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Naghmeh Rouhani
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc.(Biochemistry)
GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

High-Density Lipoproteins Regulate Displacement of Hepatic Lipase Activity

TITRE DE LA THÈSE / TITLE OF THESIS

Daniel Sparks
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

M-E. Harper

R. Milne

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

HIGH DENSITY LIPOPROTEINS REGULATE THE DISPLACEMENT OF HEPATIC LIPASE ACTIVITY

Naghmeh Rouhani

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Abstract

High density lipoproteins regulate the displacement of hepatic lipase activity

By Naghmeh Rouhani

ApoA-I and HDL readily displace cell surface-bound HL and stimulate TG-hydrolysis by HL. To evaluate the regulatory effect of HDL structure on HL displacement, cell culture experiments using the native and reconstituted HDL were undertaken. Structural features of HDL such as size, density, and chemical composition were found to be important regulators of HL displacement. The larger, more buoyant HDL were stimulatory to HL displacement, whereas the smaller denser particles were inhibitory. Apolipoprotein and lipid composition had a direct regulatory role in HL displacement. Apolipoprotein A-II increased HL displacement significantly. Apolipoprotein C-I also increased the displacement, but in a lesser degree than apolipoprotein A-II. Phospholipid content of HDL was inhibitory regardless of the electrostatic charge of the HDL particle. The triglyceride component of HDL had the most significant inhibitory role in HL displacement and blocked the displacement almost completely. In addition, TG-enriched HDL fractions from hypertriglyceridemic/hypercholesterolemic subjects were unable to displace HL from the cell surface.

In summary, these results show that the structure and composition of HDL particles in plasma are central to regulation of HL displacement and, thereby, regulate the hydrolytic activity of HL.

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Abbreviations

ABCA1, ATP-binding cassette A1 transporter

ANOVA, analysis of variance

apo, apolipoprotein

BSA, bovine serum albumin

CAD, coronary artery disease

cAMP, cyclic adenosine monophosphate

CE, cholesteryl ester

CETP, cholesteryl ester transfer protein

CHD, coronary heart disease

CHO, Chinese hamster ovary

CPM, counts per minute

EL, endothelial lipase

ELISA, enzyme-linked immunosorbent assay

EMEM, Eagle's minimal essential medium

ER, endoplasmic reticulum

FBS, fetal bovine serum

FC, free cholesterol

FCHL, familial combined hyperlipidemia

FFA, free fatty acids

HCBS, high capacity binding site

HDL, high density lipoproteins

HDL-C, high density lipoprotein cholesterol

HL, hepatic lipase

HRP, horseradish peroxidase

HSPG, heparan sulphate proteoglycans

IDL, intermediate density lipoproteins

IgG, immunoglobulin type G

kDa, kilo Daltons

KO, knock out

LCAT, lecithin: cholesterol acyltransferase

LDL, low density lipoproteins

LDL-C, low density lipoprotein-cholesterol

LDLr, low density lipoprotein receptor

LIPC, human hepatic lipase gene

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

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

LPL, lipoprotein lipase

LRP, LDL receptor related protein

mAb, monoclonal antibody

MW, molecular weight

PBS, phosphate-buffered saline

PC, phosphatidylcholine

PI, phosphatidylinositol

PK-II, protein kinase II

PL, phospholipids

PLTP, phospholipid transfer protein

POPC, 1-palmitoyl-2-oleoylphosphotidylcholine

PS-PLA$_1$, phosphotidylserine phospholipase A_1

RCT, reverse cholesterol transport

rHDL, reconstituted high density lipoproteins

rpm, revolutions per minute

RuR, ruthenium oxychloride ammoniated red

SD, standard deviation

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SFM, serum free medium

SMC, smooth muscle cells

SRB1, scavenger receptor class B type 1

T1DM, insulin-dependent diabetes mellitus

T2DM, non-insulin-dependent diabetes mellitus

TC, total cholesterol

TG, triglyceride

TGRL, triglyceride-rich lipoproteins

TK, tyrosine kinase

VHDL, very high density lipoproteins

VLDL, very low density lipoproteins

Chapter 1 – Introduction

1.1 Atherosclerosis, coronary artery disease and hepatic lipase:

Atherosclerosis and the resultant coronary artery disease (CAD) remain to be the leading cause of death among adults in developed countries[1-3]. Atherosclerosis is a complex disorder of inflammation and lipid deposition within the arterial walls [4-6], which is affected by both the lifestyle and genetic factors. However, while the progress of atherosclerosis is accelerated by the sedentary life habits, high-fat diet and smoking, it also can be slowed and controlled by a change in life style and taking advantage of medical treatments [7].

Independent risk factors for coronary heart disease (CHD) including age, gender, smoking, diabetes, hypertension, elevated levels of low density lipoprotein-cholesterol (LDL-C), decreased levels of high density lipoprotein-cholesterol (HDL-C), and increased circulating levels of inflammatory markers [4] contribute in the development of atherosclerosis [8]. A number of clinical studies have shown that the plasma concentration of triglyceride-rich lipoproteins (TGRL) is also an independent risk factor for CAD [3], since these lipoprotein particles penetrate the arterial wall easily and initiate or aggravate the atherosclerotic events [3]. Additionally, increased levels of plasma triglycerides (TG) are associated with increased risk for CAD [9] and ischemic heart disease in men [10]. Plasma lipases such as lipoprotein lipase (LPL) and hepatic lipase (HL) play important roles in the metabolism of TGRL, as well as high-density lipoproteins (HDL). Therefore, function of the lipases directly affects the atherosclerosis and CAD.

HL is a plasma lipase that influences the metabolism of plasma lipoproteins such as low-density lipoproteins (LDL) and HDL. HL is thought to modulate atherogenic risk, however its role as an anti- or pro-atherogenic factor remains unknown [11, 12]. It is well accepted that HL not only plays a role as a lipolytic enzyme, but also through its ligand-binding function facilitates the intracellular lipid metabolism both of which contribute to its anti-atherogenicity. It is known that deficiency of HL activity causes TGRL accumulation in the plasma and HDL particles that are considerably larger than normal and enriched in TG [13, 14]. On the other hand, it has been shown that increased HL activity results in the formation of atherogenic small dense lipoprotein particles [15]. In addition, recent findings indicate that HL is expressed in the arterial wall [16] which is thought to be atherogenic [17]. As a result, the classical concept of HL as a lipolytic enzyme that decreases the atherogenic risk has changed into that of a multifunctional protein with various physiological effects depending on the genetic background and sites of the lipase expression [11].

Structure of artery:

An arterial lumen is covered by the endothelial cells that are firmly adhered together through tight junctions. These cells make a strong but permeable barrier that allows the plasma lipoproteins to enter the interstitial fluid in the subendothelial space. The inner- most layer of the artery known as the "intima" is composed of collagen fibers and proteoglycans. This layer acts as a basement membrane for the endothelial cells. The middle layer "media" consists of smooth muscle cells (SMC) and is separated from the intima by a sheet of elastic fibers called the "internal elastic lamina". It limits the migration of SMC from the media to the intima. Finally, "adventitia", the outermost layer of the artery that surrounds the media is

mostly composed of connective tissue with some fibroblasts and SMC; it supports the inner parts of the artery and is separated from the media by the "external elastic lamina" [18].

Pathology of the atherosclerosis:

Atherosclerosis is defined as the excessive accumulation of cholesterol and cholesterol ester in the subendothelial space of the large and medium sized arteries due to an imbalance in the influx and efflux of cholesterol. Gradual build up of the lipid deposits inside the arterial walls narrows the arterial lumen and ultimately leads to the occlusion of the arteries, thrombosis and infarction (heart attack) [19].

The affected arteries are described by morphology of the atherosclerotic lesions and are classified into three major stages: the fatty streaks, the fibrous plaques and the complex lesions [20]. Fatty streaks, the first morphologically identifiable lesions [21], are often present since infancy [20]. These lesions are not clinically important but could be the precursors of more damaging and life-threatening lesions in the future.

Fatty streaks are characterized by the accumulation of lipid-laden macrophages known as "foam cells" within the arterial intima. These histological changes are due to the increased transport of LDL particles into, and their retention in the subendothelial space followed by LDL chemical modifications by SMC, mast cells, and macrophages [22]. Abnormal accumulation of LDL in the arterial walls is a result of imbalanced LDL flux in the arteries. LDL is either taken up and degraded by the parenchymal cells or returned into the plasma circulation via the lymphatic capillaries of the extracellular space. However, the arterial intima has structural features that make it unique in terms of lipoprotein metabolism. It lacks lymphatic capillaries that block the LDL particle return into the blood circulation. Therefore, LDL concentration in the intima increases to twice the amount of that of the

plasma [23]. In addition, there is a reduction in LDL uptake by the parenchymal SMC due to continuous high concentrations of LDL particles in the subendothelial space, which decreases the number of the cell surface receptors and, therefore, LDL metabolism [23, 24].

Oxidation, the most common form of LDL modification reported in vivo, is a prerequisite for the inflammatory response, accumulation of the lipids within the macrophages, and the formation of the foam cells. LDL oxidation is suggested to mainly occur in the subendothelial space of the arterial wall, where there is a large amount of reactive oxygen species produced by the endothelial cells and activated leukocytes [25]. Oxidized LDL causes the release of pro-inflammatory molecules such as the adhesion molecules and growth factors [26]. These factors recruit the monocytes and lymphocytes into the inflammation site [27, 28], which under the stimulation of the macrophage colony stimulating factor proliferate and differentiate into macrophages [29]. The monocyte-derived macrophages take up and accumulate the lipids via their scavenger receptors and form the foam cells which are known to be the hallmark of the fatty streaks [30].

The second stage of an atherosclerotic lesion, which is called a fibrous plaque, involves the migration of SMC from the media into the intima followed by the proliferation of the SMC and production of connective tissue matrix. Progression of the fibrous plaques leads to the formation of complex lesions. These lesions are recognized by calcification, hemorrhage, ulceration, and cellular necrosis [31].

1.2 Metabolism of the lipoproteins:

Lipids in the plasma circulate as the constituents of macromolecule complexes called lipoproteins. Due to the hydrophobicity of the lipids, they do not dissolve in the biological fluids and, therefore, need a carrier to transport them in the blood circulation. The lipoprotein complexes possess amphipatic properties that enable them to carry the lipids within the aqueous environment. Lipoproteins have a general spherical structure composed of two parts: 1) the lipid constituents such as phospholipids (PLs), cholesteryl ester (CE), TG, free fatty acids (FFA), and fat-soluble vitamins 2) the protein molecules known as apolipoproteins. All of lipoprotein particles contain a neutral lipid core of TG and CE surrounded by a phospholipid monolayer and apolipoprotein molecules at the surface of the particle [32].

Modification of the lipoprotein particles by the plasma enzymes and proteins alters their physical properties including the size, density and content as well as their function. In addition to the alterations of lipoproteins within the plasma, internalization of the lipoproteins by the cells leads to the intracellular metabolism of these particles. The complex network of lipid metabolism has been divided into two main pathways for the simplicity of the studies: the exogenous pathway and the endogenous pathway. Both metabolic sequences start with the production and secretion of lipid-rich particles [33].

Exogenous pathway: Ingestion of dietary fat, especially long chain fatty acids, leads to the synthesis of chylomicrons in the intestine. Chylomicrons, which are secreted into the lymph, finally enter the blood circulation. They function as carriers of energy and provide triacylglycerol-derived fatty acids to peripheral tissues. The lipolytic enzyme LPL, which is

bound to the luminal surface of the endothelial cells of the capillaries, extracts the fatty acids from the core of chylomicrons. Removal of TG from chylomicrons by LPL reduces the size of chylomicrons and results in the production of CE-rich lipoprotein particles known as chylomicron remnants. These remnant particles have lost some of their apolipoprotein content (apolipoprotein C) during the remnant formation. The final metabolic remnant particles are destined to the liver, where they are removed by the hepatocytes via the cellular LDL receptors (LDLr) and LDL receptor related proteins (LRP) [34].

Endogenous pathway: Synthesis of very low-density lipoproteins (VLDL) by the liver is the initial point of endogenous lipid metabolism. After the secretion of VLDL into the circulation, sequential lipolysis by plasma lipases (LPL and HL) alters the VLDL into smaller denser particles. The residence time of VLDL particles in the plasma is relatively longer than chylomicrons, as TG-hydrolysis of VLDL by LPL is less efficient than that of the chylomicrons. As a result of the lipolysis of VLDL in the plasma, intermediate density lipoproteins (IDL) and LDL particles are generated. LDL, the final product of VLDL lipolysis, is then internalized into the hepatic and extrahepatic tissues via the cell surface receptors to be further metabolized [33].

In parallel, surface components of lipoproteins such as apolipoproteins, phospholipids and unesterified cholesterol, as well as the core TG and CE, are exchanged between the particles produced in VLDL lipolysis pathway and HDL particles. These reactions are mediated by a number of the plasma proteins for instance, lecithin: cholesterol acyltransferase (LCAT) [35] and cholesteryl ester transfer protein (CETP) [36], that play significant roles in the cholesterol loading ,esterification, and transfer between the particles.

Another key component of the endogenous pathway involves HDL metabolism and reverse cholesterol transport (RCT). This metabolic circle starts with the secretion of nascent HDL (preβ-HDL) from the liver. Preβ-HDL acquires free cholesterol (FC) from the peripheral tissues and changes to very high-density lipoprotein (VHDL). In the blood circulation by the action of LCAT enzyme, VHDL acquires lipids and turns into HDL_3 and then HDL_2. This sequential lipid acquisition leads to the formation of mature HDL. HDL particles loaded with the lipids deliver their cholesterol to the liver for metabolism and excretion into the bile. Concomitantly, the TG content of HDL_2 is hydrolyzed by HL and the remainder lipid-poor apolipoprotein A-I (apoA-I) particle is either metabolized in the kidneys or remained in the circulation to acquire lipids and form HDL particles [33].

Lipoprotein classes:

Lipoproteins are classified based on their size and/or density and their apolipoprotein components. Based on the density and the size, lipoproteins are categorized into chylomicrons, VLDL, IDL, LDL, and HDL. There is an inverse relationship between the size and the density of the lipoprotein particles (e.g. chylomicrons that are the largest lipoprotein particles, have the lowest density while HDL, the smallest particle, has the highest density).

From the aspect of apolipoprotein content, there are two major groups of lipoproteins: apolipoprotein A (apoA) and apolipoprotein B (apoB) containing lipoproteins. HDL particles are apoA lipoproteins, whereas chylomicrones, VLDL, IDL, and LDL are considered apoB lipoproteins.

Separation of the lipoproteins, based on their surface electrostatic charge on agarose gel electrophoresis, is an alternative method for classification of lipoproteins. In this method,

HDL particles with the greatest mobility on the gel are defined as α- migrating particles. LDL particles that migrate a short distance above the origin of the gel are named β-migrating. Between HDL and LDL, VLDL particles that migrate a little further than LDL are called pre-β-migrating lipoproteins.

Apolipoproteins:

Apolipoproteins (or apoproteins) are important constituents of lipoproteins and determine the metabolism of lipoproteins in the plasma and the tissue-specific delivery of lipoprotein components. Hydrophilic domains of these proteins mediate the solubilization of the insoluble lipids and prevent the formation of lipid aggregates within the plasma. Apolipoproteins also mediate the interaction of lipoprotein particles with the cell surface receptors, plasma enzymes and transfer proteins. Some apolipoproteins function as cofactors for lipases while some of them are competitive inhibitors of lipid binding and uptake by the cells [37].

There are two types of apoproteins, based on their ability to shuttle between different classes of lipoproteins. ApoA, apoC and apoE are exchangeable and transfer from one lipoprotein to another lipoprotein particle [38]. ApoB, on the other hand, cannot be exchanged and does not shuttle between lipoproteins [39]. In fact, tracing the metabolic pathways of apoB is easier than exchangeable apoproteins because they tend to remain in their original lipoprotein particles.

In general, apoB lipoproteins deliver the lipids (mainly TG) from the liver and intestine to the peripheral tissues, such as muscles and adipose tissue. In contrast, apoA lipoproteins circulate the lipids (cholesterol ester) not only from the central organs to the

peripherals but also from peripherals towards the liver for metabolism and excretion into the bile.

Apolipoprotein A:

ApoA-I, II, IV and V are main subclasses of apoA group. Among these, apoA-I and apoA-II constitute the major apolipoproteins of HDL (~ 70% and 20% of the total HDL protein respectively) [40].

ApoA-I: The human apoA-I gene encodes for the major structural and functional protein component of HDL (75% of the HDL protein fraction) [41]. It is located on chromosome 11 in a cluster of genes including apoA-I, apoC-III, apoA-IV, and apoA-V genes[42, 43]. The liver and intestine are the main sites of apoA-I expression, however apoA-I expression and secretion by the heart has also been reported [41].

The apoA-I gene expression is mostly modulated at the transcriptional level by a number of hormones and metabolic signaling pathways [44]. Thyroid hormones, retinoids, estrogens, and glucocorticoids enhance apoA-I promoter activity and gene expression [45], whereas transcriptional repressors such as apoA-I repressor protein-1, hepatocyte nuclear factor-4 and thyroid hormone receptor decrease apoA-I gene expression [46]. The human apoA-I gene encodes for a 267 amino acid preproprotein, which cleaves cotranslationally and secrets a 249 amino acid proapoA-I. Finally, this proprotein is processed into a 243 amino acid apoA-I molecule (MW of 28 kDa) in the circulation [47].

The conformation of apoA-I is highly adaptable and changes from one metabolic state to another, depending on its degree of lipidation and surrounding environment (plasma or lipoprotein particle). ApoA-I exists in either a lipid-poor or completely lipidated form. At the physiological conditions, the lipid-poor form of apoA-I is unstable and rapidly becomes

lipidated [48-50]. The crystal structure of lipid-free, full-length apoA-I at 2.4Å resolution has been reported. The structure is comprised of two helical domains: a four helix bundle that is formed by the N-terminal three-quarters of the apoA-I molecule and two helices of the C-terminal quarter [51].

Structure/function studies have identified the existence of a lipid binding motif on apoA-I. This α-helix region consists of amphipathic α-helices with a tandem repeat of 11/22-mer amino acids. Many of these α-helices have a large nonpolar face with positively charged residues distributed in the polar-nonpolar interface that suits them for binding to the lipids and increases the integrity of the lipoprotein particle [52]. The exact location of the lipid binding domains have not been determined, as there is controversy whether the C-terminus is the lipid-binding domain [53, 54] and whether the N-terminus associates with lipids [55]. However, it is known that the N-terminus is responsible for the stability of lipid-free apoA-I conformation [51]. Moreover, intermolecular interactions between the N- and C-terminal domains of apoA-I are thought to enhance the stability of the lipid-bound form of the protein [56, 57].

Role of apoA-I in CHD:

The flexible conformation of apoA-I contributes to its multifunctionality and capability to function as a protective agent in cardiovascular disorders. ApoA-I has been described as a protein with a variety of physiological functions [58]. Several lines of evidence from clinical research and animal model studies have shown that HDL-cholesterol has both cardioprotecive and antiatherogenic properties [59]. These protective effects of HDL are mainly attributed to its apoA-I content [44, 60, 61]. There is evidence from some

epidemiological studies that cardiovascular disease and plasma apoA-I concentrations are inversely correlated [60].

In spite of uncertainties about the mechanisms in which apoA-I contributes to protection against CHD, several studies confirm that apoA-I prevents atherosclerosis by playing a central role in RCT pathway. In this pathway, apoA-I acts as an acceptor and carrier of phospholipids and free cholesterol from the peripheral tissues to the liver for excretion to the bile and/or steroidogenesis [51, 62, 63]. ApoA-I is involved in all three steps of RCT: 1) transfer of FC to lipid-poor pre-β HDL mediated by ATP-binding cassette A1 transporter (ABCA1), 2) esterification of FC and formation of CE catalyzed by LCAT, and 3) delivery of CE from HDL particles to the hepatocytes through scavenger receptor class B type 1 (SRB1) [51, 64-66]. In addition, apoA-I has antioxidant, anticoagulant, and anti-inflammatory activities that enhance protection against atherosclerosis [60].

ApoA-II: Human apoA-II, the second most abundant apolipoprotein in HDL, is synthesized as a pre-proprotein of 100 amino acids that is cleaved co-translationally to an 82 amino acid proapoA-II and post-translationally processed to a 77 amino acid mature apoA-II [67, 68]. Human apoA-II differs from that of other apoproteins by the presence of a cystein 6 residue that makes it appear in the plasma as an S-S linked homodimer of 17.4 kDa [69] and also heterodimers with other apos like apoE and apoD [70, 71].

The gene encoding for human apoA-II is a member of the apolipoprotein multigene superfamily and is located at chromosome 1q21 → 1q24 [40]. ApoA-II is mainly synthesized at the liver and its expression is modulated by a number of the regulatory elements in the promoter region of the apoA-II gene [72]. It was shown that nuclear receptors such as peroxisome proliferator-activated receptor (PPAR)-α, retinoid X receptor

(RXR), retinoic acid receptor-related orphan receptor (ROR)-α , and sterol regulatory element-binding protein (SREBP)-2 affect apoA-II expression [68].

ApoA-II (like most of the apolipoproteins) presents a secondary structure of 11/22mer tandem repeats that form amphipathic α-helices with large apolar patches that bind lipids [73]. Compared to apoA-I, apoA-II has larger apolar surfaces (50% of the total surface compared to 30% in apoA-I) with higher hydrophobicity of these surfaces that partly explains the stronger association of apoA-II to HDL [74, 75]. The x-ray crystal structure of lipid-free apoA-II reveals a bundle of parallel helices that open upon lipid association [76].

The physiological function of apoA-II and the association of plasma apoA-II concentrations with CHD are not clearly known. While apoA-I concentration is inversely related to the initiation and progression of atherosclerosis, the role of apoA-II in atherosclerosis remains controversial [68]. ApoA-II regulates the lipid metabolism via activation or inhibition of the essential enzymes in RCT pathway and HDL metabolism [75, 77-80]. ApoA-II attenuates the antiatherogenic effects of apoA-I by inhibition of LCAT and hepatic cholesterol uptake through the SRB1 receptor [67]. ApoA-II decreases HL activity [80] and inhibits CETP, which contribute to decreasing the atherogenicity of lipid metabolism [68]. Moreover, apoA-II has direct and indirect effects on HDL receptors [78, 81, 82], transporters [83], and HDL remodeling [79, 84].

The effect of apoA-II on apoA-I kinetics and HDL metabolism has been studied widely. It has been described that apoA-II reduces the enzymatic remodeling of HDL (like HDL fusion and apoA-I dissociation) [84-86] and inhibits apoA-I transfer among HDL particles [87, 88], which contribute to the increasing of the stability of HDL. On the other hand, it has been shown that apoA-II displaces apoA-I from the HDL particles [77, 89],

accelerates apoA-I catabolism [90], and is more strongly associated to HDL than apoA-I [75, 89]. Evidence from in vitro [82-84] and in vivo [91, 92] experiments shows that HDL particles containing both apoA-I and apoA-II (LpA-I/A-II) mediate less cholesterol transport than HDL particles containing only apoA-I (LpA-I). These data support the concept of apoA-II atherogenicity [40].

ApoA-IV: Human apoA-IV is a 376 amino acid protein associated with HDL and newly secreted chylomicrons [38]. Unlike the majority of apolipoproteins that are found in their lipoprotein-bound forms, rather than lipoprotein-free forms, apoA-IV is mainly present in its lipoprotein-free form in the plasma [93] and is displaced from the lipoprotein surfaces by other apolipoproteins. ApoA-IV is only a minor component of HDL but, similar to apoA-I, it plays a role in RCT pathway [94], activates LCAT [95], and has antioxidant properties [96]. It also plays a role as a satiety factor during the post-prandial response [97].

Apolipoprotein B

Apolipolipoprotein B, the major protein component of most of the lipoproteins, including chylomicrons, VLDL, LDL, and some subclasses of HDL, exists as two forms: the full-length apoB or apoB-100 and a truncated form known as apoB-48. Unlike the apoAs, Cs and E that are highly enriched with α-helices, apoB has less α-helical content. Instead, apoB has substantial amounts of β-sheets, β-turns, and random coils. ApoB-100 is the largest apolipoprotein with 4536 amino acids and a molecular weight of 549 kDa. ApoB-48 represents the N-terminal 48% of apoB-100 and weights 264 kDa [98]. ApoB-48 is only present in chylomicrons, whereas apoB-100 is found in VLDL, IDL, and LDL. ApoB-48 is synthesized exclusively in the intestine and is secreted on chylomicron particles post-prandially. On the other hand, ApoB-100 is secreted from the hepatocytes in the form of

VLDL particles. As it is not an exchangeable protein, it remains in remodeled lipoproteins (IDL and LDL) after the lipolysis of VLDL [38].

Each VLDL and LDL contains one molecule of apoB-100 per particle [99, 100]. Similarly, each chylomicron particle consists of only one apoB-48 molecule [101]. Since apoBs are major protein constituents of the TG-rich lipoproteins (chylomicrons and VLDL), mutations within these proteins cause significant consequences in the lipid metabolism. For example, point-mutations within the ligand-binding domain of apoB-100 impairs the affinity of apoB-100 for LDLr and causes hypercholesterolemia and premature development of atherosclerosis [102]. Metabolism of apoB-containing lipoproteins is mainly carried out through two mechanisms: 1) remodeling and hydrolysis of the TG content by plasma lipases (HL and LPL) 2) hepatic metabolism mediated by the cellular receptors such as LDLr and LRP.

Apolipoprotein C

ApoCs (C-I, C-II and C-III) are small apoproteins with a range of 6.6 to 8.8 kDa molecular mass that exist in the apolipoprotein mixture of chylomicrons, VLDL, IDL and HDL [103]. The liver is the major site of apoC proteins synthesis with the intestinal expression that contributes to a minor portion [104]. Redistribution among the lipoproteins is a common property of apoCs. Generally, they are associated with HDL particles during the fasting state. After dietary absorption of the lipids and production of chylomicrons or VLDL synthesis by the liver, apoC lipoproteins transfer to newly secreted TGRL and return to HDL after the lipolysis of chylomicrons or VLDL [38, 105]. They have individual preferences for anchoring within the lipoproteins. For instance, apoC-II tends to associate with larger TGRL, whereas apoC-III associates with smaller particles [106]. Although each

apoC has defined physiological function, all three of them have been shown to interfere with the hepatic clearance of the TGRL by displacing apo-E from the surface of lipoprotein particles [107, 108].

ApoC-I, a single chain 57 amino acid protein with a MW of 6.6 kDa, is mainly expressed at the liver and at very lower levels at the brain and a variety of other tissues [109]. The protein has a highly helical content with amphipatical regions which are suggested to be the lipid binding domains of this apoprotein [110]. ApoC-I has a direct inhibitory role in binding of the lipoproteins to the cellular receptors [111]. Either purified apoC-I or a mixture of apoCs inhibit binding of β-VLDL to LRP and decrease the apoE-mediated binding of VLDL and IDL to LDL receptor [103]. Furthermore, it is proposed that apoC-I is an activator of LCAT (to a lesser degree than apoA-I) [112], and inhibitor of phospholipase A2 [113], CETP [114], LPL [115] and HL [116]. It has been reported that overexpression of apoC-I in apoE-knock out mice results in decreasing the TG clearance and severe hypertriglyceridemia due to the inhibition of HL activity [103].

ApoC-II is a single polypeptide chain of 79 amino acids with a molecular mass of 8.8 kDa [117]. The secondary structure of apoC-II consists of three helices with amphipatic areas (thought to be the lipid binding domain) and a few β-turns in the rest of the structure [118]. ApoC-II plays an important role as a cofactor for activating the plasma LPL. Patients with apoC-II familial deficiency demonstrate severe hypertriglyceridemia and impaired lipid clearance in spite of having functional LPL [119]. The structure-function studies indicate that there are two distinctive functional domains on apoC-II structure: a region responsible for interaction with LPL and a lipid-binding domain [120]. It has also been reported that apoC-II activates LCAT and plays a role in formation of the cholesterol ester [121].

ApoC-III, the most abundant C apolipoprotein in the plasma, is a single chain 79 amino acid polypeptide with a MW of 8.7 kDa. Its gene is located on chromosome 11 in a cluster of the genes that includes apoA-I, as well [121]. ApoC-III is a sialylated protein with three subspecies (apoC-III_0, apoC-III_1, and apoC-III_2), depending on the number of sialic acid residues that are O-linked to the protein [122]. ApoC-III is known to be the major suppressor of VLDL and remnant lipoproteins lipolysis and clearance by: 1) inhibition of LPL activity and 2) decreasing the association of lipoproteins to the glycosaminoglycans at the cell surface [103]. It has also been suggested that apoC-III is inhibitory to the cofactor activity of apoC-II in activating LPL [119]. It is well known that apoC-III gene expression is down-regulated by the PPAR-α transcription factor [123]. Therefore, ApoC-III expression is affected by fibrate therapy, which activates the PPAR-α activity. It is thought that down-regulation of apoC-III is one of the mechanisms by which fibrates lower plasma TGs. In contrast to apoC-III, apoC-I levels do not change in response to the fibrates [123].

Apolipoprotein E

ApoE is a protein constituent of most of the lipoproteins, including the chylomicrons, chylomicron remnants, VLDL, LDL, and some HDL subclasses. It is a polypeptide of 299 amino acids (MW = 34.2 kDa) [123], which is highly sialylated post-translationally [124] The major portion of apoE is secreted from the liver but expression of apoE in many other tissues such as the brain, kidneys, intestine, and adrenals has been also detected [125, 126]. The apoE gene, located on chromosome 19 along with the apoC-I, apoC-II and LDLr genes, is a highly variable gene due to mutations within a single ancestral gene [127]. There are 3 different apoE isoforms in humans: apoE2, apoE3 and apoE4. The apoE3 isoform is considered to be the wild-type, whereas apoE2 and apoE4 are associated

with lipoprotein abnormalities and differ from apoE3 in single amino acid substitutions [124].

The amino acid sequence of apoE shows a highly ordered α-helical structure at the C-terminus, presenting the lipid-binding domain of the apolipoprotein. The N-terminus domain is responsible for binding to the cell surface HSPGs and LDLr through ionic interactions [38]. The major role of apoE in lipid metabolism is to mediate the binding and uptake of lipoproteins (especially VLDL and chylomicron remnants) by the cell surface receptors such as LDLr and LRP (also known as chylomicron remnant receptor or apoE receptor) [128]. Individuals homozygous for the apoE2 isoform, have defective apoE-mediated binding of lipoproteins to LDLr. A small population of these patients (<5%) develop hyperlipoproteinemia type III characterized by hypertriglyceridemia and hypercholesterolemia as a result of impaired metabolism of chylomicron remnants and VLDL [129].

1.3 HDL metabolism:

Plasma HDL-C levels are inversely correlated with the incidence of CHD in humans [130]. HDL exerts most of its atheroprotective effects through participation in RCT and transport of the lipids from atherosclerotic lesions to the liver. Therefore, in order to design new strategies to raise HDL-C levels and promote RCT, it is required to have a detailed understanding of the HDL metabolism including production and catabolism mechanisms. In spite of the fact that variation in plasma HDL-C levels, in human populations, is mostly a consequence of variations in HDL clearance rather than production [131], increasing apoA-I and HDL levels by up-regulating HDL formation pathways is still considered a potent therapeutic target.

HDL biogenesis and maturation:

HDL are spherical, heterogeneous particles with equal amounts of lipids and apoproteins in their structure. The cores of HDL particles contain neutral lipids surrounded by a monolayer of apoproteins, phospholipids, and cholesterol. Apoproteins A-I and A-II are major protein components of HDL (70% apoA-I and 20% apoA-II). Human plasma contains HDL particles of only apoA-I (LpA-I) or both apoA-I and apoA-II (LpA-I/A-II) [132].

ApoA-I, the major apolipoprotein of HDL, is primarily expressed by the liver and intestine as lipid-poor apoA-I and phospholipid-rich, lipid-poor nascent HDL particles. These newly synthesized apoA-I containing particles gain additional phospholipids and cholesterol through efflux from peripheral cells during the RCT process or from TGRL after their lipolysis by plasma lipases [131]. Cholesterol efflux from the peripheral tissues such as macrophages to the nascent HDL and lipid-poor apoA-I is mediated by a cell membrane

homotetramer protein known as ATP-binding cassette transporter A1 (ABCA1) [133]. Upon interaction of ABCA1 with apoA-I, apoA-I activates ABCA1 phosphorylation through a cAMP/protein kinase-A dependent pathway that leads to the loading of lipid-poor particles with cellular cholesterol and formation of pre-β discoidal HDL particles [134]. This step (apoA-I lipidation) is suggested to be a crucial rate-limiting step in HDL formation in contrast to the apoA-I secretion which does not appear to be rate-limiting in maintaining HDL-C levels [135].

There are different proposed mechanisms by which ABCA1 mediates apoA-I lipidation. One model suggests that interaction of lipid-poor apoA-I with ABCA1 creates a distinct apoA-I binding site on the cell membrane, where apoA-I acquires lipids locally and is released directly from there as nascent HDL particles. This high capacity binding site (HCBS) has specific affinity for binding to apoA-I compared to the apoA-I binding site on an ABCA1 molecule [134, 136]. Consistently, it is also proposed that binding of apoA-I to ABCA1 leads apoA-I to perturbed plasma membrane domains (probably created by ABCA1 phospholipid translocase activity) that allows the second step of apoA-I lipidation [137, 138]. Another theory favors endocytosis of apoA-I/ABCA1 complex to intracellular compartments rich in cholesterol and sphingomyelin followed by ABCA1 recycling and discoidal pre-β HDL formation [139].

The significant role of ABCA1 in formation of HDL is manifested in Tangier disease and familial HDL deficiency patients who lack mature HDL in their plasma due to mutations in ABCA1 gene and lacking functional ABCA1 expression [140, 141]. Nascent apoA-I containing lipoproteins in these patients fail to acquire lipids and thus are subjected to rapid clearance from the circulation.

The next step in HDL maturation involves cholesterol esterification catalyzed by LCAT in which it transforms free cholesterol to cholesterol ester. CE-rich HDL particles gain further phospholipids and TG from VLDL, expanding their size. These particles are mature spherical HDL and exhibit α-migration on agarose gel electrophoresis [88]. Simultaneously, removal of the triglycerides and surface phospholipid contents of chylomicrones and VLDL during LPL-mediated lipolysis of these particles also contributes to the formation of lipid-poor HDL particles [142]. On the other hand, remodeling of mature HDL particles by a number of the proteins and enzymes including LPL, HL, CETP, PLTP as well as SR-B1 [143] also contribute to lipid-depletion of spherical HDL and generation of lipid-poor apoA-I.

HDL speciation in the plasma:

HDL in humans is a heterogeneous mixture of particles that differ in their size, shape, electrostatic charge and composition [134]. Separation of HDL subfractions based on their surface charge on an agarose gel electrophoresis yields α, pre-α, pre-β and β migrating particles. Each population may consist of a few sub-populations. For example, pre-β particles are composed of pre-β_1, β_2 and β_3 subpopulations with each one exhibiting distinct physiological functions. In vitro studies show that pre-β_1-LpA-I particles (pre-β migrating HDL particles containing apoA-I) remove free cholesterol from cultured fibroblasts and transform into larger pre-β-LpA-I particles such as pre-β_2-LpA-I and pre-β_3-LpA-I and, finally, to pre-α-LpA-I particles [144]. Pre-β_2 particles are associated with haptoglobin [134, 145] in the plasma which may explain the antimicrobial effect of HDL and its role in human innate immunity [146]. Pre-β_3-LpA-I particles are a complex of protein components like CETP, LCAT, apo D and apoA-I that are involved in esterification and shuttling of the

cellular cholesterol [147]. ApoE and α_2-macroglobulin are associated with LCAT in this complex [148] and play a role in the regulation of LCAT's bioavailability [149]. Particles with α-migrating mobility make up the majority of the HDL population in the plasma. These are spherical particles that include HDL_2, HDL_3, apoA-I-containing and apoA-I/apoA-II-containing mature HDL subspecies. There are also minor HDL subpopulations that contain only apoE (HDL-LpE), including γ-LpE, and are suggested to play a role in RCT [150].

Placing plasma HDL on a 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by immunoblotting results in the separation of HDL particles based on their size and charge. HDL can be divided into large-sized particles (HDL_{2a} and HDL_{2b}), small-sized particles (preβ_1-HDL, HDL_{3a}, HDL_{3b} and HDL_{3c}) and preβ_2-HDL, which are very small nascent HDL [151]. While each subpopulation may have its distinct functions, these variable HDL particles cooperate with each other in order to effectively efflux cholesterol from the peripheral tissues towards the liver [152]. During the RCT, pre-β_1-HDL particles, the initial acceptors of cellular lipid, accumulate cholesterol and, by the action of LCAT, convert to large spherical HDL. It is hypothesized that pre-β_1-HDL first converts into HDL_3 and then into HDL_2 which is the largest, most lipid-rich HDL in the plasma [151].

The clinical relevance of HDL speciation has been studied in recent years and unfortunately, the results have been contradictory. Some studies indicate that increased distribution of small-sized HDL particles (pre-β) is associated with impaired HDL maturation and RCT process which contribute to the increased risk of CHD [151, 153, 154]. Controversially, data from Asztalos et al [155] shows that pre-β HDL are not significantly associated with CHD, but large-sized particles (α-HDL or HDL_{2b}) are positively related to CHD prevalence.

In spite of this disagreement, the significance of HDL speciation in cardiovascular disease is growing. It has been postulated that changes in the concentration of plasma HDL subspecies are more related to CHD risk than decreased levels of HDL-C [156]. Therefore, clinical measurements that represent plasma HDL species have been proposed to be evaluated in addition to the conventional lipid profile measurements. For instance, measuring total cholesterol (TC)/HDL-C and TG/HDL-C ratios together is an indicator of HDL subspecies distribution, as elevated ratios are correlated with the smaller-sized particles and thus increased risk of cardiovascular disorders [151].

1.4 Hepatic lipase:

The lipase family:

The lipase family belongs to a superfamily of the enzymes that includes the esterases and thioesterases. Members of this superfamily have similar tertiary structures rather than amino acid sequence homology [157].The mammalian lipase gene family includes lipoprotein lipase (LPL), hepatic lipase (HL), pancreatic lipase (PL), PL related protein 1 and 2 [158], endothelial lipase (EL) [159], phosphotidylserine phospholipase A1 (PS-PLA_1) [160, 161] and lipase H [162]. These water-soluble enzymes are synthesized in different organs and tissues and each of them plays distinct roles in lipid metabolic pathways [163] such as lipid digestion, absorption, fatty acid uptake and lipoprotein transformation [157]. All lipases except PL-related protein 1 [164] exhibit neutral and/or phospholipase activities that hydrolyze the ester bonds of lipid substrates such as triglycerides, cholesteryl esters and phospholipids [157].

Tissue specific expression of the lipases:

HL is synthesized mostly by the hepatocytes. Lower levels of HL are secreted in adrenal and ovary glands, where HL probably mediates cholesterol delivery for steroidogenesis purposes [165]. It is also secreted by macrophages and thus may play a direct role in the pathogenesis of atherosclerosis [16]. Overall, 95% of the total activity of human HL is attributed to the HL secreted from the liver [12].

LPL is expressed in the heart, skeletal muscle and adipocytes and resides at the capillary endothelium where it feeds the underneath tissues with fatty acids derived from the hydrolysis of TG core of chylomicrons and VLDL particles [166]. PL is synthesized by the pancreatic acinar cells and secreted into the intestinal lumen where it facilitates the

absorption of dietary long-chain fatty acids [167, 168]. It has a strict neutral lipid preference for hydrolysis. Two other proteins closely related to PL (PL-related proteins 1 and 2) have also been identified at the pancreas at lower concentrations than PL [169]. PL-related protein 1 has no lipase activity and its mRNA levels decrease significantly in adults compared with the neonates. In contrast, PL-related protein 2 exhibits hydrolytic activity against both TG and phospholipid substrates [170, 171]. EL is secreted from a variety of tissues including the macrophages, lungs, thyroid gland, and placenta. PS-PLA_1 is secreted from the platelets, and lipase H is expressed in the intestine [162]. The physiological role of the last three lipases is not fully understood and remains to be determined.

The lipases share several structurally conserved domains including the lipid-binding domain, heparin-binding domain, and the catalytic site. The catalytic domain is the most conserved region (Gly- Xaa-Ser-Xaa-Gly) and contains the active site serine [158]. The gene structure and sequence homology of the lipases show a common folding pattern for LPL, HL and EL. They appear to share homologous heparin binding domains at the N-terminal domain and two conserved N-linked glycosylation sites at the C-terminal domain of their structures [158, 172].

Synthesis and secretion of HL:

HL is a glycoprotein primarily synthesized by the hepatocytes and localized on the sublumenal extracellular matrix component of the endothelial cells, the microvillar surfaces of the hepatocytes in the space of Disse, interhepatocyte spaces, as well as the luminal surfaces of the hepatic sinusoidal endothelium [12, 165]. Once glycosylated, this enzyme has a molecular weight of 66 kDa and is bound to the surface of the endothelial cells through HSPGs. HL synthesis occurs in the endoplasmic reticulum (ER), where the N-terminus

leader peptide is cleaved and the protein is properly folded. After crossing the ER and during the transition through the Golgi apparatus, HL glycosylation is completed and mature HL is rapidly secreted. Therefore, two different pools of HL are identified: an intracellular pool of protein with lower molecular mass and an extracellular pool with larger molecular mass (the glycosylated form). In humans, HL presents four glycosylation sites at the 20, 56, 340 and 375 amino acid positions. It has been confirmed that the presence of the N-terminal glycosylation sites of LPL and HL is necessary for secretion of the active form of these lipases. Prior to the secretion of HL and LPL from the ER, nascent enzymes bind to the chaperon molecules, such as calnexin and calreticulin, through the innermost glucose residue of an oligosaccharide chain. Then the two outer glucose residues get cleaved by ER glucosidase I and II [173]. Glucose trimming is the final step in secretion of active HL and LPL, and drugs that inhibit this process prevent the secretion of active lipases [174].

Structural motifs:

The HL gene (LIPC) is located on chromosome 15 in humans. It is comprised of 9 exons and eight introns and spans 60 kb of DNA. It encodes a protein of 499 amino acids including a leader peptide of 22 residues so that the secreted protein contains only 477 amino acid residues [175]. The proximal promoter of HL gene contains four polymorphic sites. One of these polymorphisms is associated with low post- heparin HL activity, elevated HDL-C levels and presence of large buoyant HDL and LDL particles (featuring reduced atherogenic risk factors) [176, 177]. A comparison of the variant allele with the wild type allele in murine hepatoma cells indicates that decreased transcriptional activity of the promoter/reporter region may cause diminished HL activity [178].

HL amino acid sequence reveals the presence of several functional domains. The two most important sites consist of hydrophobic segments of 10 amino acids, each containing a serine residue (Ser146 and Ser267) which are involved in the lipase-lipid interactions. Site-directed mutagenesis studies show that the presence of Ser146 in the catalytic domain of the enzyme is essential for the activity of the enzyme. At the centre of the protein, there are 10 cysteine residues. The distances between these residues are conserved among different human lipases. This suggests that formation of the disulphide bridges is important for the conformation and catalytic activity of the lipases. Human HL also contains four putative heparin-binding domains that are involved in the binding of the enzyme to the cell surface HSPGs [179-182]. Site-directed mutagenesis studies indicate that existence of Asn56 in the N-terminus is crucial for secretion of an active enzyme. Mutation in Thr383 impairs HL secretion and is described in the rare familial HL-deficiency [183-189].

Enzymatic activity of HL:

HL is postulated to function both as a lipolytic enzyme and as a cell-surface ligand for lipoprotein uptake. HL activity is measured in the plasma after an intravenous injection of heparin which releases HL from the cell surface to the blood stream [190]. Enzymatically, HL hydrolyzes the sn-1 fatty acyl ester bonds of sn-3 phospholipid as well as the sn-1 (sn-3) ester bonds of mono-, di-, and triglycerides found in all classes of lipoproteins. Through its phosoholipase A1 and triglyceridase activities, HL contributes to the clearance of TG from the blood stream and modifies the size and density of chylomicron remnants, LDL, and IDL in addition to converting VLDL to LDL and HDL_2 to HDL_3 [12, 191, 192].

There is clear evidence that HL hydrolytic activity either in normal subjects or in patients with cardiovascular disease results in the formation of smaller, denser lipoprotein

particles which are relatively depleted in phospholipids and free cholesterol. Patients with CHD, characterized with predominance of small dense LDL (pattern B LDL subclass distribution) demonstrate significantly higher HL activity than those CHD patients with large, buoyant LDL (pattern A subclass) [193].

More evidence that suggests a central role for HL in lipoprotein metabolism is obtained from HL deficient patients (no detectable HL protein mass and no HL activity) and animal models in which the enzyme has been inactivated. In those individuals with completely absent HL activity, plasma cholesterol, TG, large buoyant β-VLDL, and less dense TG/PL enriched LDL and HDL particles are all elevated [192]. Rabbits that naturally have very low levels of active HL also exhibit a higher level of large TG-enriched HDL particles [194]. Similarly, inhibition of HL activity in vivo by intravenous injection of anti-HL antibody prevents the conversion of large HDL to smaller denser HDL particles [195]. Along with HL deficiency models, HL overexpression models have also been created. Expression of HL in mice decreased HDL size and HDL-C levels, and also decreased the formation of a homogenous HDL population [196, 197]. Similarly, overexpression of HL in transgenic rabbits caused the near absence of large HDL particles and significant reduction of HDL-C levels [198].

Ligand function of HL:

Over the last decade, an additional, separate metabolic role has been attributed to HL, in which it serves as a cell surface ligand to mediate the uptake of apoB remnant lipoproteins and HDL. The initial evidence of this ligand-binding function was obtained from the studies using heat-inactivated hepatic lipase and anti-HL antibodies [199-203]. These data were confirmed by in vivo experiments which demonstrated that the expression

of catalytically inactive HL (HL with a mutation at 145G) reduced the plasma levels of apoB lipoprotein cholesterol and HDL-C in HL-KO or apoE-KO/ LDLr-KO mouse models [204, 205].

In a clinical study by Zambon et al [199] it was shown that HL-deficient subjects who had enzymatically inactive but detectable HL levels had a much improved lipid profile than those who had no detectable HL in their plasma. In vitro studies also showed that HL promotes the selective uptake of HDL-CE via the scavenger receptor B1 in human embryonic kidney cells (HEK 293 cells) [206] and Chinese hamster ovary cells (CHO cells) [200]. In addition, involvement of HL in the uptake of chylomicrons and β-VLDL through LRP in CHO cells, human hepatoma cells, and primary human hepatocytes was described [207]. These combined data point to the essential role of HL in cellular metabolism of the lipoproteins [12] and that this ligand function is an independent phenomenon from its enzymatic activity [199, 205, 208].

Regulation of HL expression:

HL activity is regulated at the gene expression level as well as direct modulation of its activity in the plasma. HL gene expression is regulated by a number of the endogenous factors such as hormones, catecholamines, cellular demand of cholesterol and fatty acids, and the exogenous factors like diet, lifestyle, and lipid-lowering drugs. Rat studies suggest that there are a number of putative regulatory elements in the HL gene promoter that respond to these factors (e.g. hormones, cholesterol, cAMP and glucose) [209].

Hormonal regulation of HL:

Several hormones are known to impact HL activity including the steroid sex hormones, peptide hormones (insulin and leptin), and amine hormones (thyroid hormone

and catecholamines). Estrogen is suggested to reduce HL activity, since the HL activity is lower in premenopausal women than men and postmenopausal women [210]. In addition, estrogen therapy and castration are associated with decreased HL activity and mRNA levels [211, 212]. Oppositely, treatment with testosterone or synthetic anabolic steroids and ovariectomy result in upregulation of HL activity and mRNA levels [213-215].

Correlation of the peptide hormone insulin with HL expression and activity has been studied in diabetic patients, but the results are not conclusive. Insulin-dependent diabetes mellitus (IDDM) patients have decreased HL activity [216] and intraperitoneal insulin injection results in increased HL activity in these subjects [217]. Conversely, patients with non-insulin-dependent diabetes mellitus (NIDDM) have elevated HL activity [218]. In vitro studies in rat primary hepatocytes also show that HL expression increases by insulin treatment [219]. These results suggest a positive role for insulin in upregulation of HL expression and activity. However, a number of studies show that insulin therapy in NIDDM patients decreases HL activity [220, 221]. These contradictory results were explained by the fact that subcutaneous injection of insulin may cause a relative hypoinsulinization in the liver and therefore down-regulation of HL expression and activity [221, 222].

Another peptide hormone leptin (a 16 kDa protein secreted by the white adipose tissue) has also been shown to impact HL expression and activity. Ob/ob mice with defective leptin signaling pathways have a marked decrease in HL mRNA and leptin treatment of these mice results in up-regulation of HL mRNA [223]. Adrenaline (a catecholamine) has been shown to have the opposite effect of leptin through the inhibition of HL maturation and increasing the intracellular HL degradation [224].

It has been confirmed by several clinical studies that hypo- and hyperthyroidism are associated with decreased and increased HL activity respectively [225, 226] and that hormone replacement therapy in hypothyroid patients normalizes the HL activity [227, 228]. However, its mechanism is not clear yet. In hypothyroid rats, decreased HL activity is associated with HL mRNA reduction [229], suggesting that thyroid hormones regulate HL activity via a thyroid response element. In contrast, a study in HepG2 cells shows that thyroid hormone treatment increases HL activity but not mRNA levels or the rate of transcription/translation [230]. In support of the latter idea it was shown that treatment of hypothyroid rats with growth hormone results in normalization of HL mRNA levels [229, 231] suggesting that decreased thyroid hormone in hypothyroid subjects is concomitant with growth hormone deficiency and that growth hormone rather than thyroid hormones may play a role in HL regulation.

Genetic factors:

A number of the polymorphisms within the LIPC gene or its promoter have been identified that are associated with variability of HL activity in different populations. The most studied is the C-514T polymorphism in which a C nucleotide is substituted with a T nucleotide at position -514 with the respect to the transcription start site of the LIPC gene. This polymorphism is associated with reduced HL activity and accounts for 20-30% of the variance in HL activity both in men and women of different ethnic backgrounds [232-234].

Cellular supply of cholesterol:

There is evidence that HL expression responds to the cellular demand for cholesterol. It is shown that in cultured cells cholesterol levels are inversely related to the HL mRNA levels and that inhibition of the cholesterol biosynthesis up-regulates HL expression [235].

In vivo data support this idea and show that cholesterol fed rats demonstrate reduced HL activity and mRNA levels [236].

Regulation of HL activity by plasma lipoproteins and apolipoproteins:

Unlike LPL that needs apoC-II for activation, HL does not appear to have an absolute necessity for an apolipoprotein co-factor. In spite of this, studies have shown that a number of the serum factors as well as purified apoproteins impact HL activity. Several studies have suggested that various apoproteins including apoA-I/ II, apoC-I/ II/ III and apoE inhibit HL hydrolytic activity [116, 237, 238]. It has also been suggested that apoA-II [239-241] and apoE [242, 243] stimulate TG hydrolysis by HL. This inconclusive view appears due to the variability of experimental conditions such as the physicochemical state of the substrate, surface pressure of the system, and reconstituted or native particles.

Using a well-controlled lipid monolayer technique where the surface pressure and substrate composition and concentration have been carefully monitored and maintained, it was shown that TG hydrolysis by HL was inhibited by apoA-I, apoC-I and apoC-III and to a lesser degree by apoA-II [238]. Similarly, it was shown that most of the apolipoproteins (apoA-I/ II and apoC-I/ II/III) except for apoE inhibit HL as well as LPL hydrolytic activity [242]. In both studies, increasing the surface pressure caused an increase in HL inhibition because HL cannot penetrate the phospholipid monolayer that contains the apolipoproteins.

The inhibitory role of apoA-II on HL activity is in agreement with a study by Zhong et al [244] that showed HL activity in emulsion-based assays was controlled by the ratio of apoA-I/ apoA-II in HDL. Also it has been shown that apoA-II enrichment of LpA-I particles inhibits HL hydrolytic activity and affects apoA-I conformation [80]. The inhibitory effect of apoA-II on VLDL-TG hydrolysis was explained by increased interaction of HL with

HDL particles containing apoA-I/II compared to those containing only A-I. Similarly, another study by *Mowri et al* concluded that HDL_2 particles containing both apoA-I and II are better substrates for HL activity than those particles containing only apoA-I [240, 241]. ApoA-I by itself has been confirmed to regulate lipid hydrolysis by HL by liberating and activating the enzyme from cell surface HSPG and by direct modulation of lipoprotein binding and hydrolysis [245].

Hepatic lipase and atherosclerosis:

HL is involved in the metabolism of pro- as well as anti-atherogenic lipoproteins (HDL) [246, 247]. In humans an alteration in HL activity is associated with changes in the plasma lipoprotein levels and their composition which causes an increase or decrease in CHD risk [190]. During the last decade, several studies both in human and animal models have shown HL's involvement in the atherogenesis, however due to the complexity of the atherosclerosis process the role of HL as a protective or atherogenic element has not been determined yet [12, 190].

It has been shown that post-heparin HL activity is inversely related to HDL-C levels [248-250] and LDL size [251-253] and therefore increased HL activity is associated with elevation of atherogenic small dense LDL particles in the plasma.

A number of clinical studies show that elevated levels of HL activity are associated with increased CHD risk in the patients. For example, higher HDL-C levels in premenopausal women and lower incidence of CHD in this group compared to the postmenopausal women and men are in part due to the lower HL activity [254]. Moreover, conditions related to the higher risk of heart disease such as smoking [255], visceral obesity [256] and sedentary life style [257] are known to be associated with elevated HL activity. In

addition, combined familial hyperlipidemic [258] and type 2 diabetic patients [218] who are at a higher risk for CHD, have elevated levels of HL activity and atherogenic lipoprotein profile (small dense LDL particles, decreased HDL-C and increased TG levels) [259-261].

On the other hand, presence of premature cardiovascular disease in HL-deficient patients [13, 262, 263] and the inverse relationship between HL activity and the extent of CAD in men undergoing elective coronary angiography [264] suggest that HL may have anti-atherogenic properties. In support of this view, an inverse association was also found between HL activity and the extent of coronary calcification in familial hypercholesterolemia patients [265].

Similar to the clinical studies, a variety of animal model studies provides pro- as well as anti-atherogenic roles for HL. Overexpression of apoC-I [103] or apoA-II [79, 266] in apoE-null mice inhibited HL activity and increased atherosclerosis. Similarly, overexpression of HL in mice reduced the aortic cholesterol content to 42% compared to the control mice [197]. These studies suggest that HL activity reduces the atherosclerosis risk.

In contrast to the above studies, apoE-deficient mice with a background of HL deficiency were found to have reduced risk of atherosclerosis [267] and cholesterol fed HL transgenic rabbits had significantly thicker atherosclerotic lesions compared to the control rabbits [268]. Seemingly, we are still far behind understanding whether HL is a good factor for protection against CHD or it is atherogenic. Probably there are some other physiological factors that make a balance in HL activity and impairment of those factors disturbs HL function that, in turn, affects lipid metabolism and therefore CHD risk.

1.5 HL displacement from the cell surface:

Intravenous administration of heparin to individuals stimulates the liberation of HL and LPL into the blood stream and promotes the hydrolytic activity of these enzymes. Usually the peak activity of HL or LPL in the blood circulation is measurable after 10-15 minutes after the heparin infusion [269]. An increase of the post-heparin lipolytic activity is representative of an inactive pool of the lipase molecules that are attached to the HSPG in the liver or vascular endothelium. Studies have shown that the cell surface bound HL is inactive, whereas the liberated enzyme may have increased hydrolytic activity and stimulate TG hydrolysis and clearance from the blood stream [245]. Therefore, displacement of HL and factors that regulate this physiological phenomenon are important modulators of lipase activity and thus lipid metabolism.

Heparan sulphate proteoglycans (HSPGs)

HSPGs contain a core protein and one or more heparan sulphate (HS) glycosaminoglycan (GAG) chains which are linear polysaccharides composed of different N-acetylated or N-sulphated glucosamine units (*N*-acetylglucosamine, GlcNAc, or *N*-sulphoglucosamine, GlcNS) and uronic acids (glucuronic acid, GlcA, or iduronic acid, IdoA). There are three subfamilies of HSPGs: the membrane-spanning proteoglycans, the glycophosphatidylinositol (GPI)-linked proteoglycans, and the secreted extracellular matrix proteoglycans. Some HSPGs contain chondroitin sulphate or dermatan sulphate, a sugar chain that differs from heparan sulphate in its components and patterns of modification.

The chains of heparan sulphate are assembled on core proteins by Golgi enzymes. During their assembly, these chains are modified by a series of processing reactions such as

GlcNAc N-deacetylation and N-sulphation. HS are heterogenous in terms of chain length, size, and the extent of sulphation and epimerization within the modified segments. Due to its high negative charge, the HS chains bind to a variety of proteins. These protein–HSPG interactions are involved in many physiological activities. The HSPGs serve as matrix receptors, inhibit SMC proliferation and migration, control angiogenesis, exhibit anticoagulative properties and are involved in binding and internalization of lipases and lipoproteins [270].

Lipase displacement by heparin:

For over five decades, it has been known that heparin promotes the displacement of HL and LPL from the liver and vascular endothelium. Also, it has been confirmed that infusion of heparin into the rats and humans induces the lipolytic activity and plasma TG clearance [271-273]. The ability of heparin to release the cell surface-bound enzymes into the circulation is shared by other sulphated polysaccharides, such as heparin sulphate and dermatan sulphate [274, 275]. The size, degree of sulphation, and structure of the heparin and heparin-like molecules impact the lipase displacement and promotion of the lipolytic activity [276]. Heparin molecules with lower molecular weights (low-M_r heparin) induce more lipase-releasing effects in vitro, probably due to their smaller size that enables them to enter the capillaries and tissues easier [277]. However, these shorter heparins result in lower lipase activities in the plasma than conventional heparin in vivo [278-280].

Heparin is endogenously secreted in the lungs and the intestine. Trace amounts of this endogenous polysaccharide are known to be protective against atherosclerosis [281]. This atheroprotective effect is due to the activation of LPL by binding to heparin and

decreasing the hypertriglyceridemia [9], anti-chemokine effects that limit the infiltration of leukocytes into the inflammatory sites [282], and inhibition of the production of cytokines such as IL-1B, IL-6, TNF-α and NF-κB by monocytes [283]. Endogenous heparin is also known to play a role in regulation of the lipolysis process post-prandial [284].

The mechanism of HL and LPL displacement by heparin is suggested to be through the ionic interaction of negative charges on heparin with positively charged domains of HL/LPL that displace the lipases from their HSPG binding sites and form lipase-heparin complexes in the circulation [276]. There are distinct amino acid regions on HL and LPL that are responsible for the binding of lipases to the HSPG and heparin. These regions of basic amino acids determine the association of the lipases with negative clusters of heparin or heparin-like molecules.

It has also been suggested that the release of HL activity by heparin in rat hepatocytes is in part through the tyrosine kinase (TK) pathway and involves the activation of Ca^{2+}/calmadulin-dependent protein kinase II (PK-II) [285] and an increase in cytosolic phospholipase A_2 activity and leukotrienes production [286]. In addition, it was also known that the increase of HL secretion in rat hepatoma cells (Fu5AH cells) by heparin is due to the reduced degradation rate of the lipase, while the synthesis rate of HL is not affected by heparin [287].

Lipase displacement by other molecules:

A few other molecules with negative electrostatic charge are known to displace HL from the endothelial surface. Vanadate promotes the displacement of HL activity from the rat liver slices into the incubation media through the tyrosine kinase pathway [288]. Ruthenium red (ruthenium oxychloride ammoniated, RuR) stimulates the release of HL

activity from primary rat hepatocytes into the media as well. This RuR-induced HL activity was also found to be acting through a TK and PKA-activating pathway [289]. Intravenous injection of a heparin-mimicking compound called RG-13577 releases both HL and LPL into the blood and inhibits the lipase-mediated binding and uptake of LDL in vitro [290].

Polycations such as protamine (a mixture of basic proteins from salmon sperm) are known to displace the lipases by competing in binding to the cell surface HSPG. Injection of protamine into rats is reported to deplete the lipases from their binding sites and induce the release of HL and LPL activities into the plasma [291].

Role of HDL in cell surface bound-HL displacement:

ApoA-I and HDL stimulate HL displacement from the cell surface and inhibit TG hydrolysis by HL [245]. HL mass associated with the intracellular and extracellular forms of the protein decrease upon HDL or apoA-I incubation of the cells. Furthermore, various subclasses of HDL impact HL displacement differently. Larger and less buoyant HDL cause more HL to be displaced from the cells compared to the smaller denser HDL [245, 292]. The ability of different HDL particles in the blood stream to liberate the lipases from the cell surface directly influences the lipid hydrolysis and clearance suggesting that the kind and amount of HDL subclasses may determine lipase activity in the blood stream.

1. 6 Rationale and objectives:

The complex role of lipid metabolism and different classes of lipoproteins in the atherosclerosis process has been widely researched. It is well known that where LDL-C levels in the plasma are positively associated with the atherosclerosis risk, HDL-C levels are inversely related to this disease. Concentrations of these two lipoproteins in the plasma are

determined largely by the action of hepatic lipase [165]. HL activity causes more LDL production and generates smaller denser HDL species. Both of the complexes are known as proatherogenic lipoproteins. Therefore, understanding the mechanism of HL activity and the factors that regulate its activity are central to achieving a better knowledge of atherosclerosis pathogenesis and targeting new strategies towards preventing and curing this disorder.

Clinically, it has been known for over five decades that intravenous injection of heparin causes HL release into the blood stream and increases its activity [293]. In addition, in vitro studies by *Ramsamy et al* have shown that free HL in the solution exhibits higher hydrolytic activity than HSPG-bound HL [245]. Therefore, factors that release HL from the cell surface into the blood stream may be important regulators of HL activity. Purified human apoA-I and HDL directly influence the attachment of HL to the cell surface and are capable of displacing HL from the cell surface HSPG [245]. However, the mechanism of action of HDL in HL displacement is unclear and remains to be elucidated.

In order to attain a clearer understanding of HL displacement by HDL, the present study has focused on two main objectives:

1) Evaluate the mechanism of HL displacement by HDL. Determine if there is a direct interaction between the HDL particle and HL that results in displacing HL or if competitive exchange of apoproteins between HDL and HSPG cause HL displacement.
2) Evaluate the relationship between the HDL physical structure and HL displacement by HDL. Determine the impact of HDL lipid and protein composition on HL displacement.

In order to address these questions, experiments were designed and native as well as reconstituted HDL particles were used.

The experiments described in this research provide new insights into the regulation of HL displacement by various kinds of HDL in the plasma and demonstrate how the lipid and protein composition of HDL affects the displacement phenomenon. Evidence from hyperlipidemic patients shows that HDL composition may be an important regulatory factor in HL displacement.

Chapter 2: Experimental procedures

2.1 Materials

Triglyceride diagnostic kits were purchased from Roche Diagnostics (Laval, QP, Canada). Anti-mouse IgG horseradish peroxidase (HRP)-linked whole antibody (isolated from goat), broad range molecular weight markers, protein-G sepharose 4 fast Flow beads, HiTrap heparin HP columns, native gradient Phast gels, and $Na^{125}I$ were purchased from Amersham Biosciences (Baie d'Urfé, PQ, Canada). SuperSignal West Pico Chemiluminescent Substrate and Iodo-beads® iodination reagent (N-chloro-benzenesulfonamide sodium salt) were purchased from Pierce Chemical Co. (Rockford, IL). Agarose Paragon® lipo gels and the lipid stains were obtained from Beckman Coulter Inc. (Fullerton, CA). Novex polyacrylamide mini-gels, Ham's F12 medium, Eagle's minimal essential medium (EMEM), Geneticine® Selective Antibiotic (G418 sulfate), L-glutamine, and penicillin/streptomycin (pen/strep) were obtained from Invitrogen (Burlington, ON, Canada). Fetal bovine serum (FBS) and heparin (from porcine intestine) were purchased from Sigma Chemical Co. (St-Louis, MO). 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). CHO and HepG2 cell lines were obtained from ATCC (Manassa, VA). The polyclonal human anti-apolipoprotein A-I (from rabbit) was purchased from Calbiochem Biosceinces Inc (La Jolla, CA). The polyclonal human anti-HL antibody (from rabbit) was purchased from Santa Cruz (Santa Cruz, CA). Human anti-HL monoclonal antibody (mAb) XHL3-6 and anti-apoA-I monoclonal Abs (5F6 and 4H1) were obtained from Drs. Bensadoun and Marcel respectively. All other reagents were of analytical grade.

2.2 Methods

Isolation of lipoproteins by sequential ultracentrifugation

Blood samples from fasting normolipidemic/hyperlipidemic subjects were collected in Vacuett® tubes (K3E EDTA K3). One tube of the blood from each subject was sent to the pathology department of Ottawa Civic Hospital for determination of the lipid profile. Plasma was isolated from fresh blood by centrifugation at 3000 rpm in a Sorval RT6000D centrifuge for 10 minutes. VLDL, LDL, and HDL fractions were isolated from the plasma by sequential ultracentrifugation within the density ranges of $\rho < 1.006$ g/ml, $\rho = 1.019$-1.063 g/ml, and $\rho = 1.063$-1.21 g/ml respectively, using a previously published method [294]. The isolated lipoprotein fractions were dialyzed extensively against PBS (50 mM sodium phosphate, 150 mM NaCl, pH =7.2) at 4°C. Protein concentrations of lipoproteins were determined by the Markwell Lowry method [295]. Cholesterol and TG contents were measured enzymatically, using the commercial diagnostic kits (Roche Diagnostics, Laval, PQ). The homogeneity of the lipoprotein fractions was determined by agarose gel electrophoresis using pre-made Paragon® Lipogels.

Isolation of HDL fractions by sequential ultracentrifugation

Blood samples from fasting normolipidemic and dyslipidemic subjects were collected and the lipid profiles were obtained. The initial HDL fraction (ρ =1.063-1.21) of each sample was isolated by sequential ultracentrifugation as described above. Briefly, the density of HDL fraction was adjusted to 1.125 g/ml and HDL was filled into 5.1 ml ultracentrifugation tubes. The HDL was centrifuged at 100,000 rpm for 5 hour at 8°C in a TLA 110 rotor in a Beckman ultracentrifuge. Following centrifugation, the upper fraction was obtained (HDL_2) and the bottom fraction was adjusted to a density of 1.21 g/ml. Again,

5.1 ml centrifuge tubes were filled with HDL solution and centrifuged for 16 hours at 60,000 rpm. The top fraction (HDL_3) was isolated and the bottom fraction was discarded. HDL_2 and HDL_3 fractions were dialyzed against PBS (pH =7.2) and their protein concentrations were determined by the Lowry method.

HL purification: HL was purified from post-heparin human plasma by heparin affinity chromatography as previously described [245, 292]. The protein concentration of the purified HL was 0.114 mg/ml and the specific activity was determined to be 19,455 units/mg protein. SDS-PAGE and immunochemical analysis using the HL monoclonal antibody, XHL3-6a, showed a single band with an apparent molecular mass of 66 kDa.

Preparation of reconstituted HDL (rHDL) particles

Purified apoA-I, apoA-II, and apoC-I were isolated from delipidated HDL by anion exchange chromatography on a Sephacryl S-200 HR column [296]. Apoproteins were resolubilized in a 6M guanidine HCl, 10mM Tris (pH 7.2) and dialyzed extensively against PBS (pH 7.2). Reconstituted HDL complexes were prepared by sonicating palmitoyl oleyl phosphatidyl choline (POPC) with a mixture of apoproteins (apoA-I, A-II, C-I) with or without additional amounts of TG (triolein), FFA (oleic acid) or CE (cholesteryl oleate) (molar ratio of one mol protein: 60 mol lipids) as previously described [80]. POPC (3.2 mg) and other lipids in chloroform were dried under nitrogen into a 12 × 75 mm test tube and 800 µl of phosphate/saline, pH =7.4 was added. The lipid-buffer solution was initially sonicated for 1 min in a 15°C water bath and under nitrogen using a Branson 450 sonicator. The suspension was then incubated in a sealed tube for 30 min at 37°C and sonicated again for 5 min using a 95% duty cycle. ApoA-I (3 mg of a 1.8 mg protein / ml phosphate-saline solution, pH 7.4) or other apoproteins was added to the lipid suspension and the protein-lipid

mixture was sonicated for 4 × 1 min punctuated by 1-min cooling periods. All rHDL complexes were filtered through a 0.22-μm syringe tip filter. The size and homogeneity of rHDL complexes were estimated by non-denaturing gel electrophoresis on precast 8-25% acrylamide gels (Pharmacia Phastgel) after protein staining and the protein concentration was determined by the Lowry method.

Preparation of lipid-enriched HDL

Phosphatidyl inositol (PI) and phosphatidyl choline (PC) vesicles were prepared by sonication in PBS and then incubated with native HDL. Briefly, 3 mg of each lipid in chloroform was dried under nitrogen in 12 × 75 mm test tubes. 1 ml of PBS was added and the lipid-PBS mixture was sonicated for 1 min at constant duty cycle in a 15°C water bath and incubated at 37°C for 15 min. The mixture was then sonicated for 5 min at 95% duty cycle. 0.2 mg of the lipid vesicles were incubated per 1 mg fresh HDL protein for 48 hours at 4°C. Under these conditions, the entire added lipid was assimilated into the HDL particle. HDL charge was determined by electrophoretic mobilities in 0.6% agarose gels (Beckman, Paragon Lipo Kit) as previously described [297].

Preparation of apoprotein-enriched HDL

1 ml of freshly isolated HDL (5.8 mg protein / ml) from a fasting normolipidemic subject was incubated with 1.35 ml of purified and dialyzed apoA-II (2.37 mg protein / ml) for 30 min at the room temperature with constant stirring. At the end of incubation, the free apoA-I and apoA-II were isolated from HDL by ultracentrifugation. Briefly, the density of the mixture was adjusted to 1.21 mg / ml and 5.1 ml centrifugal tubes were filled with. The tubes were centrifuged in a TLA 100.4 rotor at 66,000 rpm at 8°C for 16 hours and the upper fraction was collected which contained apoA-II-enriched HDL. The total apoprotein content

of HDL was determined by SDS-PAGE in 12% precast Novex minigels follwed by silver staining. The apoA-I protein concentration of HDL was determined by ELISA (Enzyme-Linked ImmunoSorbent Assay). 96-well maxiorp immuno plates were coated with a 1:1000 dilution of monoclonal mouse anti-human apoA-I antibody (100 µl/ well) and incubated overnight at 4°C. The next day the plate was washed 3X with wash buffer (1X PBS, 0.05% tween-20), and incubated with a blocking solution (3% bovine serum albumin (BSA) in 1x PBS) at the room temperature for 1 hour. Again, the plate was washed 3X with the wash buffer. HDL samples and a series of dilutions of standard apoA-I were added to the wells (100 µl / well) and incubated at the room temperature for 2 hours. After incubation, the plate was washed 3X with the wash buffer. 100 µl of a 1:1000 dilution of detection antibody (goat anti-human apoA-I HRP) was added to each well and incubated at the room temperature for 1 hour. The plate was washed 3X and incubated at dark with 100 µl of color reagent TMB (Cedarlane) for approximately 15 min. The reaction was stopped by adding 50 µl 1N HCl to each well and the absorbance was measured at 450 nm on the microplate reader. The apoA-I concentration of HDL was calculated relative to an apoA-I standard curve. The apoA-II content of HDL was obtained by subtraction of apoA-I content from total apoproteins (obtained from silver staining) and the ratios of apoA-I/apoA-II was calculated.

Preparation of TG-enriched HDL

Plasma was obtained from a normolipidemic subject in a post-prandial state (4h after a meal). The post-prandial plasma was incubated for 5 hours at 37°C in a shaking water bath (under these conditions triglycerides from TGRL were transferred into HDL particles). Following the incubation, the HDL fraction was isolated by sequential ultracentrifugation as

described above and the total cholesterol and TG content of HDL were determined enzymatically using the commercially available Roche kits.

Hepatic lipase displacement in CHO and HepG2 cells

CHO cells stably transfected with human hepatic lipase (CHO-HL) were plated in Ham's F12 medium containing 10% FBS, 500 μg/ml G418, 1% glutamine and 1% penicillin/streptomycin in 6-well plates. HepG2 cells were plated and grown to confluence in EMEM medium containing 10% FBS, 1% glutamine and 1% penicillin/streptomycin in 6-well plates. Once confluent, cells were washed with PBS and incubated with fresh medium in the absence of FBS overnight. Next day, the medium was aspirated and the cells were washed 2X with FBS-free medium. Following the washes, the cells were incubated with FBS-free medium (2 ml medium/ well) containing HDL or rHDL samples at a concentration of 150 μg/ml for 30 min at 37°C. The medium was aspirated and collected into eppendorf tubes followed by freezing at -80°C. To prepare cell lysate, the cells were washed 2X with PBS and incubated with 100 μl of sample buffer (62.5 mM Tris-HCl, pH= 6.8, 20% glycerol, 2% SDS, 0.5 %(w/v) bromophenol blue) for 30 min at 37°C. The cell lysate was transferred into the eppendorf tubes, heated for 10 min at 90°C, and kept at -20°C until it was electrophoresed. The frozen medium was freeze-dried in a lyophilizer overnight, resolubilized in 200 μl of sample buffer, and heated at 90°C for 10 min.

SDS-PAGE and immunochemical analysis: The cell lysate and medium samples were electrophoresed on 8% polyacrylamide gels under denaturing conditions and transferred to a nitrocellulose membrane. The membrane was incubated with the blocking solution (PBS containing 1% BSA and 0.2% Tween-20) at the room temperature for 1 hour and then with the anti-HL mAb in blocking solution containing 0.02% NaN_3 at 4°C

overnight. Following 3X washes in PBS-0.2% Tween-20, a goat anti-mouse IgG HRP-linked whole antibody was used as the secondary antibody. After 1 hour incubation, the membrane was washed in PBS-0.2% Tween-20 and visualized by chemiluminescence following 5 min incubation with the Pierce Super Signal West Pico substrate. The membrane was exposed to film and the apparent molecular mass of the cell surface-associated HL as well as the liberated HL into the medium was determined using broad range molecular weight markers as a reference. The film was subsequently scanned and the mass of HL that was displaced into the medium by various types of HDL was determined relative to standard rHDL by densitometry using the BioRad Quantity One ® software.

^{125}I- labeling of LDL

1 mg of purified LDL was diluted with 500 µl of PBS, 500 µl of 1M glycine and 2 µl of 0.5M EDTA. Separately, 2.3 µl of ICl and 46 µl NaCl were mixed together on ice. 10 µl of ^{125}INa was added to the LDL solution and incubated for 5 minutes at the room temperature. Next, the ICl solution was added to quench the reaction and was incubated for 15 minutes at the room temperature. To isolate the radiolabeled LDL from free ^{125}I, a pre-equilibrated 5 ml D-salt Excellulose G-5 column (equilibrated with 50 ml of 3% BSA in PBS) was used and the sample was eluted by a 3% BSA in PBS solution. 12X of 1 ml fractions were collected and the radioactivities were counted. The two or three first fractions with the highest radioactivities were pooled together (^{125}I-LDL) and extensively dialyzed against PBS.

^{125}I- labeling of HL

Human HL isolated from post-heparin human plasma by heparin affinity chromatography as described by Ehnholm et al. [298] was purified by a 5 ml HiTrap heparin

column. Briefly, the column was pre-washed by 30 ml PBS, pH 7.2 and a total of 27 ml of HL was added to the column. Then 25 ml of PBS (binding buffer) was injected onto the column and eluted by 25 ml of 2M NaCl, 10mM sodium phosphate solution and 1 ml fractions were collected. To determine which fractions contained the highest amounts of pure HL, aliquots of the fractions were slot blotted and transferred to a nitrocellulose paper and blocked for 1 hour at the room temperature in 1% BSA, 0.2% Tween-20 solution following by probing immunochemically for the human HL. The fractions containing HL were pooled together and radiolabeled with ^{125}I. Briefly, 2 iodobeads were washed twice with 1 ml of PBS and mixed with 150 µl PBS and 10 µCi (20 µl) of ^{125}INa and incubated for 5 minutes at the room temperature. 800 µl of purified HL was added to the mixture and incubated for 45 minutes at the room temperature with gentle mixing every 10-15 minutes. The mixture was transferred to a pre-conditioned 5 ml D-salt Excellulose G-5 column (equilibrated with 15 ml of 0.1% BSA in PBS and then with 15 ml PBS alone) and 0.5 fractions were eluted by gravity by PBS. 5 µl of each fraction was counted and the first peak was pooled to obtain ^{125}I-HL.

Association of HL with HDL

HL immunoprecipitation study: 100 µg of purified HDL from human plasma was mixed with 20 µl of purified HL and 10 µl of polyclonal anti-human HL antibody raised in rabbits (Santa Cruz) and incubated overnight at 4°C on a rotor. 200 µl of a 10% protein-G Sepharose beads suspension (in 2% BSA in PBS) was added to the samples and incubated at 4°C on a rotor for 2 hours. The samples were centrifuged at maximum speed in a microfuge for 3 minute. The supernatant was discarded and following 2X gentle washes by PBS, the pellet was solubilized with SDS sample buffer. The samples were either electrophoresed on

a 12% polyacrylamide gel and probed for human apoA-I or electrophoresed on an 8% gel and probed for human HL.

Co-immunoprecipitation of HDL with ^{125}I-HL: 150 μg of purified HDL or LDL from human plasma was mixed with 20 μl of ^{125}I-HL and 200 μl of 10% protein-G Sepharose beads suspension and incubated for 2.5 hours at 4°C on a rotor (pre-clearing period). The mixture was centrifuged at maximum speed in a microcentrifuge for 3 minutes. The pellet was discarded and the supernatant was transferred into clean 1 ml minitubes. 90 μl of polyclonal anti-human apoA-I Ab raised in rabbits were added to the solution and incubated overnight at 4°C on a rotor. 200 μl of the protein-G Sepharose beads were added to the samples and incubated at 4°C on a rotor for 2 hours. After centrifugation at maximum speed for 3 minutes, the pellet was washed 2X by PBS, solubilized with 120 μl of SDS sample buffer and incubated at the room temperature for 3 hours. The samples were centrifuged at maximum speed and the supernatant was transferred into clean test tubes. Samples were electrophoresed on 12% SDS polyacrylamide gels and probed immunochemically for human apoA-I. 5 μl aliquots of the supernatant were counted in a gamma counter to determine the association of the immunoprecipitate with ^{125}I-HL.

LDL binding and uptake in CHO-HL cells

CHO-HL cells were cultured in Ham's F12 medium containing 10% FBS, 500 μg/ml G418, 1% glutamine, and 1% penicillin/streptomycin in 12-well plates. Following confluency, the cells were washed 2X with FBS-free medium and incubated with FBS-free medium containing I^{125}- LDL (10 μg/ml) ± HDL-PI (150 μg/ml) for 30 minutes at 37°C. The medium was aspirated and the cells were washed twice with PBS. To lyse the cells, 1 ml of 0.1 N NaOH was added to each well and incubated overnight rocking at room

temperature. The cell lysate was transferred into the test tubes and radioactivity associated with the samples was determined by a γ-counter to determine the total binding values. To measure the non-specific binding, the assay was performed in the presence of excessive amounts of LDL (7.5 mg LDL/ well) and the specific binding was determined by subtracting the non-specific binding from the total binding values.

Statistical analyses

Significance of differences between group mean values was calculated by Student's t-test or one-way ANOVA and Dunnett post-test analysis using GraphPad Prism® Software (Version 4.0).

Chapter 3: Results

3.1 HDL displaces cell surface-bound hepatic lipase

Displacement of hepatic lipase by heparin and HDL:

To investigate whether HDL could displace the cell surface-bound HL and how the dose of HDL affects the association of HL to the cell surface, permanently transfected CHO (Chinese hamster ovary) cells with human hepatic lipase (CHO-HL cells) were grown in complete medium in 6 well plates. Following confluency, cells were washed with PBS, the medium was replaced with FBS-free medium, and the cells were incubated overnight at 37°C. Next day, the medium was aspirated and the cells were washed and incubated with HDL preparations (75, 150, 300 or 450 μg HDL protein/ ml medium), heparin as a control (50, 100 or 200 IU of heparin/ ml of medium), or SFM (serum-free medium) alone for 30 minutes at 37°C. After incubation, the media was aspirated into clean eppendorfs and freeze-dried overnight. The powdered media were resolubilized in SDS sample buffer and heated for 10 minutes at 90°C. The samples were electrophoresed on 8% polyacrylamide gels and the released HL was detected by immunoblot analysis with an anti-HL mAb to estimate the amount of displaced HL.

It is well described that heparin displaces HL from the hepatic tissues in vivo [269]. Similarly, different doses of heparin displaced HL from the surface of cultured cells into the medium. There was very little HL released into the medium by SFM alone, but similar to heparin, HDL significantly displaced HL. The immunoblots were scanned by Quantity1® software and the percentage of released HL by HDL or heparin into the medium was determined relative to the SFM alone. Displacement of HL by heparin or HDL was dose dependent and the maximal HL displacement into the incubation medium occurred by 100

IU of heparin/ml or 150 μg of HDL protein/ml of medium (figure 1). The optimum concentration of HDL for displacing HL (150 μg/ml) is only 1/6th (~ 20%) of that of the physiological concentration of HDL in the human plasma which is 1 mg/ml. All subsequent experiments were performed under the same conditions.

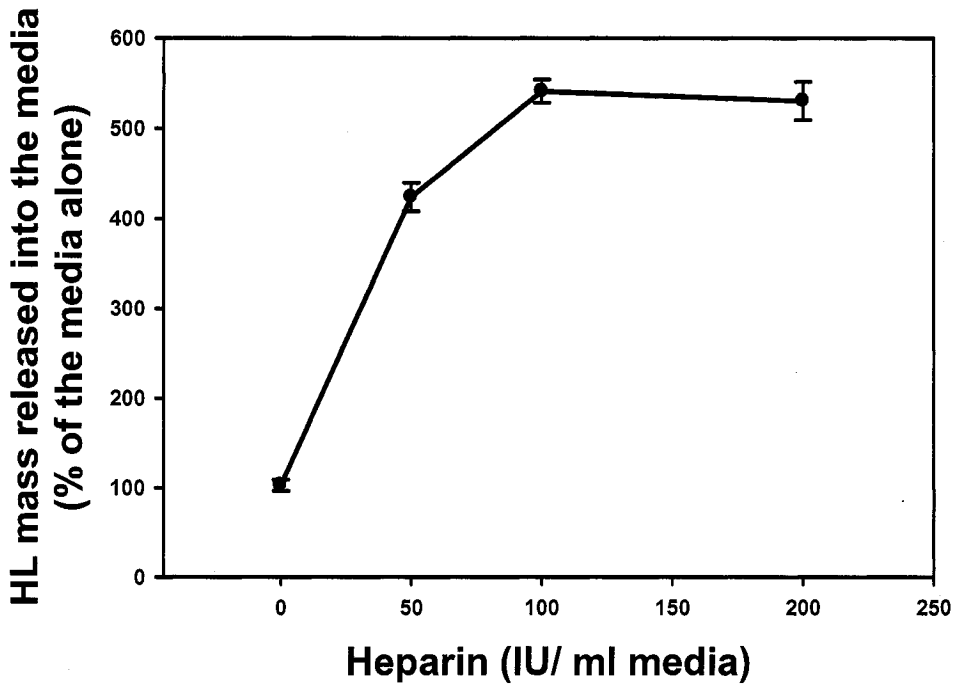
To evaluate the effect of the duration of the incubation time on HL displacement by HDL, time-course displacement experiment was carried out in CHO-HL cells under the previously described conditions. The cells were incubated with 150 μg/ml HDL protein or 100 IU of heparin/ml for various time points and amount of the released HL into the medium was measured as described above. Both HDL and heparin increased the displacement of HL during the time course and the maximum HL displacement occurred at 2 hours incubation (figure 2).

The molecular weight of HL released into the medium was estimated around 66 kDa by a standard broad range molecular weight marker and a purified human HL mixture as the standard HL (isolated from post-heparin human plasma). This molecular weight (66 kDa) is related to the cell surface-bound HL and represents the glycosylated form of the protein. The other form of HL resides inside the cytoplasm within the ER and has a lower molecular weight. These two experiments show that certain amounts of HDL or heparin affect the cell surface association of mature HL and release it into the extra-cellular space during the time.

Figure 1: Effect of heparin and HDL on HL displacement

CHO-HL cells were grown in complete medium to confluency and then the medium was changed with FBS-free medium for an overnight incubation. The cells were washed twice with FBS-free medium and incubated with increasing doses of heparin (50, 100 and 200 IU/ 1 ml of medium) and human HDL (75, 150, 300 and 400 μg HDL protein/ 1 ml medium) for 30 minutes at 37°C to liberate cell surface-bound HL into the medium. The incubating medium was collected, freeze-dried, and resolubilized in SDS sample buffer. HL mass in the medium was detected by immunochemical analysis. The western blots were scanned and the HL bands were quantified by Quantity1® software. The values of displaced HL relative to the internal control (media alone) are the mean ± S.D of triplicate determinations and are representative of three experiments.

A


HL mass released into the media
(% of the media alone)
600
500
400
300
200
100
0
0
50
100
150
200
250
Heparin (IU/ ml media)

B

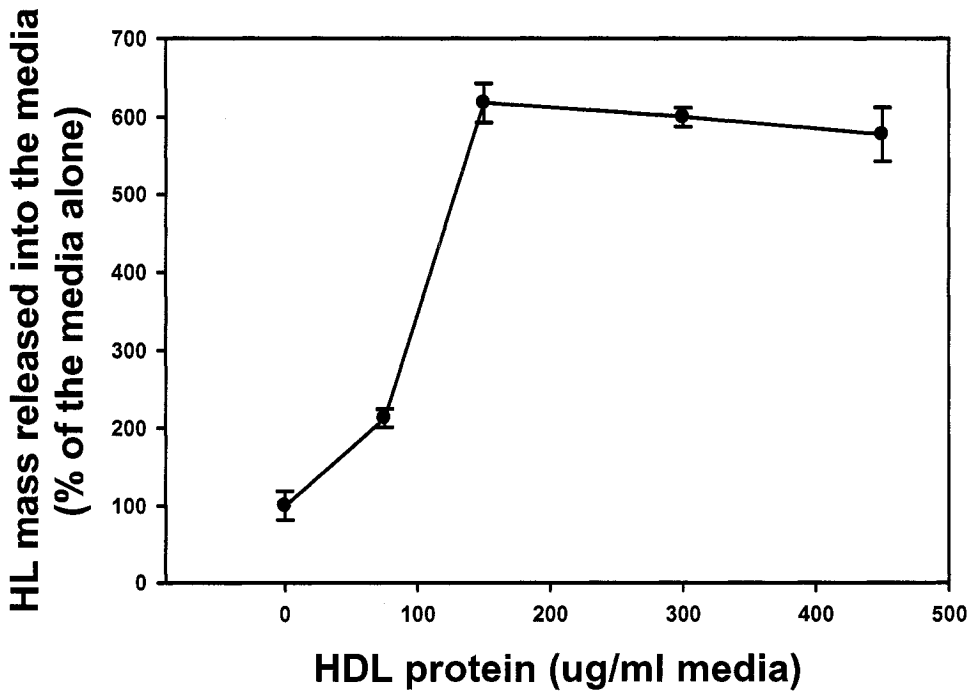
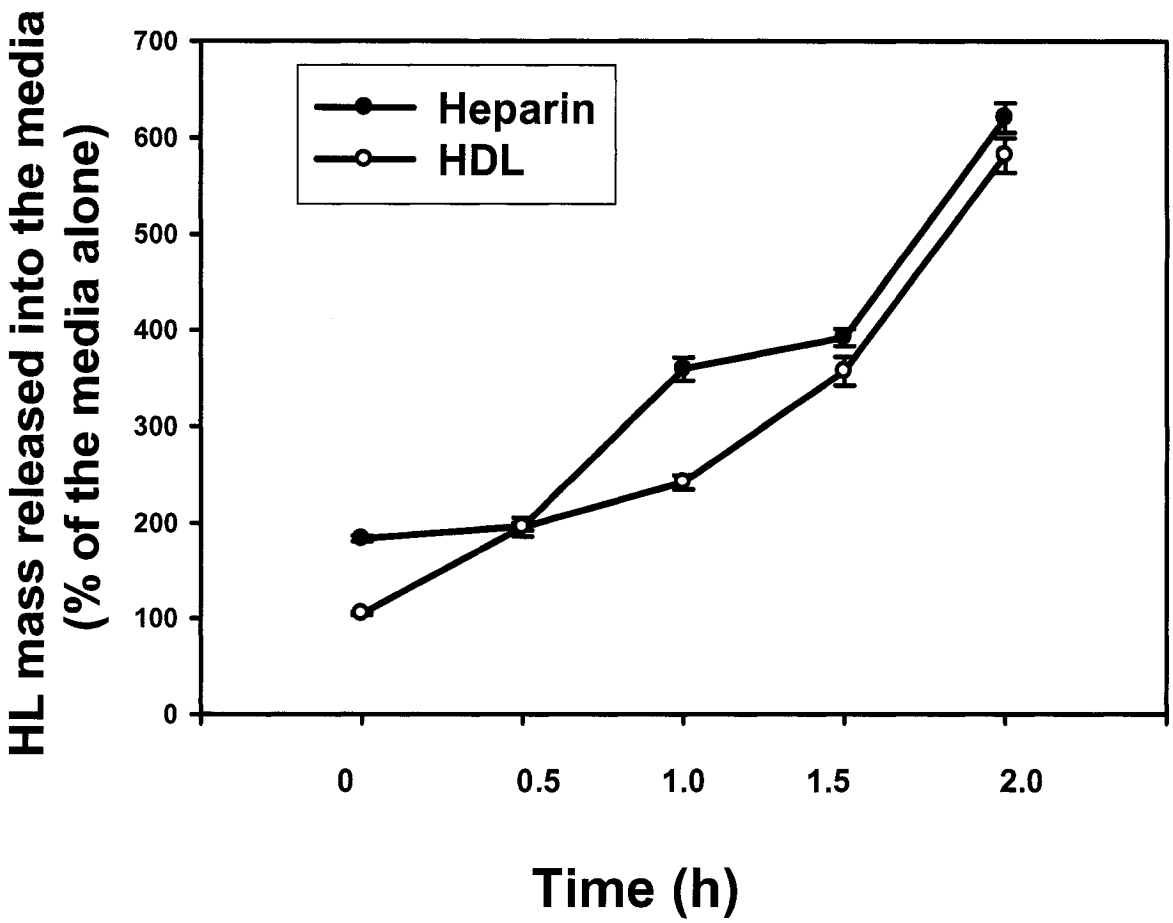

HL mass released into the media
(% of the media alone)
700
600
500
400
300
200
100
0
0
100
200
300
400
500
HDL protein (ug/ml media)

Figure 2: Time course displacement experiment in CHO-HL cells

CHO-HL cells were grown in complete medium to confluency and then the medium was replaced with FBS-free medium for an overnight incubation. Following two washes by FBS-free medium, the cells were incubated with 150 μg of HDL protein /1 ml of medium or 100 IU of heparin /1 ml of medium for various times. The incubating medium was collected and concentrated by freeze-drying and analyzed by western blots to determine the amount of HL mass released into the medium. The films of western blots were scanned and quantified by Quantity1® software. Values of released HL into the medium are relative to the internal standard (serum free media or SFM). These values are the mean ± S.D of triplicate determinations and represent two experiments.


HL mass released into the media
(% of the media alone)
700
600
500
400
300
200
100
0
Heparin
HDL
0
0.5
1.0
1.5
2.0
Time (h)

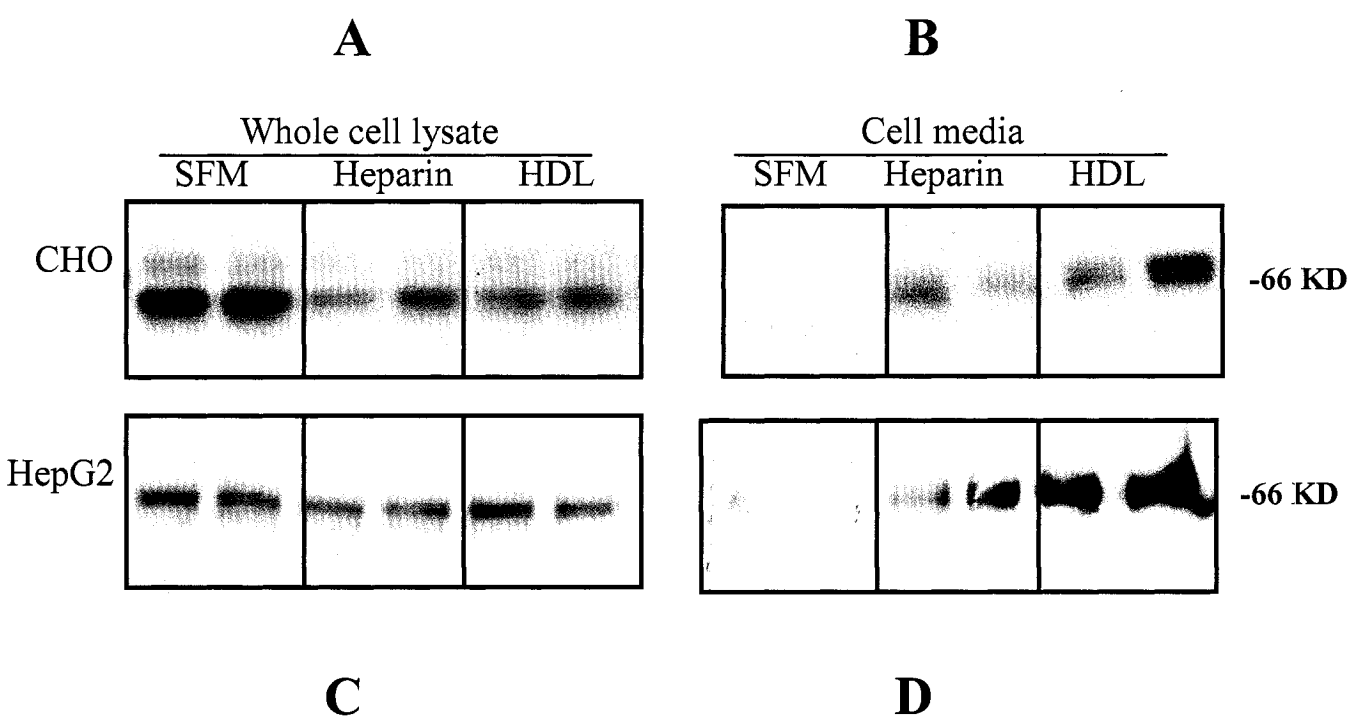
Displacement of cell surface-bound HL by HDL in CHO and HepG2 cells:

To compare the effect of HDL on HL displacement in transfected cells and cells that express HL endogenously, CHO-HL cells and HepG2 cells were grown and treated as above with HDL, heparin or SFM alone for 30 minutes. After transferring the incubation media into separate tubes for freeze-drying, the cells were washed 2X with PBS and incubated with 100 µl of SDS sample buffer at 37°C for 30 minutes. The total cell lysate in sample buffer was heated for 10 minutes at 90°C and after cooling down was electrophoresed on 8% polyacrylamide gels. Similarly, electrophoresis was performed at the same conditions with lyophilized incubation media and the intracellular and cell surface-bound forms of HL were detected by immunochemical analysis (anti-HL mAb).

There were two distinctive bands in the total extract of the CHO cells corresponding to the intra- and extra-cellular forms of over-expressed HL (figure 3-A). Addition of HDL or heparin to the incubation medium resulted in the disappearance of the HL band with the larger molecular weight (presenting the cell surface-bound HL) as well as a decrease in the intensity of the lower molecular weight band (intracellular form of HL). At the same time, a 66 kDa HL band was detected in the incubation medium of CHO cells showing that the released HL from the cell surface is directly secreted into the extra-cellular space (figure 3-B). Intra-cellular HL molecules with a lower molecular weight were not detected in the cell media, showing that these HL molecules have to be glycosylated prior to the secretion from the ER [173].

Figure 3: Displacement of cell surface-bound HL in CHO-HL and HepG2 cells

CHO-HL and HepG2 cells were grown in their complete media to confluency. Then the medium was replaced with FBS-free medium for overnight. After two washes with FBS-free medium, the cells were incubated with serum free media (SFM), heparin, and HDL for 30 minutes at 37°C. The incubating medium was aspirated, freeze-dried, and resolubilized in SDS sample buffer. The cells were washed twice with PBS to remove any remaining incubation medium and incubated with SDS sample buffer for 30 minutes at 37°C. The total cell extracts in addition to the medium samples were electrophoresed on 8% SDS polyacrylamide gels. Immunochemical analysis followed to detect the amounts of HL associated with the cells and released into the medium. The images in duplicates represent three experiments for each cell line.


A
B
Whole cell lysate
Cell media
SFM
Heparin
HDL
SFM
Heparin
HDL
CHO
-66 KD
HepG2
-66 KD
C
D

The cell extract of HepG2 cells unlike the CHO cells, only showed one band at lower than 66 kDa relating to the endogenous intracellular HL (figure 3-C). Similar to CHO cells, upon treating the cells by HDL or heparin, HL bands at 66 kDa were detected in the incubation medium (figure 3-D).

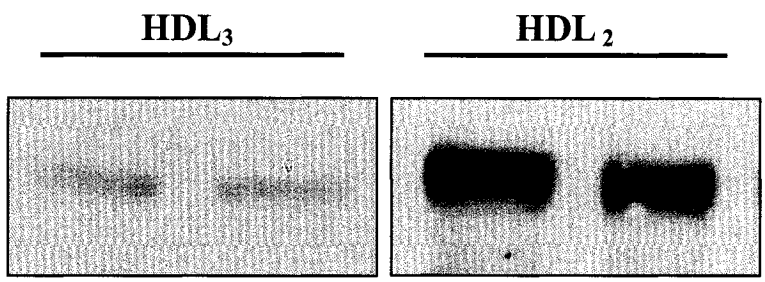
3.2 Structural properties of HDL regulate hepatic lipase displacement

Displacement of cell surface-bound HL by different HDL species:

To evaluate the effect of HDL size and degree of lipidation on HL displacement, plasma was obtained from normolipidemic subjects and HDL fractions were isolated by sequential ultracentrifugation. HepG2 cells were grown to confluency and incubated with 150 μg/ml of HDL_2 and HDL_3 fractions for 30 minutes. As described previously, the incubation media were collected, concentrated by freeze-drying and immunoblotted. Probing for HL in the medium resulted in detection of a significant amount of HL released into the medium by HDL_2 and almost no HL displacement by HDL_3 (figure 4). This data indicates that the larger species of HDL, HDL_2, are more capable of displacing HL, whereas the smaller HDL particles promote little HL displacement.

Figure 4: Displacement of cell surface-bound HL by HDL subspecies

HepG2 cells were grown in complete EMEM medium and following confluency the medium was exchanged with FBS-free medium for overnight. After two washes with FBS-free medium, the cells were incubated with **HDL_2 and HDL_3** fractions isolated from human plasma (150 µl HDL / 1 ml of medium) for 30 minutes at 37°C. The cell medium was collected and concentrated by freeze-drying. The medium samples were resolubilized in SDS sample buffer and analyzed by Western blots to determine the mass of HL released into the medium. The duplicate blots are representative of three displacement experiments.


HDL3
HDL 2

Displacement of the cell surface-bound HL by native human HDL and reconstituted HDL particles (rHDL):

In order to determine the ability of synthetic HDL particles to displace HL, rHDL particles were generated by sonication of purified apoA-I and POPC (1:60 ratio). Native HDL was also obtained from normolipidemic subjects. CHO-HL cells were grown and incubated with heparin, native HDL or rHDL for 30 minutes and the amount of HL liberated into the medium was determined by immunochemical analysis. Following quantification of the HL bands, HL mass released into the medium was calculated relative to the SFM alone (figure 5).

The result of this experiment reveals that rHDL displaces HL similar to heparin and that the ability of rHDL in displacing HL is slightly more than native HDL. It has been demonstrated previously that pure apoA-I displaces more cell surface-associated HL than does HDL [292]. Similarly, the present data show that significant amounts of HL are released by rHDL particles consisting of apoA-I and POPC. These data together suggest that apoA-I is a critical structural element of HDL involved in HL displacement.

HL displacement by native human HDL enriched with apoA-II:

In order to evaluate the regulatory effect of apolipoproteinA-II content of HDL in HL displacement, native human HDL was purified from normolipidemic plasma and enriched with pure apoA-II. Briefly, 1 ml of the freshly isolated HDL fraction (5.8 mg of HDL protein/ml) was incubated with 1.35 ml of purified apoA-II (2.4 mg protein/ml) for 30 min at the room temperature with constant stirring. It is known that during the incubation, free apoA-II displaces apoA-I molecules from HDL [86] and therefore, generates HDL particles that are depleted of apoA-I and enriched in apoA-II.

At the end of the incubation, to isolate the apoA-II-enriched HDL from free apolipoprotein molecules, the mixture was ultracentrifuged for 16 hours at a density of 1.21 and the top fraction containing HDL-apoA-II was separated from the bottom fraction (containing free apoproteins). Alterations in apolipoprotein content were detected by SDS-PAGE followed by silver staining and the total concentrations of apoA-I and apoA-II were calculated based on standard apolipoprotein references. Separately, apoA-I concentrations of HDL samples were determined by ELISA. ApoA-II concentrations were obtained by subtraction of apoA-I values (obtained by ELISA) from total protein concentration (confirmed by silver staining). Figure 6 shows that the ratio of apoA-I to apoA-II concentration has decreased by 50% in apoA-II enriched HDL. Next, to investigate the effect of apoA-II on HL displacement, CHO-HL cells were incubated with FBS-free medium containing 150 μg/ml HDL or HDL-apoA-II for 30 minutes at 37°C. The incubation media were collected and analyzed for their HL content. The HL mass released by HDL or HDL-apoA-II was calculated relative to HL displacement by standard rHDL as the internal control (figure 7). It is shown in figure 7 that incorporation of apoA-II molecules into the structure of HDL, by displacing apoA-I from the complex, causes a 2.5 fold increase in HL displacement from the cell surface. This data suggests that apoA-II is a structural component of HDL that plays a significant role in displacing cell surface-bound HL.

Figure 5: Displacement of HL by native and reconstituted HDL (rHDL)

CHO-HL cells were grown in complete medium to confluency then the medium was replaced with FBS-free medium for an overnight incubation. Following two washes with FBS-free medium, the cells were incubated with 150 μg/ ml of native HDL isolated from fresh human plasma or reconstituted HDL (POPC/apoA-I) for 30 minutes at 37°C. The incubating medium was collected, freeze-dried, and resolubilized in SDS sample buffer. Western blot analysis was performed on the medium samples to detect HL released into the medium by native and synthetic HDL. HL bands were scanned and quantified using Quantity1® software. The values of released HL are mean ± SD of three determinations and represent two displacement experiments. (Significance of difference from heparin by one-way ANOVA and Dunnett post-test analysis,* $p < 0.01$)

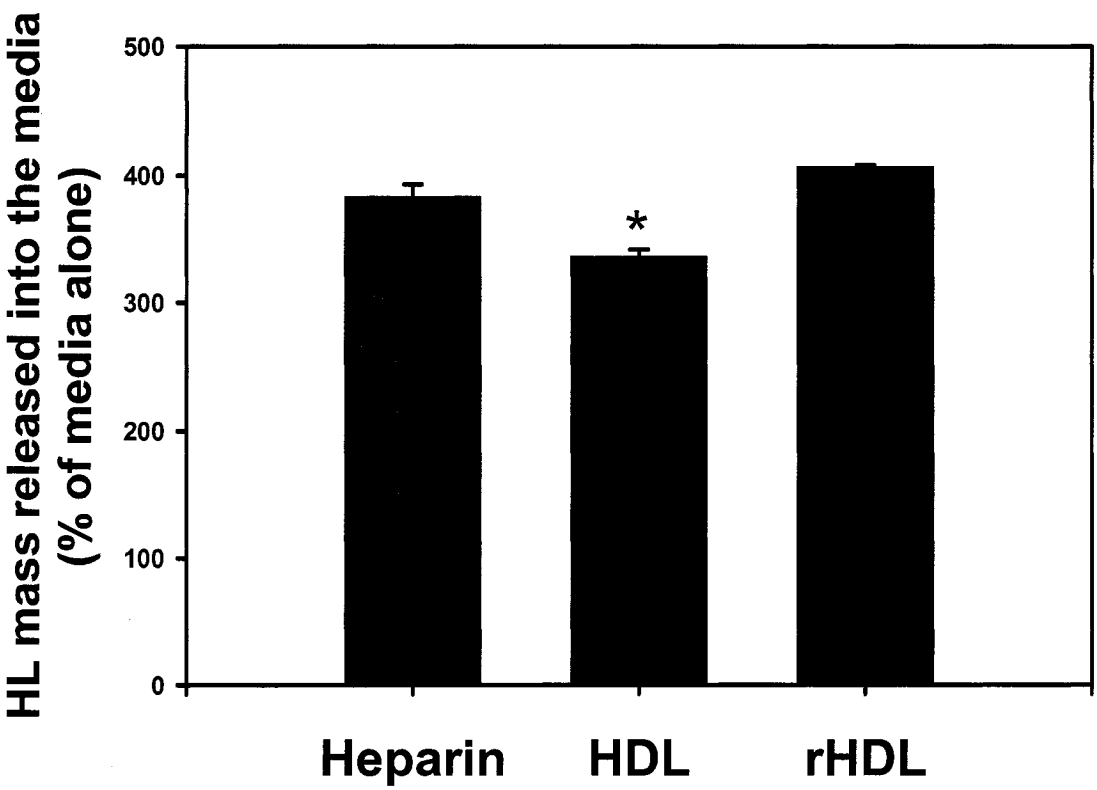

HL mass released into the media
(% of media alone)
500
400
300
200
100
0
*
Heparin
HDL
rHDL

Figure 6: Enrichment of native HDL by apoA-II

HDL fraction was isolated from normolipidemic fasting human plasma and 5.8 mg of that was incubated with 2.37 mg of pure human apolipoproteinA-II for 30 minutes at the room temperature with constant stirring. At the presence of excessive amounts of apoA-II during the incubation, apoA-I is displaced from HDL structure and replaced by apoA-II molecules. To isolate free apoA-II molecules from HDL, the density of the mixture was adjusted to 1.21 mg/ml (HDL's density) and the mixture was centrifuged at 66,000 rpm for 16 hours. The upper fraction containing apoA-II-enriched HDL was isolated. ELISA assay was performed in order to determine the concentration of apoA-I in HDL and HDL-apoA-II. SDS-PAGE followed by silver staining was also performed to determine the ratio of apoA-I to apoA-II content. The values of apoA-I: apoA-II ratios are mean ± SD of triplicate determinations and represent two experiments. (Significance of difference by Student's t-test, $^{**}p < 0.001$)

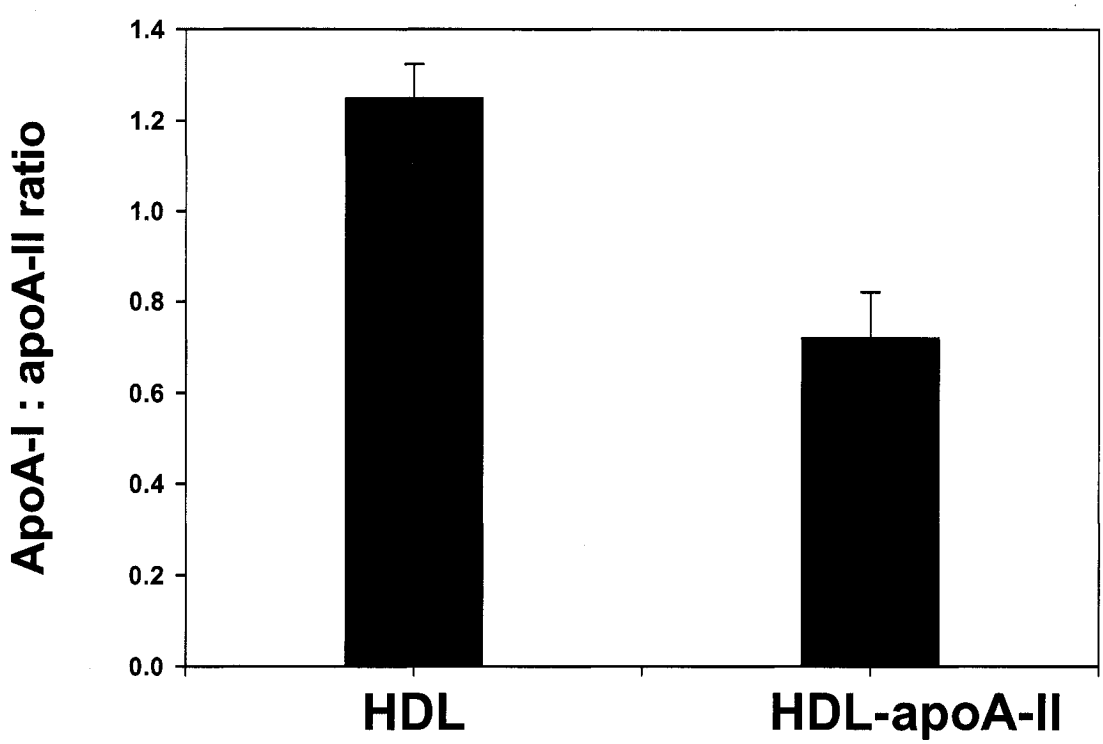
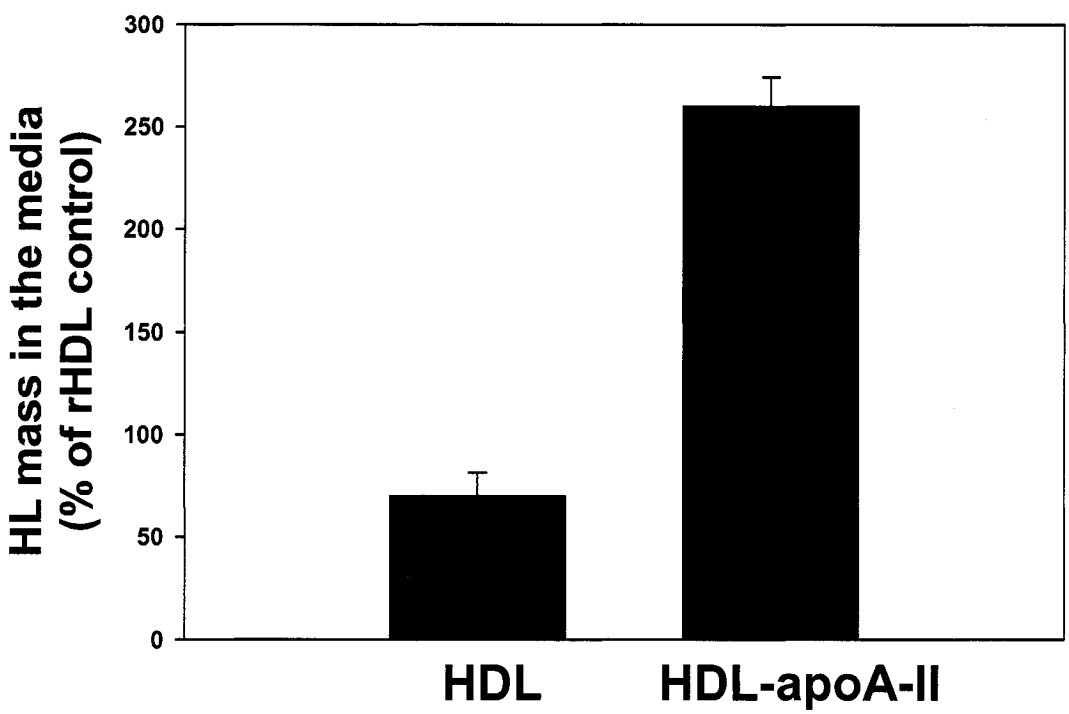

ApoA-I : apoA-II ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HDL
HDL-apoA-II

Figure 7: HL displacement in CHO-HL cells by native and apoA-II-enriched HDL

CHO-HL cells were grown in complete medium and following confluency; their medium was exchanged with FBS-free medium overnight. After two washes by FBS-free medium, the cells were incubated with 150 μg/ ml of native or apoA-II-enriched HDL for 30 minutes at 37°C. The cell medium was collected, freeze-dried, and resolubilized in SDS sample buffer. The cell media samples were electrophoresed on 8% polyacrylamide gels and immunochemical analysis followed to determine the amount of HL released into the medium. The blots were scanned and quantified by Quantity1® software and the mass of liberated HL into the medium was calculated relative to the control (rHDL particles). The values of HL in the medium are mean values ± SD of triplicate determinations and represent two displacement experiments (Significance of difference by Student's t-test, ***$p < 0.0001$).


HL mass in the media
(% of rHDL control)
300
250
200
150
100
50
0
HDL
HDL-apoA-II

HL displacement by reconstituted HDL particles enriched with apolipoproteins:

In order to assure that apoA-II has a positive impact on HL displacement, and to estimate the role of other structural apoproteins of HDL in HL displacement, reconstitution of various HDL particles was carried out. A number of purified apoproteins including A-I, A-II, C-I and apoHDL (a mixture of all apoproteins of HDL) that were previously isolated from delipidated HDL by anion exchange chromatography, resolubilized in 6M guanidine HCl, 10mM Tris (pH 7.2) and dialyzed extensively against PBS (pH 7.2). 3.2 mg/ml of POPC in chloroform was dried under nitrogen and resolubilized in 800 µl of PBS. As described in section 2.2, the apoproteins and POPC were incorporated together by sonication and the rHDL complexes were generated. The molar ratio of the lipid and protein components of each particle is shown in table below.

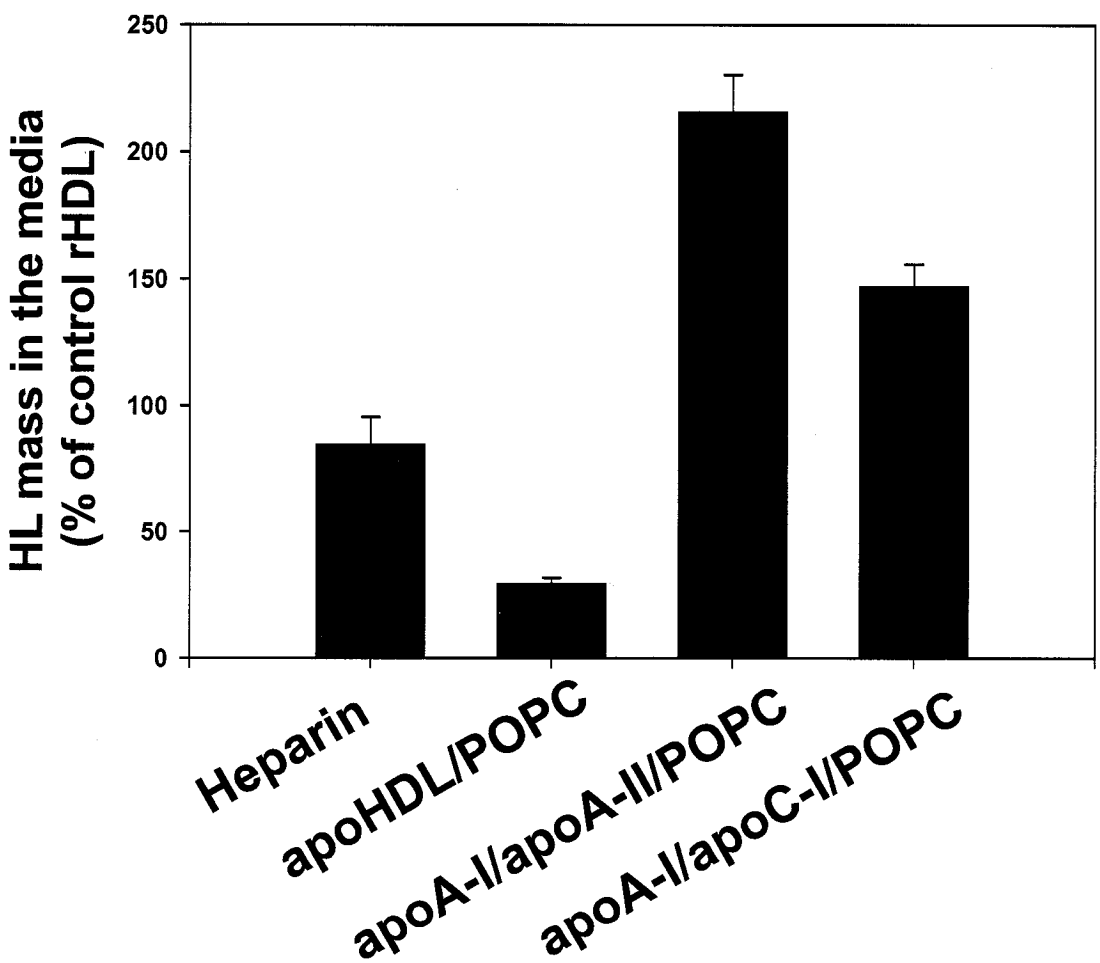
rHDL particle	POPC (mol)	apoA-I (mol)	apoA-II (mol)	apoC-I (mol)	apoHDL (mol)	rHDL Protein: lipid (mol:mol:mol)
ApoA-I/POPC	60	1.0	0.0	0.0	0.0	2:120
ApoA-I/A-II/POPC	60	0.33	0.66	0.0	0.0	1:2:120
ApoA-I/C-I/POPC	60	0.33	0.0	0.66	0.0	1:2:120
ApoHDL/POPC	60	0.0	0.0	0.0	1.0	nd

rHDL prepared from apoA-I alone contained 2 moles of apoA-I / particle, while those prepared with apoprotein mixtures contained one mole of apoA-I and two moles of the test apoprotein.

HL displacement assay in CHO-HL cells was performed using the reconstituted HDL particles (150 μg/1 ml of incubation medium) and the incubation media were analyzed for HL content. Figure 8 presents the percentage of released HL into the medium by various kinds of rHDL particles and heparin relative to the standard rHDL particle (apoA-I/POPC). The figure indicates that apoA-I/apoA-II/POPC particles increase HL displacement by >2 fold relative to control rHDL confirming the results of the previous experiment (native HDL-apoA-II had an increasing effect on HL displacement). ApoA-I/apoC-I/POPC particles increased the HL displacement as well, but only up to 1.5 fold control, suggesting that apoC-I like apoA-II has a stimulatory role in HL displacement. ApoHDL/POPC particles, which contained the whole variety of HDL apoproteins, had a lesser effect on HL displacement (25% of HL displacement by control rHDL). This effect may be a result of the inhibitory role of other apoproteins of HDL such as apoC-III, apoA-IV, or apoE that decrease the stimulatory effect of apoA-I, apoA-II, or apoC-I in HL displacement.

Figure 8: HL displacement by rHDL particles containing various apolipoproteins

Synthetic HDL particles were generated by sonicating phospholipids and pure apoproteins together. CHO-HL cells were grown in complete medium and following the confluency, their medium was exchanged with FBS-free medium for overnight. After two washes by FBS-free medium, the cells were incubated with 150 μg/ ml of POPC/A-I, POPC/A-I/A-II, POPC/A-I/apoC-I, and POPC/apoHDL particles for 30 minutes at 37°C. The incubating medium was aspirated, concentrated by freeze-drying, and solubilized in SDS sample buffer. The western blot analysis followed to determine the mass of HL released into the medium. The blots were scanned and quantified and the values of HL in the medium were calculated relative to the control (rHDL). The values of HL are mean ± SD of triplicate determinations and represent three displacement experiments (Significance of difference from heparin by one-way ANOVA and Dunnett post-test analysis, $^{**}p < 0.001$, $^{*}p < 0.01$).


HL mass in the media
(% of control rHDL)
250
200
150
100
50
0
Heparin
apoHDL/POPC
apoA-I/apoA-II/POPC
apoA-I/apoC-I/POPC

HL displacement by reconstituted HDL particles enriched with specific lipids:

HL displacement experiments with different kinds of rHDL particles enriched in lipids were performed to determine whether various lipid constituents of HDL impact HL displacement. Using the previously described sonication technique, rHDL particles enriched in cholesterol ester (CE), triglyceride (TG), and free fatty acid (FFA) were generated. These particles contained apoA-I, POPC and CE, TG or FFA (the ratios are shown in table below).

rHDL Particle	ApoA-I (mol)	POPC (mol)	CE (mol)	TG (mol)	FFA (mol)	rHDL Protein:lipid (mol:mol:mol)
ApoA-I/POPC	1.0	60.0	0.0	0.0	0.0	2:120
ApoA-I/POPC/CE	1.0	60.0	15.0	0.0	0.0	2:120:30
ApoA-I/POPC/TG	1.0	60.0	0.0	15.0	0.0	2:120:30
ApoA-I/POPC/FFA	1.0	60.0	0.0	0.0	15.0	2:120:30

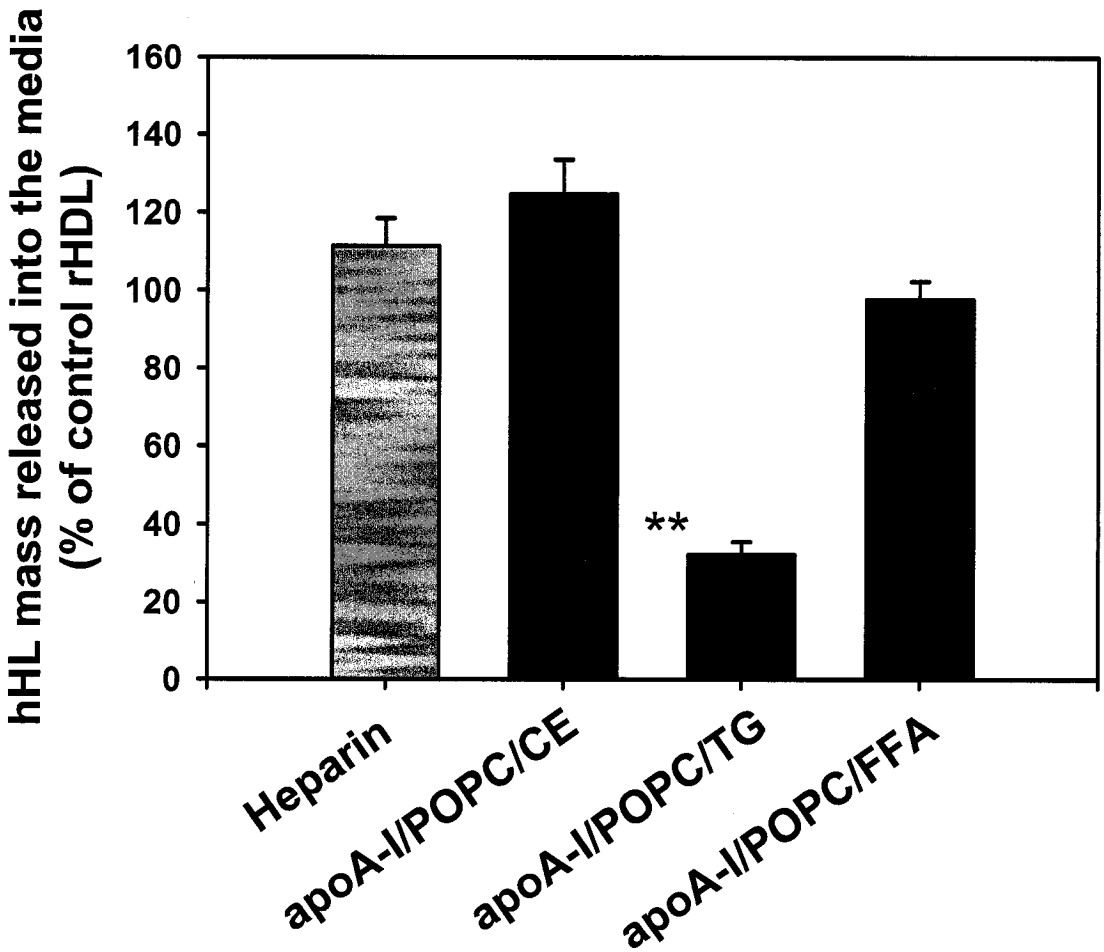
rHDL particles contain 2 moles of apoA-I / particle.

CHO-HL cells were incubated with lipid-enriched rHDL particles (150 μg/ 1 ml of medium) or heparin (100 IU/ 1 ml medium) for 30 minutes at 37°C and the incubating media were analyzed immunochemically to determine the amount of released HL into the media. The results of this experiment (figure 9) demonstrate that CE- enriched particles slightly increased HL displacement (1.2 fold control), whereas the particles enriched with TG significantly decreased HL displacement to 25% of control. FFA-enriched particles had slight inhibitory effect on HL displacement. These results suggest that the TG component of HDL particles is an important regulator of HL displacement from the cell surface. Although

the CE and FFA constituents of HDL have a stimulatory and inhibitory effect on HL displacement respectfully, their effects are not significant.

Figure 9: HL displacement by rHDL particles enriched by various lipids

Synthetic HDL particles enriched in lipids were generated by sonicating POPC and CE/ TG/ FFA with pure apoA-I. CHO-HL cells were grown in complete medium to confluency and then their medium was exchanged with FBS-free medium for an overnight incubation. After two washes by FBS-free medium, the cells were incubated with 100 IU/ ml of heparin or 150 μg/ ml of apoA-I/POPC, apoA-I/POPC/CE, apoA-I/POPC/TG, and apoA-I/POPC/FFA particles for 30 minutes at 37°C. The incubating medium was aspirated, concentrated by freeze-drying, and solubilized in SDS sample buffer. The western blot analysis followed to determine the mass of HL released into the medium. The blots were scanned and quantified and the values of HL in the medium were calculated relative to the control (apoA-I/POPC). The values of HL are mean ± SD of triplicate determinations and represent three displacement experiments with each particle (Significance of difference from heparin by one-way ANOVA and Dunnett post-test analysis, $^{**}p < 0.001$).


hHL mass released into the media
(% of control rHDL)
160
140
120
100
80
60
40
20
0
**
Heparin
apoA-I/POPC/CE
apoA-I/POPC/TG
apoA-I/POPC/FFA

HL displacement by native HDL enriched with phospholipids:

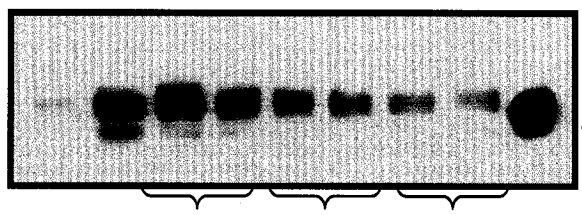
In order to study the role of surface phospholipids of HDL in HL displacement, similar experiments with native HDL enriched by phospholipids were performed. Following the isolation of fresh HDL from normolipidemic subjects, phospholipid vesicles were prepared by sonication and incubated with HDL. In summary, 3 mg of phospholipids (PI or POPC) in chloroform were dried under nitrogen, resolubilized in 1 ml PBS, and sonicated to generate PI or POPC vesicles. Then, 0.2 mg of each vesicle solution was incubated with 1 mg of fresh HDL for 48 hours at 4°C. During the incubation, all of the phospholipids were transferred into HDL particles. HDL, HDL-PI, and HDL-POPC were electrophoresed on agarose gels to assess the phospholipid enrichment and electrostatic charge modifications [299]. The agarose gel electrophoresis showed that PI-enrichment of HDL makes the lipoprotein particles negatively charged, whereas POPC vesicles make them positively charged (*Boucher*, unpublished data).

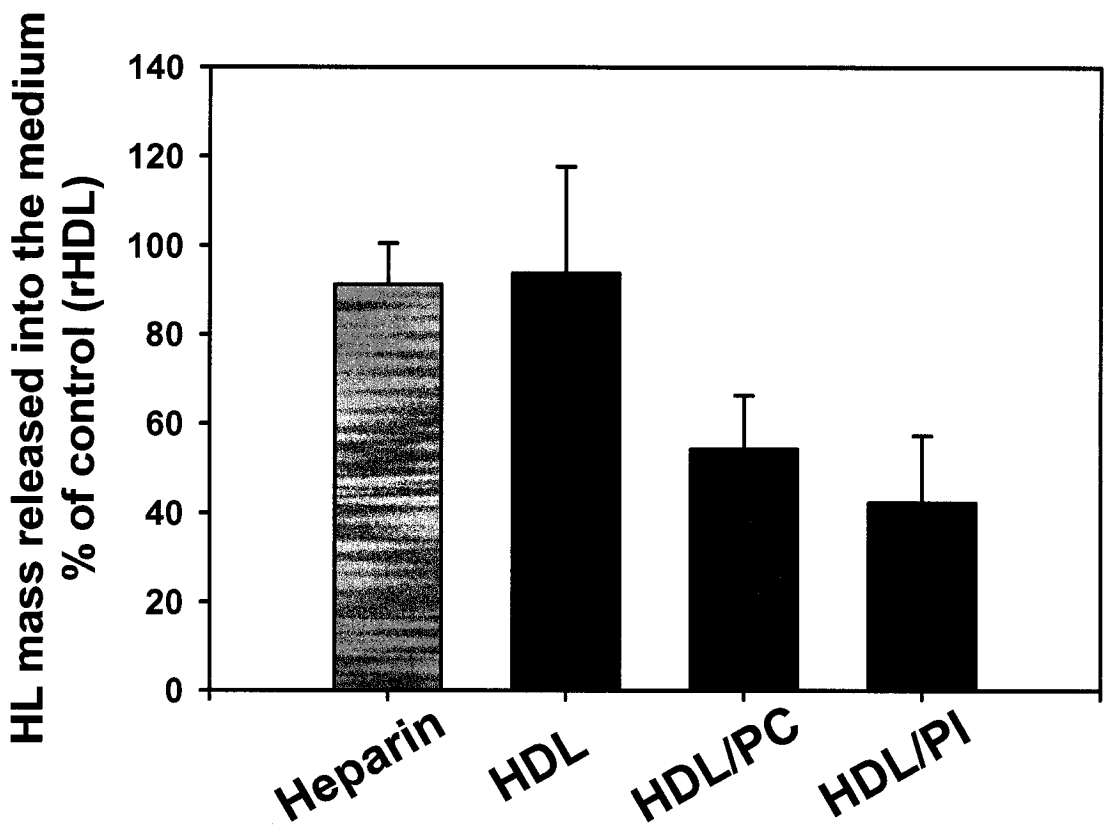
HL displacement assay with native HDL, HDL-PI, and HDL-POPC, followed by analysis of the incubation medium revealed that both kinds of phospholipids were able to decrease HL displacement relative to native HDL or heparin (~ 50% that of control rHDL) (figure 10).

Figure 10: HL displacement by phospholipid-enriched native HDL

Native HDL obtained from normolipidemic human subjects was enriched with phosphotidyl choline (PC) or phosphotidylinosytol (PI) by sonication. CHO-HL cells were grown in complete medium to confluency. Then the medium was exchanged with FBS-free medium for an overnight incubation. After two washes by FBS-free medium, the cells were incubated with 100 IU/ ml of heparin or 150 μg/ ml of HDL, HDL/PC, or HDL/PI for 30 minutes at 37°C. The incubating medium was aspirated, concentrated by freeze-drying, and solubilized in SDS sample buffer. The western blot analysis followed to determine the mass of HL released into the medium. The blots were scanned and quantified and the values of HL in the medium were calculated relative to the control (apoA-I/POPC). The values of HL are mean ± SD of triplicate determinations and represent three displacement experiments with phospholipid-enriched HDL (Significance of difference from heparin by one-way ANOVA and Dunnett post-test analysis, *$p < 0.01$).

Western blots of the cell medium





HL displacement by fasted and TG-enriched normolipidemic HDL:

In order to confirm the modulatory role of TG component of HDL in HL displacement, HL displacement assays were carried out using HDL isolated from pre- and post-prandial plasma from a normolipidemic subject. Plasma from the post-prandial sample contained elevated levels of TG and was incubated for 3 hours at 37°C to let the plasma TG assimilate into HDL particles. Total HDL fractions were isolated from plasma samples and the HDL-TG levels were determined by commercial kits. Figure 11 shows that the TG level in the post-prandial HDL (TG-enriched HDL) is elevated by 1.3 fold compared to the fasting HDL.

The results show that the ability of TG-enriched HDL in displacing HL is decreased significantly compared to the fasting state HDL (figure 12). This data confirms the results of experiments with rHDL-TG, suggesting that the TG content of HDL particles play an inhibitory role in HL displacement.

Figure 11: HDL-TG levels in fasting and TG-enriched states

Plasma samples were obtained from a normolipidemic subject at the fasting and TG-enriched states. The post-prandial plasma was incubated for 3 hours at 37°C in a shaking water bath in order to enrich lipoprotein particles with plasma triglycerides. HDL fractions were isolated from pre- and post-prandial plasma and the TG concentration of HDL samples were determined by commercial kits (Roche TG assay kit). The values of TG (mg) per 1 ml of HDL are mean ± SD of triplicate measurements and represent only one blood sample. A Student's t-test was performed and results were considered statistically significant ($*p < 0.01$).

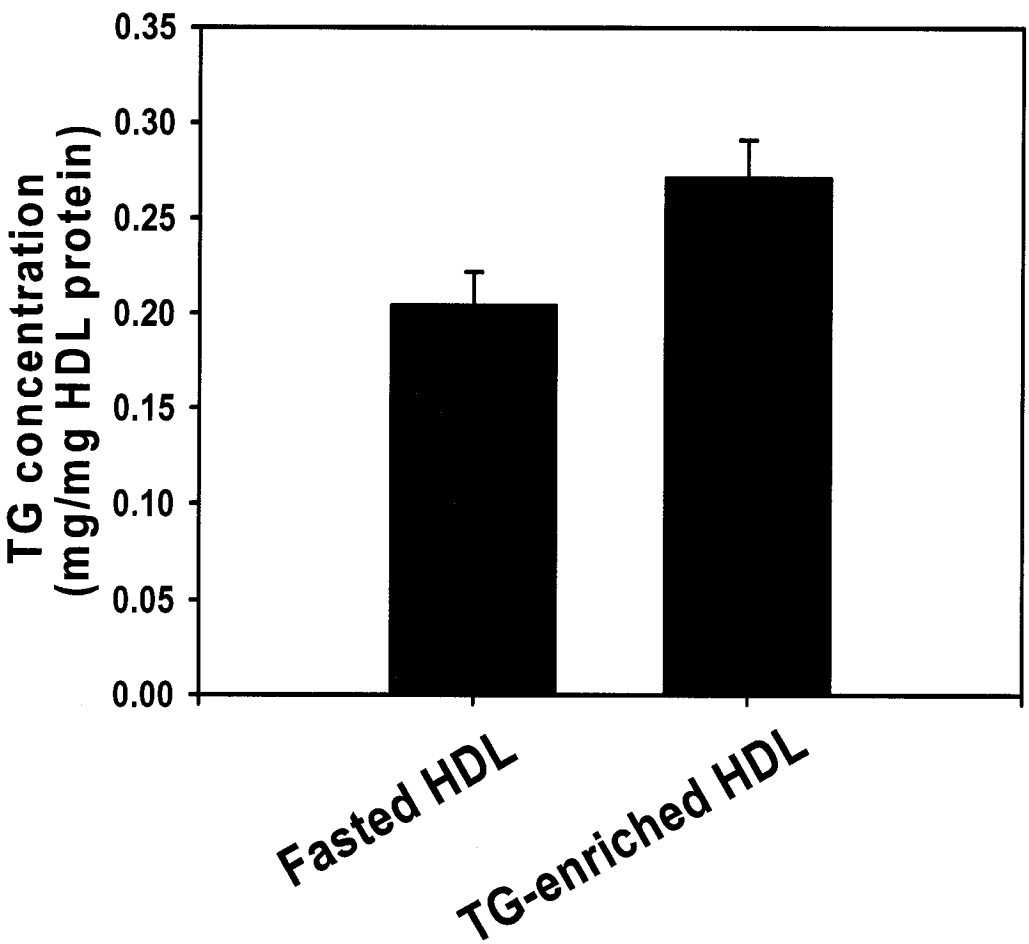
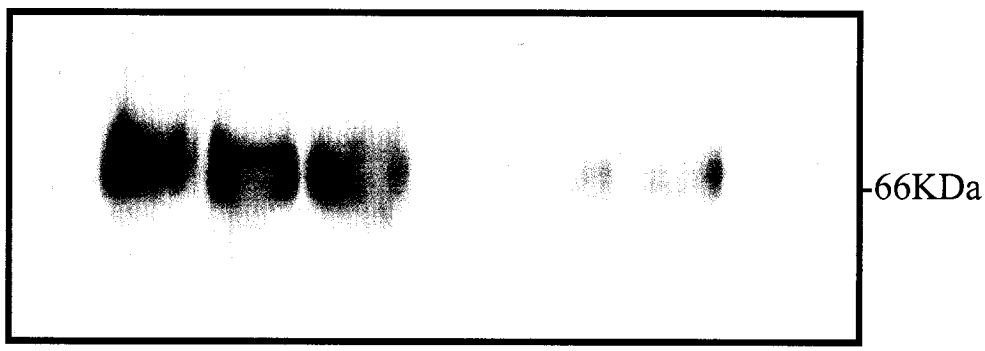

TG concentration
(mg/mg HDL protein)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Fasted HDL
TG-enriched HDL

Figure 12: HL displacement in CHO-HL cells by fasted and TG-enriched HDL

Normolipidemic plasma from the fasting and post-prandial states was obtained and the post-prandial sample was incubated at 37°C for 3 hours. During the incubation time, TG from plasma assimilated into the HDL particles and enriched them. CHO-HL cells grown to confluency were incubated with 150 μg/ ml of HDL (fasting state) or HDL-TG for 30 minutes at 37°C. The incubating medium was aspirated, concentrated by freeze-drying, and solubilized in SDS sample buffer. Western blots and immunochemical analysis followed to determine the mass of HL released into the medium by various types of HDL. The triplicate images are representative of two displacement experiments.

HL Western blots: Cell medium



Fasted HDL **TG-enriched HDL**

HL displacement by HDL fractions from hyperlipidemic patients:

To determine whether HDL from hyperlipidemic subjects has the same effect as normal HDL in displacing HL, preliminary experiments were undertaken. Blood samples were obtained from two mildly hypercholesterolemic patients as well as a normal individual. The total HDL fraction was isolated from fresh plasma and TG levels were determined by commercial TG test kits (Roche). The HDL-TG levels were significantly higher in the hyperlipidemic subjects compared to the normal subject (figure 13 representing one normal subject versus one patient). Subsequently, HDL_2 and HDL_3 subfractions were isolated from total HDL by ultracentrifugation. The CHO-HL cells were incubated with total HDL, HDL_2, and HDL_3 fractions as explained previously and the incubation media were analyzed for their HL content. The western blots of the cell media (shown in figure 14) reveal that HDL samples from the hyperlipidemic patient are not capable of displacing cell surface-bound HL. Western blots of the normolipidemic subject indicate that the total HDL, as well as the HDL_2 fraction, displaces HL readily. As was expected, HDL_3 fraction from both subjects had very little or no ability to displace HL.

Figure 13: HDL-TG levels in normo- and hyperlipidemic subjects

Plasma samples were obtained from normolipidemic and mild hypercholesterolemic subjects. HDL fractions were isolated from plasma by sequential ultracentrifugation and the TG content of HDL samples was determined by commercial kits (Roche TG assay kit). The values of TG (mg / mg HDL protein) are mean ± SD of triplicate measurements and represent only one series of blood samples (Significance of difference by Student's t-test, **p < 0.001).

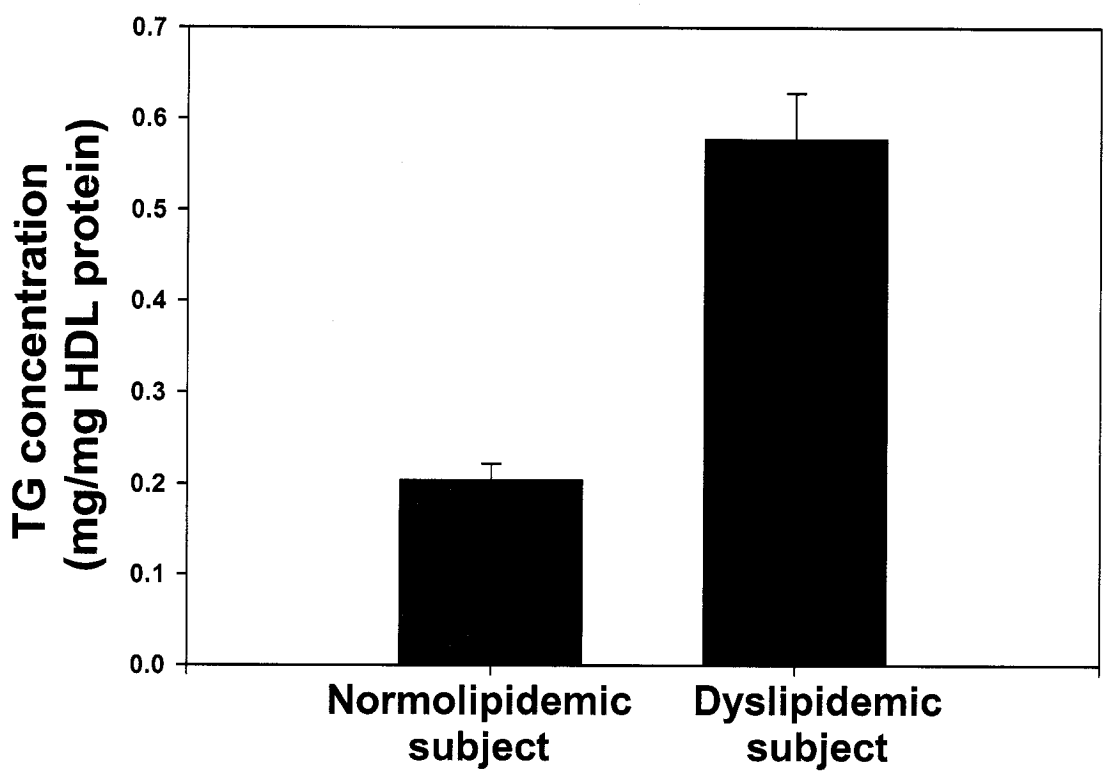
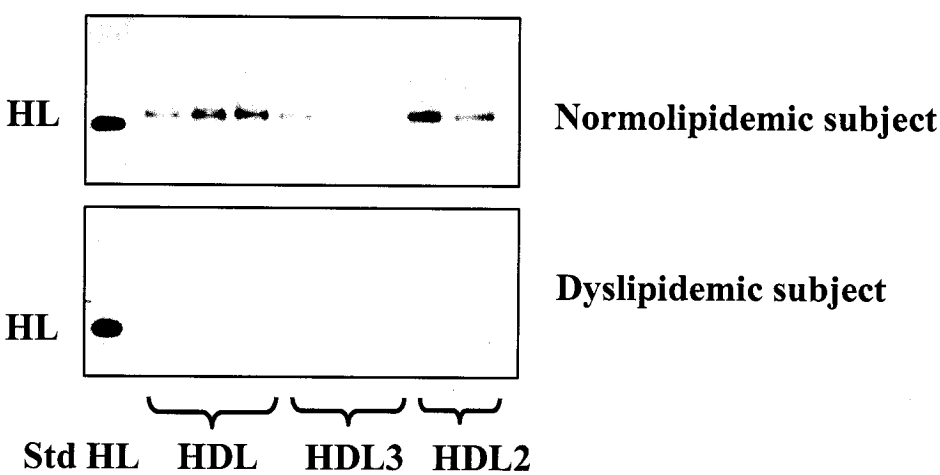

TG concentration
(mg/mg HDL protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Normolipidemic subject
Dyslipidemic subject

Figure 14: HL displacement in CHO-HL cells by HDL fractions from normal and hyperlipidemic subjects

HDL subfractions (HDL_2 and HDL_3) were isolated from total HDL of normolipidemic and mild hypercholesterolemic subject by sequential ultracentrifugation. CHO-HL cells were grown in complete medium to confluency. Then the medium was exchanged with FBS-free medium for an overnight incubation. After two washes by FBS-free medium, the cells were incubated with 150 μg/ ml of total HDL, HDL_2, and HDL_3 for 30 minutes at 37°C. The incubating medium was aspirated, concentrated by freeze-drying, and solubilized in SDS sample buffer. Western blot analysis followed to determine the mass of HL released into the medium. The images are representative of three experiments for each subject.


HL
Normolipidemic subject
HL
Dyslipidemic subject
Std HL
HDL
HDL3
HDL2

3.3 Mechanism of hepatic lipase displacement by HDL

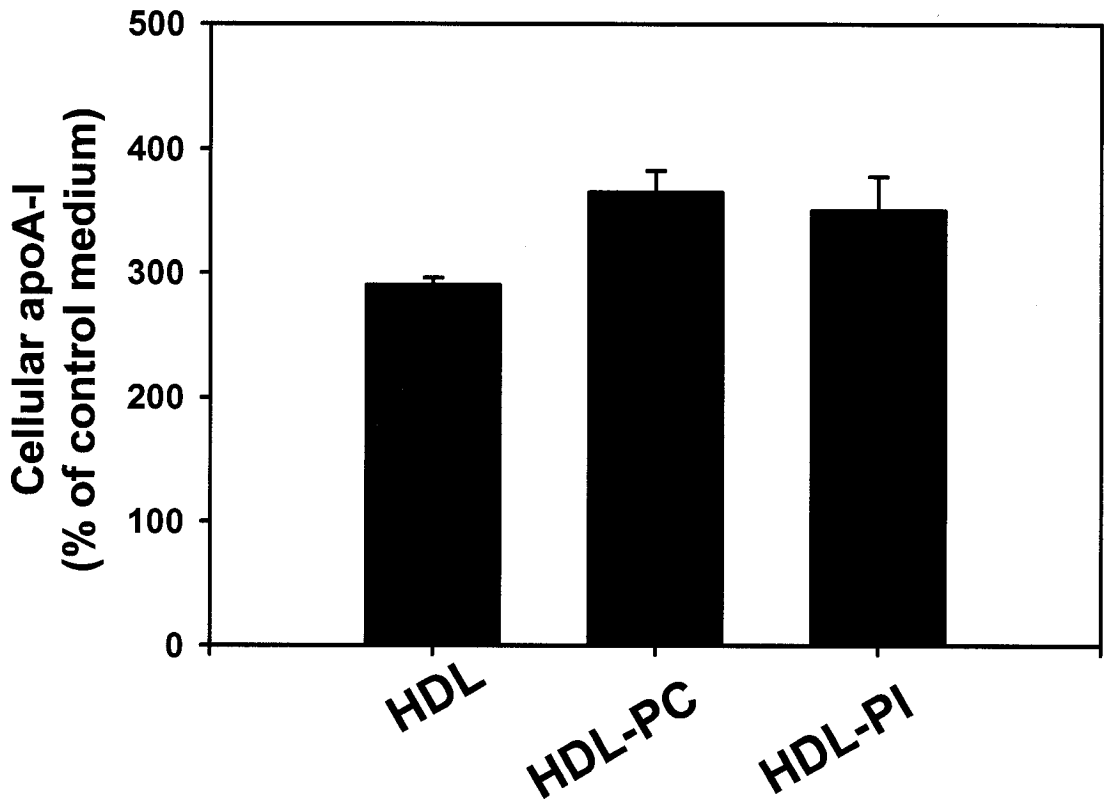
As described in section 3.2, cell culture studies in CHO-HL and HepG2 cells showed that displacement of cell surface-bound HL by HDL particles in the plasma is a dependent of the structural properties of HDL. However, these experiments revealed that the protein components, core lipids, and surface phospholipids of HDL regulate HL displacement; the mechanism of HL displacement by these factors was not known. To address the underlying mechanism of HL displacement by HDL, experiments were undertaken and association of HL with HDL was assessed in vitro.

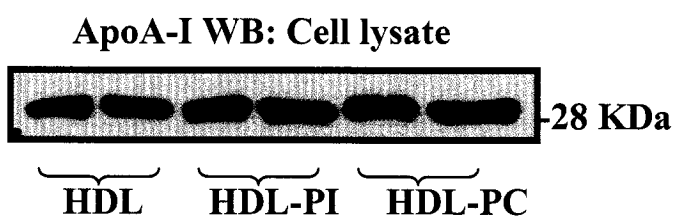
Association of apoA-I with the cells upon incubation with HDL:

To investigate whether HL displacement by HDL is a consequence of the exchange of apoA-I between the cell surface HSPG and HDL particles, cell culture assays were performed. CHO-HL cells were grown in 12 well plates and after confluency; the complete medium was replaced with FBS-free medium for one overnight. The cells were washed twice with FBS-free medium following by incubation with HDL and PI or POPC-enriched HDL (150 μg HDL protein/ 1 ml medium) for 30 minutes at 37°C. The incubation medium was removed and the cells were washed gently by PBS twice. 60 μl of SDS sample buffer was added to each well and the plate was incubated at 37°C for 30 minutes to lyse the cells. The cell lysate was then heated for 10 minutes at 90°C. The cell lysate samples in SDS buffer were electrophoresed on 12% SDS polyacrylamide gels and blots were probed for apoA-I with a mixture of anti-human apoA-I monoclonal antibodies. The western blot film was scanned and apoA-I content of the cells was quantified by the Quantity1® software.

Figure 15: ApoA-I association with CHO-HL cells upon HDL incubations

CHO-HL cells were grown in complete medium to confluency and then the medium was replaced with FBS-free medium for an overnight incubation. Following two washes by FBS-free medium, the cells were incubated with 150 μg / ml of freshly isolated human HDL or PI/POPC-enriched HDL for 30 minutes at 37°C. The incubating medium was aspirated and the cells were washed by PBS twice to remove any remainders of the medium. The cells were then incubated with 60 μl /well of SDS sample buffer for 30 minutes at 37°C. The total cell lysate was electrophoresed on 12% polyacrylamide gels and the blots were probed with an anti-human apoA-I mAb to estimate the amounts of apoA-I associated with the cells. The western blot films were scanned and quantified by Quantity1® software and the cellular apoA-I content was calculated relative to the medium alone. The values of cellular apoA-I represent mean ± SD of duplicate determinations and are representative of three experiments (Significance of difference from HDL by one-way ANOVA and Dunnett post-test analysis, *$p < 0.01$).


Cellular apoA-I
(% of control medium)
500
400
300
200
100
0
HDL
HDL-PC
HDL-PI


ApoA-I WB: Cell lysate
28 KDa
HDL
HDL-PI
HDL-PC

The results (figure 15) indicate that the cellular content of apoA-I increases modestly upon incubation of the cells by phospholipid-enriched HDL compared to HDL. If HL displacement by HDL was mediated by apoA-I exchange between the HDL particle and cell surface HSPG, it was expected to see less apoA-I cell association upon inhibition of HL displacement. However, the results of this experiment show that phospholipid enrichment of HDL which was earlier shown (figure 10) to inhibit HL displacement, increase apoA-I cell association, suggesting that apoA-I exchange is may not be involved in HL displacement process.

Association of HDL with hepatic lipase:

It was postulated that HL displacement by HDL might be mediated by direct interaction between HDL complexes and cell surface-bound HL molecules. In order to assess such a theory, preliminary co-immunoprecipitation studies were undertaken in vitro. Fresh HDL was isolated from normolipidemic human plasma and incubated (100 μg of purified HDL protein) ± 20 μl of pure human HL with 10 μl of an anti-human HL polyclonal Ab. The mixtures were incubated overnight at 4°C on a rotor. Next day, 200 μl of a 10% protein-G Sepharose beads suspension was added to the samples and incubated at 4°C on a rotor for 2 hours. The samples were centrifuged and the immunoprecipitate (the pellet) was isolated and washed 2 times gently by PBS. The samples were solubilized in SDS sample buffer followed by electrophoresis. The western blot analysis was performed both for detection of human apoA-I and hepatic lipase (figure 16). The blot probed for human apoA-I (figure 16-A) displays that HDL particles are immunoprecipitated along with the HL molecules (HL + HDL sample, lane 4). HDL alone (lane 3) is also slightly precipitated by anti-HL Ab showing that trace amounts of HL are naturally associated with HDL particles in the plasma.

The blot probed for HL (figure 16- B) indicates the same. HDL is pulled down along with HL (HL + HDL sample, lane 4) and very little HL is precipitated by the anti-HL Ab when the HDL is not supplemented with HL (panel B, lane 3). In summary, the results of the above study suggest that there are molecular interactions between HDL and HL that may mediate HL displacement in vitro.

Association of HDL with ^{125}I-HL:

To assure that HDL and HL interact in vitro, purified HL was labeled with ^{125}I and immunoprecipitation experiments were performed. Normolipidemic plasma was obtained and LDL and HDL fractions were isolated. LDL, HDL (150 μg of purified lipoprotein) or PBS were incubated with 20 μl of ^{125}I-HL and 90 μl of polyclonal anti-human apoA-I Ab overnight at 4°C. 200 μl of the protein-G Sepharose beads was added to the samples and incubated at 4°C on a rotor for 2 hours. After centrifugation at maximum speed for 3 minutes, the pellet was washed two times by PBS, solubilized with 120 μl of SDS sample buffer, and incubated at the room temperature for 3 hours. Then the samples were centrifuged at maximum speed and the supernatant was electrophoresed on 12% SDS polyacrylamide gels and probed for human apoA-I. Five μl aliquots of the supernatant were counted in a gamma counter to determine the radioactivity associated with the immunoprecipitate (representing the amount of HL associated with the pellet).

ApoA-I western blot of the pellet of the immunoprecipitation experiment (figure 17-A) shows that a significant amount of apoA-I is precipitated in HDL but not LDL or PBS samples. The radioactivities of the same samples (panel B) indicate that ^{125}I-HL associated with LDL is as much as the ^{125}I-HL associated with PBS (background control). Association of ^{125}I-HL with HDL is increased by 1.2 fold compared to LDL or PBS.

Figure 16: HL immunoprecipitation study (association of HDL and HL)

100 µg of HDL isolated from normolipidemic human plasma was incubated with or without 20 µl of pure human HL and 10 µl of anti-human HL polyclonal Ab for overnight at 4°C on a rotor. 200 µl of a 10% protein-G Sepharose beads suspension was added to the samples and incubated at 4°C on a rotor for 2 hours. The samples were centrifuged and the pellet was isolated and washed twice by PBS. The pellet was solubilized in SDS sample buffer following by electrophoresis. The western blot analysis of the immunoprecipitate was performed both for detection of human apoA-I and hepatic lipase. The western blot images are representative of three immunoprecipitation experiments.

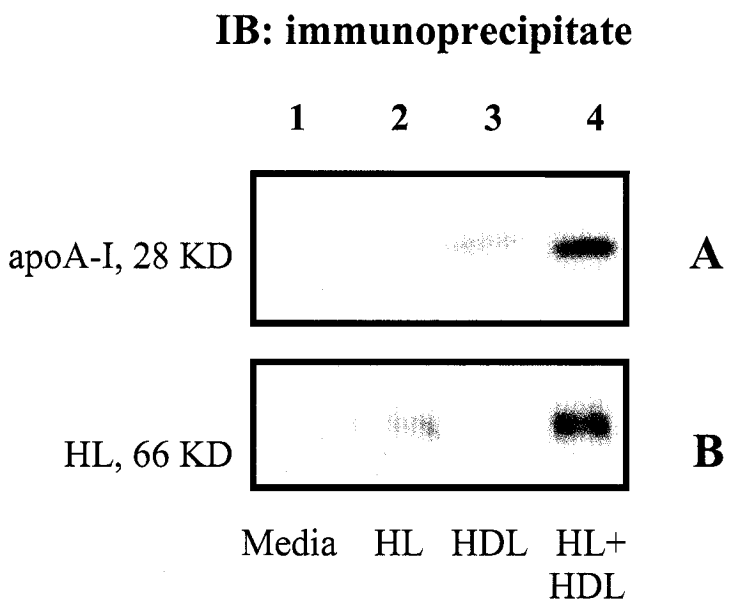
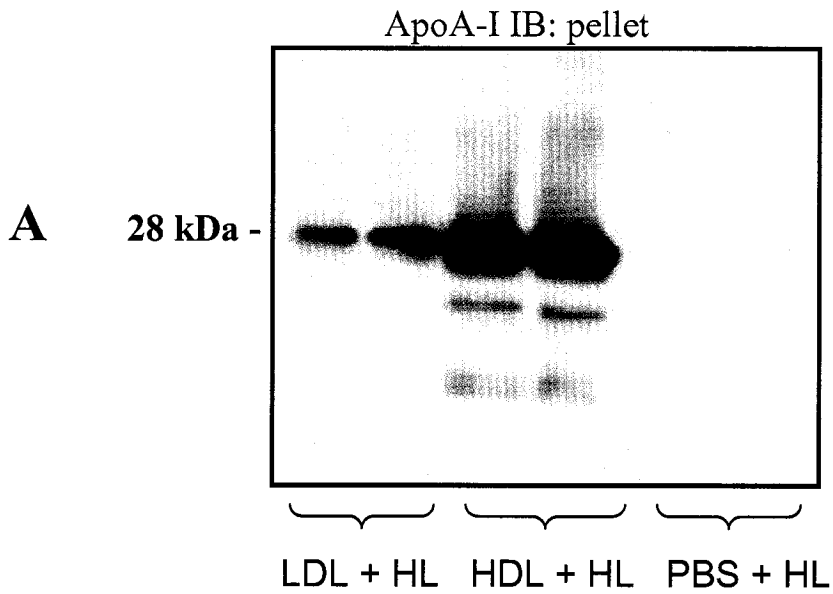
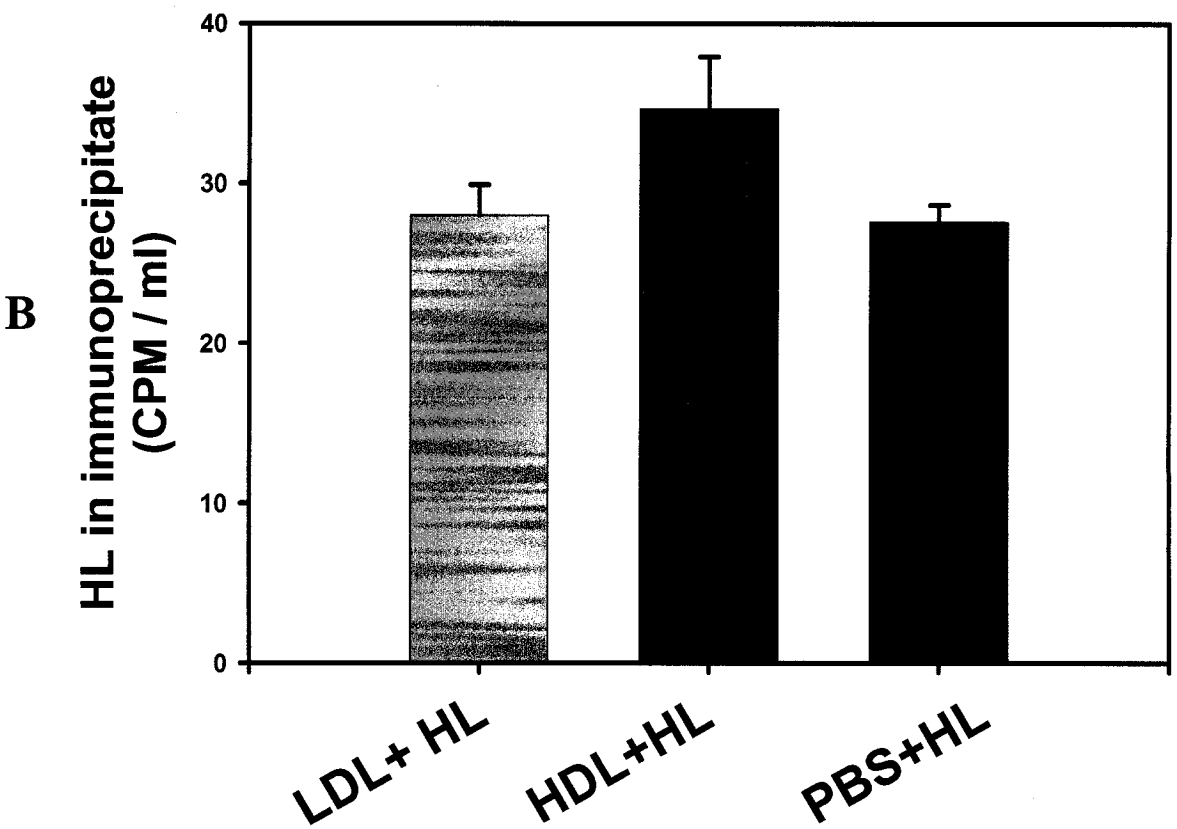

IB: immunoprecipitate
1
2
3
4
apoA-I, 28 KD
A
HL, 66 KD
B
Media
HL
HDL
HL+
HDL

Figure 17: ApoA-I immunoprecipitation study (association of HDL and ^{125}I-HL)

Purified HL was labeled with ^{125}I, and LDL and HDL fractions were isolated from normolipidemic plasma. 150 μg of purified LDL or HDL was incubated with 20 μl of ^{125}I-HL and 90 μl of polyclonal anti-human apoA-I Ab overnight at 4°C on a rotor followed by 2 hours incubation with 200 μl of protein-G Sepharose beads. After centrifugation at maximum speed for 3 minutes, the pellet was washed twice by PBS, solubilized with 120 μl of SDS sample buffer, and incubated at the room temperature for 3 hours. The samples in SDS buffer were centrifuged at maximum speed and the supernatant was electrophoresed on 12% SDS polyacrylamide gels and probed for human apoA-I (A). Five μl of the supernatant was counted in a gamma counter to determine the radioactivity associated with the pellet (B).The apoA-I immunoblots represent one experiment. The cpm values represent mean ± SD of triplicate determinations of one immunoprecipitation experiment (Significance of difference from LDL by one-way ANOVA and Dunnett post-test analysis, *$p < 0.01$).


A
ApoA-I IB: pellet
28 kDa -
LDL + HL
HDL + HL
PBS + HL


B
HL in immunoprecipitate
(CPM / ml)
40
30
20
10
0
LDL+ HL
HDL+HL
PBS+HL

3.4 Regulation of apoB lipoprotein binding and uptake by HDL:

In order to address the impact of HL displacement by HDL on the binding and uptake of apoB lipoproteins, preliminary binding studies were undertaken in CHO-HL cells. LDL and HDL fractions were isolated from normolipidemic human plasma. The LDL fraction was radiolabeled with I^{125} and HDL was enriched with PI. Cells were grown in 12 well plates, and upon confluency, the medium was replaced with FBS-free medium for overnight. Next day the cells were washed by FBS-free medium twice and incubated with FBS-free medium containing I^{125}- LDL (10 μg/ml) ± HDL or HDL-PI (150 μg HDL protein /1 ml medium) for 30 minutes at 37°C. After aspirating the medium, the cells were washed twice with PBS and 1 ml of 0.1N NaOH added to each well and incubated overnight rocking at the room temperature. The total protein concentration of the cell lysate was measured by commercial kits (Roche BCA protein assay kit) and the radioactivity of the cell lysate was determined on a γ-counter. The concentration of LDL protein was obtained from LDL's specific activity which represents the total binding values per mg of the cell protein. To measure the non-specific binding, the experiment was performed in the presence of excessive amounts of LDL (7.5 mg LDL/ well) and the specific binding was determined by subtracting the non-specific binding from the total binding values.

The results of the binding assay in CHO-HL cells (figure 18) indicate that HDL inhibits LDL binding in these cells by 40% and that PI-enrichment of HDL decreases LDL binding even further (63% decrease). Similar experiments were performed to examine the effect of HDL on LDL and VLDL binding during a time course.

Figure 18: Regulation of LDL binding by HDL in CHO-HL cells

LDL and HDL fractions were obtained from normolipidemic human plasma. LDL was labeled by I^{125} and HDL was enriched with PI. CHO-HL Cells were grown in complete medium to confluency and then the medium was replaced with FBS-free medium for an overnight incubation. The cells were washed by FBS-free medium twice and incubated with 10 μg / ml of I^{125}- LDL ± 150 μg / ml HDL or HDL-PI for 30 minutes at 37°C. After aspirating the medium and 2 washes by PBS, 1 ml of 0.1 N NaOH added to each well and incubated overnight rocking at the room temperature. Radioactivity of the cell lysates was measured in a γ-counter to determine the total binding values. To determine the non-specific binding values, the experiment was performed in the presence of 7.5 mg LDL/ well and the specific binding was calculated by subtracting the non-specific binding from the total binding values. The cell association values are mean ± SD of triplicate determinations and represent two binding assays (Significance of difference from LDL by one-way ANOVA and Dunnett post-test analysis, $*p < 0.01$, $**p < 0.001$).

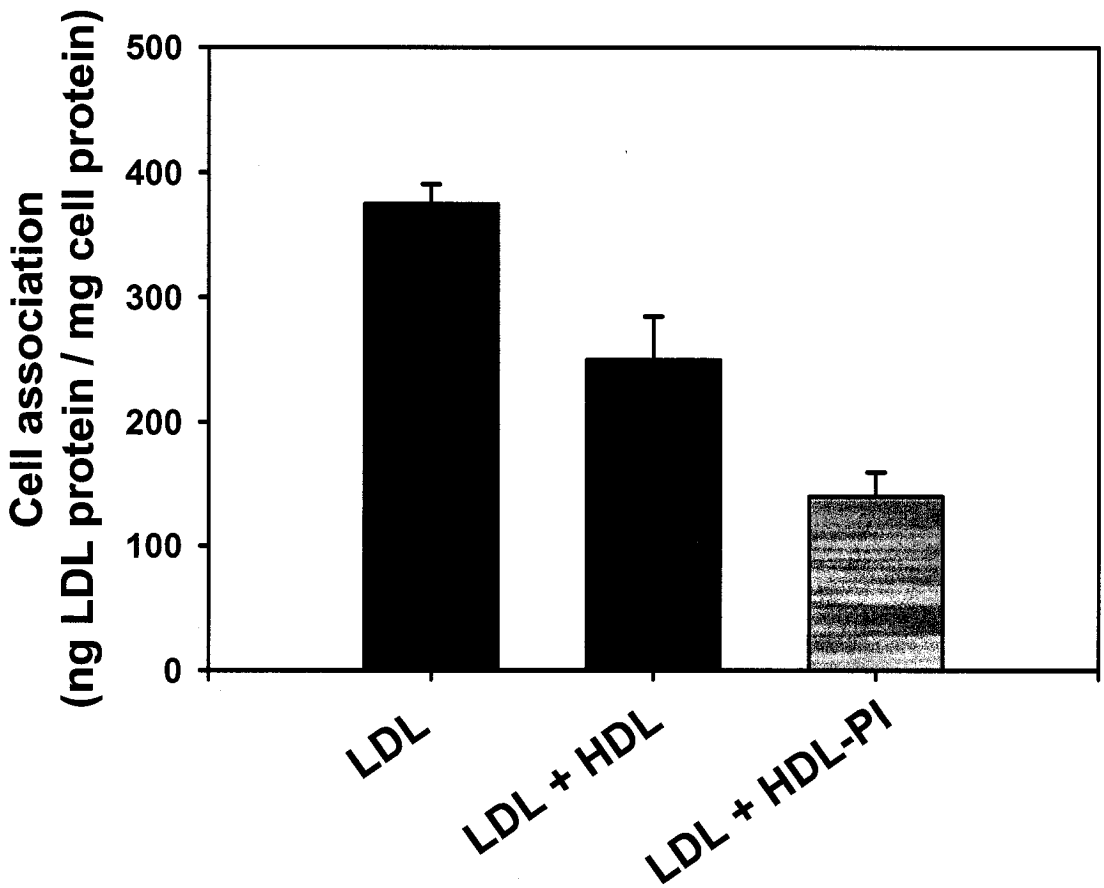
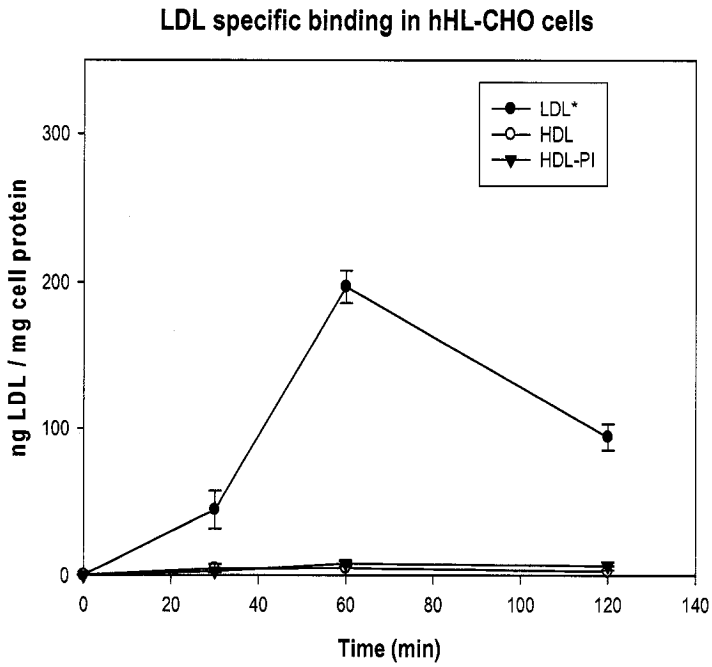

Cell association
(ng LDL protein / mg cell protein)
500
400
300
200
100
0
LDL
LDL + HDL
LDL + HDL-PI

Figure 19- A shows the specific binding of I^{125}-LDL in CHO-HL cells at the presence of HDL or HDL-PI over a 2-hour period. The figure shows that while the binding of LDL reaches a peak by the first hour and drops ~ 50% after the second hour, both HDL and HDL-PI completely block LDL binding. The specific binding of VLDL is also decreased significantly by HDL and HDL-PI (figure 19- panel B).

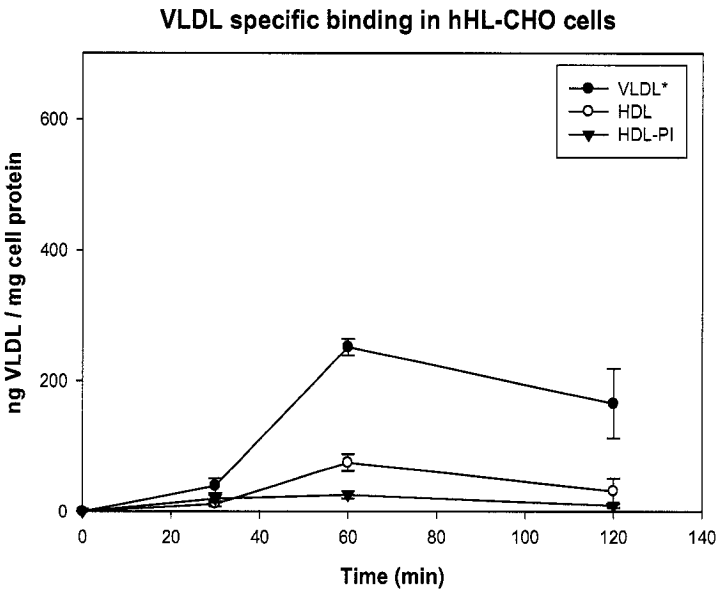
Figure 19: Time course study of regulation of apoB-lipoproteins binding by HDL in CHO-HL cells

VLDL, LDL, and HDL fractions were obtained from normolipidemic human plasma. VLDL and LDL were labeled by I^{125} and HDL was enriched with PI. CHO-HL Cells were grown in complete medium to confluency. Then the medium was replaced with FBS-free medium for an overnight incubation. The cells were washed by FBS-free medium twice and incubated with 10 μg / ml of I^{125}- LDL/VLDL ± 150 μg / ml HDL or HDL-PI for various times (0, 30, 60 and 120 minutes) at 37°C. After aspirating the medium and 2 washes by PBS, 1 ml of 0.1 N NaOH was added to each well and incubated overnight. Radioactivity of the cell lysates was measured to determine the total binding values. To determine the non-specific binding values, the experiment was performed in the presence of 7.5 mg LDL or VLDL per well and the specific binding was calculated by subtracting the non-specific binding from the total binding values. The cell association values are mean ± SD of triplicate determinations and represent two binding assays.

A


LDL specific binding in hHL-CHO cells
LDL*
HDL
HDL-PI
ng LDL / mg cell protein
300
200
100
0
0
20
40
60
80
100
120
140
Time (min)

B


VLDL specific binding in hHL-CHO cells
VLDL*
HDL
HDL-PI
ng VLDL / mg cell protein
600
400
200
0
0
20
40
60
80
100
120
140
Time (min)

Chapter 4: Discussion

4.1 Introduction

HL is a lipolytic enzyme from the lipase family that is mainly secreted from the liver. This enzyme is anchored at the surface of hepatic endothelial cells through HSPGs. HL assists in the clearance of plasma lipids by two separate mechanisms: 1) catalyzing the hydrolysis and remodeling of plasma lipoproteins and 2) increasing the intracellular metabolism of lipoproteins by its ligand-receptor function.

Postheparin plasma HL activity varies widely in the general population. A number of factors such as, HL gene promoter polymorphism, gender, insulin resistance, and obesity [192] are associated with HL activity. Evidence from clinical [300, 301] and animal [302, 303] studies indicates that HL activity is linked to CAD. In this regard, HL activity has been shown to demonstrate both pro- and anti- atherogenic effects. For example, cardiovascular patients characterized by the predominance of smaller denser LDL particles, compared to the normal subjects, have higher HL activities [193]. On the other hand, HL-deficient patients, who present elevated plasma TG and cholesterol levels, and large buoyant lipoproteins are also predisposed to premature cardiovascular disease [251].

The underlying mechanisms that regulate HL activity within the plasma are the interest of the present study. Hereby, we show that plasma HDL is a crucial element to the liberation of HL activity in the plasma and that HDL structure plays an important role in regulation of the HL activity. The anti-atherogenic role of apoA-I as the basic component of RCT pathway has been widely investigated and HDL has been well known for transporting the cholesterol from peripheral tissues to the liver [304, 305]. Recent studies have shown a

different mechanism by which HDL and apoA-I contribute to the lipid metabolism and prevention of heart disease. In vitro and cell culture studies demonstrated that exogenous HDL and apoA-I but not other lipoproteins can displace HL from purified HSPG [245] or cell surface [292] and increase VLDL-TG hydrolysis mediated by HL.

HL displacement and a consequent increase in HL activity was demonstrated as early as 1970 [272]. Intravenous injection of heparin to both humans [306] and animal models [307] results in displacement of HL from the capillary endothelium and releases HL activity into the blood stream. In vivo evidence [308] shows that expression of human HL, that is deficient in binding to HSPG, in mice elevates pre-heparin HL activity and leads to enhanced catabolism of lipoproteins. The rapid increase in lipid hydrolysis and clearance resulted from circulating HL suggests that blood-borne HL activity plays an essential role in lipid metabolism and that HSPG-bound HL does not function efficiently. Therefore, high postheparin HL activity may reflect an inactive pool of HL attached to the endothelium that is released after the heparin infusion.

Post-heparin HL activity and plasma HDL-C levels have been shown to be inversely correlated. Clinical studies indicate that patients with elevated post-heparin HL activity often have low HDL-C levels [309, 310]. It is also known that postheparin HL activity is almost twice as high in men as in premenopausal women [192], and that plasma HDL-C levels are lower in men than in women [311]. Abnormal low HDL concentration is usually associated with other lipid abnormalities such as low TG hydrolysis and hypertriglyceridemia [312]. For instance, patients with familial combined hyperlipidemia (FCHL), who have elevated levels of TG and total cholesterol, also have low HDL-C and HDL_2 levels and enhanced postheparin HL activities [313]. All of this clinical data suggest

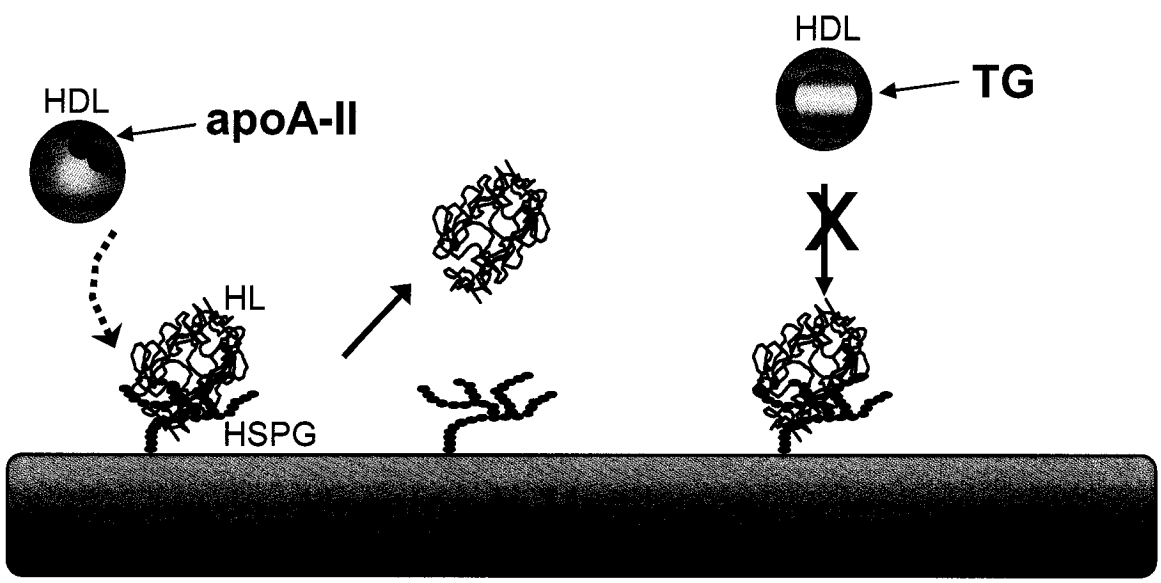
that HDL levels and composition are crucial to the regulation of HL activity and TG metabolism.

The relationship between displacement of HL activity and regulatory role of HDL have been previously investigated [292]. HDL and apoA-I liberate cell surface-bound HL from hepatocytes and CHO cells and thereby increase VLDL-TG hydrolysis by HL. The same study showed that the larger, more buoyant subfractions of HDL had the greatest effect in displacing cell surface-bound HL. The data suggest that structural differences in HDL have a regulatory effect on HL displacement. Related studies have demonstrated that apoA-I [245] and apoA-II [80] components of HDL decrease HL activity by inhibiting VLDL-TG hydrolysis. These data together suggest that plasma HDL regulates HL at two stages: 1) displacement of cell surface-bound HL and 2) modulation of the activity of displaced HL by binding to it and inhibiting VLDL-TG hydrolysis. This regulatory effect of HDL appears to be controlled by the physical and structural properties of HDL particle. In the present study, experiments were undertaken to elucidate the linkage between structural properties of HDL and liberation of HL activity. The mechanism of displacement is also examined to determine if HDL directly interacts with the enzyme and detaches it from HSPG or if a competitive exchange of apoA-I between HSPG and HDL leads to HL liberation.

Cell culture experiments using the native and reconstituted HDL particles revealed that each structural component of HDL has a unique impact on HL displacement and activation. For instance, apoA-II content of HDL is stimulatory to HL displacement, whereas the TG component is inhibitory (figure 20). The inhibitory effect of TG is shown by HDL from

Figure 20: Schematic model of regulation of HL displacement by protein and lipid components of HDL

Displacement of HL from cell surface is modulated by the structural composition of HDL. Each component of HDL plays a unique role to regulate HL displacement. The apoA-II content of HDL stimulates HL displacement (left) and causes HL to be detached from HSPG. Oppositely, the TG content of HDL inhibits HL displacement and HL stays anchored at HSPG molecule (right).


HDL
apoA-II
HL
HSPG
HDL
TG

hyperlipidemic patients as well as TG-enriched HDL and rHDL. Immunoprecipitation studies show that the displacement of HL by HDL is likely mediated by a direct interaction between HDL and HL rather than apoA-I exchange between HSPG and HDL. This direct association of HL and HDL may in fact be a consequence of activation of signaling pathways within the cell.

Results of the present study provide insight on the mechanism of lipase activation by plasma HDL that in turn is a potential therapeutic target in prevention and treatment of lipid disorders and atherosclerosis. This study also highlights that the quality of HDL classes in the plasma is a very important risk factor for CAD and that not all species of HDL are beneficial and anti-atherogenic.

4.2 HL displacement from the cell surface by heparin and HDL

HL activity is detectable in the postheparin plasma and in heparin perfusates of the liver. The enzyme bound to the endothelial surfaces is rapidly released by heparin from the capillary beds into plasma. The peak of lipase activity and TG clearance can be measured 10 to 15 minutes after heparin injection [269].

The mechanism of heparin-mediated displacement of HL has not yet been clearly elucidated. It is thought that heparin directly interacts with the enzyme or competes with it to bind to the binding sites on the cell surface HSPGs. It has been suggested that interaction of heparin with lipases or other biological molecules such as antithrombin-III is due to association of charged groups of heparin with unique sequences of amino acid residues that are potentially involved in heparin binding [307, 314]. *Tagashira et al* [285] reported that heparin may act on arachidonate signaling pathways to stimulate the release of HL activity from rat hepatocytes. This pathway involves increases in cytosolic phospholipase (PL) A_2 activity and leukotriene (LT) B_4 content in the hepatocytes. Another study [285] showed that HL displacement by heparin involves Ca2+/calmodulin-dependent protein kinase II activity.

Heparin infusion with or without insulin is used to treat acute pancreatitis caused by hypertriglyceridemia (over 1,000 mg/dl) [315]. This method is an effective alternative to reduce TG levels in patients quickly. Heparin is also administered in haemodialysis patients to prevent clotting in the extracorporeal devices [316]. This injection causes a quick increase in plasma LPL activity during the first hour. During the remaining session, LPL activity decreases to a stable plateau while TG increases towards and beyond baseline [317], suggesting that heparin infusion depletes the endothelial stores of lipases. Clinical studies indicate that low molecular weight heparins disturb the lipase system much more than

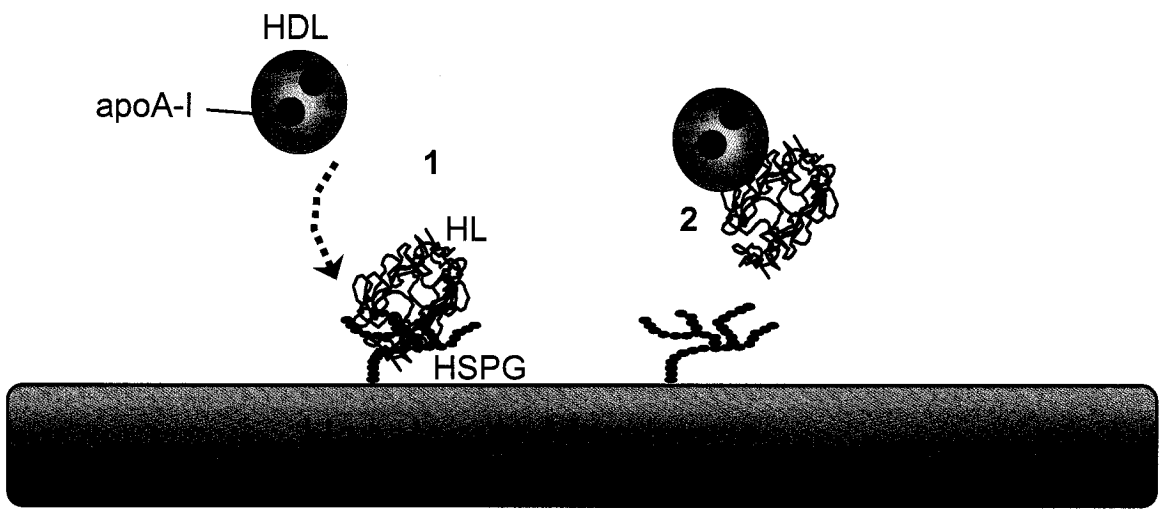
conventional heparins [316, 317]. The endogenous heparin level has also been described to be involved in lipid metabolism. Individuals with lower endogenous circulating heparin activity have more serum lipid abnormalities and are more susceptible to atherosclerosis [281]. Interestingly, post-heparin HL activity in familial low HDL patients is higher than normal subjects [318]. This indicates that plasma HDL plays a role in activation of HL and mimics the action of heparin in the body.

The mechanism of HL displacement mediated by HDL has not been studied. However, one can speculate that HDL may act in a similar manner to heparin to displace HL from the cell surface. Our early work had shown that upon HDL incubations, apoA-I binds to HSPG. Thus, we proposed that HDL may displace HL from HSPG by competitive exchange of apoA-I [245]. However, experiments in the present study revealed that apoA-I cell association does not parallel HL displacement and therefore may not be linked. Instead, data suggest that HDL directly interacts with the HL molecule (figure 21). This association probably involves the ionic interactions between negative amino acid regions of apolipoproteins in HDL particle and positively charged domains in HL. It is not known which structural component(s) of HDL is (are) responsible for interaction with HL, but it is more likely that apoprotein components with negatively charged regions be responsible for interaction with HL.

Another possibility is that binding of HDL to HL triggers a signaling event such as activation of the arachidonic pathway and leukotriene production which mimics the action of heparin [286]. Alterations in production of cytosolic proteins may lead to conformational changes of HL in a way that HL is no longer able to bind to HSPG binding sites and therefore, detaches from the cell surface.

Figure 21: Schematic model of HL displacement by HDL through HDL association mechanism

HDL displaces cell surface-bound HL into the circulation through a mechanism that involves direct binding to the enzyme.


HDL
apoA-I
1
HL
2
HSPG

4.3 HDL structure regulates HL displacement

Data from our study indicate that in order to displace HL, HDL must possess specific qualities including normal levels of TG content, proper size, and density. Abnormal TG-enriched HDL from hypertrigyceridemic patients or postprandial healthy subjects appears unable to displace HL (model shown in figure 20). Similarly, HDL size directly influences HL displacement and activity. HDL_2 have a greater capacity to displace HL from the cell surface, while HDL_3 is inhibitory to HL displacement [292]. This observation is consistent with the inverse relationship between the magnitude of plasma HDL_2 levels and postprandial lipemia [319]. The apolipoprotein components of HDL are important factors in displacement of HL. Our study shows that apoA-I and apoA-II both stimulate HL displacement, however the stimulatory role of apoA-II is more significant (model shown in figure 20). Previous work has shown that apoA-II inhibits VLDL-TG hydrolysis by HL [80]. This may be explained by the fact that apoA-II affects the conformation of apoA-I on HDL [80] and significantly increases the binding of HL with HDL particles [241]. Thus, one may conclude that HDL conformational alterations by apoA-II directly enhance HDL and HL association. The increased binding of HL to HDL increases HL displacement but may affect HL ability to shuttle between the lipoprotein substrates and hydrolyze them. ApoA-II appears to enhance HL displacement, but inhibits HL activity.

The results of our studies point to the fact that the regulatory effects of apolipoprotein components of HDL are combined with other structural factors of HDL like particle size. HDL_3 contain a higher ratio of apoA-II/apoA-I than HDL_2 particles [320] . Since apoA-II stimulates HL displacement, it would be expected to see more HL displacement with HDL_3, relative to HDL_2. However, our experiments reveal that HDL_3

particles are almost unable to displace HL. This may be explained by the fact that due to the larger size of HDL_2 particles, they contain more apoA-II than HDL_3 particles, despite having a smaller ratio of apoA-II/apoA-I. Therefore, functionality of HDL in displacement of HL is controlled by the net mass of apoA-II in HDL particles rather than the apoA-II/apoA-I ratio. ApoC-I also increases HL displacement but in a lesser degree than apoA-II. Other apoproteins like apoE and apoC-III may have inhibitory effects and need to be further investigated.

The HDL lipid components also regulate HL displacement. TG content of HDL is a potent inhibitor of HL displacement. This effect was demonstrated with the native and reconstituted HDL particles that were enriched in their TG content and with HDL fractions that were taken from hypercholesterolemic patients. It has been shown earlier that modifications in the neutral lipid content of HDL give rise to specific changes in the structure of apoA-I and stability of the particle [296]. A decrease in CE/TG ratios in LpA-I particles decreases the integrity of the particle and stability of apoA-I α-helical segments [296]. This kind of alteration in the composition of HDL, which is associated with high HDL-TG levels, has been observed in the plasma of hypertriglyceridemic subjects [105, 321]. Abnormalities in HDL composition and size directly affect the physiological functions of HDL by altering the interaction of HDL with LCAT [322], CETP [323] and the cell surfaces [324]. Therefore, an increase in TG levels of HDL that disturbs the stability of the particle could also affect HL displacement by HDL. Free fatty acid content of HDL has a slight inhibitory role on HL displacement, whereas the CE content increases HL displacement moderately. It is known that the core TG-enrichment and CE depletion of HDL associated with conformational changes of apoA-I are closely related to acceleration of

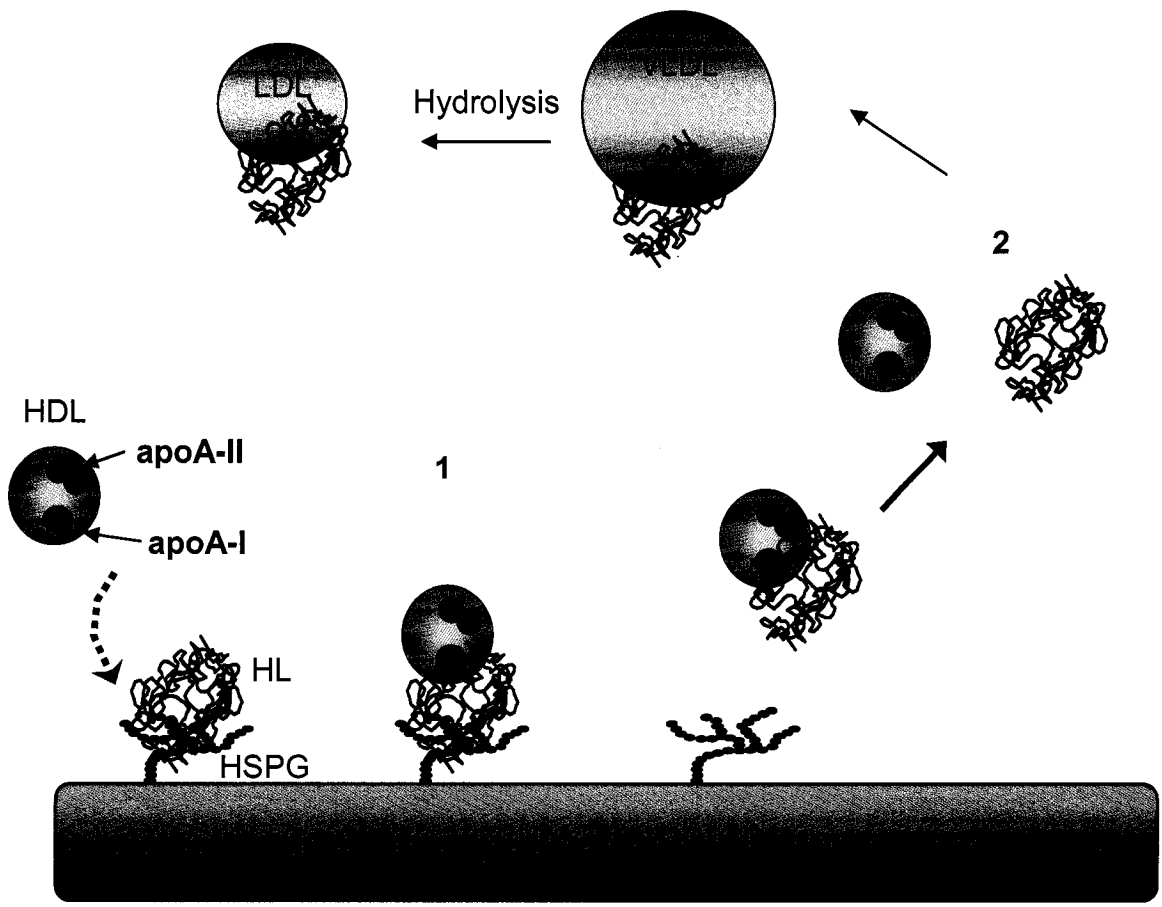
atherosclerosis in metabolic diseases [325]. Thus, normal CE content of HDL seems to be essential for proper function of HDL. This perspective appears to be consistent with enhancement of HL displacement by larger classes of HDL, which contain more CE in their structure [326].

The phospholipid content of HDL, like its TG content, also inhibits HL displacement. PI has been shown to increase the hydrolytic activity of HL by reducing the binding affinity of HL for HDL (*Boucher,* unpublished results). Enrichment of native HDL with anionic phospholipids significantly stimulates VLDL hydrolysis by HL, relative to that observed for HDL enriched with uncharged lipids (*Boucher*, unpublished results). Therefore, it appears that the electrostatic charge of HDL regulates HL activity. In the present study it was shown that, phospholipid-enrichment of HDL particles regulates displacement of HL activity by inhibition of HL liberation. However, the electrostatic charge of the particles does not make a significant difference in HL displacement and both of negatively and positively charged particles inhibit HL displacement. This suggests that phospholipid components of HDL affect the structure and therefore, the function of HDL in an independent manner of charge modifications.

Combined data from our structural experiments suggest that two conditions are necessary for the maximum activity of HL: first, liberation from the cell surface HSPG mediated by HDL and second, dissociation of HL from HDL particle in order to shuttle between the lipoprotein substrates (figure 22). Dissociation of HDL particle from HL has been earlier shown to be stimulated by FFA and phospholipids in the plasma (*Boucher*, unpublished data). These negatively charged molecules render HDL a negative surface charge that decreases the binding affinity of HDL to HL and increases HL activity. This

Figure 22: Schematic Model of HL displacement and activation by HDL

The maximum activity of HL occurs within the plasma. The cell surface-bound HL is displaced from HSPG by the action of HDL. Some molecules on HDL such as apoA-I and apoA-II stimulate the HL displacement event. It is proposed that HDL displaces HL into the circulation by direct binding to it. After displacement by HDL, HL is still inactive, since it is bound to HDL and cannot easily move between the substrates (step 1). Dissociation of HL from HDL complex activates HL. The unbound HL can shuttle between the lipoprotein molecules and catalyze TG-hydrolysis (step 2).


LDL
Hydrolysis
VLDL
2
HDL
apoA-II
apoA-I
1
HL
HSPG

shows that there is an inverse relationship between the regulation of HL displacement and activation by HDL. The structural elements that enhance HL displacement from the cell surface (such as apoA-II) do not stimulate the hydrolytic activity due to increasing the binding affinity of the enzyme to HDL and vice versa.

Abnormal HL displacement in dyslipidemic patients may in fact be causative to their hyperlipidemia. The inability of HDL to displace HL from the cell surface locations appears concomitant with elevated levels of HDL-TG in these subjects and may indicate that TG content of HDL plays a key role in regulation of HL displacement. This effect is also observed in postprandial HDL samples from healthy subjects. During a postprandial response, LPL activity increases to the peak as a physiological response to increased plasma lipids and generates considerable amounts of TG in the blood stream. At this state the exchange of core lipids between circulating lipoproteins is also increased [327]. As a result, HDL particles that are enriched with the TG produced by lipolysis of chylomicrons and VLDL will inhibit HL displacement. In disease conditions such as hypertriglyceridemia, post-prandial lipemia is prolonged and results in TG-enrichment of LDL and HDL particles [327]. As a result, HL displacement does not occur to initiate HL activity and the next cycle of lipolysis. In spite of high TG levels that inhibit HL displacement by HDL, other metabolic factors such as apolipoprotein exchange between circulating lipoproteins stimulate HL liberation from the endothelial surfaces. Liberated HL in the circulation would then be expected to be stimulated by increased levels of anionic lipids (PI and fatty acids) coming from the actions of LPL. It has been shown that free fatty acids and phospholipids stimulate dissociation of HL form HDL and activate lipid hydrolysis by HL (*Boucher et al, unpublished data*).

Illustrations in Figure 23 show that HL displacement and activation are regulated by structural modifications of HDL in the blood stream. The initial events that turn HDL into its active form and facilitate HL displacement are thought to be the apolipoprotein exchanges between HDL and apoB lipoproteins such as chylomicrons and VLDL (step 1). Acquiring specific apolipoproteins like apoC-I and apoA-II by HDL causes HL displacement from the capillary endothelial cells (step 2). At this state HL is not active yet due to association with HDL complex. Parallel to HL displacement, lipolysis of chylomicrons and VLDL by LPL produces FFA in the circulation. FFA stimulates the dissociation of HL from HDL particle that in turn increases the activity of HL (step 3).

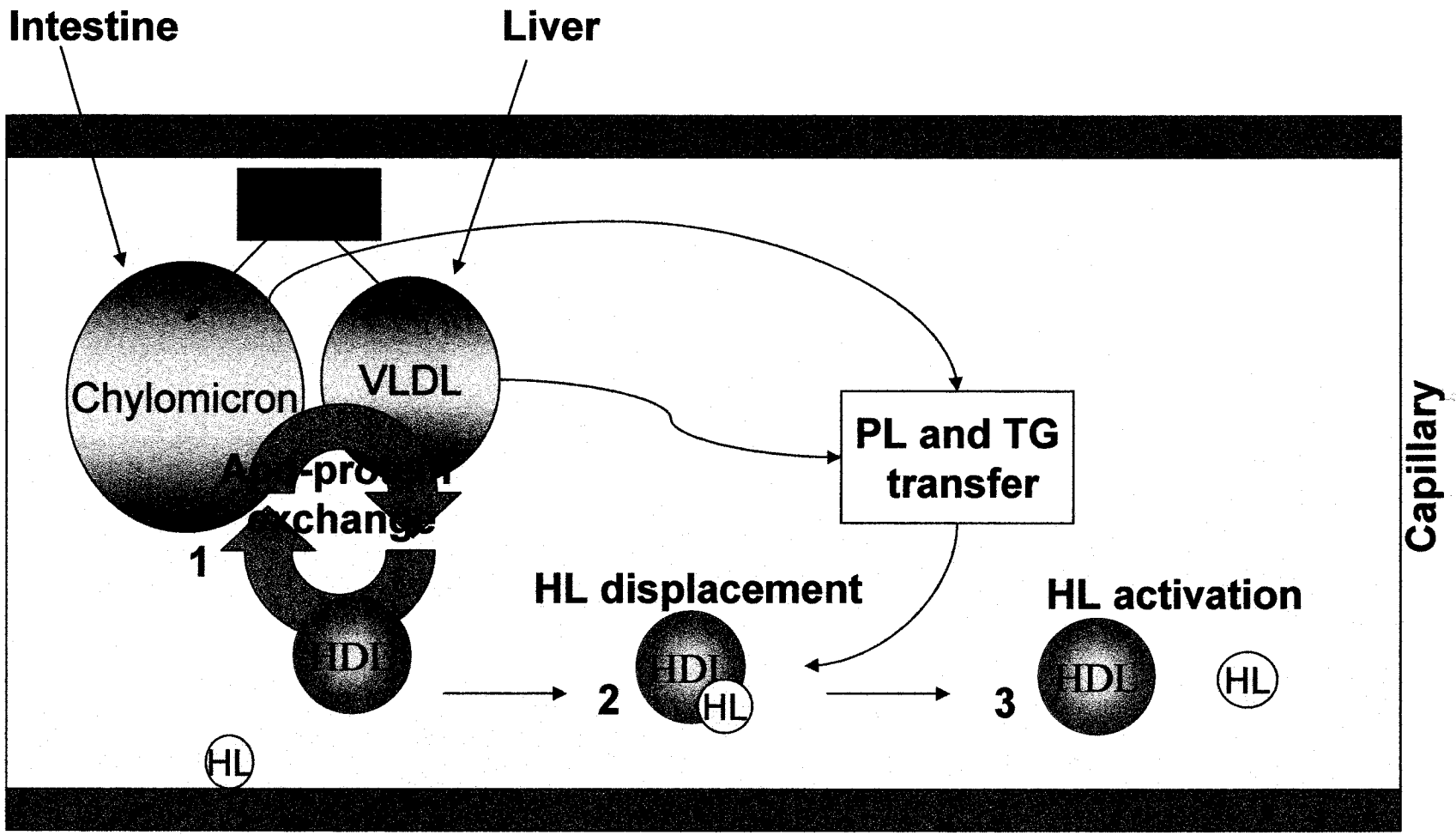
Displacement of HL by HDL not only modulates the activity of the enzyme but also affects the clearance of apoB lipoproteins by the liver. Uptake studies in CHO cells overexpressing HL show that HL displacement by HDL decreases the binding and uptake of LDL and/or VLDL. This effect is aggravated by the abnormal HDL enriched with phospholipids. These data suggest that the optimal location of HL for its ligand function differs from that of its maximum hydrolytic activity. While HL needs liberation from HSPG into the plasma to demonstrate hydrolytic activity, it has to be anchored at the cell surface in order to mediate the binding and uptake of apoB lipoproteins.

Inhibition of HL displacement by phospholipid enrichment of HDL further inhibits the clearance of apoB lipoproteins. Thus, it seems that abnormal structure of HDL affects both activity and ligand function of HL. Further experiments with wild type CHO cells (non-transfected) are required to evaluate the effects of HDL on the cellular metabolism of apo B lipoproteins, independent of HL, mediated by cell surface receptors such as LDLr and LRP.

Taken together, the results of displacement and uptake experiments suggest that maintenance of normal HDL concentrations and structure in the plasma are crucial to modulation of HL lipolytic activity and ligand function. It is also noteworthy to mention that combined data from the present research and studies that investigated the role of HDL structure in regulation of HL activity (*Boucher et al*) show that HL activity and displacement are inversely modulated by HDL structural components. Further investigation is needed to elaborate the underlying mechanisms that account for this difference.

Figure 23: Structural modifications of HDL in the plasma cause HL displacement and activation

During post-prandial state considerable amounts of apoB lipoproteins are secreted into the blood circulation. Chylomicrons are produced from the intestine and VLDL is secreted from the liver. These apoB lipoproteins tend to exchange their surface apoproteins with HDL (step 1). Exchange of apoproteins and structural modifications in HDL convert HDL to a state that readily displaces HL from the endothelial surface (step 2). The FFA that is generated in the plasma because of LPL action on chylomicrons and VLDL stimulates the dissociation of HL from HDL complex and activates HL (step 3).


Intestine
Liver
Chylomicron
VLDL
PL and TG transfer
1
HL displacement
HL activation
HDL
HL
2
3
Capillary

4.4 Conclusion

HDL is a plasma lipoprotein that like heparin displaces HL into the circulation and increases HL-mediated TG hydrolysis. HDL structure plays a pivotal role in its regulation of HL liberation. Various structural elements in HDL affect HL displacement uniquely. Some structural components such as apoA-II, apoC-I, and CE increase HL displacement, whereas others like TG, FFA, and phospholipids decrease it. Among the inhibitory factors, TG plays a key role in blocking HL displacement. This effect has been observed with TG-enriched HDL from dyslipidemic patients, which is incapable of displacing HL activity. The size of HDL particles also determines HL displacement, as only larger and more buoyant classes of HDL displace HL activity. The lipase displacement event appears to be mediated by a direct interaction between HDL particles and the enzyme.

Further studies are needed to elucidate which structural components of HDL are directly involved in HL interactions and whether these components function through activating a signaling cascade that eventually results in liberation of the enzyme. It is also of great value to determine alterations of HDL structural components in disease states like FCHL or metabolic syndrome and evaluate the impact of these alterations on HL displacement and lipid clearance.

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Naghmeh Rouhani – *Curriculum vitae*

University of Ottawa Heart Institute
40 Ruskin Street
Ottawa, K2Y 4W7
Ontario, Canada
Email: nrouhani@ottawaheart.ca

Education

University of Ottawa **2004-present**
Ottawa, Ontario

Masters of Science (MSc) in Biochemistry: Program consists of laboratory research at the University of Ottawa Heart Institute in the Lipoprotein and Atherosclerosis research group. Practical lab work is complemented by participating in scientific seminars and graduate courses.
MSc supervisor: Dr. Daniel L Sparks

Baha'i Institute for Higher Education **1992- 1997**
Iran, Tehran

Bachelor of Pharmaceutical Sciences: The five-year program consists of courses related to biology, microbiology, parasitology, chemistry, and pharmacy. Theory was complemented with laboratory courses in microbiology, parasitology, chemistry, and pharmacognosy as well as working at local pharmacies. The program included a Bachelors research thesis. The thesis focused on preparation of an herbal pharmacopoeia that included various methods for extraction of 64 herbal plants. It also consisted of a practical section on preparation of a powdered dosage form from "*Valeriana Officinalis*" plant.

Research Experience

Lipoprotein and Atherosclerosis research Group **2004- Present**
University of Ottawa Heart Institute, Ottawa, Ontario

MSc thesis: High density lipoproteins regulate hepatic lipase displacement and activity.
Techniques: Cell culture, immunochemistry, lipid biochemistry, generation of synthetic lipoproteins, and use of radioactivity.

Baha'i Institute for Higher Education **1996- 1997**
Iran, Tehran

Bachelor's thesis: Preparation and quality control of the herbal extracts.
Techniques: Extraction of medicinal plants, identification, and quantifying the medicinal ingredients, preparation, and quality control of pharmaceutical dosage forms.

Teaching Experience

Baha'i Institute for Higher Education **2000- 2002**
Iran, Tehran

Coordinated and taught 4th and 5th year undergraduate courses including: Introduction to Pharmaceutical Dosage Forms, Basics of Radiopharmacy, and Biopharmaceuticals.

Pre-University Preparation Classes **1999- 2000**
Iran, Esfahan

Instructed high school 12th year students general and molecular biology.

Women's Health workshops **1997- 2000**
Iran, Esfahan
Lectured and counseled on family and women's health care as a pharmacist.

Publications

Naghmeh Rouhani, Elizabeth Young, Cynthia Chatterjee, and Daniel L. Sparks. HDL Composition Regulates Displacement of Cell- Surface Bound Hepatic Lipase. Manuscript in preparation (2007)

Boucher J, Bamji M, **Rouhani N** and Sparks DL. Hepatic lipase and HDL metabolism, Review. Manuscript in preparation (2006).

T Momeni, P Aghsani, and **Rouhani N**. Herbal Extracts (2000). Rahnemaye Chitsaz A (Ed), Shahid Farhad Reza Publications, Tehran, Iran.

Scholarships

2004- 2007: BIHE (Baha'i Institute for Higher Education) scholarship for Master's studies in Canada.

Conferences

N Rouhani and Sparks DL. HDL structure regulates the displacement of cell surface hepatic lipase and its ability to promote HL secretion in HepG2 cells – Poster. 30th annual Canadian Lipoprotein Conference. Published abstract (2005).