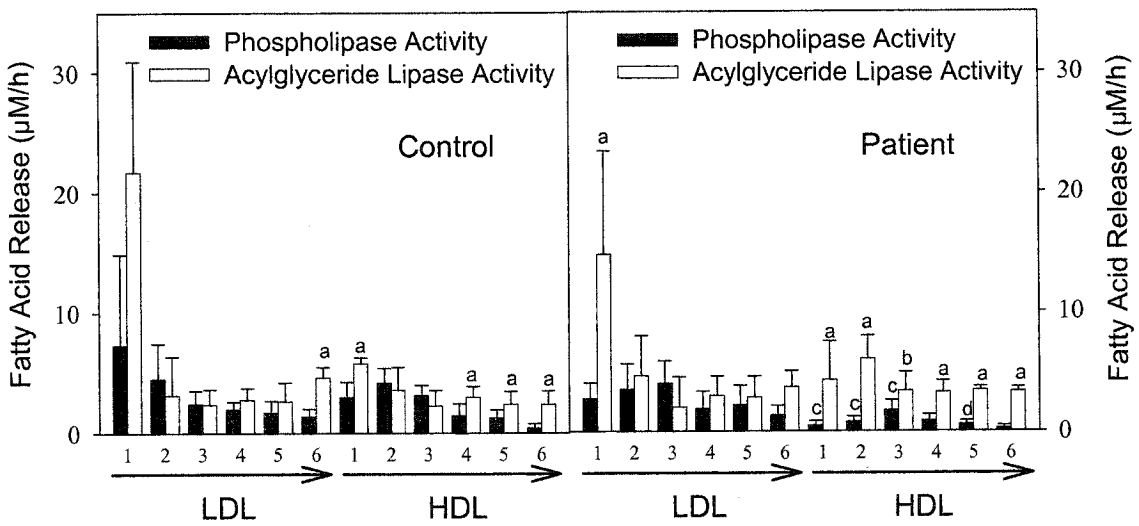

Figure 3.1.4 Phospholipid and acylglyceride hydrolysis of control and FCHL LDL and HDL fractions

[^{3}H]-DPPC-labelled LDL (0.15 mg/ml protein) or HDL (0.35 mg/ml protein) fractions from control (left panel) and patient (right panel) groups were incubated with HL for 3 hours at 37°C. Total fatty acid release was measured enzymatically. PL hydrolysis was determined by TLC and HPTLC (solid bars). Acylglyceride hydrolysis was determined by subtracting PL hydrolysis from total hydrolysis (open bars). Hydrolytic values are the mean ± S.D. of six patients or control each determined in triplicate. Hydrolysis for each patient fraction in the right panel was compared to the corresponding control value in the left panel for statistical significance using the Student's t-test ($^{c}p < 0.05$ or $^{d}p < 0.1$). In addition, acylglyceride hydrolysis for each fraction in each group was compared to the corresponding PL hydrolysis for statistical significance using the Student's t-test ($^{a}p < 0.05$ or $^{b}p < 0.1$). Bars with no symbols are not statistically significant ($p > 0.1$).

different between the two groups. However, PL hydrolysis differed significantly ($^{c}p < 0.05$) for the 3 most buoyant HDL fractions (ρ = 1.115-1.155 g/ml) and the trend was present in one of the most dense HDL fractions (ρ = 1.195 g/ml) ($^{d}p < 0.1$).

3.1.5 Diglyceride and triglyceride content of LDL and HDL derived from control subjects and patients with FCHL

In order to determine whether the differences in the acylglycerol and PL hydrolysis observed were related to DG content and/or the DG to TG ratio, three LDL (ρ = 1.019, 1.048, 1.077 g/ml) and HDL (ρ = 1.134, 1.173, 1.195 g/ml) fractions were sent to the laboratory of Dr. X. Collet (Toulouse, France) to have their DG and TG content analyzed by

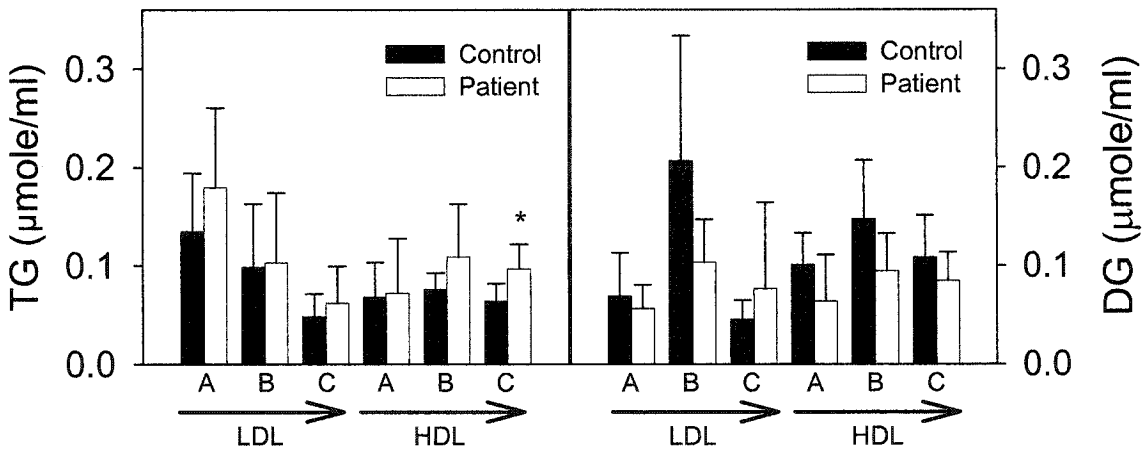

Figure 3.1.5 Triglyceride and diglyceride levels in control and FCHL LDL and HDL fractions

The TG and DG content of LDL and HDL fractions was determined by gas-liquid chromatography and are shown in the left and right graphs, respectively, for control (solid bars) and patient (open bars) groups. Lipid values are the mean ± S.D. of six patients or controls each determined in singlicate. DG and TG values for each patient sample were compared to the corresponding control sample for statistical significance using the Student's t-test (*$p < 0.05$). Bars with no symbols are not statistically significant ($p > 0.05$).

gas-liquid chromatography. Results shown in figure 3.1.5 demonstrate that the DG and TG levels in LDL and HDL did not differ significantly between the two groups with the exception of the TG levels in the most dense HDL fraction, which was statistically elevated in the patient group (* $p < 0.05$).

3.2 ApoA-I and HDL regulate the displacement of hepatic lipase

3.2.1 Activity of HSPG-bound hepatic lipase

Removawells were coated with pure HSPG (5 μg/Removawell), incubated with FAF-BSA to block any non-specific binding of HL and with excess purified human HL (120 U) for 2 hours to ensure maximum association with HSPG. Control incubations (without HSPG) showed that negligible amounts of HL bound to albumin treated plates. Unbound HL was removed by washing with PBS and 60 μl of SDS sample buffer was added and incubated for 30 minutes at 37°C to elute the HSPG-bound HL. Eluted samples were subjected to SDS-PAGE followed by immunoblot analysis with an anti-HL mAb to estimate the amount of HL bound. Maximal binding occurred within 2 hours and approximately 26 U of HL associated with 5 μg HSPG. All subsequent experiments were carried out under these conditions, followed by a brief wash to remove excess unbound HL.

To evaluate whether the association of HL to HSPG alters the catalytic activity of the enzyme, VLDL, LDL or HDL (350 μM TG) were added to the wells containing HSPG-bound HL for 3 hours at 37°C. These incubation times (3 hours) and high substrate (TG) concentrations were required to promote enough hydrolysis to allow for comparison of hydrolytic rates for the best (VLDL and LDL) and poorer (HDL) HL substrates (figure 3.2.1). Figure 3.2.1 shows that the association of HL with HSPG has a significant inhibitory affect on VLDL and LDL lipid hydrolysis, as compared to the control (BSA coated) Removawells (26 U of HL but no HSPG). In contrast, when HDL was evaluated as a substrate for HL, in the presence and absence of HSPG, the difference in total hydrolytic activity of the enzyme was much less (figure 3.2.1). Association of HL with HSPG inhibited LDL and VLDL hydrolysis by > 80%, and inhibited HDL hydrolysis by approximately 40%.

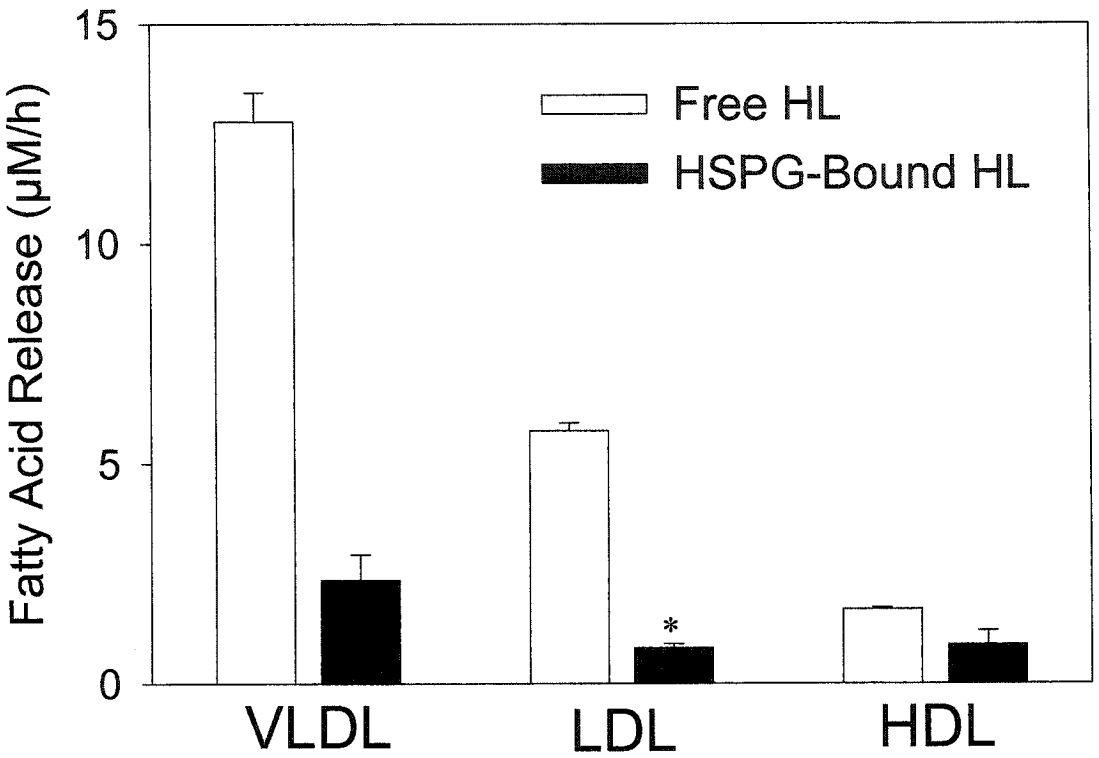

Figure 3.2.1 Hydrolysis of VLDL, LDL and HDL by HSPG-bound HL

Removawells were incubated with 5 μg HSPG for 2 hours at 24°C. HSPG-coated (solid bars) and -deficient (open bars) wells were washed 3 times with PBS and pre-incubated with 1% FAF-BSA in PBS overnight at 4°C. The HSPG-coated wells were incubated with 120 U of purified HL in PBS for 2 hours at 24°C and washed to remove any unbound HL. HSPG-deficient wells were incubated with 26 U of HL for 2 hours and then all Removawells were incubated with VLDL, LDL or HDL (350 μM TG) isolated by ultracentrifugation for 3 hours at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of two different experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Bonferroni Multiple Comparisons Test was performed comparing each lipoprotein sample incubated with the HSPG-bound HL to the corresponding sample incubated with free HL for statistical significance (*$p < 0.001$).

3.2.2 Displacement of hepatic lipase from proteoglycans by lipoproteins

To investigate whether the various lipoproteins affected the binding/association of HL with the HSPG, Removawells were coated with HSPG, pre-incubated with FAF-BSA, incubated with excess HL for 2 hours, washed and then incubated with different lipoproteins. After a standard 3 hours incubation, the supernatant was removed and the Removawells were washed with PBS. SDS sample buffer was added and the eluants were analyzed by SDS-PAGE and by Western blot. The amount of HL that remained bound to HSPG after a 3 hour incubation with VLDL, LDL and HDL was estimated, relative to a control Removawell

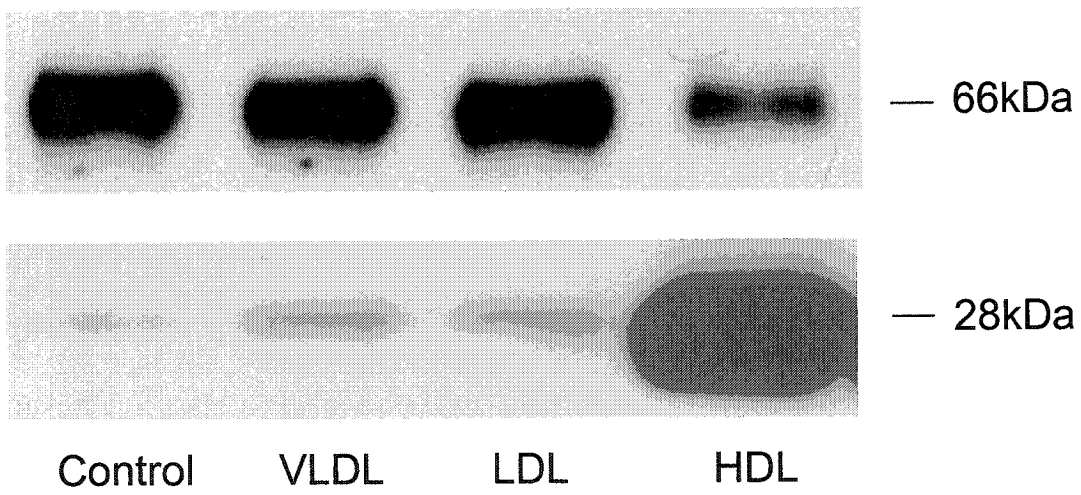

Figure 3.2.2 Effect of lipoproteins on the association of HL with HSPG

Removawells were coated with HSPG and HL (described in figure 3.2.1) and incubated with various plasma lipoproteins or PBS (control) for 3 hours at 37°C. Wells were washed and the HSPG-bound proteins were eluted from the Removawells by incubating with 60 µl of SDS sample buffer at 37°C for 30 minutes. The eluants were electrophoresed on SDS-PAGE and electrotransferred to nitrocellulose. Samples were probed with an anti-HL mAb (upper panel) or with anti-apoA-I mAbs (lower panel) and with an anti-mouse IgG HRP-linked secondary antibody. Apparent molecular mass determinations were derived from broad range molecular weight markers. Images are representative of triplicate determinations of two different experiments.

incubated with PBS. Figure 3.2.2, upper panel, shows that LDL and VLDL were poor at displacing HL from pure HSPG (> 90% of the HL remained associated). In contrast, HDL readily displaced the enzyme and only approximately 40% of the HL remained bound after a 3 hour incubation. Displacement of HL was time dependent with minimal displacement observed by 30 minutes and maximal displacement by 3 hours (data not shown). Experiments were also carried out with lipoproteins from other subjects and a substantive variation in displacement was evident (data not shown). Some HDL preparations almost completely dissociated HL from HSPG while some VLDL caused slightly more displacement (5-10%) than that illustrated in figure 3.2.2.

3.2.3 Displacement of hepatic lipase from HSPG by HDL and apoA-I

To determine if apolipoprotein components of the different lipoproteins were able to displace HL through association with HSPG, the eluant was probed with mAbs to specific apolipoproteins. While some apoB retention to the HSPG was observed with LDL and VLDL, as others have reported (584-586), no detectable amounts of apoE were found associated with the HSPG after incubation with any of the lipoproteins (data not shown). In contrast, large amounts of apoA-I were retained on the HSPG when HDL was incubated with the HSPG-bound HL (figure 3.2.2, lower panel). Furthermore, small amounts of apoA-I were also detectable in incubations with LDL and VLDL, suggesting that some apoA-I had dissociated from these lower density lipoproteins. In figure 3.2.3, an experiment was undertaken to determine if HDL and pure lipid-free apoA-I were equally effective at displacing HL from HSPG. When equal amounts of protein were incubated with HSPG-bound HL, apoA-I was able to promote approximately 1.5 times the HL release, as compared to HDL (figure 3.2.3, upper panel). In addition, twice as much apoA-I was retained on the

HSPG with incubations of the lipid-free protein, as compared to HDL (figure 3.2.3, lower panel). ApoA-I titration experiments were next performed to assess the effect of apoA-I concentration on HL displacement from HSPG. The results showed that even very low concentrations of apoA-I (14 μg/ml) promoted maximal displacement of HL and that a 10-fold increase in apoA-I concentration had no additional effect on the liberation of HL from HSPG (data not shown).

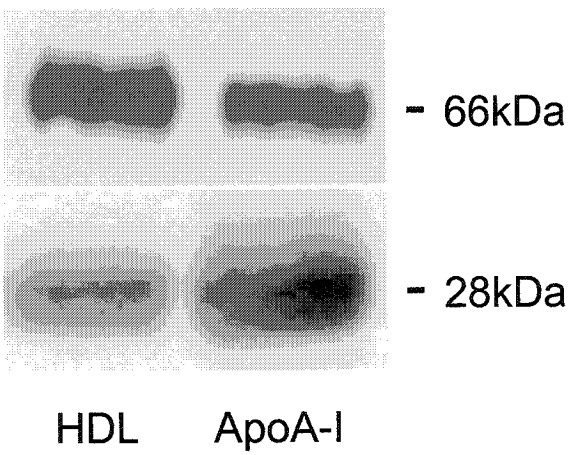

Figure 3.2.3 Effect of HDL and apoA-I on the association of HL with HSPG

Removawells were coated with HSPG and HL (described in figure 3.2.1) and then incubated with HDL or apoA-I (164 μg protein) for 3 hours at 37°C. Wells were washed and the HSPG-bound proteins were eluted and subjected to SDS-PAGE. Samples were transferred to nitrocellulose and probed with an anti-HL mAb (upper panel) or with anti-apoA-I mAbs (lower panel). Images are representative of triplicate determinations from three different experiments.

3.2.4 Characterization of hepatic lipase in CHO-hHL and HepG2 Cells by Western blot analysis

The results in figures 3.2.2 and 3.2.3 show that HDL and apoA-I displaced HL from pure HSPG. In order to extend these studies to a cell culture system, CHO-hHL and HepG2 cells were characterized and then utilized to explore the ability of HL to be displaced from the cell surface. CHO-hHL and HepG2 cells were grown to confluence in their respective complete medium and the adherent cells were either solubilized in SDS sample buffer to produce whole cell lysates or treated with cytochalasin B to separate the intact cells from their ECM (587) and allow for differentiation of both intracellular and cell surface-

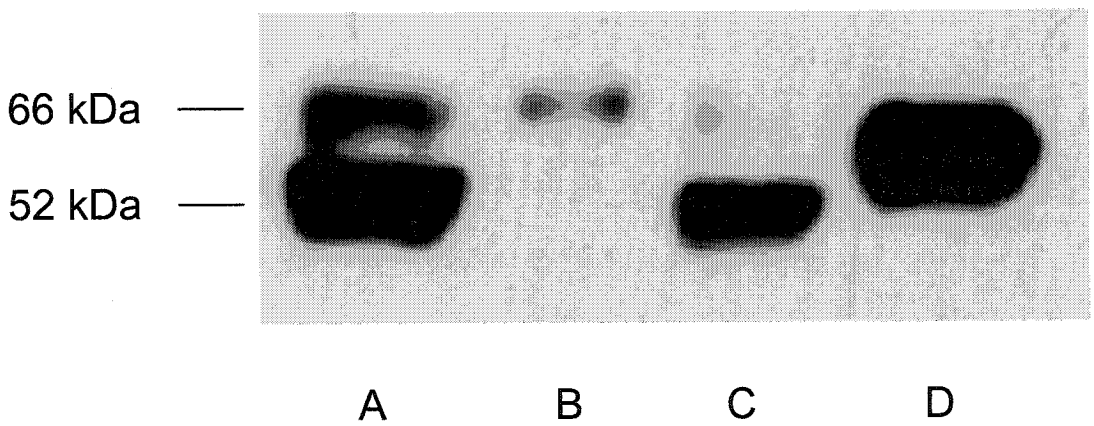

Figure 3.2.4 Characterization of HL in CHO-hHL by Western blot analysis

CHO-hHL cells were grown to confluence in complete medium. The adherent cells were either lysed with SDS sample buffer (whole cell lysate) or treated with complete medium containing cytochalasin B (10 μg/ml) for 1 hour at 37°C in order to separate the cells from the ECM. The cells and the ECM were separately treated with SDS sample buffer. Whole cell lysate (lane A), ECM extract (lane B), cell extract (lane C) and purified HL (lane D) were electrophoresed on an 8% Novex gel and transferred to nitrocellulose. Samples were probed with an anti-HL mAb and with an anti-mouse IgG HRP-linked secondary antibody. Apparent molecular mass determinations were derived from broad range molecular weight markers. The image is representative of duplicate determinations from three different experiments.

associated HL. Whole cell lysates, ECM and cell extracts were individually treated with SDS sample buffer, and then electrophoresed and probed for HL. Results showed that HL from whole cell lysates of both cell lines exhibited two distinct bands, one at 66 kDa and the other at 52 kDa, which appear to represent two differently glycosylated forms of the enzyme. The HL associated with the cell surface ECM is similar in size to that of purified, human HL obtained from post-heparin plasma and has an apparent molecular mass of 66 kDa. The HL present in the cell extract appears to be an intracellular form that is smaller and has an apparent molecular mass of 52 kDa (figure 3.2.4).

3.2.5 Displacement of hepatic lipase from CHO-hHL and HepG2 cells by HDL and apoA-I

To determine if HDL and apoA-I could displace HL from the cell surface, CHO-hHL and HepG2 cells were treated with apoA-I or HDL (150 μg protein/ml of medium) for various times at 37°C. Figure 3.2.5 shows the HL present in whole cell lysates of CHO-hHL cells following treatment with either apoA-I (A) or HDL (B) for the indicated times. Although both forms of HL decrease as a function of time when treated with either HDL or apoA-I, the 66 kDa cell surface-associated enzyme is depleted much faster than is the smaller 52 kDa, intracellular HL. Panel C is a composite graph of the Western blots shown in panel A and B, after band quantification by densitometry. The displacement curves show HL to be more efficiently displaced from the cell surface by apoA-I than by HDL; HL displacement by apoA-I is complete after 1 hour, while only 60% of HL is displaced by HDL by 2 hours. Panel D shows that both HDL and apoA-I displaced HL from the surface of HepG2 cells. Displacement curves appear similar to that observed with the CHO-hHL cells; apoA-I promoted complete displacement by 1 hour, while HDL required 2 hours to completely liberate cell surface HL.

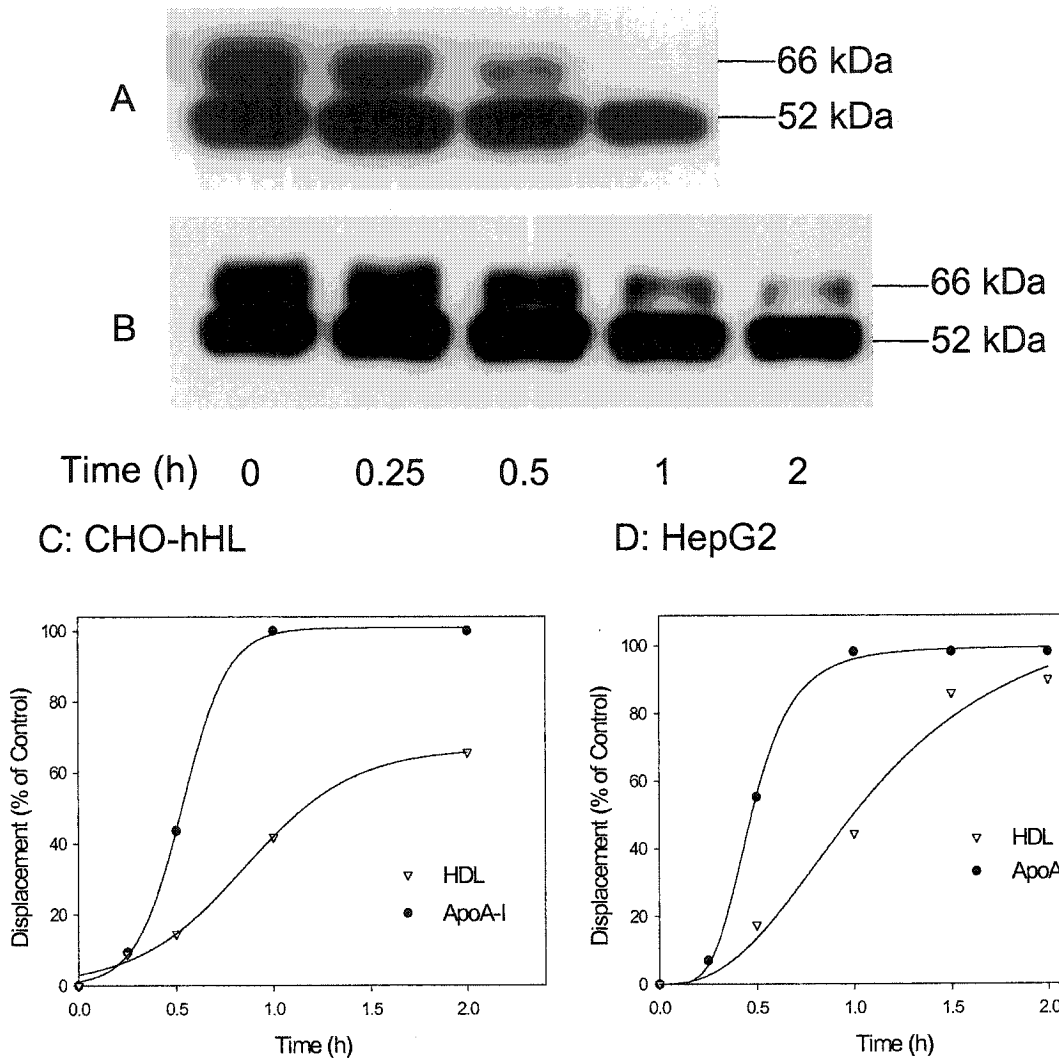

Figure 3.2.5 Displacement of HL from the cell surface of CHO-hHL and HepG2 cells by HDL and apoA-I

CHO-hHL (panels A-C) and HepG2 (panel D) cells were grown to confluence in complete medium. Cells were washed and incubated with serum-free medium ± apoA-I or HDL at 37°C for the times indicated. Once the medium was removed, the cells were washed with PBS and incubated with SDS sample buffer overnight at 24°C. The cell lysates were electrophoresed and transferred to nitrocellulose membranes. Samples were probed with an anti-HL mAb and with an anti-mouse IgG HRP-linked secondary antibody. Western blots of apoA-I and HDL displacement of HL in CHO-hHL cells are shown in Panel A and B, respectively. Apparent molecular mass determinations were derived from broad range molecular weight markers. HL displacement by apoA-I (●) and HDL (▽) relative to HL displacement in the absence of HDL or apoA-I at time zero was quantified by densitometry and is graphically shown in panel C for CHO-hHL cells and Panel D for HepG2 cells. Data are representative of duplicate determinations from three different experiments for CHO-hHL cells and duplicate determinations from two different experiments for HepG2 cells.

3.2.6 Effect of HDL density on hepatic lipase displacement from the cell surface

Experiments were also undertaken to determine whether various HDL density subclasses differed in their abilities to displace HL. HDL was isolated from plasma by sequential ultracentrifugation in order to remove VLDL, LDL and albumin fractions (ρ < 1.063 g/ml and > 1.25 g/ml). The HDL was then separated into fractions of varying densities (ρ = 1.064-1.25 g/ml) by discontinuous density gradient ultracentrifugation and the abilities of the various HDL to displace HL were investigated. CHO-hHL cells were grown to confluence in complete medium and the cells were then incubated for 1 hour with serum-free medium ± HDL fractions (ρ = 1.1-1.22 g/ml) at 37°C. As detailed in figure 3.2.6, there was an inverse relationship between HDL density and HL displacement. While the smaller HDL (ρ = 1.22 g/ml) displaced approximately 50% of the HL from the cell surface,

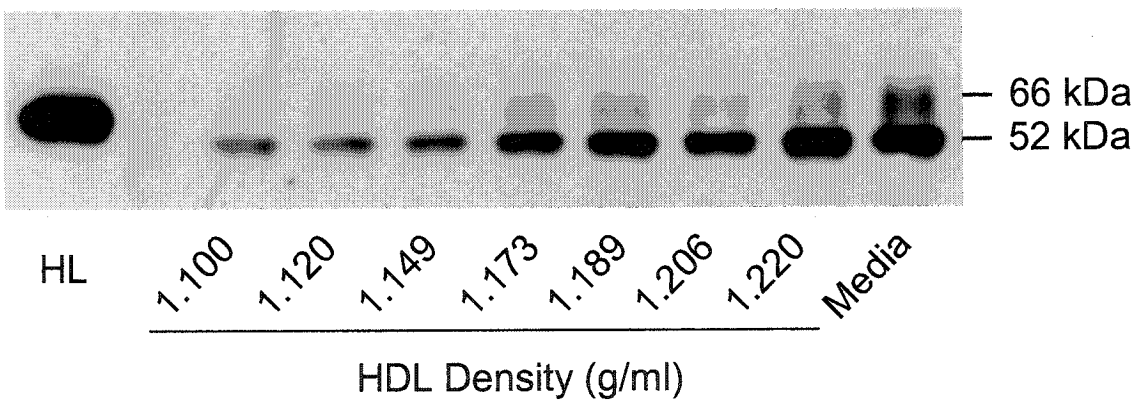

Figure 3.2.6 Displacement of HL from the cell surface of CHO-hHL by HDL fractions

CHO-hHL cells were grown to confluence in complete medium. Cells were washed and incubated with serum-free medium ± HDL density fractions (150 μg protein/ml medium) at 37°C for 1 hour. Cells were washed with PBS, incubated with SDS sample buffer overnight and then the cell lysates were electrophoresed and transferred to a nitrocellulose membrane. Samples were probed with an anti-HL mAb and with an anti-mouse IgG HRP-linked secondary antibody. The image is representative of duplicate determinations from three different experiments.

the larger HDL (ρ = 1.10 g/ml) displaced > 95% the enzyme. Furthermore, the more buoyant HDL also caused a greater depletion in the intracellular stores of the enzyme. Taken together, these data indicate that HDL composition influences the displacement of HL from cell surface proteoglycans and mobilization of the enzyme from the cell.

3.2.7 Effect of VLDL on HDL and apoA-I-mediated displacement of hepatic lipase from HSPG

Since HL is inactive when bound to pure HSPG and apoA-I liberates the enzyme using both the pure HSPG system and cell culture models, experiments were performed to determine if the liberation of HL from pure HSPG by apoA-I stimulates the hydrolysis of VLDL. Incubations were carried out with free and HSPG-bound HL as described in figure legend 3.2.1, however, in some wells, VLDL was co-incubated with either pure apoA-I or HDL. While the displacement of HSPG-bound HL by heparin recovers the activity of the enzyme (data not shown), figure 3.2.7 shows that addition of apoA-I or HDL to incubations of HSPG-bound HL and VLDL had little effect on the rate of lipid hydrolysis, relative to that expected for a displaced enzyme. Figure 3.2.8 further shows that this "retained inhibition" was partly due to an impaired displacement of HL by either apoA-I or HDL, in the presence of VLDL. In this experiment, apoA-I and HDL were able to displace between 40 and 50% of the HSPG-bound HL. In the presence of VLDL, HL displacement fell to less than 10%. However, it is evident that even though some HL displacement had occurred in these incubations, hydrolytic rates remained unaffected (figure 3.2.7). These data suggest that apoA-I may have a secondary effect on HL-mediated hydrolysis.

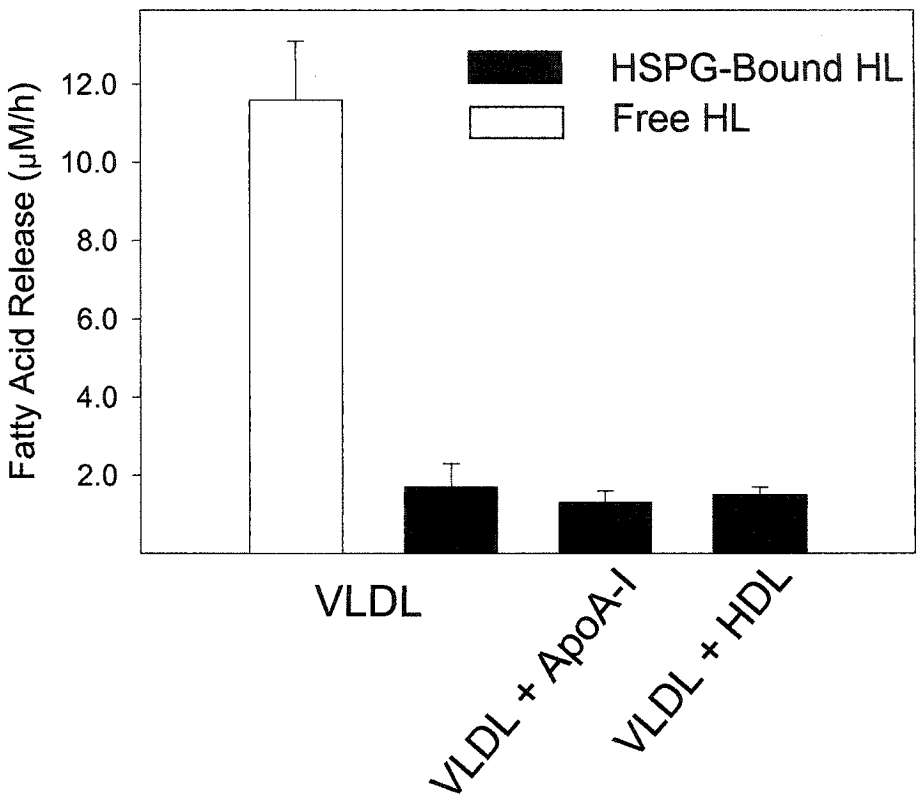

Figure 3.2.7 Effect of apoA-I and HDL on the hydrolysis of VLDL by HSPG-bound HL

Removawells were coated with HSPG and HL and then incubated with VLDL isolated by ultracentrifugation, in the presence or absence of HDL (130 μM TG) or apoA-I (equivalent HDL protein concentration) for 3 hours at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of two different experiments. The hydrolytic values obtained for the VLDL sample incubated with HSPG-bound HL in the presence of HDL or apoA-I were compared to the hydrolytic value obtained for the VLDL sample incubated with HSPG-bound HL in the absence of HDL or apoA-I for statistical significance using a one-way ANOVA. Results were determined not to be significant ($p > 0.05$).

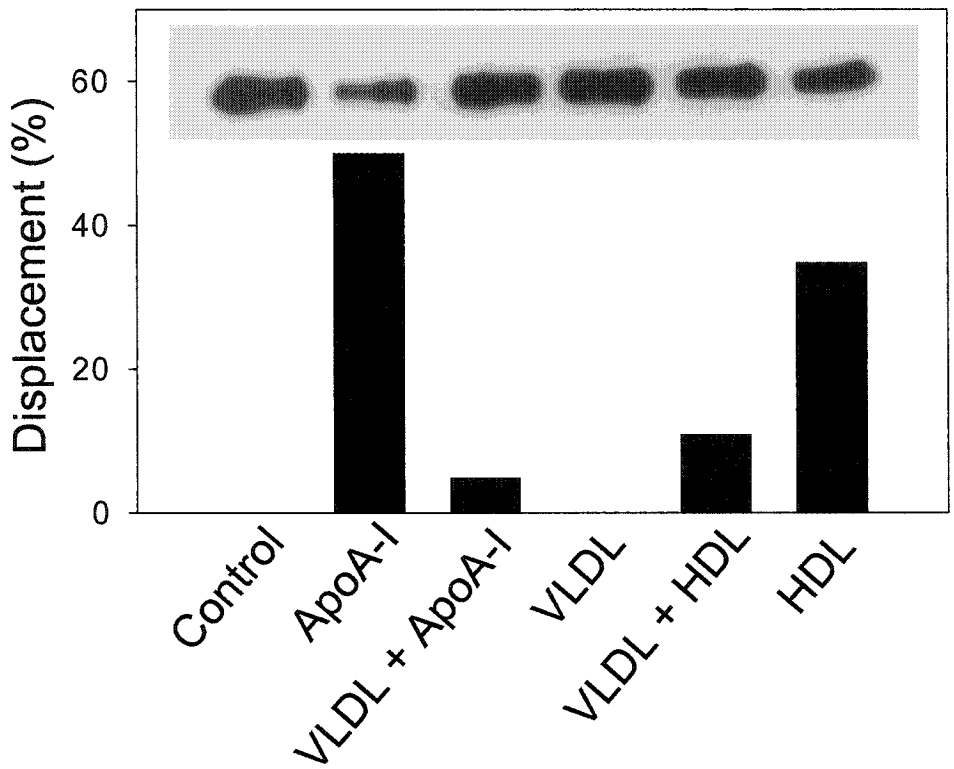

Figure 3.2.8 Effect of VLDL on the dissociation of HL by HDL and apoA-I

Removawells were coated with HSPG and HL (described in figure 3.2.1) and incubated with VLDL (350 μM TG), HDL (130μM TG), apoA-I (equivalent HDL protein concentration) or VLDL plus HDL or apoA-I for 3 hours at 37°C. Wells were washed and the HSPG-bound proteins were eluted and subjected to SDS-PAGE. Samples were transferred to nitrocellulose and probed with an anti-HL mAb. The histogram shows the percent of HL displaced from the HSPG (relative to the control PBS incubation) and were estimated from the Western blot shown. Values are representative of two different experiments.

3.3 HDL regulates hepatic lipase activity

3.3.1 Effect of HDL and apoA-I on VLDL hydrolysis by hepatic lipase

To determine whether apoA-I or total HDL could directly affect the hydrolysis of VLDL by HL, standard hydrolytic assays, in the absence of HSPG, were performed. Figure 3.3.1 shows that increasing the concentration of apoA-I or total HDL significantly inhibited

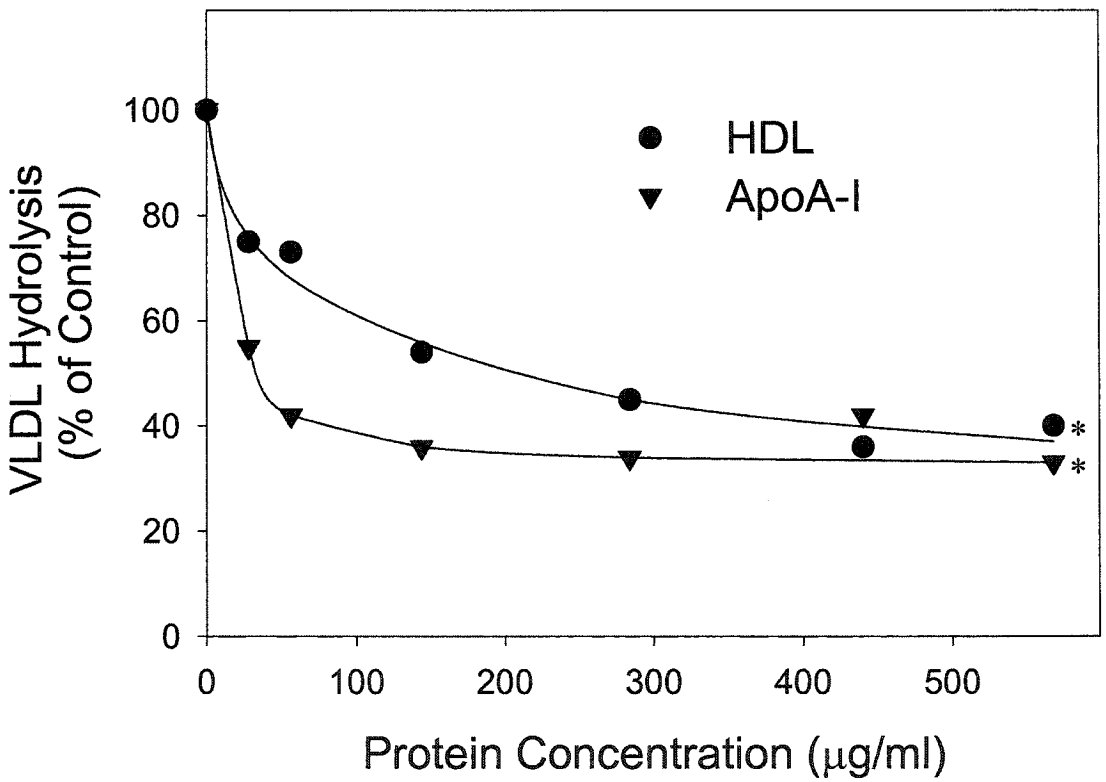

Figure 3.3.1 Effect of varying inhibitor concentration on the hydrolysis of VLDL by HL

VLDL (350 μM TG) was incubated with HL (26 U) and increasing amounts of HDL or apoA-I for 30 minutes at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of triplicate determinations and representative of two experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest HDL or apoA-I concentration to the hydrolytic value obtained in the absence of HDL or apoA-I for statistical significance (*$p < 0.01$).

the hydrolysis of VLDL, up to a maximum of 60%. The amount of inhibition observed by apoA-I appears to be dependent on the source of VLDL since some incubations have shown greater inhibition by apoA-I (figure 3.3.5). In addition, at the lower concentrations, apoA-I

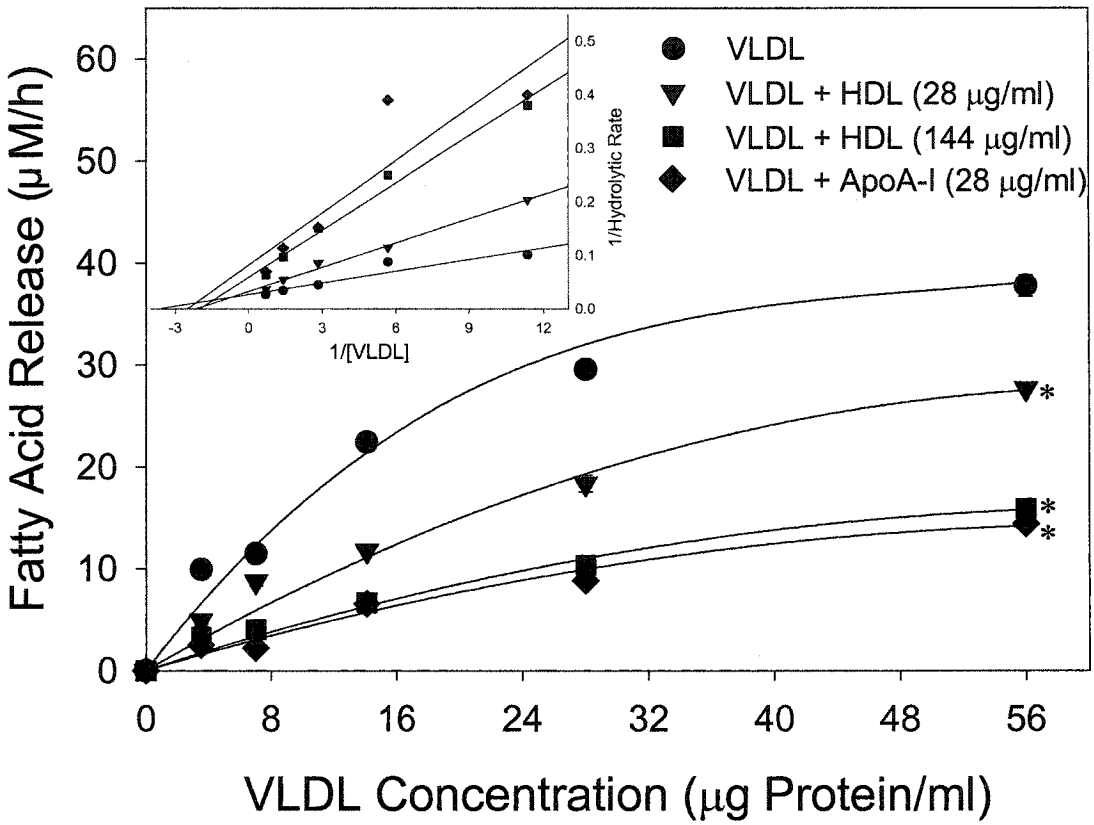

Figure 3.3.2 Effect of apoA-I and HDL on the hydrolysis of VLDL by HL

Various amounts of VLDL were incubated with HL (26 U) and a constant amount of HDL or apoA-I (as indicated) for 30 minutes at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of triplicate determinations. Inset: Double reciprocal plots are shown where reciprocals of hydrolytic rates (μM/h) are plotted against reciprocals of VLDL concentration (μg protein/ml). A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest VLDL concentration in the presence of HDL or apoA-I to the corresponding hydrolytic value obtained in the absence of HDL or apoA-I for statistical significance (*$p < 0.01$).

was up to 5 times more inhibitory to HL than HDL. This is further evident in figure 3.3.2, which shows HL hydrolytic rates as a function of VLDL concentration, but at a constant inhibitor concentration. 5 times more HDL is required to accomplish the equivalent inhibition as a given amount of apoA-I. The inset of figure 3.3.2 shows that inhibition of HL is associated with an increase in the apparent Michaelis-Menten constant (K_m) values (inverse of x intercept) and a concomitant decrease in the maximum velocity (V_{max}) of the enzyme. Bolin *et al.* and Sparks *et al.* (510, 588) have previously shown that this apparent K_m for an interfacial enzyme, when represented as a protein concentration, indicates the amount of VLDL particles required for a half maximal velocity and therefore reflects a measure of binding affinity. The data therefore suggest that apoA-I may inhibit HL by decreasing its binding affinity to VLDL.

3.3.2 Effect of HDL on hepatic lipase activity in CHO-hHL and HepG2 cell medium

To determine whether HL displacement resulted in an increase in HL activity in our cell culture models, the medium of CHO-hHL and HepG2 cells pre-incubated with either HDL or heparin was tested for HL activity. The cells were washed and incubated in serum-free medium containing either HDL (150 μg protein/ml of medium) or heparin (200 U/ml of medium) for various times at 37°C. The medium was removed and HL activity was measured by tracking the hydrolysis of a standard [^{3}H]-TG emulsion. Figure 3.3.3 shows that in the absence of HDL or heparin, only a small amount of HL activity is observed in the medium of both the CHO-hHL and the HepG2 cells (inset of figure 3.3.3). As expected, incubation of CHO-hHL cells with heparin for times ranging from 0-2 hours resulted in a progressive increase of HL activity in the medium. Similarly, treatment of the cells with HDL also resulted in an increase of HL activity in the medium, however hydrolytic rates

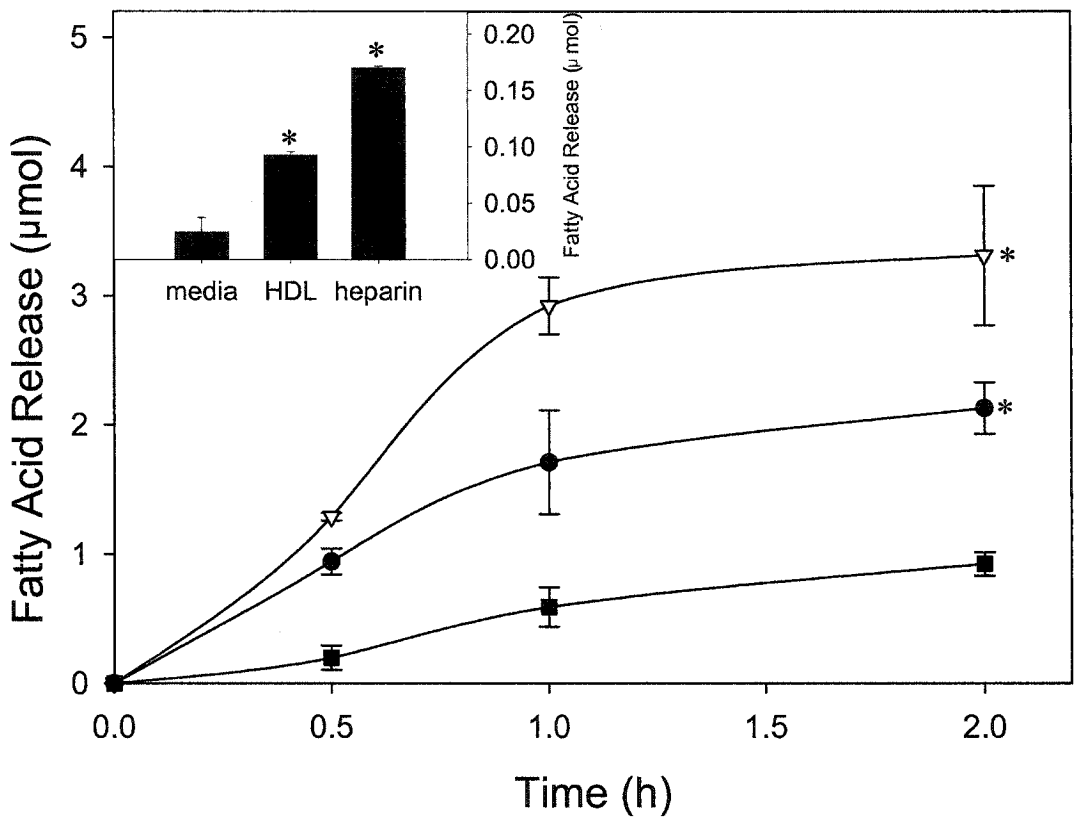

Figure 3.3.3 Effect of HDL on HL activity in CHO-hHL and HepG2 cell medium

CHO-hHL and HepG2 (inset) cells were grown to confluence in complete medium. Cells were washed and incubated with serum-free medium (■), + HDL (●) or + heparin (▽) at 37°C for various times. An aliquot of the medium was removed and triglyceride hydrolytic rates were measured after incubation with a [^{3}H]-triolein emulsion. The graph shows [^{3}H]-fatty acid released during a 1 hour hydrolytic assay using CHO-hHL cell medium that had been incubated with HDL or heparin for various times as indicated in the figure. Inset shows [^{3}H]-fatty acid released during a 3 hour hydrolytic assay using HepG2 cell medium, which had been incubated with HDL or heparin for 4 hours. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of two experiments for each cell line. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values obtained for serum-free medium in the presence of HDL or heparin at the 2 hour time point for CHO-hHL cells and the 4 hour time point for HepG2 cells to the corresponding hydrolytic value obtained for serum-free medium for statistical significance (*$p < 0.01$).

were less than that observed for heparin. Results obtained using HepG2 cells (inset) paralleled those obtained with CHO-hHL cells. These data show that HDL acts much like heparin and can release catalytically active HL from the surface of both CHO-hHL and HepG2 cells into the medium. The fact that more activity was detectable when the cells were incubated with heparin, relative to HDL, reflects an inhibitory effect of HDL on HL, much as we see with the pure HSPG system and/or that heparin caused a more enhanced release of HL.

3.3.3 Effect of plasma HDL concentration on lipid hydrolysis by hepatic lipase

To determine the physiological relevance of HL inhibition by HDL, experiments were performed to estimate the effect of HDL concentration in plasma on lipid hydrolysis by HL. Plasma lipoproteins from fasting normolipidemic subjects were isolated within the density ranges, $\rho < 1.063$ g/ml and $\rho = 1.063$-1.25 g/ml and then recombined in various ratios. Mixtures contained the equivalent amount of apoB-containing lipoproteins, to that found in plasma, and progressively increasing amounts of HDL, representing zero to 100% of the original amount of HDL. Plasma and the different lipoprotein mixtures were then incubated with a constant amount of purified HL for various times. Figure 3.3.4 shows that HL-mediated lipid hydrolysis in plasma is almost completely inhibited. Hydrolysis was maximal for the apoB-containing lipoproteins alone and was progressively inhibited by increasing amounts of HDL. These results appear consistent with previous work that showed that serum, HDL and the $\rho > 1.21$ bottom fraction could inhibit HL activity (512, 515, 518). Our experiment further showed that approximately 70% of the HL inhibition in plasma is likely due to HDL/apoA-I specific effects, with other components in plasma accounting for the remaining inhibitory effects.

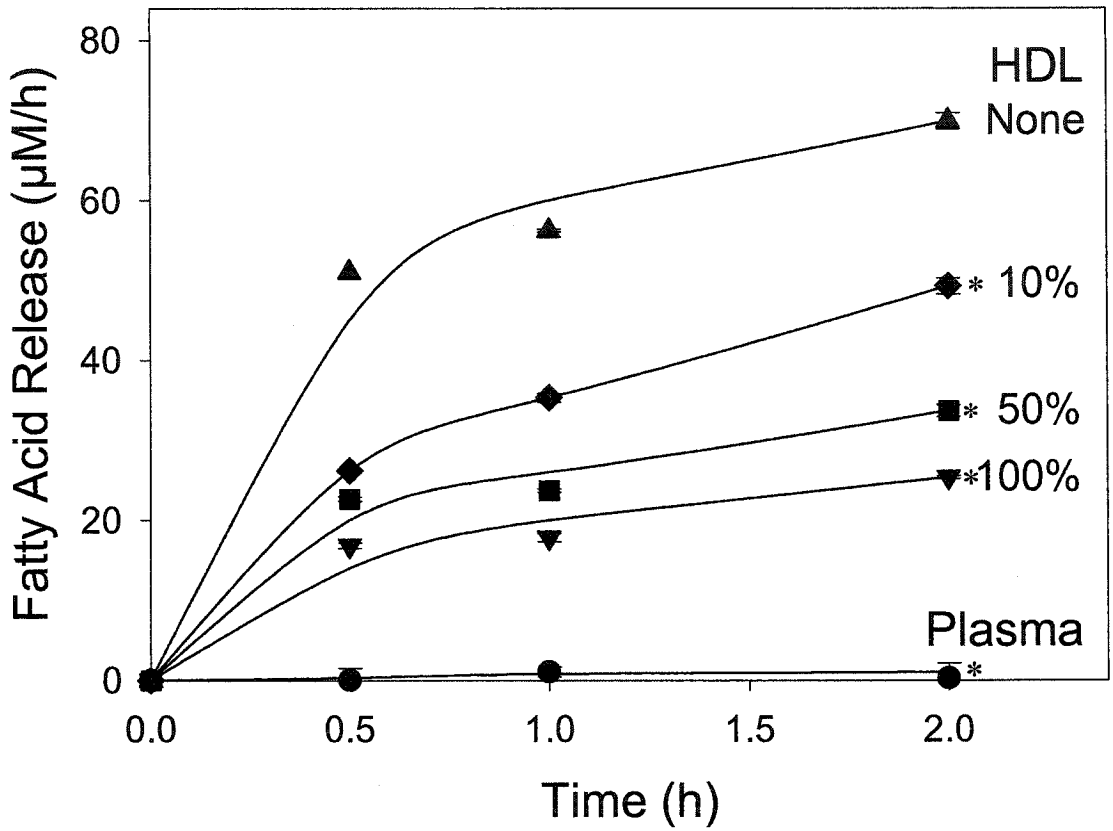

Figure 3.3.4 Effect of HDL concentration on lipid hydrolysis in plasma

Plasma lipoproteins from a fasted normolipidemic subject were isolated within the density ranges, $\rho < 1.063$ g/ml and $\rho = 1.063$-1.25 g/ml. Mixtures of the plasma lipoproteins were prepared to contain the equivalent amount of apoB-containing lipoproteins, to the original plasma concentration, and progressively increasing amounts of HDL. HDL concentration is illustrated as zero to 100% of the original amount of HDL in the plasma sample. Unmodified plasma and lipoprotein mixtures were then incubated with a constant amount of purified HL (26 U) for 0-2 hours at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean $\pm$ S.D. of triplicate determinations. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values obtained for whole plasma or the reconstituted plasma containing increasing amounts of HDL at the 2 hours time point to the corresponding hydrolytic value obtained for the reconstituted plasma containing no HDL for statistical significance (*$p < 0.01$).

3.3.4 Effect of HDL subfractions on VLDL hydrolysis

Hydrolytic assays were performed to determine whether HDL fractions differed in their abilities to affect the hydrolysis of VLDL by HL. The results in figure 3.3.5 show the relative total lipid hydrolytic rates for VLDL ± HDL or apoA-I and are expressed as percent

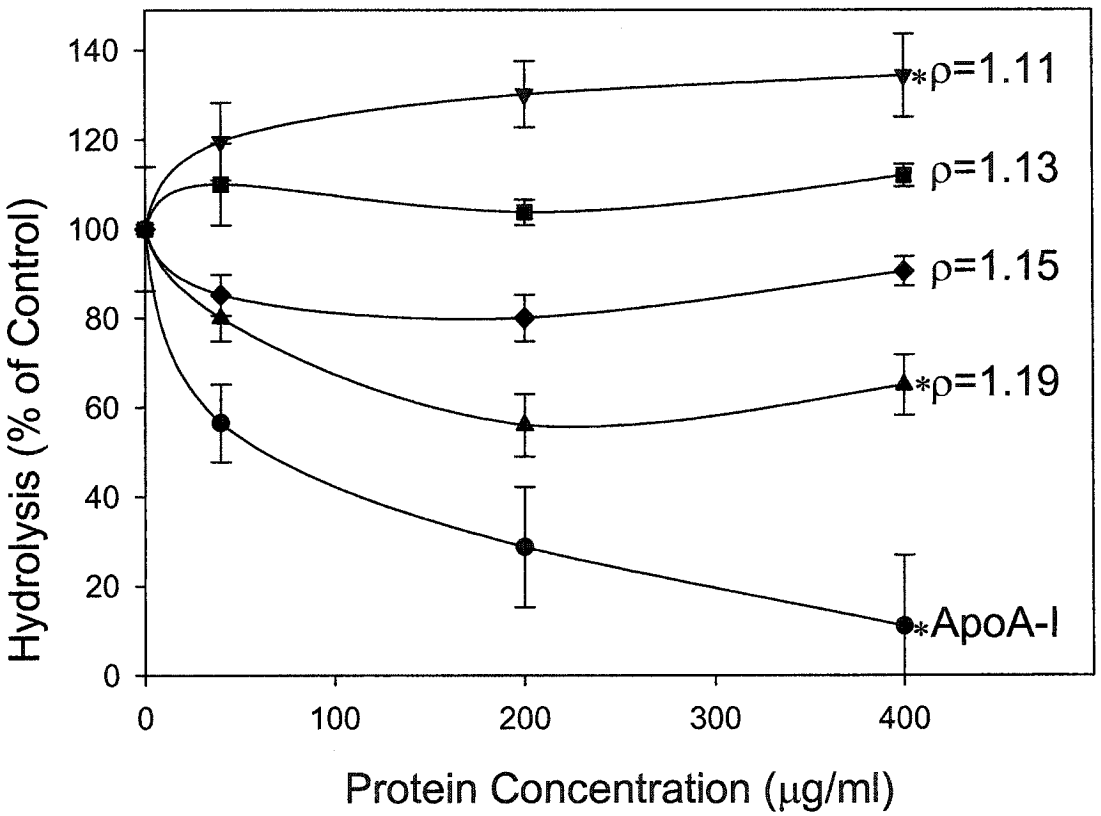

Figure 3.3.5 Effect of HDL fractions on the hydrolysis of VLDL by HL

VLDL (350 μM TG) was incubated with HL (26 U) and increasing amounts of different HDL density fractions (ρ = 1.11 g/ml (▼), ρ = 1.13 g/ml (■), ρ = 1.15 g/ml (◆), ρ = 1.19 g/ml (▲)) or apoA-I (●) for 30 minutes at 37°C. An aliquot was removed and fatty acid release was measured. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of two experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest HDL (for each fraction) or apoA-I concentration to the hydrolytic value in the absence of HDL or apoA-I for statistical significance (*$p < 0.01$). Curves with no symbols are not statistically significant ($p > 0.05$).

of hydrolysis of VLDL alone. The figure shows that HDL density has significant effects, both stimulatory and inhibitory, on HL-mediated VLDL hydrolysis. As the density of the HDL increases, progressively less stimulation and more inhibition of HL-mediated VLDL hydrolysis can be observed. While the most buoyant HDL fraction (ρ = 1.11 g/ml) was found to stimulate HL activity by approximately 30%, the more dense HDL fractions (ρ = 1.19 g/ml) inhibited the enzyme's activity by up to 50%. This is consistent with the previous findings of Mowri *et al.* (529) that HDL_3 inhibited VLDL hydrolysis to a greater extent than HDL_2. As previously shown, apoA-I inhibits HL-mediated VLDL hydrolysis by 80-90%. The data show that HDL composition plays an important role in determining whether HDL stimulates or inhibits HL-mediated VLDL hydrolysis.

3.3.5 HDL derived apoA-I binding to VLDL

The data shown in figure 3.2.8 suggested that with co-incubations, VLDL interfered with the ability of apoA-I and HDL to displace HL from HSPG. One way this could have occurred is if apoA-I associated directly with VLDL instead of interacting with the pure HSPG. This may also account for the inhibitory effects of apoA-I. Several experiments were performed to determine whether the ability of HDL to either stimulate or inhibit VLDL lipolysis was related to the transfer of apoA-I to VLDL. Figure 3.3.6 shows VLDL-apoA-I association by a solid phase radio-immunometric assay. Plates coated with a mAb to apoB (1D1) were incubated with VLDL that had been incubated (3 hours) with HL $\pm$ HDL or apoA-I. ApoA-I-associated VLDL was then measured with a pooled mixture of two ^{125}I-labeled mAbs to apoA-I (4H1 and 5F6). As seen from figure 3.3.6, significant amounts of apoA-I were found associated with VLDL that had been incubated with either apoA-I or the

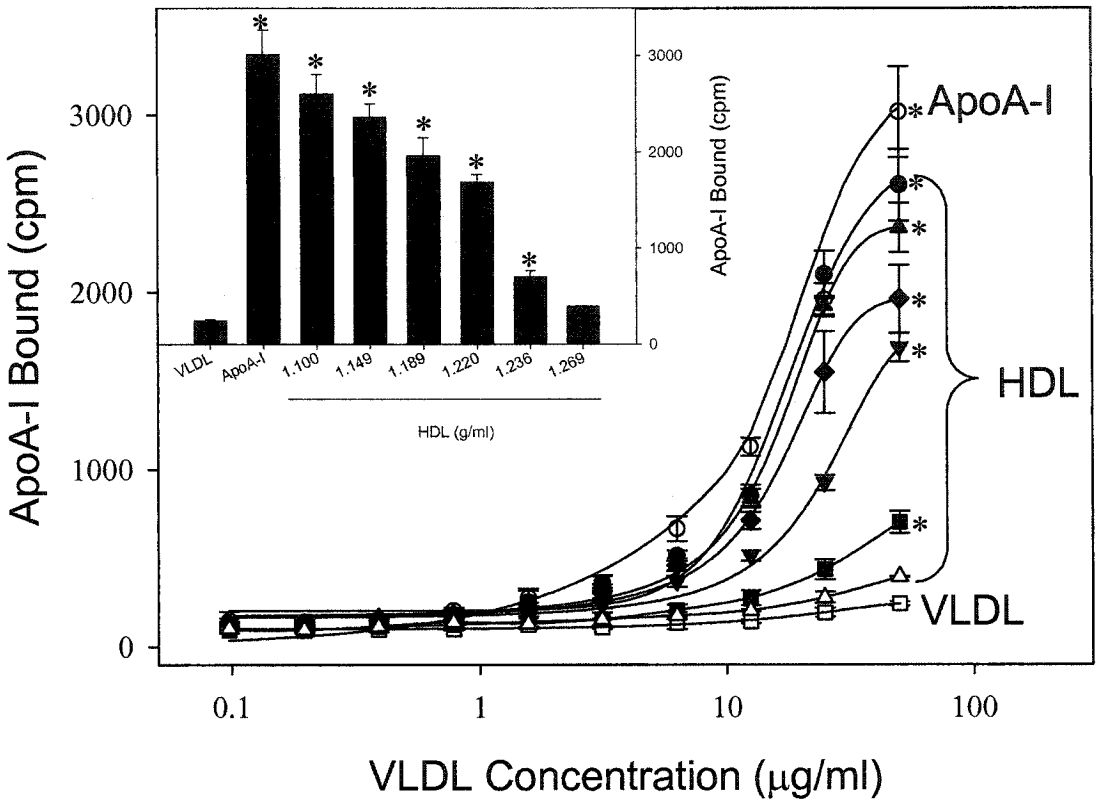

Figure 3.3.6 VLDL-apoA-I association by a solid phase radio-immunometric assay

Removawells coated with a mAb to apoB (1D1) were incubated with VLDL that had been pre-incubated (3 hours) with HL ± apoA-I or HDL density fractions (ρ = 1.100 g/ml (●), ρ = 1.149 g/ml (▲), ρ = 1.189 g/ml (◆), ρ = 1.220 g/ml (▼), ρ = 1.236 g/ml (■) ρ = 1.269 g/ml (△). ApoA-I association with the VLDL was then measured by the addition of two ^{125}I-labeled mAbs to apoA-I (4H1 and 5F6), followed by washing and radioactivity measurement. Values were corrected for background association and are the mean ± S.D. of triplicate determinations and are representative of two different experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the values representing apoA-I-associated VLDL (based on cpm) at the highest VLDL concentration in the presence of apoA-I or the various HDL fractions to the apoA-I-associated VLDL in the absence of apoA-I or the various HDL fractions for statistical significance (*$p < 0.01$). Curves with no symbols are not statistically significant ($p > 0.05$).

different HDL fractions, but not with the native VLDL, which was not incubated with apoA-I or HDL. Omitting HL from these incubations had no effect on apoA-I association with VLDL (data not shown). The inset shows that an inverse relationship exists between HDL density and apoA-I transfer to VLDL. As the HDL density increased (towards the small HDL), there was progressively less apoA-I transferred to the VLDL. It is also of note that similar amounts of apoA-I were transferred to VLDL in incubations with pure apoA-I as with incubation with the $\rho = 1.100$ g/ml HDL density fraction (HDL_2). Even though similar amounts of apoA-I associated with VLDL, lipid-free apoA-I was inhibitory to HL hydrolysis of VLDL while the buoyant HDL were stimulatory.

3.3.6 Effect of apoA-I lipidation on VLDL hydrolysis

In order to determine whether the stimulatory/inhibitory effects seen with the HDL fractions were related to the particle physical properties, hydrolytic assays were performed to evaluate the effect of apoA-I lipidation on VLDL hydrolysis. Figure 3.3.7 shows that the amount of PL associated with apoA-I directly affects its ability to both stimulate and inhibit HL. Addition of a few molecules of PL to apoA-I (POPC:apoA-I, 5:1, mol:mol) converted the apolipoprotein from an inhibitor of HL activity, to a stimulator. However, while the poorly-lipidated 5:1 particle, which contains one molecule of apoA-I per particle, profoundly stimulated HL-mediated VLDL hydrolysis, additional lipidation of apoA-I negated this stimulatory effect. The 35:1 particle, which contains two molecules of apoA-I per particle (Lp2A-I), stimulated HL to a much lesser extent than the 5:1 particle. Furthermore, the 60:1 particle, also an Lp2A-I particle, inhibited the enzyme activity by almost 50%. This therefore suggests that the lipidation-dependent physical properties of HDL affect its ability to regulate HL.

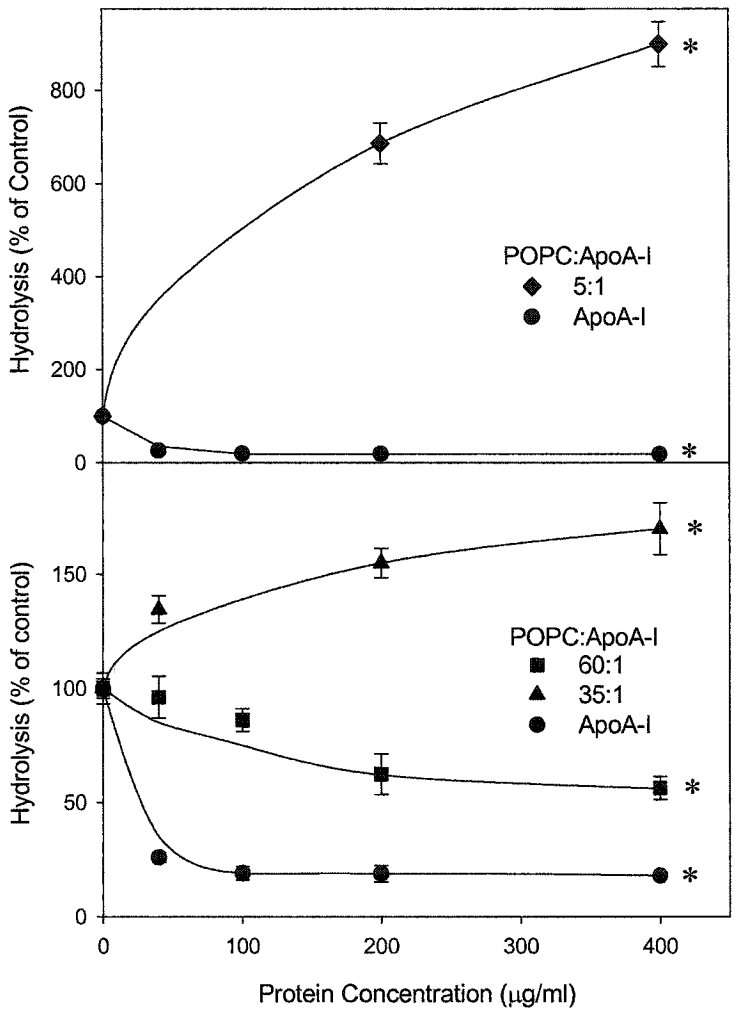

Figure 3.3.7 Effect of rHDL on the hydrolysis of VLDL by HL

VLDL (350 μM TG) was incubated with HL (26 U) and increasing amounts of rHDL particles (POPC:apoA-I; 5:1 (◆), 35:1 (▲), 60:1 (■)) or apoA-I (●) for 30 minutes at 37°C. An aliquot was removed and fatty acid release was measured. Hydrolytic values are the mean ± S.D. of triplicate determinations and representative of two experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest apoA-I or particle concentration to the hydrolytic value in the absence of apoA-I or particles for statistical significance (*$p < 0.01$).

3.4 Displacement and regulation of lipoprotein lipase activity

The work presented thus far suggests that the displacement of HL and the activity of the enzyme are regulated, in part, by HDL, and particularly, by apoA-I. Since LPL and HL are related enzymes with overlapping functions in lipoprotein metabolism it is conceivable that HDL and apoA-I could have a similar effect on LPL activity. Earlier reports have suggested that LPL association to proteoglycans influences its activity. A number of investigations have suggested that HSPG association may also have inhibitory effects on LPL activity (536, 537), and one group further extended these studies by demonstrating that the binding of LPL to HSPG partially inhibited the lipolysis of VLDL (540). Based on the results obtained with HL, preliminary studies were performed to determine the effect of apoA-I and HDL on the activities of bLPL and human LPL (hLPL) and the association of the enzyme with HSPG.

3.4.1 Activity of HSPG-bound bovine lipoprotein lipase

Removawells were coated with pure HSPG (5 µg/Removawell), incubated with FAF-BSA to block any non-specific binding of the enzyme and with excess bLPL (11 U) for 1 hour to insure maximum association with HSPG. bLPL was initially used in these studies since it is readily available commercially and is commonly used as a surrogate for hLPL. Control incubations (without HSPG) showed that negligible amounts of bLPL bound to albumin treated plates (data not shown). Unbound bLPL was removed by washing with PBS and 60 µl of SDS sample buffer was added and incubated for 30 minutes at 37°C to elute the HSPG-bound bLPL. Eluted samples were subjected to SDS-PAGE followed by immunoblot analysis with an anti-LPL mAb to estimate the amount of bLPL bound. Maximal binding was shown to occur within 1 hour and approximately 2.9 U ± 5% of bLPL was shown to

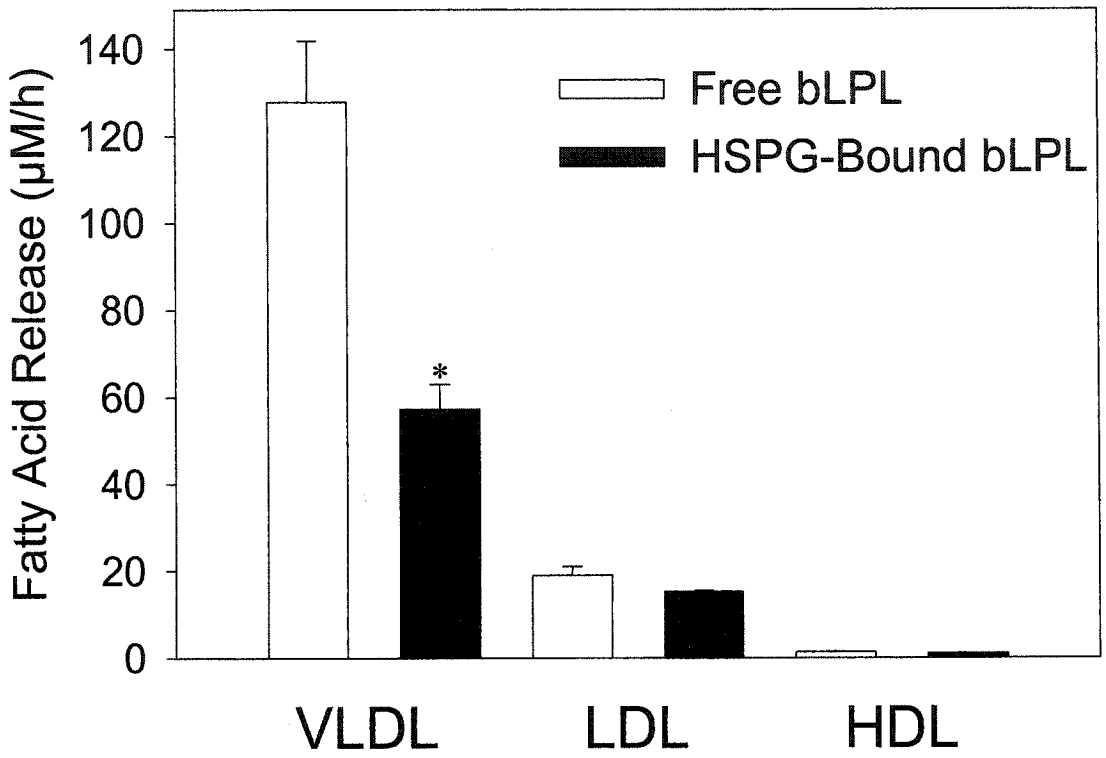

Figure 3.4.1 Hydrolysis of VLDL, LDL and HDL by HSPG-bound bLPL

Removawells were incubated with 5 µg HSPG for 2 hours at 24°C. HSPG-coated (solid bars) and -deficient (open bars) wells were washed 3 times with PBS and pre-incubated with 1% FAF-BSA in PBS overnight at 4°C. The HSPG-coated wells were incubated with 11 U of bLPL in PBS for 1 hour at 24°C and washed to remove any unbound bLPL. HSPG-deficient wells were incubated with 2.9 U of bLPL for 1 hour and then all Removawells were incubated with VLDL, LDL or HDL (350 µM TG) isolated by ultracentrifugation for 30 minutes at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean ± S.D. of triplicate determinations and are representative of two different experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Bonferroni Multiple Comparisons Test was performed comparing each lipoprotein sample incubated with the HSPG-bound bLPL to the corresponding sample incubated with free bLPL for statistical significance (*$p < 0.001$). Bars with no symbols are not statistically significant ($p > 0.05$).

associate with 5 μg HSPG (data not shown). All subsequent experiments were therefore carried out with a 1 hour association of bLPL with HSPG, followed by a brief wash to remove excess unbound bLPL.

To evaluate whether the association of bLPL to HSPG alters the catalytic activity of the enzyme, Removawells were coated with HSPG, pre-incubated with FAF-BSA, incubated with excess bLPL for 1 hour and washed. VLDL, LDL or HDL (350 μM TG) were added to the wells and incubated for 30 minutes at 37°C. As with the HL studies, high substrate (TG) concentrations were used to promote sufficient hydrolysis to allow for comparison of hydrolytic rates for the best (VLDL) and poorer (LDL and HDL) bLPL substrates (figure 3.4.1). Figure 3.4.1 shows that the association of bLPL with HSPG has an inhibitory effect on VLDL and LDL lipid hydrolysis, as compared to the control Removawells containing bLPL but no HSPG. Furthermore, since HDL is not a substrate for the enzyme, very little bLPL-mediated hydrolysis of HDL was obtained in either the presence or absence of HSPG. VLDL hydrolysis was inhibited by 55%, LDL by 20% and HDL by 5% when bLPL was associated with HSPG.

3.4.2 Displacement of bovine lipoprotein lipase from HSPG by lipoproteins

Since the binding of bLPL to HSPG only partially inhibited bLPL-mediated lipoprotein hydrolysis, this suggests that all the lipoproteins should be able to displace bLPL to some extent. We therefore investigated whether various lipoprotein classes also affected the binding/association of the enzyme with HSPG. Removawells were coated with HSPG, pre-incubated with FAF-BSA, incubated with excess bLPL for 1 hour, washed and then incubated with the different lipoproteins. After a 30 minute incubation, the supernatants were removed and the Removawells were washed with PBS. SDS sample buffer was added

and the eluants were analyzed by SDS-PAGE and immunoblotted with an anti-LPL mAb. The amount of bLPL that remained bound to HSPG after a 30 minute incubation with VLDL, LDL and HDL was estimated, relative to a control Removawell incubated with PBS. Figure 3.4.2, shows that VLDL, LDL and HDL displaced bLPL from pure HSPG. When either LDL or VLDL was incubated with HSPG-bound bLPL, > 75% of the LPL remained associated. HDL readily displaced bLPL and only approximately 5% of the enzyme remained bound after a 30 minute incubation. In contrast to the results obtained with HL, all lipoproteins were shown to displace some bLPL from the HSPG.

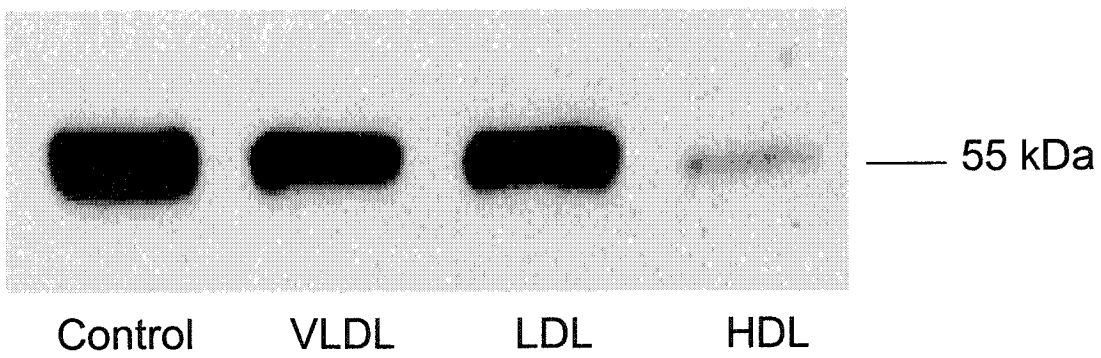

Figure 3.4.2 Effect of lipoproteins on the association of bLPL with HSPG

Removawells were coated with HSPG and bLPL (described in figure 3.4.1) and incubated with various plasma lipoproteins or PBS (control) for 30 minutes at 37°C. Wells were washed and the HSPG-bound proteins were eluted from the Removawells by incubating with 60 µl of SDS sample buffer at 37°C for 30 minutes. The eluants were electrophoresed on SDS-PAGE and electrotransferred to nitrocellulose. Samples were probed with an anti-LPL mAb and with an anti-mouse IgG HRP-linked secondary antibody. Apparent molecular mass determinations were derived from broad range molecular weight markers. Images are representative of triplicate determinations of two different experiments.

Similar experiments were next performed using a TG emulsion instead of lipoproteins. Results obtained show that the TG emulsion could not displace the bLPL from the HSPG (data not shown). Furthermore, this lack of displacement was associated with the absence of hydrolytic activity (data not shown). Consistent with the results obtained with HL, these results suggest that the displacement of bLPL is required for enzyme activity.

3.4.3 Effect of HDL and apoA-I on VLDL hydrolysis by bovine lipoprotein lipase

In section 3.3, we showed that HDL and apoA-I inhibited HL-mediated VLDL hydrolysis. To determine whether apoA-I and/or HDL can also directly affect the hydrolysis of VLDL by bLPL, standard hydrolytic assays, in the absence of HSPG, were performed. Figure 3.4.3 shows that, similar to what was observed with HL, increasing the concentration of HDL progressively inhibited the hydrolysis of VLDL by bLPL, to a maximum of 30%. However, although apoA-I was shown to have a greater inhibitory effect than HDL on HL-mediated VLDL hydrolysis, figure 3.4.3 shows that apoA-I had little effect on bLPL-mediated VLDL hydrolysis. These results suggest that HL and bLPL activity are regulated differently.

3.4.4 Effect of apoA-I on VLDL hydrolysis by hepatic lipase, bovine and human lipoprotein lipases

Although bLPL is commonly used to study LPL activity due to its availability, it is possible that the bLPL enzyme may be regulated differently than its human ortholog. To determine whether bLPL and hLPL behave differently, hLPL was purified from plasma. Next, VLDL hydrolysis by HL and these two enzymes were measured in the presence of increasing apoA-I concentrations. Figure 3.4.4 shows that increasing the concentration of

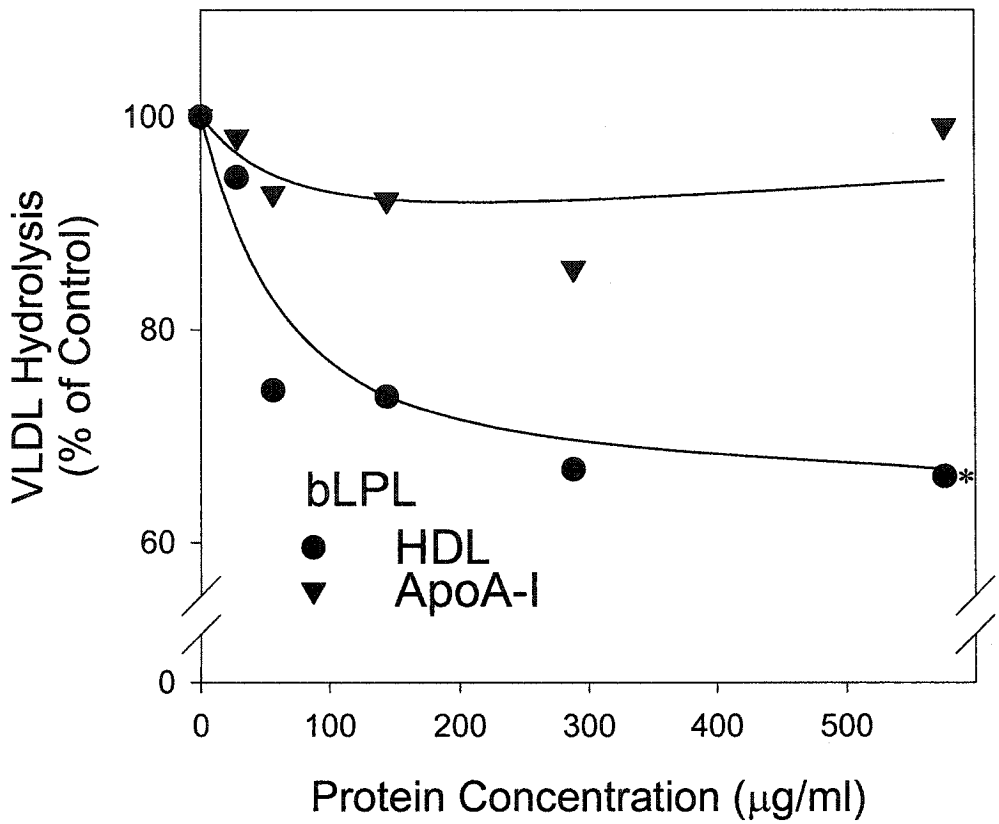

Figure 3.4.3 Effect of HDL and apoA-I on the hydrolysis of VLDL by bLPL

VLDL (350 μM TG) was incubated with LPL (2.9 U) and increasing amounts of HDL or apoA-I for 30 minutes at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean of triplicate determinations and representative of two experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest HDL or apoA-I concentration to the hydrolytic value obtained in the absence of HDL or apoA-I for statistical significance (*$p < 0.01$).

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apoA-I had different effects on the activities of the HL, bLPL and hLPL. Whereas apoA-I inhibited HL- and hLPL-mediated hydrolysis of VLDL, up to a maximum of 60% and 30% respectively, no such inhibition was apparent for bLPL. These results suggest that important regulatory differences exist between species and that bLPL is not an appropriate alternative for the human enzyme.

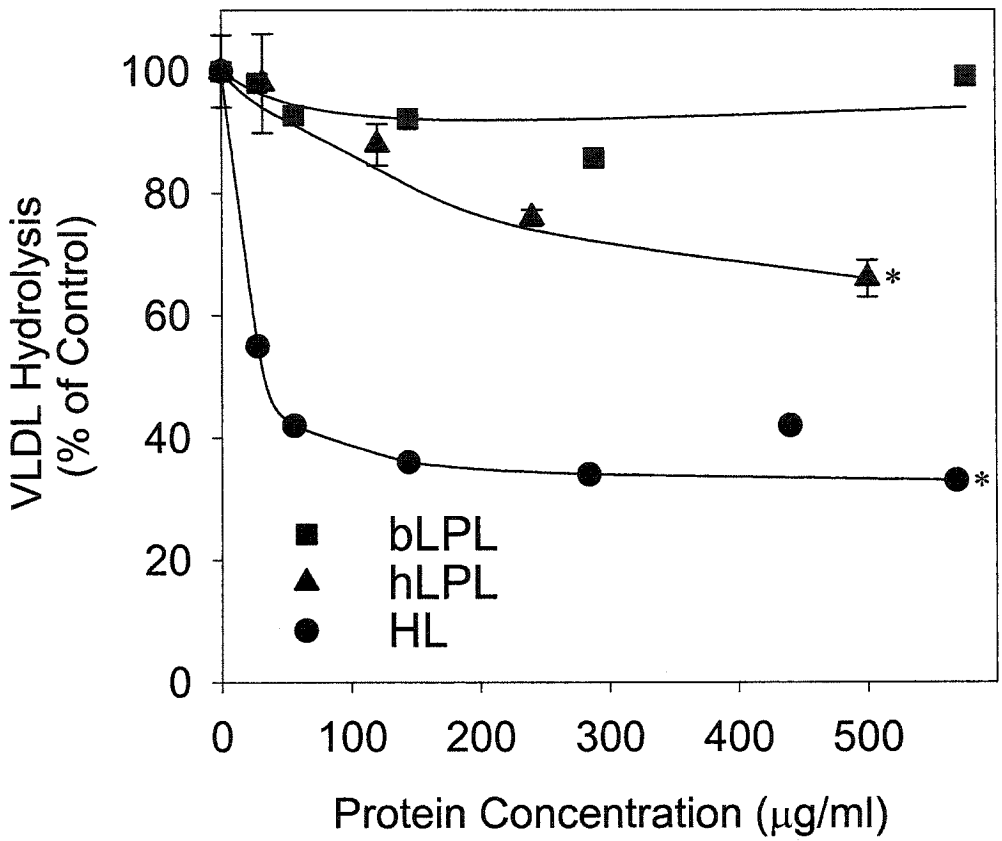

Figure 3.4.4 Effect of apoA-I on the hydrolysis of VLDL by HL, bLPL and hLPL

VLDL (350 μM TG) was incubated with increasing amounts of apoA-I and bLPL (2.9 U) or HL (26 U) for 30 minutes or hLPL (13.5 U) for 10 minutes at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean of triplicate determinations and representative of two experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest apoA-I concentration for each enzyme to the corresponding hydrolytic value obtained in the absence of apoA-I for statistical significance (*$p < 0.01$). Curves with no symbols at the highest concentration are not statistically significant ($p > 0.05$).

3.4.5 Effect of HDL_2, HDL_3 and apoA-I on VLDL hydrolysis by human lipoprotein lipase

To determine whether HDL could directly affect the hydrolysis of VLDL by hLPL as was shown with HL (figures 3.3.1 and 3.3.5), standard hydrolytic assays, in the absence of HSPG, were performed using purified hLPL, HDL_2 and HDL_3. Figure 3.4.5 shows that increasing the concentration of apoA-I inhibited the hydrolysis of VLDL, up to a maximum

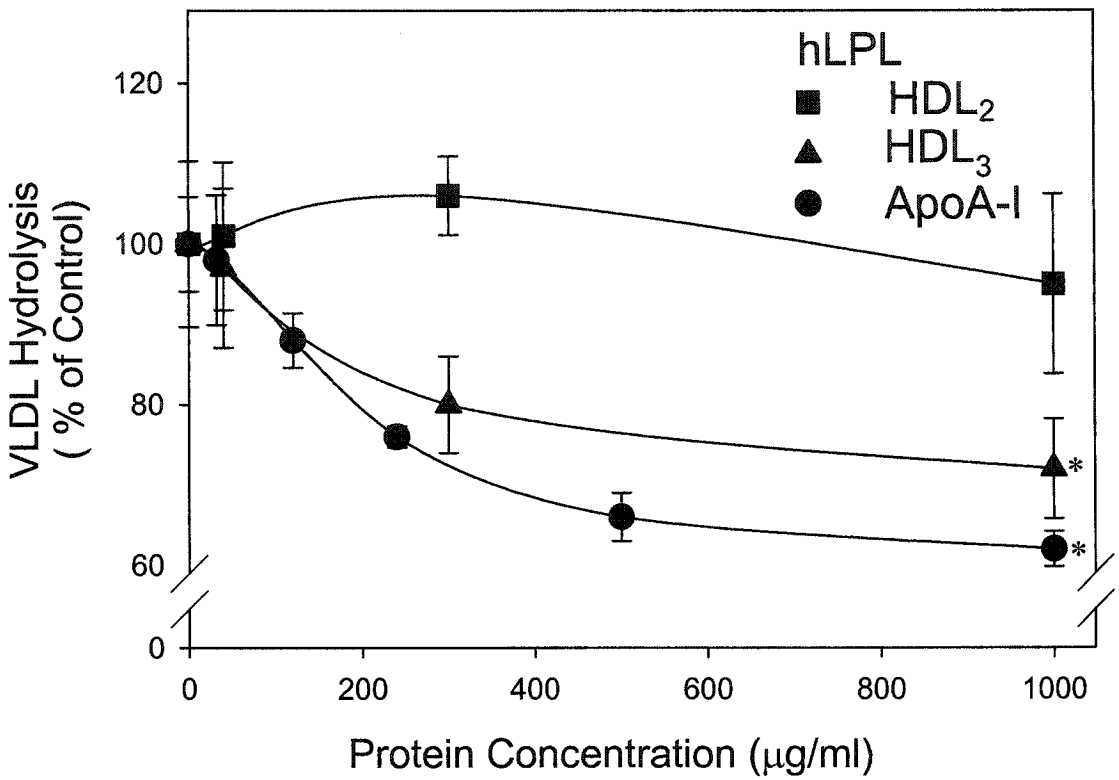

Figure 3.4.5 Effect of HDL_2, HDL_3 and apoA-I on the hydrolysis of VLDL by hLPL

VLDL (350 μM TG) was incubated with hLPL (13.5 U) and increasing amounts of HDL_2, HDL_3 or apoA-I for 10 minutes at 37°C. An aliquot was removed and fatty acid release was measured enzymatically. Hydrolytic values are the mean of triplicate determinations and representative of two experiments. A one-way ANOVA was performed and results were considered highly significant ($p < 0.0001$). Post-test analysis using the Dunnett Multiple Comparisons Test was performed comparing the hydrolytic values at the highest HDL_2, HDL_3 or apoA-I concentration to the hydrolytic value obtained in the absence of HDL_2, HDL_3 or apoA-I for statistical significance ($*p < 0.01$). Curves with no symbols at the highest concentration are not statistically significant ($p > 0.05$).

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of 40%. HDL_2 and HDL_3, on the other hand, differed in their abilities to regulate VLDL hydrolysis by hLPL. Whereas HDL_2 did not appear to have any effect, HDL_3 inhibited the hydrolysis of VLDL by 30%. Of note, it is clear that even though apoA-I and HDL subfractions can affect hLPL hydrolysis, the effects observed are much less than that observed for HL.

Chapter 4 – Discussion

4.1 Introduction

The majority of plasma LDL is thought to be produced by the combined actions of HL-mediated hydrolysis of VLDL and IDL and by the transfer/exchange of lipids and apolipoproteins with HDL (414, 415). The ability of HL to promote the production of potentially atherogenic lipoprotein particles has led to the view that increased HL activity may promote atherosclerosis (542, 543). This is consistent with observations from a number of laboratories that have identified an inverse relationship between HL activity and plasma HDL levels. Studies have shown that HDL_2 levels in the plasma are inversely related to post-heparin plasma HL activity (350, 544, 545) and to the risk of developing CAD (589). While several anti-atherogenic HDL functions have been proposed, some views have maintained that HDL levels are merely reflective of an efficient lipolytic system and through this link, a marker for the risk of developing CAD (590). The present work, however, suggests quite the opposite and shows that HDL and/or apoA-I levels may play an active role in regulating TG metabolism.

4.2 Hepatic lipase substrate specificity and patient studies

Independent of the lipoprotein class studies, HL activity is highly influenced by the nature of the lipids and the apolipoproteins present in the lipoprotein (discussed in sections 1.3.7 and 1.3.8). Although HL is often referred to as hepatic triglyceride lipase, the substrate specificity of the enzyme is broader as a number of studies have shown that HL can hydrolyze not only TG but DG, MG as well as various PL with varying preferences

(discussed in section 1.3.7). Therefore, this demonstrates that the concentration and type of lipids in the lipoprotein influence HL-mediated hydrolysis of lipoproteins.

Collet *et al.* found that traditional methods of measuring TG content misrepresented and actually overestimated the HDL-TG content by neglecting to consider contributions of DG and MG (492). Using gas-liquid chromatography, they found that DG is a major lipid constituent of HDL, which suggests that DG may play an important role in HDL metabolism. Our laboratory has obtained similar results (2.2:1, DG:TG for HDL_3) and further observed that the majority of the fatty acids released from normolipidemic HDL_3 were derived from PL and DG with only 1% of the fatty acids being derived from TG (figure 3.1.2 right pie chart). This suggests that DG is the preferred acylglyceride substrate in HDL for HL. Since Collet *et al.* also demonstrated that a significant proportion of the DG is located on the HDL surface (492), this, combined with our results, suggests that HL primarily acts as a surface lipid lipase, predominantly hydrolyzing the surface PL and DG and not the TG sequestered within the core of the HDL. Subsequent studies have since suggested that the DG content in HDL regulates HL-mediated hydrolysis of both PL and TG possibly by changing the structural organization of the surface lipids and therefore the structural properties of HDL. This, in turn, affects the ability of HL to interact with the interfacial surface of the lipoprotein and act as an acylglyceride lipase (360).

TG-enrichment of HDL has been observed in subjects with FCHL (591) and has been credited for stimulating HL-mediated lipolysis (592), which results in the reduced HDL-C levels often observed in these patients. Since traditional methods of measuring TG neglected to account for DG content (492), we measured the DG content of HDL and LDL derived from subjects with FCHL and control subjects matched for age and gender. Here, I demonstrated (albeit in a small sample size) that there was no statistically significant

elevation in the DG content of the LDL or HDL patient samples (figure 3.1.5). Yet, despite the lack of correlation between HL-mediated hydrolysis and DG content, it is likely that we would observe a shift from PL hydrolysis in the control samples to acylglyceride hydrolysis in patient samples. Using a reconstituted lipoprotein system, Coffill *et al.* have shown that TG-enrichment of Lp2A-I particles stimulates an increased rate of both TG and DG hydrolysis by HL at the expense of PL hydrolysis (360). Similarly, Azema *et al.* (508) also showed that TG-enrichment of HDL stimulated TG hydrolysis. Since the HDL from both hypertriglyceridemic and combined hyperlipidemic patients are enriched in TG (496), this compositional modification may partially account for the increased HL hydrolytic rates observed in incubations with HDL from these patients.

FCHL is a common inherited dyslipidemia afflicting 1-2% of the general population. Although some studies found significantly elevated HL activities in these patients, none have attempted to characterize the ability of lipoproteins from these individuals to act as substrates for the enzyme. In a small cohort of French Canadian FCHL families, subjects with FCHL recruited for our study presented with decreased HDL-C levels, primarily in the HDL_2 density range. Previous reports concerning HDL-C levels in FCHL patients have been inconsistent. For example, Brunzell *et al.* observed comparable levels of HDL-C in subjects affected with FCHL and control subjects (593), while others have observed decreased levels of HDL_2-C in affected individuals (591, 594). The causative factor(s) for the decrease in HDL-C in some FCHL patients has yet to be elucidated. However, previous studies have suggested that the observed reduction in HDL-C was not due to a defect in the secretion or catabolism of apoA-I or apoA-II since the levels of these apolipoproteins did not differ significantly between patient and control groups (591, 593). Since there is increased residence time for TG-rich lipoproteins in the circulation of individuals with FCHL, it has

been suggested that TG-enrichment of HDL via the actions of CETP produces lipoproteins that are hydrolyzed by HL at a higher rate (592). Consequently, the increased lipolysis of these TG-rich HDL by HL reduces the levels of HDL-C. Similar to the work of Soro *et al.* (591), our study also observed TG-enrichment of HDL, although the increase was not statistically significant. However, total hydrolytic values suggested that the various lipoprotein classes isolated from normal subjects were equally good substrates as the corresponding lipoproteins from patients with FCHL despite the TG-enrichment of HDL (figure 3.1.3). However, in a detailed analysis of PL versus acylglyceride (TG, DG and MG) hydrolysis, differences in substrate preferences were present. Figure 3.1.4 shows that HL-mediated hydrolysis of PL and acylglyceride was comparable for control HDL in the HDL_2 density range whereas in the HDL_3 density range, acylglyceride hydrolysis was preferred. In contrast, acylglyceride hydrolysis predominated with all HDL classes from patients with FCHL. This is an interesting finding and suggests that HDL composition and hence structure plays a role in regulating the activity of the enzyme.

This study suggests that the HDL composition can be a determining factor in deciding whether HL will function as a phospholipase or an acylglyceride lipase. However, abnormal lipoprotein composition does not appear to affect the overall hydrolysis of the various lipoprotein fractions (figure 3.1.3). This suggests that structural changes to the lipoproteins, which are a result of abnormal lipoprotein composition, may play a role in the interlipoprotein regulation of HL-mediated hydrolysis.

4.3 HDL regulates hepatic lipase activity

The studies of Ehnholm *et al.* (512), pointed to the regulatory effect of serum on HL activity. Whereas HL activity is slightly stimulated by the addition of serum at low

concentrations, it is progressively inhibited at higher concentrations. These observations are also consistent with those of Kubo *et al.* (515), which showed that HL inhibition was mediated by HDL, in addition to the $\rho = 1.21$ g/ml bottom fraction of plasma. These data are consistent with our findings (figure 3.3.4), which show that the progressive addition of HDL (0-100% of the original amount of HDL in the plasma) to apoB-containing lipoproteins (equivalent to its plasma concentration in the absence of the $\rho > 1.21$ g/ml fraction) progressively inhibited the activity of exogenously added HL to a maximum of 80% whereas the addition of whole plasma completely inhibited the activity of the enzyme. Cheung *et al.* (518) also obtained similar results and subsequently identified the inhibitory component of the lipoprotein-free fraction ($\rho > 1.21$ g/ml) as an apoA-I containing particle of pre-ß-electrophoretic mobility and minimal lipid content (pre-ß-HDL). In addition, they found that the greater inhibition of lipase activity by the uremic serum compared to control serum was due to an increased concentration of this particle in the non-lipoprotein fractions as well as an increased inhibition by uremic lipoproteins (518). The elevated levels of pre-ß-HDL in chronic renal failure has previously been observed (519, 520) and appears to be a direct result of renal impairment of the uptake of these particles (519).

While loosely-bound apoA-I appears to indirectly affect the actions of HL by displacing it, the protein also has a direct affect on lipid hydrolysis by HL. The addition of purified apoA-I or HDL to incubations of HL and VLDL in the absence of HSPG significantly inhibited lipid hydrolysis (figures 3.3.1 and 3.3.2). This is consistent with the work of Kubo *et al.* (515), which showed HL inhibition by HDL, and the work of Zhong *et al.* (87), which observed that murine HDL from non-transgenic and a variety of transgenic mice inhibited the hydrolysis of a TG emulsion by murine HL. This latter study further demonstrated that the HDL containing apoA-II were more inhibitory to the enzyme than

those devoid of this apolipoprotein. Similarly, Mowri *et al.* (529) showed that HDL_3 but not HDL_2 could inhibit HL-mediated hydrolysis of VLDL triglycerides. In contrast, they found that the presence of apoA-II in HDL subpopulations stimulated HDL hydrolysis and inhibited VLDL hydrolysis to a greater extent than HDL devoid of apoA-II. Immunoradiometric analysis further suggested that the partitioning of the enzyme between VLDL and HDL was not influenced by the density of the lipoprotein but rather by the apoA-II content of the HDL. The authors concluded that HDL_2 and HDL_3 have similar binding affinities for the enzyme but differ in their efficiency of lipolysis. This is consistent with the work of Hime *et al.*, which also showed a reduced HL affinity for apoA-I enriched HDL, relative to apoA-II containing particles (531). However, this latter report also showed that the apoA-II HDL were substantially poorer substrates for HL than apoA-I HDL and promoted significantly reduced rates of hydrolysis of both TG and PL. Their data suggest that apoA-II may also be an inhibitor of HL and this agrees with the view of Plump *et al.* that the mild hypertriglyceridemia in apoA-I knock-out mice may be partially due to the apoA-II-enriched HDL being poorer substrates for HL (595). However, it appears unlikely that apoA-II would have contributed significantly to the HL inhibitory capacity of HDL on VLDL hydrolysis, since in our experiments, at low inhibitor concentrations, apoA-I was more effective than HDL (containing both apoA-I and apoA-II) at inhibiting HL, but at higher concentrations both apoA-I and HDL were very similar in their abilities to inhibit this enzyme (figure 3.3.2).

In our study, the larger HDL fractions also appeared to directly stimulate VLDL-TG hydrolysis by HL (figure 3.3.5). Therefore, the well-described inverse relationship between HDL_2 and postprandial lipemia may be due to both an enhanced liberation of HL into the plasma compartment and a direct stimulation in TG hydrolysis. Conversely, the smaller

HDL fractions were much less able to liberate HL and actually inhibited HL activity. While it is known that the apoCs readily transfer between HDL and VLDL during lipolysis (596, 597), differences do not appear to be due to unique apolipoprotein effects as both stimulation and inhibition of HL could be demonstrated with different kinds of rHDL containing only apoA-I. Of note, the lipid poor rHDL stimulated HL to a greater extent than the PL enriched particles (figure 3.3.7). The most stimulatory of the recombinant particles was the 5:1 PC:apoA-I particle, a complex that was previously shown to also be highly reactive with LCAT and similar in structure and charge to the pre-β-HDL subfractions described by others (598). Previous studies by Barrans *et al.* and Guendouzi *et al.* have shown that HL has the ability to generate pre-β-HDL from TG-enriched HDL_2 (599, 600). HDL_2 may therefore stimulate HL by generating this novel substrate that catalyzes TG hydrolysis.

It is less clear how the smaller HDL particles act to inhibit HL-mediated hydrolysis of VLDL. We had initially thought that this inhibition may be due to the transfer of apoA-I from the HDL fraction to VLDL, however, this does not appear the case as HDL_2 had a greater propensity to transfer apoA-I to VLDL than did the smaller HDL classes (figure 3.3.6). Since inhibition of VLDL hydrolysis by HL did not appear to be a function of apoA-I association, it suggests that the different classes of HDL may be affecting VLDL hydrolysis through a regulation of interfacial association. Our data suggest that HL preferentially binds to HDL over VLDL and that inhibition may be associated with a very high affinity binding and impaired interlipoprotein shuttling of HL. Both crosslinking and apoA-I immunoprecipitation studies suggested that HL associated with HDL particles and that this association was unaffected by the presence or absence of VLDL (Ramsamy *et al.*, unpublished observation). It therefore appears that with the smaller HDL particles, high

affinity HL association may affect interlipoprotein movement of the enzyme and thereby inhibit VLDL hydrolysis.

The larger HDL particles may bind HL with a lower affinity and as with apoA-I, HL may more easily dissociate from this lipoprotein. In addition, the pre-β-like-HDL, potentially generated from the HDL_2 particle, may have a lower binding affinity for HL and allow a greater interlipoprotein shuttling with VLDL. This is consistent with a previous study, which showed that stimulation in LCAT reactivity with the small pre-β-HDL particles was also associated with a reduced binding affinity for the particles (598). This previous work has suggested that binding affinity is related to lipoprotein electrostatic properties and therefore it may be that HDL charge affects VLDL hydrolysis through a modulation of HL binding affinity. Pre-β-HDL is, by definition, more positively charged than the alpha migrating classes. Our laboratory has shown that lipidation of pre-β-rHDL increases the negative charge on the lipoprotein particle (598). In this study, lipidation of the rHDL inhibited HL. In addition, it is also well established that smaller native HDL particles are progressively more negatively charged than the larger HDL classes (601) and we now show that they are also more inhibitory to HL. Taken together, there appears to be an inverse relationship between HDL charge and HL activity, which suggests that HDL particle charge influences the VLDL hydrolytic activity of HL.

4.4 HDL and apoA-I regulate the displacement of hepatic lipase

HL is anchored by HSPG to the lumenal and sublumenal surfaces of liver sinusoidal endothelial cells, on the external surfaces of hepatocyte microvilli located in the space of Disse and in the interhepatocyte space (362). It is bound preferentially to glycosaminoglycans, which are long, unbranched, highly charged carbohydrate side chains

composed of repeating disaccharide subunits (described in detailed in section 1.2.10) (602). Although human HL is primarily found anchored to cell surfaces, most of the studies to date have evaluated the hydrolytic activity of this enzyme when free in solution. Early studies (603-605) identified HL as a triglyceride lipase activity that was not detectable in regular plasma samples but was present in post-heparin plasma, heparin perfusates of liver and in heparin-treated rat liver plasma membranes. Waite *et al.* (366) later reported a change in the substrate specificity of HL after it was released by heparin and suggested that the function/activity of an HSPG-bound enzyme may differ. Similar investigations with LPL suggested that HSPG association may have inhibitory effects on this enzyme. Posner *et al.* (536) and Clark *et al.* (537) immobilized LPL onto heparin sepharose and found significant reductions in Michaelis-Menten constants (K_m) and maximum velocities (V_{max}) for the heparin-bound enzyme when compared to LPL in solution. More recently, de Man *et al.* demonstrated that the binding of LPL to HSPG also partially inhibited the lipolysis of VLDL (540). These findings have been indirectly confirmed *in vivo* in studies showing significantly increased rates of TG hydrolysis when HL and LPL are released from cell surface HSPG by heparin administration (572, 606). Consistent with these observations, our data show that VLDL hydrolysis by HL or by LPL was also significantly inhibited when HL or LPL were bound to HSPG (figures 3.2.1 and 3.4.1). These data suggest that lipolytic activities of these enzymes are impaired when HSPG-bound. Efficient lipolysis therefore requires the displacement of these lipases from the cell surface matrix and the factors that regulate displacement are critical to achieve efficient lipolysis.

In the study of Posner *et al.*, the authors concluded that the lower V_{max} values obtained for heparin sepharose-bound LPL might have been partly due to a reduced accessibility of VLDL for the bound LPL (536). In the present study, we observed

significantly decreased hydrolysis of both LDL and VLDL when HL was bound to HSPG (figure 3.2.1). This reduced hydrolytic activity observed for the HSPG-bound HL supports the view that lipoproteins are unable to gain access to the HSPG-bound enzyme. This hypothesis is further confirmed by the observation that neither LDL nor VLDL could significantly displace HL from the proteoglycans (figure 3.2.2). These data show that the HSPG-bound HL is inactive and suggests that hydrolysis of the larger TG-enriched lipoprotein substrates requires the dissociation of HL (and perhaps LPL (figure 3.4.2)) from cell surface HSPG and helps explain the increased rates of lipid hydrolysis evident in post-heparin plasma samples (572, 606).

On the other hand, this work shows that association of HL with HSPG has much less effect on the hydrolysis of HDL lipids by HL (figure 3.2.1). This reduced inhibition of the catalytic activity of HL suggests that either the HSPG-bound enzyme is readily accessible to the smaller lipoproteins or that some component of HDL may act to liberate HL and stimulate its activity. All lines of evidence support this latter perspective. With all lipoproteins studied, the rate of hydrolysis in the presence of HSPG closely correlated to the capacity of the lipoprotein to displace HL from the HSPG. Minimal displacement by LDL and VLDL paralleled maximal inhibition, while 60% displacement by HDL was associated with only a 40% inhibition of HL activity (figures 3.2.1 and 3.2.2). HDL preparations from different subjects sometimes exhibited a greater ability to displace HL, and in these instances, less inhibition was observed. These data show that some component/constituent of HDL, such as apoA-I, can directly displace the bound HL. ApoA-I appears to mediate HL liberation from the HSPG by binding to sites on the proteoglycan and dislodging the bound HL or by directly binding to and displacing HL. Purified apoA-I was in fact more effective than heparin (data not shown) and some HDL fractions at HL displacement when normalized

to protein content (figure 3.2.3), which suggests that only a fraction of HDL-apoA-I is functional in this respect. This may indicate that HL displacement from HSPG is facilitated by a more exchangeable/loosely-bound fraction of apoA-I on HDL (figure 4.1, panel A). Several earlier studies have shown that a loosely-bound fraction of apoA-I does indeed exist on HDL particles (598, 607) and higher levels have been identified in hypertriglyceridemic subjects (522). Since co-incubations of the HSPG-bound HL with VLDL in the presence of either apoA-I or HDL inhibited HL displacement, it appears that VLDL may compete with HSPG and preferentially bind this loosely-bound apoA-I fraction or that VLDL may sterically hinder HL displacement by HDL or apoA-I (figure 3.2.8).

Studies in this laboratory have shown that VLDL from different subjects also vary in their abilities to displace HL from HSPG and be hydrolyzed by this enzyme. As shown in figure 3.2.2, a small amount of HL displacement by VLDL also coincided with the association of endogenous apoA-I on the HSPG. ApoA-I has been well described to be a constituent of VLDL (608-610) and these data suggest that apoA-I associated with VLDL particles may play a role in regulating HL displacement and lipid hydrolysis. Some data actually suggest that apoA-I may be a substantial component of VLDL (609), but that its contribution to VLDL protein may be underestimated by its highly exchangeable and dissociable nature during ultracentrifugation (608). In unpublished work, we too have shown that significant amounts of apoA-I were pelleted when VLDL was washed by ultracentrifugation. Therefore, substantial amounts of loosely-bound apoA-I may also exist on VLDL (and possibly LDL) and this component may control the displacement of HL from HSPG and indirectly affect the remodeling of these lipoproteins.

The use of pure proteoglycans to study the displacement of HL and the effect of HSPG association on hydrolysis is a convenient system that eliminates the uncertainty

associated with variables in more complicated *in vivo* systems. Although control studies suggested that HL did not bind to the FAF-BSA-coated Removawells, we could not eliminate the possibility that other artifactual interactions may have contributed to the displacement or inhibition of the enzyme. We also attempted to reproduce the results using a cell-derived ECM, however, this system proved problematic since treatment of the cells with the various reagents required to produce an ECM, such as TritonX-100 and Cytochalasin B, generated highly variable results. In order to extend this study to a more physiologically relevant system we opted to evaluate the factors that regulate HL displacement in two different cell lines; HepG2 cells that endogenously express HL and a CHO cell line stably overexpressing human HL. We chose to study displacement in a CHO cell culture model in which human HL was under control of a constitutively active promoter (cytomegalovirus) to circumvent any possible gene regulatory affects of lipoproteins or apoA-I and consequential changes in the production and secretion of the expressed protein. Western blot analysis showed that the CHO-hHL (figure 3.2.4) and HepG2 (data not shown) cell extracts exhibited two immunoreactive HL proteins, one that represents mature HL and is associated with the cell surface matrix and a second lower molecular mass intracellular species (figure 3.2.4). This smaller intracellular HL protein is likely to be a less glycosylated form of the enzyme similar to that observed by others (378).

Using the CHO-hHL cell culture model, we showed that both apoA-I and HDL readily displaced cell surface HL and that, as with the pure HSPG displacement studies, apoA-I was > 2-fold more able to disassociate cell surface HL than was HDL (figure 3.2.5 panel A-C). Experiments in HepG2 cells produced almost identical results (figure 3.2.5 panel D), which suggests that HDL predominantly acts by displacement of the surface enzyme and may have less effect on the regulation of the HL gene. Displacement of cell

surface HL was further shown to be concomitant with the liberation of an active enzyme and increased lipid hydrolysis (figure 3.3.3). Furthermore, HL displacement paralleled decreases in intracellular HL levels. This implies that apoA-I-mediated HL displacement from the cell surface is more rapid than its replenishment by the newly synthesized and secreted HL. It also suggests that the displaced HL remains tightly bound to components (lipoproteins) in the medium and does not re-associate with the cell surface.

We showed that the lowest density HDL fractions have the greatest capacity to remove HL from both the cell surface and intracellular compartments (figure 3.2.6). It is striking that almost the entire cell surface and a significant amount of the intracellular HL was removed from the CHO-hHL cell line after a 1 hour incubation with an HDL_2 fraction. The depletion appeared to be an acute phenomenon, as both intracellular and cell surface HL were replenished after extended incubations (data not shown). In contrast to HDL_2, the smaller, higher density HDL fractions were much less able to displace HL. Since HL was readily displaced by lipid-free apoA-I, the ability of HDL to displace HL from the cell surface may be a function of the amount of loosely-bound or exchangeable apoA-I on the surface of the larger HDL_2 particles. It has been previously reported that HDL_2 may allow for a greater dissociation of lipid-poor apoA-I than the smaller HDL particles (599, 600) and this is consistent with our VLDL-apoA-I association studies, which showed that more apoA-I can be transferred to the VLDL surface from large and less dense HDL fractions than from the smaller particles (figure 3.3.6). These data suggest that higher levels of HDL_2 may promote a greater displacement of cell surface HL and enhanced TG hydrolysis by HL (figure 4.1). This appears consistent with a number of studies that have shown that there is an inverse relationship between the magnitude of postprandial lipemia and plasma levels of HDL_2 (496,611). However, the data does not appear consistent with reports that have

identified an inverse relationship between plasma HDL_2 levels and post-heparin HL activities (612, 613). However, with the new view that HDL acts like heparin and displaces cell surface HL, it follows that if HDL_2 levels are high and HL is readily displaced from cell surface HSPG, lower amounts of HL would be liberated by heparin and in turn may result in reduced post-heparin activities. This may suggest that post-heparin HL activities actually reflect an inactive pool of cell surface-bound HL.

The displacement studies presented, using both the pure proteoglycan and the cell culture systems, support the hypothesis that HDL and apoA-I displace HL from HSPG and the surface of the cell and further suggest that this displacement would/should "activate" the enzyme. Yet, the addition of purified apoA-I or HDL to incubations of VLDL and HSPG-bound HL resulted in "retained inhibition" of the enzyme (figure 3.2.7). Western blot analysis suggested that this was partly due to the reduced ability of HDL and apoA-I to displace HL from the pure HSPG when co-incubated in the presence of VLDL (figure 3.2.8). However, despite co-incubations with VLDL, some displacement was still achieved by HDL and apoA-I in the absence of any HL stimulation. This confirms the view that HDL and apoA-I have direct regulatory effects on HL-mediated hydrolysis and suggests that the net hydrolytic activity will depend both on the ability of the HDL pool to displace/activate HL and on the ability of the VLDL pool to modulate this event.

These studies also show that the hydrolysis of TG-enriched lipoproteins requires the displacement of HL from cell surface HSPG. It appears that this phenomenon may have significant consequences on lipid metabolism *in vivo*. It is conceivable that if the abilities of different lipoproteins to displace lipolytic enzymes affects the plasma clearance of TG, this pathway may play an important role in regulating the duration and magnitude of the postprandial response. It is now well established that efficient TG hydrolysis and clearance

from the blood stream is directly related to the amount and kinds of HDL in the plasma. My current work helps explain some of these poorly understood relationships between HDL and TG metabolism. The well described inverse relationship between HDL_2 and postprandial lipemia may in fact be a direct relationship resulting from enhanced liberation of HL into the plasma compartment by this lipoprotein (figure 4.1 panel A) and a direct stimulation of VLDL-TG hydrolysis (figure 4.1 panel B). Conversely, low HDL_2 and high levels of small HDL particles in the blood would be expected to reduce the displacement of cell surface HL and to directly inhibit the enzyme. This helps explain the link between low HDL_2 levels and high post-heparin HL activity measurements and impaired VLDL hydrolysis consistent with observations in numerous laboratories (611-613).

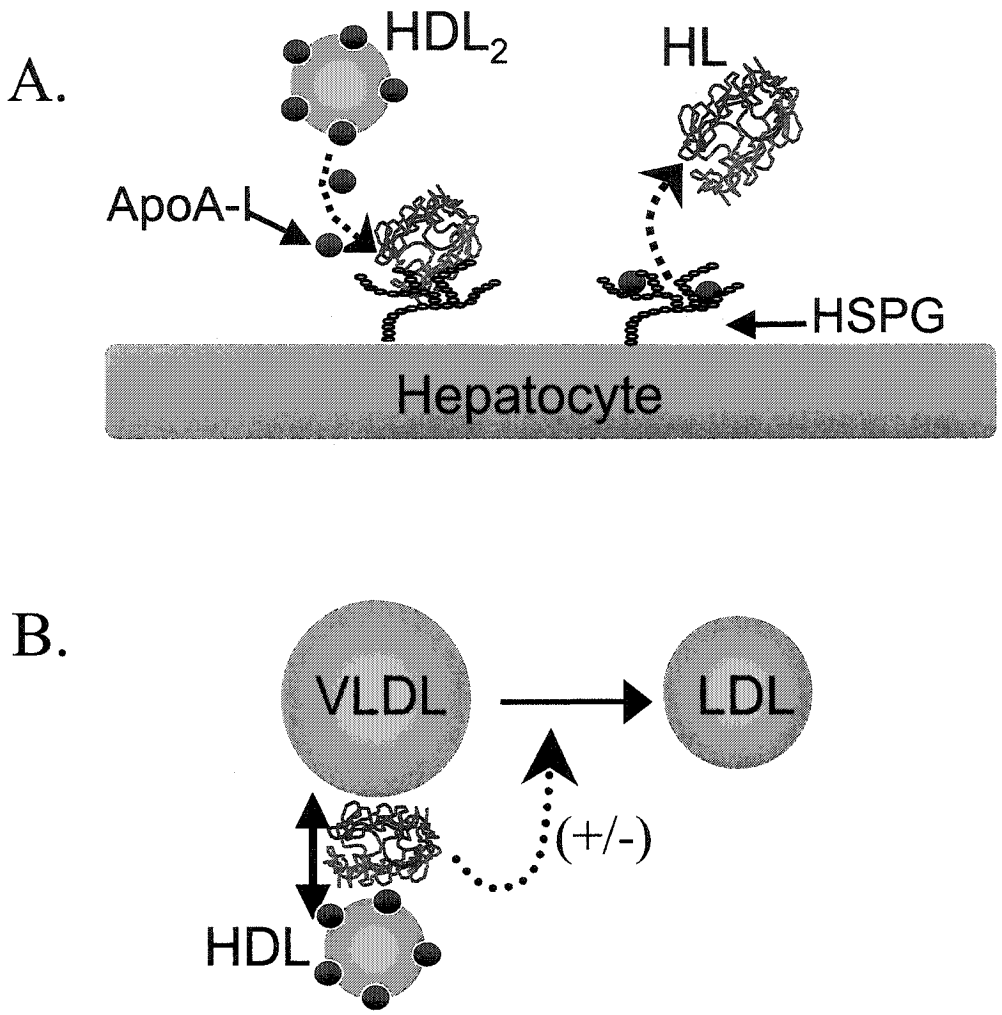

Figure 4.1 Effect of HDL on the displacement and catalytic activity of HL

ApoA-I molecules on the more buoyant HDL are less tightly bound and can become dissociated from the lipoprotein particle. These dissociated apoA-I molecules bind to cell surface HSPG and cause the displacement of cell surface-bound HL (panel A). The activity of HL is then dependent on the kinds of HDL in the plasma. The smaller HDL_3 and lipid-free apoA-I are inhibitory to HL, while the more buoyant HDL_2 stimulate VLDL-TG hydrolysis (panel B).

4.5 The regulation of lipoprotein lipase activity

Studies with LPL suggest that the regulation of the activity of this enzyme shares some similarities with HL. In our work with HL, we showed that apoA-I and HDL affect this enzyme by liberating the inactive HL from pure HSPG and by directly inhibiting/stimulating lipid hydrolysis by HL. In agreement with results that were previously reported by de Man *et al.* (540), our preliminary results using bLPL suggest that the enzyme is only partially inhibited when HSPG-bound (figure 3.4.1). However, our results further showed that all lipoproteins studies (VLDL, LDL and HDL) liberated the enzyme to some degree (figure 3.4.2). VLDL and LDL displaced 25% of the bLPL, while HDL displaced almost all of the enzyme from HSPG. Combined, these results suggest that the hydrolysis observed in the presence of the HSPG-bound bLPL may be related to the ability of all the lipoproteins to displace bLPL from HSPG. This was confirmed using a TG emulsion, which showed that the inability of the emulsion to displace bLPL from HSPG is associated with the absence of enzyme activity.

ApoA-I and HDL affect bLPL activity differently than HL activity. HDL was shown to inhibit HL and bLPL-mediated lipolysis, but while apoA-I profoundly inhibited HL activity, the apolipoprotein had no effect on bLPL activity (figures 3.3.1 and 3.4.3). In contrast, the effects of apoA-I and HDL on hLPL paralleled the results obtained with HL (figures 3.3.1 and 3.4.5). The observation that hLPL and bLPL differed in their abilities to be regulated by apoA-I and HDL (figures 3.4.3, 3.4.4. and 3.4.5) suggests that interspecies differences exist. Species differences in LPL activity have been previously noted by a number of investigators (614-617). Furthermore, in a comparison of LPL derived from various species, Raisonnier *et al.* (618) observed that although the main domains (catalytic,

N-glycosylation and putative heparin binding sites) are well conserved between species, exon 10 contains species-characteristic deletions, insertions or elements rich in A or A + T, which the authors suggest may impact on the activity of the enzyme derived from various species.

The results presented suggest that efficient lipolysis appears to require the displacement of HL and LPL from the cell surface HSPG. Indeed, a number of reports have already indirectly confirmed this *in vivo*. Significantly increased rates of TG hydrolysis *in vivo* were routinely observed when HL and LPL were released from the endothelium cell surface HSPG by administration of heparin (572, 606). Our results therefore imply that the factors (HDL and apoA-I) that regulate the displacement of these enzymes (HL and LPL) are an essential requirement for efficient lipolysis.

4.6 Concluding remarks and future experiments

HL is a key enzyme in determining the plasma concentration of LDL and HDL. These, in turn, play a significant role in the development of atherosclerosis either by contributing or impeding the progression of the disease. Consequently, knowledge of the factors that regulate the activity of this enzyme is central in obtaining a better understanding of how HDL and LDL levels are regulated and hence how they contribute to this disease.

Although the studies in this thesis presented a novel role for HDL and apoA-I in the regulation of HL activity via displacement and inhibition of the enzyme, much work has yet to be done to gain a complete understanding of the factors involved in the regulation of HL activity. Although I proposed that the electrical properties of HDL may play a role in regulating the activity of the enzyme, this has yet to be confirmed. More detailed analysis, using reconstituted HDL particles as a model system, is presently underway to determine if

modifying HDL charge can alter its ability to stimulate or inhibit HL activity and its effects on displacement.

While my work demonstrated that HDL and apoA-I could displace HL from pure HSPG, the displacement/activation effects of different apolipoproteins on the cell surface-bound HL and the resulting consequences on hydrolysis have yet to be established. In addition, further *in vitro* experiments are required to characterize the effect of abnormal lipoprotein composition on the displacement of HL from HSPG and on lipolytic activation or inhibition on HL. Recent studies have suggested that HDL from different subjects vary considerably in their abilities to modulate this enzyme and therefore the ratio of HDL_2/HDL_3 may regulate lipid hydrolysis by HL. Studies are presently underway to characterize the significance of this in patients.

The importance of the factors that promote HL-cell surface displacement/activation on TG metabolism *in vivo* is also an avenue that should be explored. ApoA-I-knock-out mice have been shown to be hypertriglyceridemic, however, the cause of this hyperlipidemia has never been resolved. *In vivo* experiments can be designed to determine how apoA-I deficiency affects the regulation of plasma lipolytic activity and how apoA-I may affect HL synthesis and catabolism. In addition, studies involving the administration of HDL into humans would help elucidate the *in vivo* consequences of HDL administrations on human HL activity.

In addition to its role as a catalytic enzyme, HL has been postulated to function as a ligand in the uptake of lipoproteins by the liver. Further investigations should focus on the consequences of apoA-I-mediated HL displacement on the binding and uptake of lipoprotein particles by HL possibly in CHO cells expressing HL or in hepatocytes.

As shown in section 3.4, preliminary studies with LPL suggest that the regulation of this enzyme is similar to HL albeit to a lesser extent. Studies are presently underway to determine if HDL and apoA-I, in addition to the effect of charge on the displacement/inhibition of this enzyme, regulate hLPL expressed in a Chinese Hamster Lung cell line in a similar manner.

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Contribution of collaborators

Section 3.1 Hepatic lipase substrate specificity and patient studies

A number of people have contributed to the work presented in section 3.1 including Cynthia Coffill, Tracey Neville, Dr. Sylvie Braschi, Dr. Xavier Collet and Dr. Daniel Godet. Cynthia Coffill was the primary author on the work that focused on the effects of DG on HL-mediated lipolysis. Dr. Daniel Godet recruited and obtained plasma samples from patients with FCHL and matched controls. Dr. Xavier Collet determined the DG and TG content of HDL fractions derived from patient and control samples using gas-liquid chromatography. Dr. Sylvie Braschi assisted with the compositional analysis of lipoprotein fractions. Tracey Neville assisted with the compositional analysis, HPTLC and HL assays of lipoprotein fractions.

Section 3.2 ApoA-I and HDL regulate the displacement of hepatic lipase

Robert Brown created the CHO-hHL cell line used throughout this section. Tracey Neville, Jonathan Boucher, Bobby Chauhan and Dhiraj Aggawal assisted in the development of the systems and the experimental work presented in this section.

Section 3.3 HDL regulates hepatic lipase activity

Tracey Neville, Jonathan Boucher and Bobby Chauhan assisted in the experimental work presented in this section.

Section 3.4 Displacement and regulation of lipoprotein lipase activity

Bobby Chauhan and Jonathan Boucher assisted in much of the developmental and preliminary work presented. Jonathan Boucher has since taken over the project.

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1. Canadian Institutes of Health Research/Heart and Stroke Foundation of Canada Combined Doctoral Scholarship: Jul. 2000, Jul. 2001 and Jul. 2002.

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3. 1st place Biochemistry Ph.D. Best Poster Presentation Apr. 2001. Title: Apolipoprotein A-I regulates lipid hydrolysis by hepatic lipase.

4. Natural Sciences and Engineering Research Council of Canada Ph.D. Post Graduate Scholarship: Sept. 1999.

5. Ontario Graduate Scholarship: Sept. 1998, Sept. 1999 declined.

6. University of Ottawa Excellence Scholarship: Sept. 1998, Sept. 1999, Sept. 2000, Sept. 2001 and Sept. 2002.

7. Cum Laude citation upon graduating with Honours B.Sc.

8. Inscription to the Dean's Honour list during the 1995-1996 academic year.

9. Finalist in the biochemistry research presentation day Apr. 1996. Title: The role of HDL structure and stability in the regulation of hepatic lipase.

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MANUSCRIPTS PUBLISHED OR ACCEPTED IN REFEREED JOURNALS:

Brown R.J., Schultz J.R., Ko K.W., Hill J.S., **Ramsamy T.A.,** White A.L., Sparks D.L. and Yao Z. The amino acid sequences of the carboxyl termini of human and mouse hepatic lipase influence cell surface association. Journal of Lipid Research 2003 44(7):1306-14.

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T.A. Ramsamy, and D.L. Sparks. Apolipoprotein A-I Regulates Lipid Hydrolysis by Hepatic Lipase. Canadian Lipoprotein Conference, Digby, Nova Scotia, Oct. 2001.

T.A. Ramsamy, T. A.-M. Neville, B.M. Chauhan, Aggarwal D. and D.L. Sparks. Apolipoprotein A-I Regulates Lipid Hydrolysis by Hepatic Lipase. Canadian Federation of Biological Sciences, Ottawa, Ontario, Jun. 2000.

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CONFERENCES – ORAL PRESENTATIONS:

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