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Elizabeth Young
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biochemistry)
GRADE / DEGREE

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

HDL-APOE Content Regulates the Cell Surface Displacement of Hepatic Lipase

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

Rosalind Labow

Yves Marcel

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

HDL-APOE CONTENT REGULATES THE CELL SURFACE DISPLACEMENT OF HEPATIC LIPASE

Elizabeth Young

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the Masters Degree in Biochemistry (MSc)

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine, University of Ottawa

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ABSTRACT

Human hepatic lipase (HL) is an interfacial enzyme that must be liberated from cell surface proteoglycans to hydrolyze lipoprotein triglyceride. HDL and apolipoprotein (apo)A-I can displace HL from cell surface proteoglycans, much like heparin. HL displacement is inhibited by HDL-apoE content. Postprandial HDL is ~2-fold better at displacing HL than fasting HDL, but only has about half the apoE content. Enriching native HDL with triglyceride decreases HDL-apoE content and increases HL displacement. In contrast, enriching synthetic HDL with apoE significantly inhibits HL displacement. HDL from fasted female normolipidemic subjects displaces HL ~2-fold better than HDL from male subjects. HDL from female subjects also has significantly less apoE than HDL from males. Normolipidemic females have increased circulating HDL-bound HL. Hyperlipidemia has little effect on the HL displacement ability of HDL from men, while HDL from hypercholesterolemic females exhibits impaired HL displacement. HL displacement from liver HSPG therefore appears to be linked to interlipoprotein apoE exchange. Decreased HL displacement is associated with higher HDL-apoE levels and may impact vascular triglyceride hydrolysis.

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List of Abbreviations

ABCA1:	ATP-Binding Cassette transporter type A1
ANOVA:	Analysis of variance
Apo:	Apolipoprotein
CE:	Cholesterol Ester
CETP:	Cholesterol Ester Transfer Protein
CHD:	Coronary Heart Disease
CHO:	Chinese Hamster Ovary cells
CHO-hHL:	Chinese Hamster Ovary Cells transfected with human HL
CVD:	Cardiovascular Disease
DG:	Diglyceride
EL:	Endothelial Lipase
ELISA:	Enzyme-linked Immunosorbent Assay
ER:	Endoplasmic Reticulum
FBS:	Fetal Bovine Serum
HDL:	High Density Lipoprotein
HL:	Hepatic Lipase
HSPG:	Heparan Sulfate Proteoglycans
IDL:	Intermediate Density Lipoprotein
LCAT:	Lecithin:Cholesterol-acyltransferase
LDL:	Low Density Lipoprotein
LDL-R:	LDL receptor
LPL:	Lipoprotein Lipase
LRP:	LDL receptor- related protein
NGGE:	Non-denaturing gradient gel electrophoresis
PL:	Phospholipid
POPC:	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PPAR:	Peroxisome-Proliferator Activator Receptor
RAP:	Receptor Associated Protein
RCT:	Reverse Cholesterol Transport
rHDL:	Reconstituted High Density Lipoprotein
SR-BI:	Scavenger Receptor-BI
TG:	Triglyceride
VLDL:	Very Low Density Lipoprotein

Chapter 1 – Introduction

Cardiovascular Disease (CVD), also referred to as coronary heart disease (CHD), is the leading cause of death in the developed world among both men and women regardless of ethnic background (*1*). The primary cause of heart disease is atherosclerosis, which is characterized by the accumulation of lipids and inflammation in the arteries. An atherogenic lipid profile involves high levels of LDL and low levels of HDL (*2*). HDL is considered an anti-atherogenic lipoprotein because of its antioxidant properties and its ability to remove cholesterol from macrophages preventing foam cell formation, also called reverse cholesterol transport (RCT).

In contrast to HDL, LDL is a pro-atherogenic lipoprotein. It can get trapped in the artery wall by passively diffusing through the endothelium where it can become oxidized (*3*). The presence of oxidized LDL in the artery leads to inflammation (*4*), the formation of foam cells through oxidized LDL recruitment by scavenger receptors and the formation of fibrous plaques (*5*). These plaques can lead to narrowing of the arteries, thrombosis, and possibly myocardial infarction (*6*)

High levels of triglycerides (TG) are also considered a risk factor for heart disease, although their contribution is sometimes overshadowed by the effect of cholesterol levels (*7*). Lipases are interfacial enzymes involved in the hydrolysis and clearance of triglycerides from the bloodstream. One type of lipase, hepatic lipase (HL), not only participates in the hydrolysis and clearance of VLDL, LDL, and triglycerides but also plays a crucial role in reverse cholesterol transport (*8*). To understand the importance of HL, it is important to understand the role this protein plays in lipoprotein metabolism and reverse cholesterol transport.

1.1 Lipoprotein Metabolism

1.1.1 Exogenous and Endogenous Lipid Transport

Neutral lipids, such as cholesteryl esters (CE) and TG, can only exist in the bloodstream as components of lipoproteins because they are insoluble in aqueous solutions. Lipoproteins have neutral cores composed of TG and CE surrounded by a monolayer of amphipathic molecules, mainly phospholipids, free cholesterol and apolipoproteins (*9*). Once formed, lipoprotein size, density, function and composition can be modified by the actions of various enzymes and proteins. Enzymes, apolipoproteins, and lipoproteins work together to transfer lipid and cholesterol to specific tissues. There are two different pathways involved in the transport of lipids and cholesterol throughout the bloodstream, the exogenous pathway and the endogenous pathway (*10*).

Exogenous Pathway: The intake of dietary fat from a meal, especially long-chain fatty acids, results in the formation of chylomicrons in the intestine. These chylomicrons are secreted from the intestines into the lymph and eventually into the bloodstream. Chylomicrons transport triacylglycerol-derived fatty acids to the peripheral tissues, which provide a source of energy. Chylomicrons also contain apoB48 which is synthesized in the intestines. Lipoprotein Lipase (LPL), a triglyceride lipase bound to the surface of endothelial cells in the capillaries, removes the fatty acids in the core of the chylomicrons resulting in the formation of CE-rich chylomicron remnants. The hydrolysis of TG in the chylomicrons promotes the formation of chylomicron remnants and leads to the loss of apolipoproteins, specifically apoCs (*9;10*). These chylomicron remnants are eventually

transported to the liver where they are taken into the hepatocytes by the LDL receptor (LDL-R) and the LDL receptor-related protein (LRP) (*11*).

Endogenous Pathway: The liver is also capable of synthesizing its own form of TG-rich lipoprotein, VLDL. Once the VLDL has been synthesized and secreted from the liver into the bloodstream, it undergoes lipolysis by LPL and HL. These lipases transform the VLDL into smaller, denser IDL and eventually LDL particles. LDL is the final product of VLDL hydrolysis. VLDL and LDL are taken up by the hepatic and extrahepatic tissues by LDL-R and LRP (*10*). VLDL and LDL contain one molecule of apoB100. ApoB100 acts as a ligand for LDL-R, but only in LDL and not VLDL (*10*). The time required to clear VLDL from the bloodstream is greater than that required to remove chylomicrons because LPL is more efficient at hydrolyzing the TG in chylomicrons (*10*).

While the VLDL particle is being hydrolyzed, other lipoprotein components are modified as well. The apolipoproteins, phospholipids, unesterified cholesterol, core TG and CE, are exchanged with other lipoprotein particles during VLDL lipolysis (*10*). The exchanges impact the actions of plasma proteins such as lecithin cholesterol acyltransferase (LCAT) (*12*) and the cholesterol ester transfer protein (CETP) (*13*). These proteins participate in cholesterol loading, esterification and the transfer between particles. The exchange of TG and CE between TG-rich lipoproteins and HDL is an important step in the maturation of HDL and the clearance of cholesterol from the bloodstream (*10*).

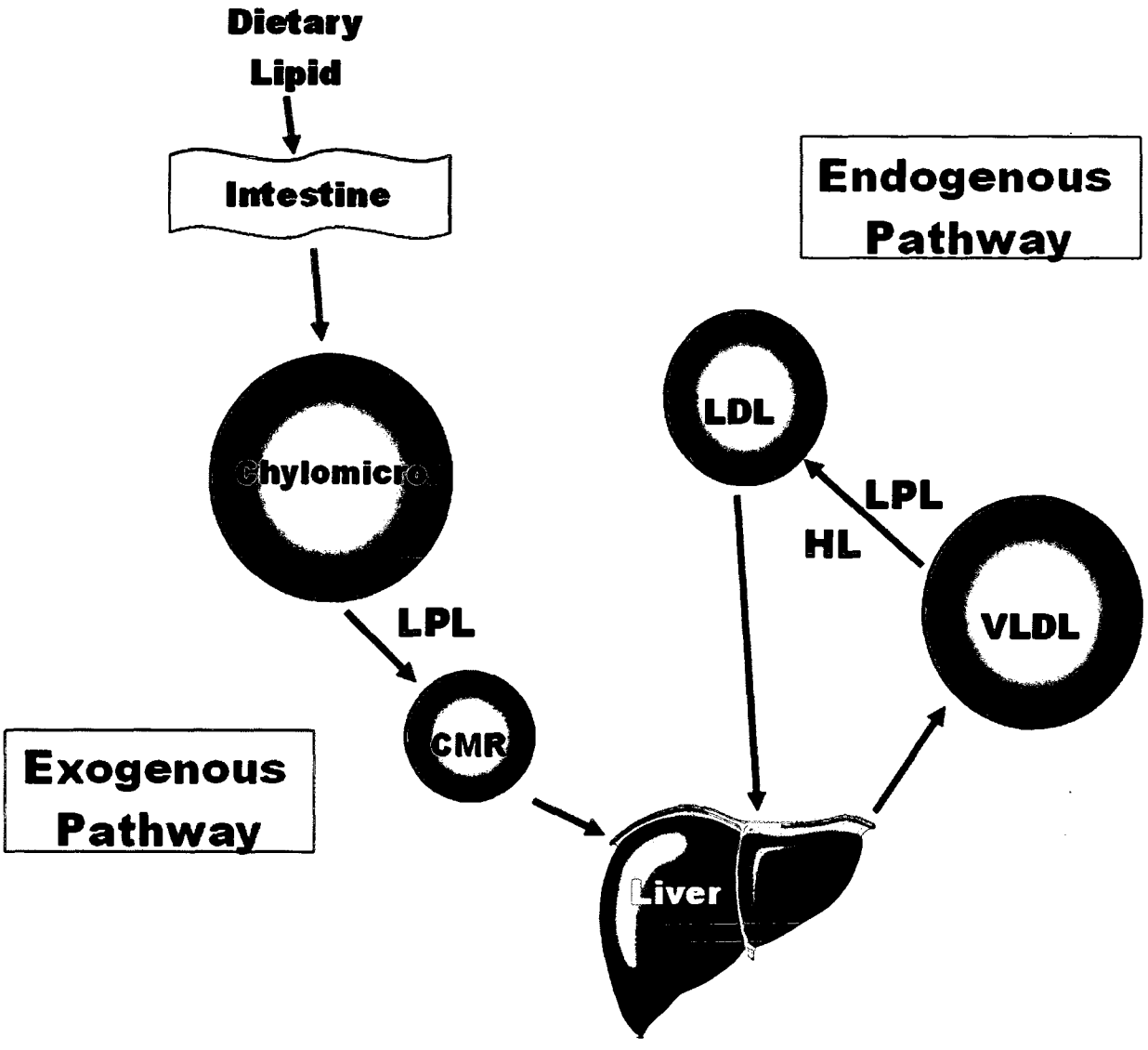


Figure 1.1.1: Endogenous and Exogenous Lipid Transport
Once lipids are digested in the intestine, they are absorbed and secreted into circulation as chylomicrons. LPL hydrolyzes the lipids from chylomicrons and produces chylomicron remnants (CMR). The remnants are taken up by the liver through apoE-dependent receptor uptake pathways. VLDL is produced in the liver and secreted into circulation as the major carrier of cholesterol. HL and LPL hydrolyze the TG in VLDL and convert it to LDL. LDL can then deliver cholesterol to peripheral cells or be taken back up by the liver via the LDL-R.

1.1.2 HDL Metabolism and Reverse Cholesterol Transport

HDL is believed to be atheroprotective because it has the ability to transport cholesterol from atherosclerotic lesions to the liver through a process called reverse cholesterol transport (RCT). High levels of HDL and its primary constituent, apoA-I, are believed to protect against atherosclerosis because of their involvement in removal of cholesterol from tissues during RCT (*10*). For this reason, raising HDL has become a therapeutic target in the fight against atherosclerosis (*14*).

ApoA-I is expressed by the liver and intestine. ApoA-I can either bind to VLDL or chylomicrons and be secreted, or it can be secreted in a lipid-poor form called preβ-HDL (*15*). The apoA-I particles, once secreted, can then obtain phospholipids and cholesterol through an efflux from peripheral cells by ABCA1 (*16*) or from TG-rich lipoproteins, which have been hydrolyzed by lipases (*17*). The efflux of the cholesterol from cells is thought to be an atheroprotective step in RCT. The next step in the maturation of HDL involves the esterification of the newly obtained free cholesterol by LCAT to form HDL_3 (*18*). The CE-rich HDL_3 can then exchange the CE for TG with apoB containing lipoproteins for clearance in exchange for triglycerides. This process is mediated by CETP (*10*). As the HDL_3 particle acquires more cholesterol and TG, it increases in size to form a mature HDL_2 particle. The HDL_2 particle is recognized by the scavenger receptor BI (SR-BI) on the cell membrane of hepatocytes (*19*). The HDL_2 particle can also be hydrolyzed by lipases such as HL and endothelial lipase. As the size of the HDL_2 particle decreases and it reverts back to an HDL_3 particle, apoA-I molecules are released. These lipid-poor apoA-I molecules can then return to the beginning of the RCT cycle to remove more cholesterol from cells and continue to deliver it to the liver for excretion (*10*).

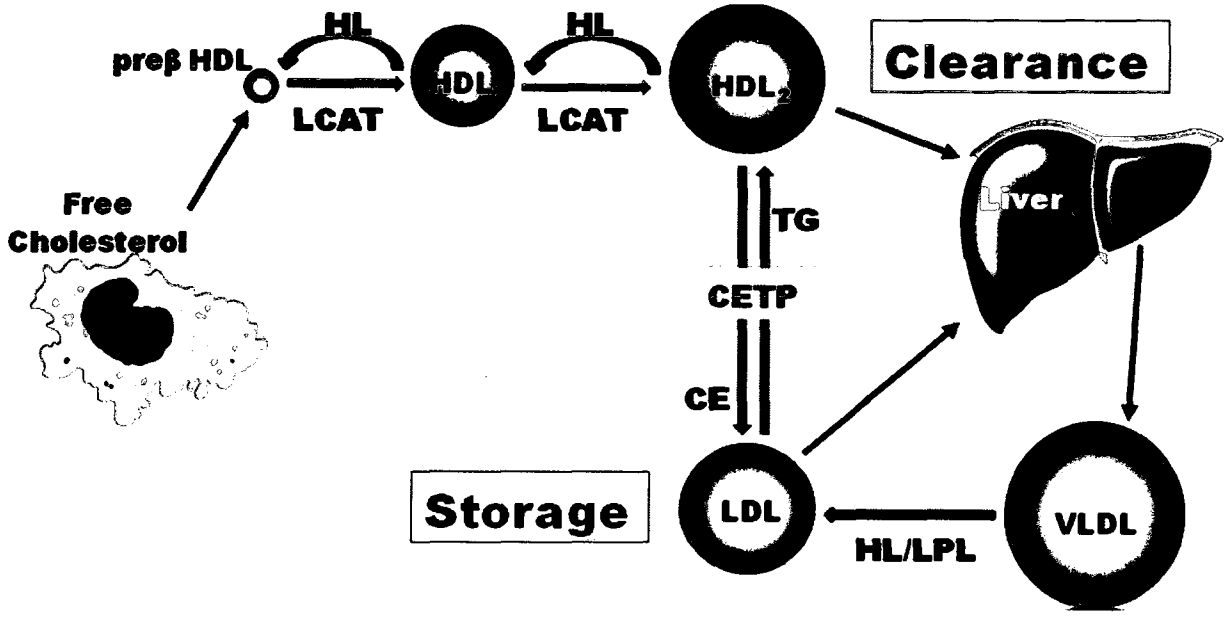


Figure 1.1.2: HDL Metabolism and Reverse Cholesterol Transport
Lipid-poor apoA-I can bind to transporters (ABCAI) on peripheral cells and extract free cholesterol and phospholipids. The action of LCAT converts preβ HDL to HDL_3 and then HDL_2. This molecule can then be taken up by the liver, via SR-BI and through this route cholesterol is returned to the liver. HDL_2 can also exchange TG for CE with LDL or VLDL by the action of CETP.

1.2 HDL Composition and Subclasses

1.2.1 Apoproteins

Apolipoproteins, also called apoproteins, are important in the composition of lipoprotein particles. They are involved in the metabolism of lipoproteins in the plasma and they help mediate the delivery of lipoprotein components to specific tissues (*20*). Apoproteins are also involved in the solubilization of insoluble lipids and prevent the formation of lipid aggregates within the plasma. Apoproteins play a role in the interaction of lipoproteins with cell surface receptors, plasma enzymes and transfer proteins. Some apoproteins function as cofactors for lipases and others inhibit the binding and uptake of lipoproteins by cells (*20*).

There are two types of apoproteins, exchangeable and non-exchangeable. Exchangeable lipoproteins, such as the apoAs, apoCs and apoE, are able to shuttle between different classes of lipoproteins (*21*). ApoB, however, is a non-exchangeable apoprotein and remains bound only to TG-rich lipoproteins. The apoB lipoproteins deliver lipids, mainly TG, from the liver and intestine to peripheral tissues such as muscles and adipose tissue (*10*). ApoA-I containing lipoproteins, ie. HDL, are involved in the transport and circulation of CE, not only from the central organs to the peripheral organs, but also from the peripherals to the liver for metabolism and excretion into the bile (*10*).

ApoA-I: ApoA-I makes up 70% of the apoprotein concentration on HDL (*22*). It is a major structural and functional component and as described earlier, plays a critical role in HDL metabolism and reverse cholesterol transport. The gene for apoA-I is located on

chromosome 11 in a cluster of lipoprotein-related genes including apoC-III, apoA-IV, and apoA-V (*23;24*). Although the main sites of apoA-I expression are the liver and the intestines, apoA-I expression and secretion has also been detected in the heart (*23*).

The expression of apoA-I can be effected by many things including hormones and signaling pathways (*25*). It has been shown that thyroid hormones, retinoids, estrogens, and glucocorticoids increase apoA-I promoter activity and gene expression (*26*). Repressors of apoA-I gene expression include the apoA-I repressor protein-1, hepatocyte nuclear factor-4 and the thyroid hormone receptor (*27*). The apoA-I gene encodes for a 267 amino acid preprotein, which is then cleaved co-translationally and secreted as a 249 amino acid proprotein. The proapoA-I is then processed to form a 243 amino acid apoA-I molecule with a molecular weight of 28 kDa. This is the form of apoA-I found in circulation (*28*).

ApoA-I is comprised of multiple amphipathic helices which are formed by 22mer repeats separated by proline residues. The amphipathic nature of these helices allows them to interact with both phospholipids through their hydrophobic face and the aqueous phase with their hydrophillic face (*29*). It has also been suggested that the two extreme outer helices of apoA-I initiate binding to the phospholipid, which is then followed by the cooperative binding of the other helices. The structure of apoA-I contains two helical domains. The first domain includes a four helix bundle that is formed by the N-terminal three quarters of apoA-I. The second domain is comprised of two helices formed by the remaining C-terminal quarter (*30*). The N-terminal and C-terminal domains are both involved in the binding of lipids, which is one of the most important functions of apoA-I (*31;32*). Also, the interaction between the N-terminal and C-terminal domains is thought to

enhance the stability of the lipid bound form of apoA-I (*33;34*). The C-terminal domain is also required for the initial association of lipid with apoA-I (*35-37*), whereas the N-terminal domain of apoA-I is involved in maintaining the stability of lipid free apoA-I (*30*).

The central domain (aa 100-186) has been shown to be responsible for the plasticity of apoA-I. The plasticity of this domain allows for the binding of varying amounts of lipid (*38*). The first few amino acids of the central domain (aa 100-121) are believed to play an important role in the stabilization of the lipid/apoA-I complex. The second section of the central domain (aa 122-186) are thought to contribute to the initial rates of lipid/apoA-I association (*38*). ApoA-I exists in either a lipid-poor or completely lipidated form; however, under physiological conditions, the lipid-poor apoA-I is unstable and rapidly becomes lipidated (*39;40*). Another important function of the central apoA-I domain (aa 144-186) is its involvement in the activation of LCAT, a critical step in the formation of mature HDL particles as well as RCT (*41-44*). Its lipid binding and LCAT activating ability makes apoA-I critical in the RCT process and its ability to accept an efflux of lipids, specifically cholesterol, from tissues helps protect against the development of atherosclerosis. Besides removing cholesterol from tissues, apoA-I also has antioxidant, anticoagulant, and anti-inflammatory properties, which help protect against atherosclerosis as well (*45*).

ApoA-II: ApoA-II makes up 20% of the apoprotein composition of HDL. It is also synthesized as a pre-proprotein, which is 100 amino acid residues in length. The pre-proapoA-II is then cleaved co-translationally to 82 amino acids. Post-translationally, the proapoA-II is cleaved again to form a 77 amino acid, 17.4 kDa, mature apoA-II molecule (*46*). Human apoA-II is also unique among apoproteins because it possesses cysteine, which

causes the formation of disulfide bonds between two apoA-II molecules (*47*). For this reason, apoA-II usually exists as a homodimer in plasma. ApoA-II can also form heterodimers with other apoproteins such as apoE and apoD (*48;49*).

The apoA-II gene is located at chromosome 1q21 →1q24 (*22*) and it is mainly synthesized in the liver. Its expression can be affected by a number of regulatory elements in the promoter region of the apoA-II gene. It has been demonstrated that nuclear receptors, including PPAR-α, RXR, and SREBP-2 can affect apoA-II expression (*46;50*). The mature apoA-II protein contains 11/22-mer tandem repeats that form amphipathic α-helices with large apolar regions that bind to lipids (*51*). ApoA-II also has a stronger association with HDL than apoA-I due to its larger apolar surfaces and higher hydrophobicity (*52*).

It has been shown that apoA-II has both pro and anti-atherogenic properties (*46*). ApoA-II is known to inhibit LCAT and hepatic cholesterol uptake (*53*) and apoA-II is also thought to inhibit CETP (*46*). ApoA-II also plays an important role in HDL metabolism. ApoA-II is known to have direct and indirect effects on HDL receptors (*54-56*) and transporters (*57*). HDL remodeling, by HDL fusion and apoA-I dissociation, are affected by apoA-II (*58;59*). Also, the apoA-I transfer among HDL particles is inhibited by apoA-II (*60;61*). Studies have shown that HDL particles that contain both apoA-I and apoA-II mediate less cholesterol transport than HDL particles that contain only apoA-I (*22*). It has also been shown that apoA-II has the ability to displace apoA-I from HDL particles increasing the amount of apoA-I available for RCT (*60;61*). The regulatory effects of apoA-II in HDL metabolism and RCT may contribute to its theoretical atherogenicity.

ApoE: ApoE is present on chylomicrons, chylomicron remnants, VLDL, LDL, and HDL. It is a polypeptide of 299 amino acids with a molecular weight of 34.2 kDa (*62*)). Most of apoE is secreted from the liver; however, apoE is found in many other tissues including the brain, kidneys, intestines, and adrenals (*63;64*). The gene for apoE is located on chromosome 19 with the genes for apoC-I, apoC-II and LDL-R (*65*). The apoE protein contains a highly ordered α-helical structure at the C-terminus, which contains the lipid binding domain. The N-terminus is responsible for the binding of apoE to cell surface receptors including HSPG, LDL-R and LRP (*21*). The main function of apoE is to act as a ligand to bind lipoproteins to these receptors, which are then taken up by the cell (*66*).

Unlike some of the other apoproteins, there are three common apoE isoforms found in humans classified as apoE2, apoE3, and apoE4. The most common isoform is apoE3 and the metabolic effects of the other two isoforms are often compared to apoE3. ApoE2 contains a cysteine residue at amino acid 158, where in apoE3 there is an arginine. ApoE4 contains an arginine at amino acid 112, where in apoE3 there is a cysteine (*67*). These mutations can significantly affect the function of apoE.

The single amino acid mutations in apoE2 and apoE4 are associated with lipoprotein abnormalities and affect lipoprotein metabolism. ApoE2 only binds approximately 2% of the LDL-R receptors compared to apoE3 and apoE4 (*68*). ApoE4 binds preferentially to VLDL unlike apoE2 and apoE3, which preferentially bind to HDL. ApoE4, therefore, leads to an increase in apoB and cholesterol levels and a decrease in apoE (*69;70*). ApoE2, on the other hand, has the opposite effect on apoB, cholesterol and apoE levels (*70*). The preferential distribution of apoE4 to TG-rich lipoproteins leads to the acceleration of their

uptake. This leads to a down regulation of hepatic LDL-R expression and an increase in LDL levels. The low affinity of apoE2, in comparison, would lead to a decrease in cellular cholesterol influx and lower levels of cholesterol as well as upregulating LDL-R (*71-73*). ApoE4 is considered a dangerous isoform because it has been shown to be associated with the early onset of both Alzheimer's Disease and CHD (*74;75*)

1.2.2 HDL Subclasses

All of the exchangeable apoproteins can be found as components of HDL. HDL can be divided into different subclasses by density. These different subclasses have different apoprotein and lipid compositions. HDL_2 is the least dense subclass of HDL and has a density ranging from ρ = 1.065-1.125mg/mL. HDL_3, on the other hand, has a density between ρ = 1.125-1.21 mg/mL (*76*). HDL_2 is formed from HDL_3 as a result of an increase in lipids. HDL_3 is formed from preβ HDL though an accumulation of lipid and apoproteins. When HDL_2 is hydrolyzed by HL, HDL_3 is also formed (*77*).

During the process of HDL metabolism, the HDL particle is also constantly exchanging and losing apoproteins. HDL_2 contains less overall apoproteins and has a lower ratio of apoA-I to apoA-II than HDL_3, even though HDL_2 is larger in size (*78*). HDL_3, however, contains lower concentrations of apoE (*79*). HDL_2 is also more TG enriched due to the exchange of CE for TG through CETP (*80*). There has been some dispute as to whether smaller, more dense, HDL are more atheroprotective than larger HDL. Some studies report that smaller HDL (preβ) impair HDL maturation and RCT, which would increase the risk of atherosclerosis (*80;81*). Other studies, however, show that it is the larger

HDL particles that are negatively associated with CHD (*82-84*). The differing compositions of the HDL subclasses can clearly affect their atheroprotective ability.

1.2.3 Involvement of ApoE in Lipolysis

ApoE and lipoprotein exchange: During lipoprotein metabolism, the exchangeable apoproteins are constantly being shuttled from TG-rich lipoproteins to HDL and back. During a postprandial period, the period of time following a meal when the TG concentration in the plasma is increasing, apoE is shuttled from HDL to the TG-rich lipoproteins, particularly chylomicrons and VLDL. ApoE is shuttled to the TG-rich lipoproteins during a postprandial response to act as a ligand and bind LRP and LDL-R (*85*). It has been shown that apoE mediates the uptake of lipoproteins through its ability to bind to LDL-R (*71*) and LRP (*86;87*). Once the postprandial period is over, the apoE is then shuttled back to higher density particles where it is stored until it is needed again (*85*). It has been demonstrated that apoE also has a heparin binding site and is able to bind heparan sulphate proteoglycans (HSPG). The interaction between apoE and HSPG is another way in which lipoproteins can bind to the cell surface and be taken up into the cell (*88*). It would appear that because apoE can aid in the binding, uptake and subsequent clearance of cholesterol, it would be anti-atherogenic; however, many studies have found that patients with high levels of apoE also tend to be hypercholesterolemic and hypertriglyceridemic (*89*). A study by Mooijaart *et al.* also found that high levels of plasma apoE levels in seniors, correlates to an increase in the severity of CHD (*90*).

Interactions of apoE with other apoproteins: ApoE is also known to interact with other apoproteins such as apoA-II and the apoCs. ApoE can form a heterodimer through disulfide

bonds with apoA-II on HDL (*91*). Less than 20% of the apoE on HDL is in a free form, the rest is bound to apoA-II (*92*). The formation of the heterodimer inhibits the ability of apoE to bind to the cell surface receptors (*91;92*) as well as shuttle between HDL and the TG-rich lipoproteins (*92*). The apoE on VLDL and chylomircrons is in a monomer form and thus interacts normally with receptors. ApoE4, however, cannot form the disulfide bonds with apoA-II and thus does not form the apoE – apoA-II complex. This may partially explain why apoE4 is found to be associated with VLDL more than HDL (*92*).

The ability of apoE to bind to LDL-R and LRP is critical to cholesterol metabolism. A family of smaller apoproteins, called the apoCs, can affect the ability of apoE to mediate lipoprotein binding and uptake. ApoC-I, apoC-II, and apoC-III have all been shown to inhibit the ability of apoE to bind to cell surface receptors. It has been demonstrated that all of the apoCs displace apoE from VLDL and chylomicrons, inhibiting the binding of these TG-rich lipoproteins to lipoprotein receptors. Also, apoC-I can inhibit the binding of apoE to cell surface receptors, like LRP, by masking or altering the conformation of apoE instead of displacing it (*93*).

1.3 Hepatic Lipase

1.3.1 Hepatic Lipase and the Lipase Family

Hepatic lipase is an interfacial enzyme that is involved in the hydrolysis of TG and phospholipids (PL). HL can be found in hepatocytes, adrenal glands and the ovaries; however, most of the HL found in circulation is expressed by the liver (*94*). HL belongs to a family of lipases that are involved in the hydrolysis of sn-1 fatty acyl ester bonds of sn-3

phospholipids and mono, di and triglycerides (*95*). Other lipases in this family include LPL, pancreatic lipase and endothelial lipase (EL). LPL is a TG lipase and is involved in the lipolysis of chylomicrons and VLDL to IDL and LDL, which can then be cleared by the liver (*96*). It also requires a co-factor, apoC-II, for activation. HL is both a TG and phospholipid lipase and is involved in the lipolysis of TG-rich lipoproteins as well as the hydrolysis of HDL_2 to HDL_3 (*97*). EL is specifically a phospholipase and is involved in the lipolysis of phospholipid in HDL (*98*). Unlike LPL, neither HL nor EL require a co-factor for activation (*99*).

Lipases share a similar structure including independently folded N- and C-terminal domains and an active site enclosed by a lid or loop domain (*100;101*). This lid domain is responsible for substrate specificity. For example, when the lid domain of HL was replaced with the lid domain from LPL, HL preferentially hydrolyzed TG over PL (*102*). HL and LPL have also been shown to function as ligands and can aid in the binding and uptake of lipoproteins by LDL-R, LRP, and HSPG, enhancing lipoprotein clearance (*88;103;104*).

1.3.2 Hepatic Lipase Structure

HL is a 477aa, 66 kDa, serine hydrolase that exists in homodimers in circulation. The N- and C-termini are both involved in the formation of head-to-tail homodimers of HL as well as the substrate specificity of HL (*105*). HL also contains a lipid binding domain, a heparin binding domain and an active site. The active site contains a Gly-X-Ser-X-Gly motif where 'X' represents any amino acid. The Ser residue is an activated serine and is responsible for the lipolytic activity of HL (*105;106*).

It is believed that both the N- and C-termini of HL are involved in heparin binding. These heparin binding sites are responsible for the interaction between HL and HSPG. HL is bound to the cell surface by HSPG through electrostatic interactions between the negatively charged sulphate groups and positively charged amino acids on HL (*107*). It is bound to HSPG and is localized in the subluminal extracellular matrix component of endothelial cells and microvillar surfaces of hepatocytes in the space of Disse, interhepatocyte spaces and luminal surfaces of hepatic sinusoidal endothelium (*94;108*). Two possible heparin binding motifs include BBBxxB and BBxB, where B represents basic amino acids (*107*). Another study found that the orientation of the basic amino acids in the protein is also important for heparin binding (*109*). Similar heparin-binding motifs have been found in other heparin-binding proteins such as apoE, apoB-100, and LPL (*110-112*). Hill *et al.* also discovered that the last 60 amino acids at the C-terminus of HL are important for heparin binding (*113*). This observation was confirmed by Brown *et al.* when they replaced that last 70aa of human HL with the corresponding section of mouse HL. They also created an HL protein missing the final 5aa and looked at the heparin-binding activity of both mutants. They found that the heparin-binding ability of the human/mouse chimera and the truncated human HL was significantly less than that of wild-type human HL. This observation demonstrated that the last 70aa in human HL, specifically the final 5 amino acids, are critical to its heparin-binding ability (*114*).

1.3.3 Hepatic Lipase Synthesis and Expression.

The synthesis of HL occurs mainly in liver parenchymal cells (*115*); however, HL mRNA has been detected in steroidogenic organs in rats and humans (*116*). Once

synthesized and secreted, the HL is then transported through circulation where it can bind to tissue specific sites (*117;118*). The HL gene, LIPC, is located on chromosome 15 in humans. This gene contains 9 exons, 8 introns, and 60 kb of DNA (*119*). HL is synthesized in the ER with an N-terminal leader peptide. The leader peptide is cleaved and the HL protein acquires N-linked oligosaccharides translationally at 4 N-linked glycosylation sites (aa 20, 56, 340, 375) (*120;121*). The glucose is then cleaved through a series of steps, and once the HL protein is folded correctly, the mature HL protein is secreted from the Golgi apparatus into circulation. Mature HL is 66 kDa and 477 amino acids in length (*122*).

The expression of HL has often been related to its circulating activity. To measure plasma HL activity, an injection of heparin is required. Heparin releases HSPG-bound HL from the cell surface resulting in an increase in TG catabolism. The HL activity measured is referred to as post-heparin HL activity (*123-125*). An injection of heparin, however, does not measure the amount of HL mass in circulation. Several lines of evidence show that there is some circulating HL mass that does not have activity, due to an inhibition by HDL (*126*).

Many different factors can affect the expression/activity of HL including, hormones, catchecholamines, cellular demand for cholesterol and fatty acids, and exogenous factors like diet, lifestyle and lipid lowering drugs (*127*). Estrogen, for example, has been shown to decrease HL expression, and premenopausal women seem have less HL expression compared to men and postmenopausal women (*128*). Estrogen therapy and castration have also been shown to decrease HL post-heparin activity and HL mRNA levels (*129;130*). Testosterone, in contrast, has been shown to increase HL expression (*131;132*). In diabetic patients, insulin treatment is also known to increase post-heparin HL activity (*133*). Ob/ob

mice treated with leptin show an increase in HL mRNA (*134*). In contrast, adrenaline seems to have the opposite effect and an increase in adrenaline inhibits HL maturation and increases degradation (*135*).

1.3.4 Ligand Function of Hepatic Lipase

As mentioned earlier, HL binds to cell-surface HSPG. The binding of HL to HSPG is involved in the uptake and clearance of lipoproteins. ApoE can also act as a ligand to bind lipoproteins to HSPG through its heparin binding domain (*136*). Treatment of cells with heparinase released surface-bound HL and inhibited the binding and uptake of chylomicron remnants, VLDL, and HDL, indicating that HL and/or HSPG may mediate the uptake of lipoprotein particles (*88;137;138*). Interestingly, inactive HL is still able to promote the binding and uptake of lipoproteins (*139*). Also, patients who have mutations in HL, making it inactive, have better lipid profiles than those who have no HL expression (*140*).

HSPG are not the only cell-surface molecules to which HL can bind. HL has been found to act as a ligand for lipoproteins to bind to HSPG, LDL-R, and LRP. HL acts as a bridge between the HL-lipoprotein complex and the cell-surface receptors (*108*). It has also been shown that HSPG can act to sequester lipoproteins to the cell surface through HL and apoE binding and then the lipoproteins can be taken up by LDL-R and LRP (*86*). HL has been shown to be involved in chylomicron and VLDL uptake through LRP in Chinese Hamster Ovary cells (CHO), human hepatocytes and primary liver cells (*141*). The receptor-associated protein (RAP) is a LRP antagonist and treatment with RAP was found to partially inhibit HL mediated binding and uptake of lipoproteins (*88;142*). RAP treatment also partially inhibited the clearance of chylomicrons in the liver (*104*). Not all HL-mediated

binding and uptake was inhibited when LRP was inhibited, indicating the involvement of another cell-surface receptor. The overexpression of anchored HL (*143*) and rat HL (*144*) increased the binding and degradation of LDL through LDL-R and this increase was inhibited by LDL-R specific antibodies. Clearly, HL-mediated binding and uptake of lipoproteins also involves LDL-R.

HL appears to act with SR-BI to promote lipoprotein clearance as well. Wang *et al.* discovered that female HL knock-out mice had a decrease in adrenal cholesterol stores even when adrenal SR-BI expression was increased, indicating a role of HL in CE clearance through SR-BI (*145*). Also, the expression of HL and SR-BI in kidney 293 cells increased HDL cell association and CE uptake compared to cells that were only transfected with either SR-BI or HL alone (*146*). Both of these observations indicate that HL may be involved in SR-BI-mediated selective uptake of cholesterol by the adrenals and that HL levels may be linked to SR-BI expression.

1.3.5 HL Substrate Specificity and Activity

HL Substrate Specificity: HL is an acylglyceride lipase and hydrolyzes TG in HDL and TG-rich lipoproteins. HL, however, preferentially hydrolyzes diglyceride (DG) as a substrate, over TG (*147;148*). The DG content of the HDL regulates HL-mediated hydrolysis of TG by changing the structural organization on the surface of HDL as well as the properties of HDL (*149*). High concentrations of DG can also affect the ability of HL to interact with the interfacial surface of HDL and act as an acylglyceride lipase (*147*). HL also appears to be more active in the presence of short-chain acylglycerides even though its preferred substrate is long-chain fatty acids (*150;151*). The increased activity of HL on

shorter and moderate chain length fatty acids is believed to be caused by the increased mobility of shorter chain fatty acids in the PL monolayer compared to fatty acids with longer chain lengths (*152*).

HL is also a phospholipase and is involved in the hydrolysis of PL on the surface of HDL. Its preferred PL substrate is phosphatidylethanolamine. The phospholipase activity of HL is affected by the phospholipase head group (*153*). Similar to TG hydrolysis, HL activity increases as the chain length decreases. Acyl group hydrolysis by HL also increases as the degree of unsaturation of the PL increases (*150*). Although HL is both a phospholipase and a TG lipase, its substrate specificity is affected by its loop or lid domain. This domain covers the active site on the HL molecule and dictates the type of lipid that can be hydrolyzed. As mentioned earlier, the PL hydrolysis activity of HL was significantly reduced when its lid domain was switched with the lid domain of LPL, a TG specific lipase (*102*).

HL Activity: HL and LPL work together to form LDL through the hydrolysis of VLDL and IDL and in the transfer of lipids and apoproteins with HDL. Unlike LPL, however, HL does not require a co-factor for activation. Whereas LPL is mainly responsible for converting VLDL into IDL (*154*), HL plays a small role in the conversion of VLDL to IDL and a large role in the conversion to LDL (*96;155*). Patients who have an HL deficiency have shown a decrease in the conversion of VLDL to IDL by 50% and a decrease in IDL to LDL conversion by 90% (*96*). HL is also responsible for the conversion of HDL_2 to HDL_3 (*77*). Through its phospholipase A1 and TG hydrolysis activities, HL is also able to clear TG and PL from the bloodstream and modify the size and density of lipoprotein particles

(*108;156;157*). HL activity has an inverse relationship with LDL size and buoyancy, which is a reflection of IDL catabolism (*158*). Increased post-heparin HL activity leads to the formation of smaller, dense and more atherogenic LDL particles. Also, an increase in HL activity may result in a decrease of IDL (*159*).

HL activity not only affects the hydrolysis of apoB containing lipoproteins, it also has a significant effect on HDL hydrolysis. An increase in HL activity leads to a decrease in HDL cholesterol, as well as a decrease in size and buoyancy of HDL particles and a more homogenous HDL population (*96;159*). Normally, the hydrolysis of HDL to smaller less stable particles is not beneficial as it is associated with an increased clearance of HDL and lower plasma HDL levels (*8*). An increase in small dense HDL particles is therefore considered atherogenic compared to an increase in larger more buoyant HDL. In HL-deficient patients there is a redistribution of HDL and an increase in the generation of large, TG-enriched, HDL_2 is observed (*96*).

As mentioned earlier, HL activity is only measurable after an intravenous injection of heparin. Heparin releases HL into circulation from the cell surface where it is bound to HSPG. Following heparin treatment, a significant increase in TG metabolism is observed (*123-125*). Post-heparin HL activity is considered a risk factor for CHD. Subjects with high post-heparin HL activities are at a higher risk for CHD than those with lower activities (*160;161*).

In studies from this laboratory, it has been shown that HL is relatively inactive when bound to HSPG and must be released from the cell surface in order to become active (*126*). This means that a post-heparin HL activity measurement is only measuring the inactive store

of HL on the cell surface and not the amount of HL circulating in the bloodstream. ApoA-I and HDL can release HL from the cell surface in a similar manner to heparin (*126;162;163*). Once released, the HL then has the potential to become active and hydrolyze TG and PL. The composition of HDL has been shown to affect this displacement. The presence of apoC-I and apoA-II in synthetic HDL particles increases the displacement of HL from the cell surface when compared to synthetic HDL containing only apoA-I (*163*). These studies have indicated that it may not be high levels of HL that are a risk factor for CHD, but a high level of liver-bound, inactive HL that is unable to aid in the clearance of TG.

1.3.6 Mechanism of HL Displacement from the Cell Surface by HDL

The displacement and activation of HL appears to be regulated by a three step process (*162;163*). The first step involves both lipid and apolipoprotein exchanges between HDL and the apoB containing lipoproteins. The second step of this is the displacement of HL from the surface of the liver (*163*). High affinity association of HL with HDL has been shown to inhibit its lipolytic activity (*148;164*) and therefore, once displaced from the surface of the liver, HL remains bound to the HDL in an inactive form.

The final step in HL activation is dissociation from HDL (*148*). This step may be due to altered electrostatic interactions that result from anionic lipids released when VLDL and chylomicrons are degraded by LPL. An increase in vascular fatty acid levels results in an increased negative charge in the interfacial environment around the HDL, which could result in the release and the activation of HL (Figure 1.3.1) (*148;165*). It has previously been shown that an increase in the net negative charge on HDL results in a decreased affinity of HL for HDL and an increase in TG lipolysis (*148*).

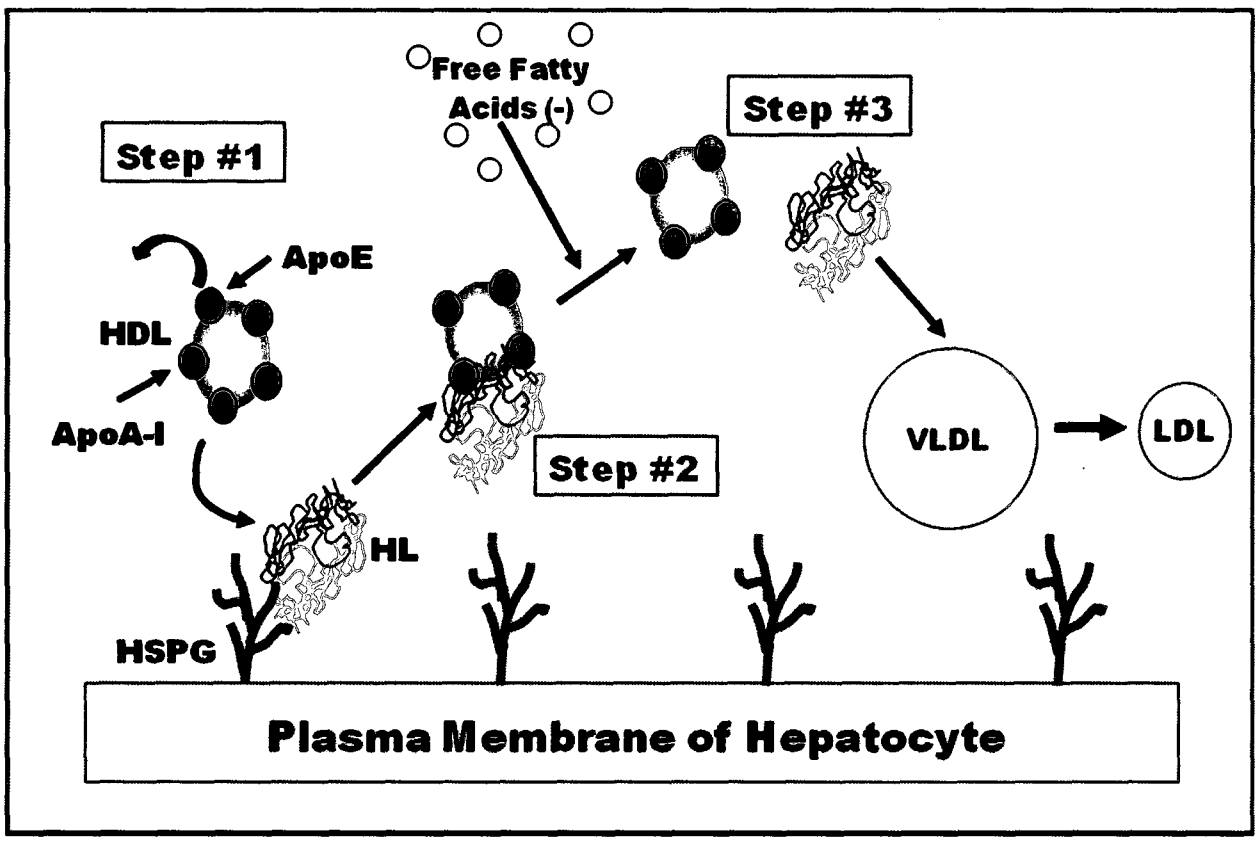


Figure 1.3.1: The mechanism of HL displacement from the cell surface by HDL. The first step in HL displacement from the cell surface involves the lipid and lipoprotein exchanges between HDL and the apoB-containing lipoproteins. The HDL then interacts with the HL on the cell surface and displaces it. The HL remains bound to the HDL in an inactive form (Step 2). The HL needs to be released from the HDL to become active (Step 3). This may occur due to altered electrostatic interactions caused by the release of anionic lipids during the hydrolysis of TG-rich lipoproteins by LPL.

1.3.7 HL and Atherosclerosis

Atherosclerosis is the leading cause of death of adults in developed countries (*1*). Studies have linked high post-heparin HL activity to an increase in risk for atherosclerosis. A high HL activity results in an atherogenic lipid profile including a decrease in HDL (*166*), which is an atheroprotective lipoprotein, and an increase in atherogenic small dense LDL (*167*). To support this conclusion, patients with CHD and an increased risk of CHD have been shown to have increased post-heparin HL activities. Patients with familial combined hypercholesterolemia (*168*) and type 2 diabetes (*133*), who are also at a higher risk for CHD, also show elevated post-heparin HL activity. The presence of other heart disease risk factors, such as smoking (*169;170*), visceral obesity (*161;171;172*) and sedentary lifestyle (*173;174*) have been linked to high post-heparin HL activities as well. Premenopausal women are protected from heart disease because they have higher HDL levels than men or postmenopausal women. This increased HDL may be partially due to a lower post-heparin HL activity (*175*). After LDL from HL-deficient patients has been depleted of TG by incubation with HL, the LDL was degraded at twice the rate of native LDL by macrophages and arterial smooth muscle cells. This suggests that the modifying effect of the lipase on LDL can cause significant cholesterol accumulation in these cells, contributing to foam cell formation and CHD development (*176*).

HL hydrolyzes TG and an increased TG level is a risk factor for CHD (*177*). An increase in HL activity would, therefore, increase TG hydrolysis and decrease the chance of CHD in subjects. The ability of HL to act as a ligand and facilitate intracellular lipid metabolism is also atheroprotective. When the activity of HL is inhibited or absent, an increase in atherosclerosis is observed. The overexpression of apoC-I (*178*) or apoA-II

(*58;179*) in apoE-null mice, resulted in an inhibition of HL activity and an increase in atherosclerosis compared to the control mice. To support the atheroprotective role of HL, when HL was overexpressed in mice, a 42% decrease in aortic cholesterol compared to control mice was observed (*180*). Human patients with HL deficiency also show an increase in the prevalence of premature heart disease, indicating that a lack of HL may be atherogenic (*158;181;182*). An inverse correlation was also found between the extent of CHD in men undergoing elective coronary angiography and post-heparin HL activity (*183*).

HL is a multifunctional enzyme and currently its involvement in the onset and progression of atherosclerosis is not understood. Many different factors could be regulating the atherogenic nature of HL; however, the identity and effect of all of these factors on CHD risk has yet to be elucidated.

1.4 Rationale and Objectives

Lipid metabolism and its role in the development and progression of atherosclerosis have been widely studied. It has been shown that LDL and HDL concentrations in plasma are predictors of atherosclerosis and risk factors for CHD. High levels of LDL increase the risk for CHD whereas high HDL levels decrease CHD risk. The concentrations of these two lipoproteins are greatly influenced by the actions of HL (*94*). Understanding the mechanism of action of HL and what regulates its activity is therefore crucial to understanding its role in lipoprotein metabolism and the progression of atherosclerosis. A complete knowledge of the involvement of HL in atherosclerosis pathogenesis could lead to the development of new strategies to treat and prevent the onset of this disease.

It has been known for six decades that an injection of heparin releases HL from the cell surface, and stimulates an immediate TG hydrolysis (*184;185*). There is a prevailing notion, however, that HL and LPL are catalytically active when bound to HSPG and therefore an increase in HSPG-bound HL and LPL is believed to be related to increased TG hydrolysis (*154*). This view is counter-intuitive and in disagreement with what is known of interfacial enzymes. An interfacial enzyme must be free to shuttle between substrates in order to attain optimal hydrolysis (*186;187*). Ramsamy *et al.* determined that HL becomes active when it is displaced from the cell surface. Both apoA-I and HDL have the ability to displace HL from the cell surface similar to heparin (*162;163*). Besides apoA-I, HDL contains other apoproteins and lipids that affect its ability to displace HL. Rouhani *et al.*, for example, determined that the presence of apoA-II and apoC-I on reconstituted HDL particles greatly enhanced HL displacement compared to the apoA-I control particle (*163*). HDL

composition may be affected by gender, hyperlipidemia and postprandial lipemia. If this is the case, then HL displacement would be expected to be affected as well. The hypothesis of this study is that lipemia promotes changes in the lipid and apoprotein composition of HDL, which regulates HL displacement. To understand what regulates the displacement of HL by HDL, this study focused on two main objectives:

1. Determining the effect of HDL composition on HL displacement

2. Determining the effect of lipemia on HL displacement

The experiments detailed within this study provide insight into how HL displacement is regulated by HDL composition and that this displacement correlates to HDL-bound HL levels in human plasma. This study reveals that gender, postprandial lipemia, and hyperlipidemia can affect HL displacement as well. Evidence is also provided that indicates a direct role for apoE in the inhibition of HL displacement.

Chapter 2 – Experimental Procedures

2.1 Materials

The mouse monoclonal HL antibody was received from Dr. Bensadoun, Cornell University. The mouse monoclonal apoA-I and apoE antibodies were obtained from Dr. Marcel, University of Ottawa Heart Institute. The colorimetric triglyceride and cholesterol assays were obtained from Wako Chemicals USA (Richmond, VA). The recombinant apoE3 protein was obtained from Abcam Inc. (Cambridge, MA). The apoA-II and apoC-III antibodies were purchased from Biodesign International (Saco, ME). The Fetal bovine serum (FBS), heparin (from porcine intestine), alkaline avidin phosphatase, alkaline phosphatase yellow liquid substrate and Intralipid were from Sigma-Aldrich (St. Louis, MO). The mouse anti-human capture antibody, horseradish peroxidase-linked goat anti-human apoA-I antibody and the K-Blue MAX substrate (TMB) were purchased from Cedarlane (Hornby, ON). The SuperSignal West Femto and West Dura Chemiluminescent Substrates were purchased from Pierce Chemical Co. (Rockford, IL). The Novex polyacrylamide 4-20% gels, Ham's F12 medium and the Geneticin® Selective Antibiotic (G418 sulfate) were obtained from Invitrogen (Burlington, ON). 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL). 6-well and 12-well plates were purchased from Corning (New York, NY). The goat anti-mouse horseradish peroxidase-linked secondary antibody was purchased from KPL (Gaithersberg, MD). The Immuno Maxisorp 96-well plates were purchased from Nunc (Rochester, NY). Laemmli sample buffer, 30% acrylamide, ammonium persulfate (APS), TEMED, resolving gel buffer and stacking gel buffer were purchased from Biorad (Hercules, CA). The CHO-hHL cells were received from Drs. Robert Brown and Zemin Yao (University of Ottawa, Ottawa, ON). Unless otherwise stated, all other reagents were of analytical grade.

2.2 Methods

2.2.1 Lipoprotein Isolation using Sequential Ultracentrifugation

The blood samples were obtained from patients after 12 hours of fasting. The postprandial blood samples were obtained 4 hours after the subjects had consumed an 1800 calorie meal. The blood samples were obtained in Vacuette® EDTA coated tubes, for lipoprotein isolation from plasma. Blood samples obtained for the isolation of serum were collected in Vacuette® SST tubes. VLDL/LDL and HDL were isolated by sequential ultracentrifugation according to the procedure described by Havel *et al.* from fasting and postprandial human plasma samples using density ranges ρ = 1.006-1.065 g/mL and ρ = 1.065-1.25 g/mL respectively (*188*). VHDL was included in the HDL fraction to ensure that all apoA-I containing forms of HDL were used for displacement. Briefly, the plasma was isolated by centrifuging the tubes for 15min at 3000 rpm. The plasma was removed and its density was adjusted to 1.065 g/mL using dry KBr. The plasma solution was sealed in Quick-Seal centrifuge tubes (25mm X 89mm)(Beckman, Palo Alto, CA). The tubes were spun using the L8-70M Beckman ultracentrifuge for 20 hours at 40000 rpm in a 70Ti rotor. The VLDL and LDL were in the top fraction of the solution and the bottom fraction was collected to spin for HDL. The density of the bottom fraction was increased to 1.25g/mL using dry KBr and was spun for 40 hours at 40000rpm. Again the top fraction, containing the HDL, was isolated. All of the lipoprotein samples were dialyzed extensively against phosphate buffered saline (PBS)(50mM sodium phosphate, 150mM NaCl, pH 7.2). The heterogeneity of the lipoprotein samples was determined using agarose gel electrophoresis on Lipogels (Beckman-Coulter, Mississauga, ON) using neutral lipid staining. The protein

concentrations were determined using the Lowry method as modified by Markwell *et al.* (*189*). Cholesterol and triglyceride concentrations were determined using commercial diagnostic assays from Wako Chemicals USA (Richmond, VA).

2.2.2 Triglyceride Enrichment of HDL Samples

The blood samples were obtained from patients after 12 hours of fasting. The blood samples were collected in Vacuette® EDTA coated tubes, for lipoprotein isolation. VLDL/LDL were isolated by sequential ultracentrifugation according to the procedure described by Havel *et al.* from fasting plasma samples using the density range ρ = 1.006-1.065 g/mL (*188*). Once the VLDL and LDL had been removed, half the remaining fraction with ρ = 1.065g/mL was incubated with Intralipid, a 20% TG emulsion (Sigma-Aldrich, St. Louis, MO) for 3 hours at 37°C. The non-absorbed Intralipid was then removed using sequential ultracentrifugation using a density of ρ = 1.065g/mL as previously described. HDL from both the fasted and TG enriched samples was then isolated using a density range of ρ = 1.065-1.25 g/mL (Figure 2.2.1). All of the lipoprotein samples were dialyzed extensively against phosphate buffered saline (PBS)(50mM sodium phosphate, 150mM NaCl, pH 7.2). The heterogeneity of the lipoprotein samples was determined using agarose gel electrophoresis on Lipogels (Beckman-Coulter) using neutral lipid staining. The protein concentrations were determined using the Lowry method as modified by Markwell *et al.*(*189*). Cholesterol and triglyceride concentrations were determined using commercial diagnostic assays from Wako Chemicals USA (Richmond, VA).

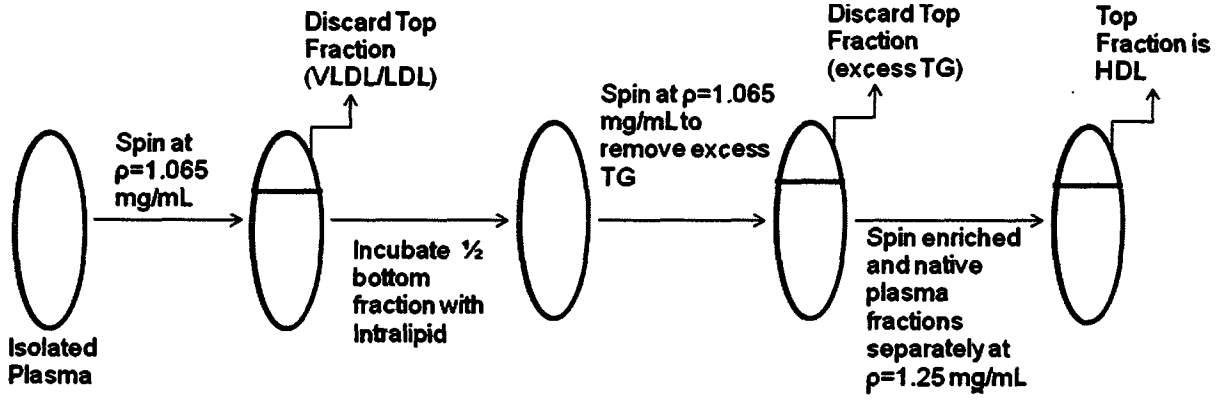


Figure 2.2.1: Lipoprotein isolation and triglyceride enrichment of HDL with Intralipid. The isolated plasma was spun at $\rho = 1.065$ and the top VLDL/LDL fraction was discarded. The $\rho > 1.065$ fraction was incubated for 3 hours at 37°C with Intralipid. The excess TG from the Intralipid was removed by spinning the plasma fraction at $\rho = 1.065$mg/mL and discarding the top fraction. The remaining TG enriched plasma fraction was then spun at $\rho = 1.25$mg/mL to isolate the HDL fraction.

2.2.3 Purification of ApoA-I

ApoA-I was isolated from human HDL that had been delipidated in chloroform:methanol as previously described (*190*). ApoA-I was isolated using size exclusion chromatography on a Sephacryl S-200 HR column (*191*). Purified protein samples were lyophilized and stored at -80°C until needed. For use, apoA-I was re-solubilized in 6M guanidine hydrochloride (GdnHCl), 10mM Tris (pH 7.2) and dialyzed extensively against PBS.

2.2.4 Cell Culture Techniques

Chinese hamster ovary cells overexpressing human HL (CHO-hHL) were obtained from Drs. Robert Brown (University of Ottawa) and Zemin Yao (University of Ottawa). CHO-hHL cells were maintained in Ham's F12 medium containing 10% fetal bovine serum (FBS) and 500 μg/mL geneticin selective antibiotic (G418 sulfate) in a 37°C incubator with 5% CO_2. Once the cells were approximately 90-95% confluent in a 10cm plate, they were split 1 in 4 and plated onto 6-well plates. The cells were then allowed to reach ~85% confluency before treatment. The cells were pre-incubated in serum-free medium for 3 hours prior to use in an experiment. The cell surface HL displacement ability of HDL was determined by incubating the CHO-hHL cells with 150 μg/mL HDL or apoA-I in FBS-free medium for 45 minutes at 37°C. After the incubation the medium was collected and the amount of HL released was measured using immunochemical analysis (Refer to section 2.2.6).

2.2.5 Preparation of Reconstituted HDL (rHDL) Particles

Reconstituted HDL complexes were prepared by sonicating 1-palmitoyl-2-oleyl-phosphatidylcholine (POPC) with a mixture of apolipoproteins (apoA-I and apoE) at a molar

ratio of one mole protein : 60 moles lipids as previously described by Boucher *et al.* (*164*). Briefly POPC (1 mg) in chloroform was dried under nitrogen in a 12 × 75 mm test tube and 800 µl of PBS, pH 7.4 was added. The lipid-buffer solution was initially sonicated for 1 min at a 100% duty cycle. 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 and apoE were added to the lipid suspension and the protein-lipid mixture was sonicated for 4 × 1 min punctuated by 1 min cooling periods. The size and homogeneity of the rHDL complexes were estimated by non-denaturing gel electrophoresis (NGGE) on a 4-20% gel (Invitrogen, Carlsbad, CA). The protein concentration was determined by the Modified Markwell Lowry method (*189*).

2.2.6 Quantification of Cell-surface HL Displacement by HDL

The media samples were combined in a 1:1 ratio with Laemmli sample buffer (Biorad, Mississauga, ON) containing 5% β-mercaptoethanol. The samples were electrophoresed on an 8% polyacrylamide gel under denaturing conditions at 125V. The proteins were immunoblotted onto PVDF membrane (Biorad). The blots were then blocked with 1% BSA in TBST and probed using a 1:5000 dilution of the mouse monoclonal anti-human HL antibody. The blots were then washed three times with TBST. Finally the blots were incubated with a 1:20,000 dilution of the HRP-linked goat anti-mouse IgG secondary antibody (KPL, Gaithersburg, MD) in 1% BSA/TBST. The blots were developed using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) on the Fluorchem AlphaImager instrument. The band intensities were analyzed with spot-densitometry using the AlphaEase© software provided with the AlphaImager. The band intensities of the blots

were normalized to total cell protein (from BCA assay) and expressed as a percent relative to apoA-I control displacement values, derived from the same experiment.

2.2.7 Non-denaturing Electrophoresis and HL Immunoblotting

Isolated HDL samples were diluted with Ham's F12 media to a protein concentration of 150μg/mL. The samples were then diluted 1:1 with non-denaturing gradient gel tris/glycine sample buffer. The samples were electrophoresed on a 4-20% non-denaturing gradient gel (Invitrogen) under non-denaturing conditions for 19 hours at 100V. The gel was incubated in 0.1% SDS solution for 15 minutes to ensure the proteins had enough negative charge to transfer unidirectionally toward the anode. The proteins were transferred onto a PVDF membrane (Biorad) at 125V for 4 hours and immunoblotted. The membrane was blocked with 1% BSA and probed using the monoclonal anti-human HL XHL3-6a antibody from mouse (1:5000). A goat anti-mouse IgG HRP-linked whole antibody (1:20,000) was used as the secondary antibody (KPL). The blots were washed in between each step with TBST. The membrane was visualized by chemiluminescence following a 5 minute incubation with the Pierce Super Signal West Dura substrate. The molecular weight and the densitometry profiles were generated using AlphaEase© software.

2.2.8 Quantification of ApoE in HDL Samples

The HDL samples were diluted in serum free F12 media to a protein concentration of 150 μg/mL. The diluted HDL samples were combined in a 1:1 ratio with Laemmli sample buffer (Biorad) containing 5% β-mercaptoethanol. The samples were electrophoresed on a 12% polyacrylamide gel under denaturing conditions. The proteins were transferred onto

PVDF membrane (Biorad), blocked with 1% BSA and probed using a 1:2000 dilution of both mouse anti apoE monoclonal primary antibodies 3H1 and 6C5 and a 1:20,000 dilution of the HRP-linked goat anti-mouse IgG secondary antibody (KPL) in 1% BSA/TBST. The blots were washed with TBST between each step. The blots were developed using the West Dura Sensitivity Substrate (Pierce) on the Fluorchem AlphaImager. The band intensities were analyzed with spot-densitometry using the AlphaEase© software.

2.2.9 Determination of HDL in the Media Before and After HL Displacement

In this study, apoA-I concentration was used as a measure of HDL mass. An apoA-I ELISA was used to measure the amount of HDL in the media prior to its use as treatment in the HL displacement experiment. HDL mass of the media after the displacement was also measured. Briefly, the ELISA was performed on a 96-well plate (Nalge Nunc International, Rochester NY) that had been coated overnight with the mouse anti-human apoA-I capture antibody (1:1000) (Cedarlane, Hornby, ON) in PBS. Samples and standards were incubated in the wells for 2 h. The plate was then washed in PBST followed by an hour incubation with a horseradish peroxidase-linked goat anti-human apoA-I antibody (1:1000) (Cedarlane) in PBS. 100μL of K-blue MAX TMB substrate (Cedarlane) was added to each well and allowed to incubate for 30min. The reaction was then stopped using 50 μL of 1N HCL. The absorbance was recorded at 450nm.

2.2.10 ApoA-II and ApoC-III ELISA

ApoA-II and apoC-III protein content from each isolated HDL sample was analyzed by ELISA on a 96-well plate. Briefly, the Nunc Immuno-maxisorp 96-well plates (Nalge Nunc

International) were coated with a mouse anti-human apoA-II or mouse anti-human apoC-III polyclonal antibody in PBS (10μg/mL) (Biodesign, Saco, ME) and incubated at 37°C for 3 hours. The plate was then stored overnight at 4°C. Samples and standards were incubated in the wells for 2 h at 37°C, followed by a 2 hour incubation with a biotin conjugated goat anti-human apoA-II or apoC-III antibody (Biodesign) at 37°C. The plate was then incubated for 1h with avidin alkaline phosphatase (Sigma-Aldrich). Alkaline phosphatase yellow liquid substrate (Sigma-Aldrich) was added to the plate and the absorbance was recorded at 415nm. The plate was washed with PBST after each step in the procedure.

2.2.11 Study Population

The study was approved by the Human Research Ethics Board of the University of Ottawa Heart Institute. The normolipidemic cohort consisted of healthy men (n = 9) and women (n = 10) with fasted cholesterol levels between 3.5mmol/L and 5.2mmol/L and TG levels below 2.0 mmol/L. The combined hyperlipidemic cohort (n=8) consisted of patients with fasted cholesterol levels above 5.2 mmol/L and TG levels above 2.0 mmol/L. The hypercholesterolemic cohort (n=14) consisted of patients with fasted cholesterol levels above 5.2mmol/L and TG levels below 2.0 mmol/L. No patients were on any medication that would alter their lipid profiles at the time of the study.

2.2.12 Statistical Analysis

Values are shown as mean ± SD and $P<0.05$ was considered significant. Differences between mean values were evaluated using a one-way analysis of variance (ANOVA) and the Student-Newman-Keuls Method (SNK) (SigmaStat; Systat Software, Inc., SanJose, CA).

Chapter 3 - Results

3.1 HL Displacement by HDL and ApoA-I

Experiments have shown that apoA-I and heparin affect the cell surface association of HL with CHO-hHL cells, resulting in the release of HL into the media (Figure 3.1.1). HDL also has the same ability to release HL (Figure 3.1.2). Normally, very little HL is released into the media when the cells are cultured in FBS-free medium. When HDL or apoA-I are added to the medium, HL is readily displaced, similar to treatment with heparin. Maximal HL displacement occurs with 200 IU of heparin/ml or 150 μg of HDL or apoA-I protein/ml of media (Figures 3.1.1 and 3.1.2). The difference between the amount of HL displaced by heparin and that of apoA-I was not significant. HDL displaces significantly less HL than both apoA-I and heparin. Interactions between the apoprotein and lipid constituents of HDL and HL may be responsible for the decrease in HL displacement observed.

3.2 Postprandial Lipemia Affects HL Displacement

To determine the effect of a postprandial response on HL displacement, a subject was given an 1800 calorie, high fat meal and blood samples were drawn after fasting and 3, 5, and 7 hours postprandial. Displacement experiments were performed using the obtained blood samples. CHO-hHL cells were treated with ultracentrifugally isolated HDL (150 μg HDL protein/ml). Displacement is presented relative to control apoA-I displacement values. The highest level of HL displacement by HDL was obtained from HDL isolated 5 hours postprandial although the difference in HL displacement over time does not appear to be

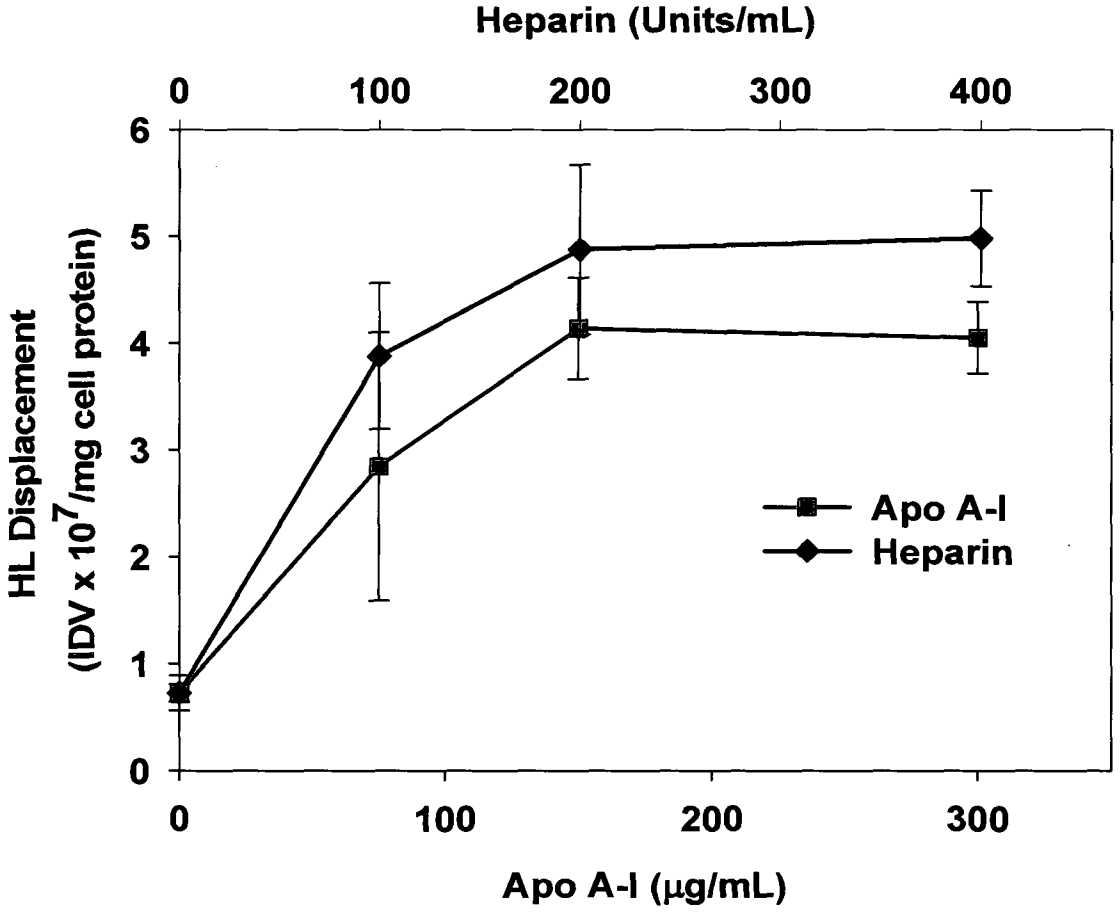


Figure 3.1.1: ApoA-I displaces HL similar to heparin. CHO-hHL cells were cultured in 6-well plates and treated with increasing concentrations of heparin and apoA-I. The medium was collected 45 minutes later and probed using Western blot analysis and densitometry to determine the amount of HL liberated from the surface. All values are the mean of triplicate determinations ± SD and are representative of three displacement experiments.

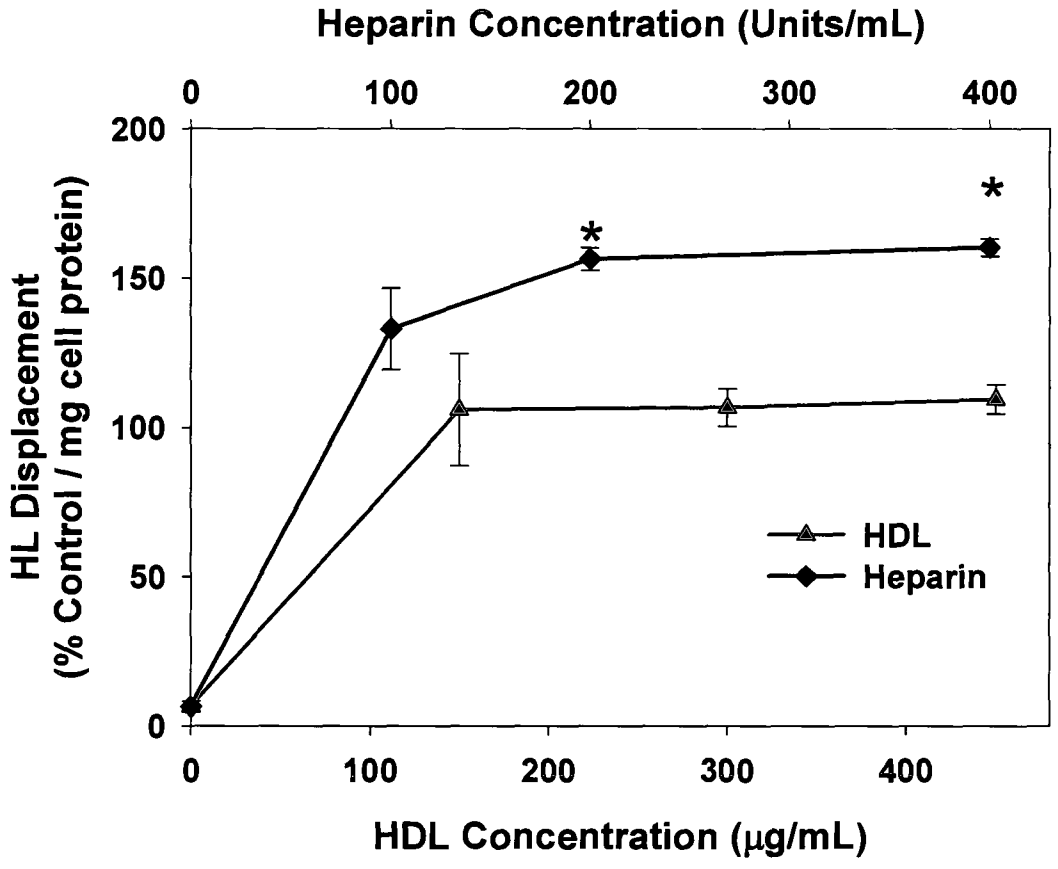


Figure 3.1.2: HDL displaces HL similar to heparin. CHO-hHL cells were cultured in 6-well plates and treated with increasing concentrations of heparin and HDL. The medium was collected 45 minutes later and probed using Western blot analysis and densitometry to determine the amount of HL liberated from the surface. All values are expressed as a percent of the apoA-I control and are the mean of triplicate determinations ± SD. Data are representative of three displacement experiments. Significant difference relative to HDL *$p<0.05$.

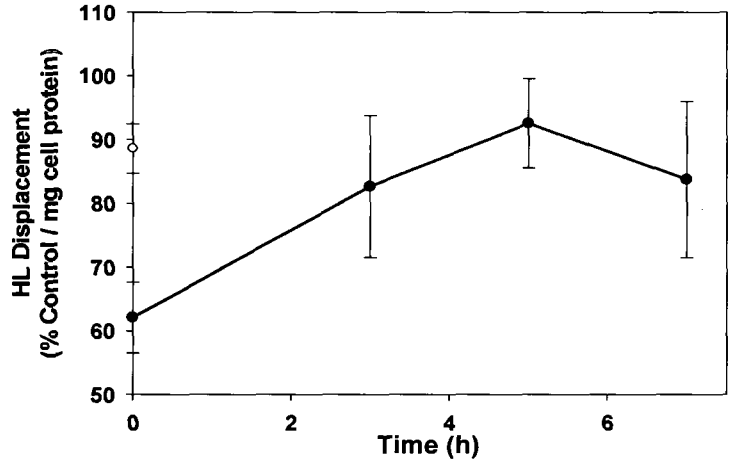
significant (Figure 3.2.1A). TG levels were also measured during the postprandial response and they reached their peak value at 3 hours postprandial (Figure 3.2.1B). Subjects (n=3) were given the same high fat meal (1800 calories) and blood samples were drawn. Displacement experiments were performed with fasted blood samples and with samples drawn 4h after the meal. CHO-hHL cells were then treated with serum or ultracentrifugally isolated HDL (150 μg/ml). HDL isolates contained small amounts of endogenous HL and HDL isolated from the postprandial blood samples contained higher levels of endogenous, HDL-bound HL (~2-fold), than fasted HDL (Figure 3.2.2A). The amount of HL present in the HDL isolates was generally ~10% of the amount in the media after incubation of HDL with the CHO-hHL cells (Figure 3.2.2A). In Figure 3.2.2B, displacement is presented relative to control apoA-I displacement values. HDL isolated at the peak of a postprandial response displaces ~50% more HL than fasting HDL samples. Serum samples showed a similar magnitude of displacement and postprandial stimulation of HL displacement. The amount of apoE in fasted and postprandial HDL samples was quantified using western blot analysis and densitometry and is shown in Figure 3.2.3. The results showed that HDL-apoE content decreased during a postprandial response.

3.3 ApoE Influences HL displacement

3.3.1 ApoE Removal from Native HDL Increases HL Displacement

To determine if the apoE concentration of HDL influences HL displacement, experiments were performed with both native and synthetic HDL preparations containing variable apoE levels. For native HDL studies, the ρ = 1.065-1.25 g/mL fraction was

A.



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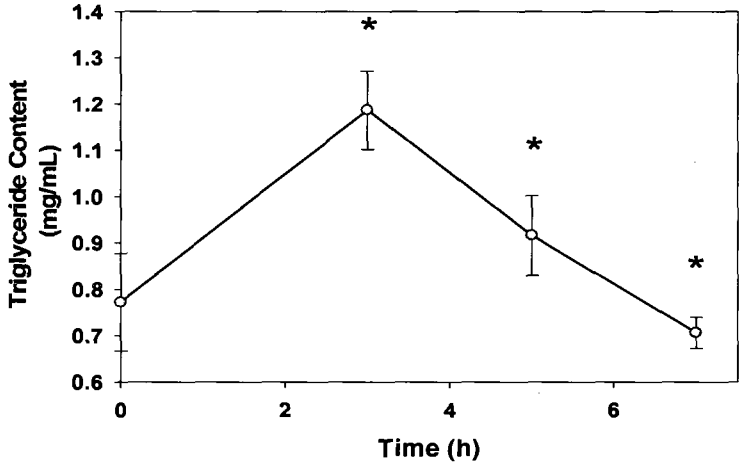
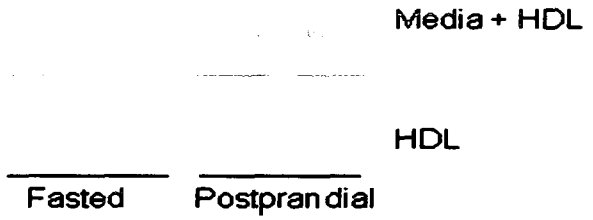


Figure 3.2.1: A postprandial response affects HL displacement. A single normolipidemic subject was fed a high calorie meal (1800 cal) and blood samples were obtained after fasting and 3, 5 and 7 hours after the meal. The HDL was isolated using sequential ultracentrifugation. (Panel A) The HDL was used to treat CHO-hHL cells, cultured in 6-well plates, at a concentration of 150 μg/mL. After 45 minutes the medium was collected from the cells and probed for HL Western blot analysis and densitometry. All values are the mean of triplicate determinations ± SD. (Panel B) The triglyceride levels of the isolated HDL were measured using a colorimetric TG assay to determine the timing of the postprandial peak during the response. Signifiant difference compared to previous time points *$p<0.05$. All of the values are the mean of triplicate determinations ± SD.

A.



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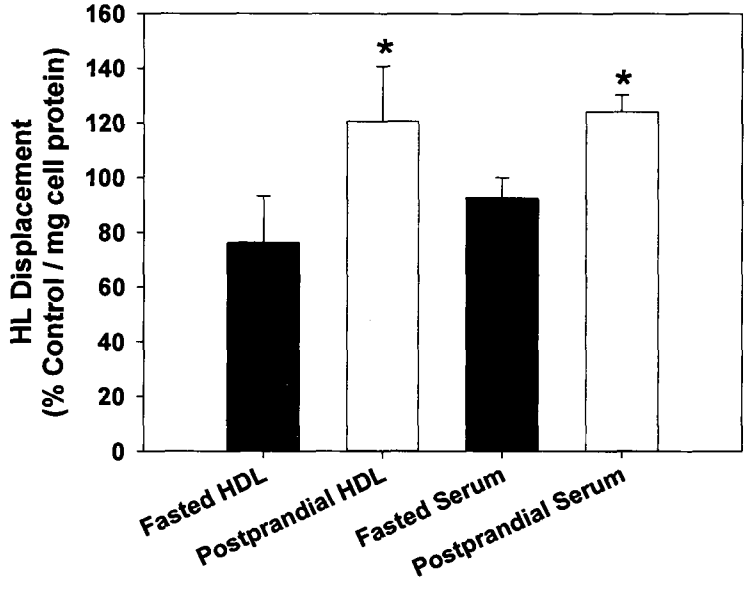


Figure 3.2.2: Postprandial lipemia affects HL displacement by HDL. CHO-hHL cells were seeded in 6-well plates and treated with serum and HDL isolated from normolipidemic subjects (n=3) for 45 minutes. (Panel A) Representative HL Western blot images are shown for fasted and postprandial HDL samples and CHO-hHL media samples, after incubation with HDL. (Panel B) Averaged values are shown for serum and HDL samples obtained from 3 subjects after fasting and 4h postprandial, following an isocaloric meal. HL displacement from the cell surface was analyzed using Western blots and densitometry. All values were calculated as a percentage of the HL displaced by the apoA-I control and were normalized to total cell protein. Values are the mean ± SD of triplicate determinations from one assay and represent three displacement experiments. Significance of difference relative to fasted HDL *$p<0.01$.

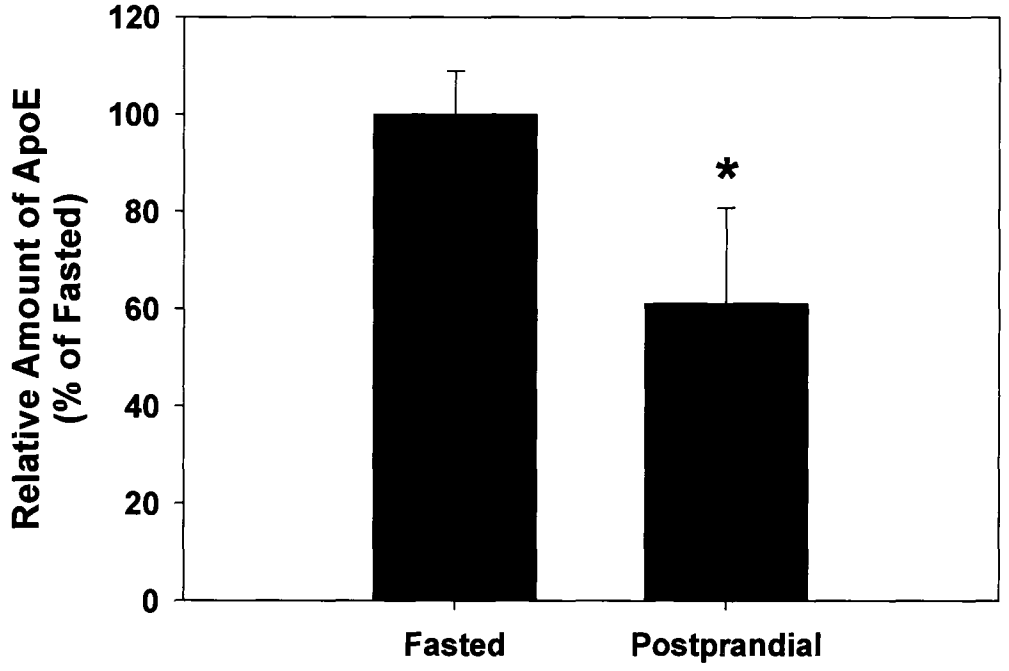


Figure 3.2.3: Postprandial HDL contains less apoE than fasted HDL. HDL samples isolated after fasting and 4 hours postprandial were probed for apoE using Western blot analysis and densitometry. The HDL samples had previously been used in displacement experiments with CHO-hHL cells (Figure 3.2.3). All values are the mean of triplicate determinations from three separate subjects $\pm$ SD. Statistical significance compared to fasted *$p < 0.05$.

obtained from fasted normolipidemic subjects (n=3) and then dialyzed. HDL was enriched in TG and depleted in apoE by incubating the ρ >1.065 g/mL with Intralipid (20% TG emulsion, Sigma), at a ratio of 1:2 by volume (ρ>1.065:Intralipid), for 3h at 37°C. HDL was then isolated by sequential ultracentrifugation. HDL-TG content was increased by 5-fold. TG enrichment of native HDL stimulates HL displacement (Figure 3.3.1A). This stimulation is associated with a significant reduction in HDL-apoE content in the TG-enriched HDL (Figure 3.3.1B).

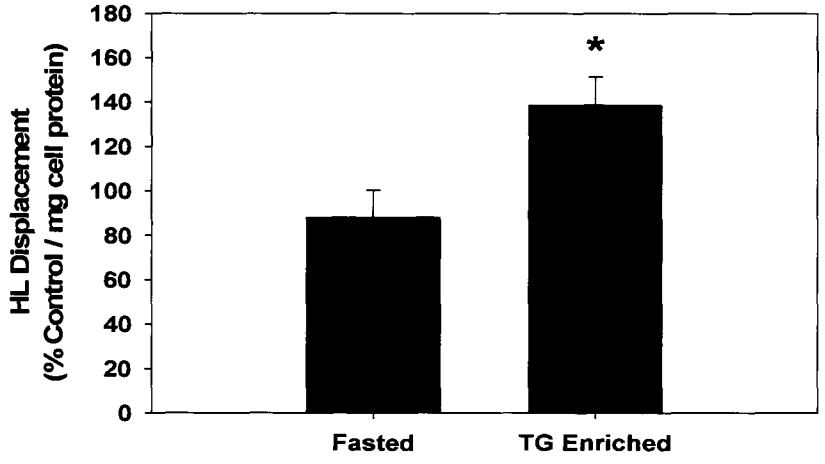
3.3.2 ApoE in Reconstituted Particles Decreases HL Displacement

Reconstituted HDL (rHDL) particles were synthesized using POPC and varying amounts of apoA-I and apoE. These particles were then used to treat CHO-hHL cells and the amount of HL displaced was measured. Inclusion of apoE into the rHDL complexes decreased HL displacement. Particles with 1.3:0.4 and 1:1 apoA-I:apoE (mol:mol) displaced 50% and 60% less HL than the control rHDL containing only apoA-I (Figure 3.3.2).

3.3.3 Antibodies to Amino Acids 1-15 in the N-terminus of ApoE Influence HL Displacement

Antibodies to apoE also affected the ability of HDL to displace HL. HDL was incubated with an anti-apoE antibody (450μg/mL) for 2 hours prior to its use in a displacement experiment. Three different antibodies were tested, which were specific for three distinct apoE epitopes; 6C5 (aa 1-15), 1D7 (aa 140-160), and 3H1 (aa 243-272). 1D7 and 3H1 had no significant affect on HL displacement; however, when HDL was incubated with 6C5, HL displacement increased by ~85% (Figure 3.3.3).

A.



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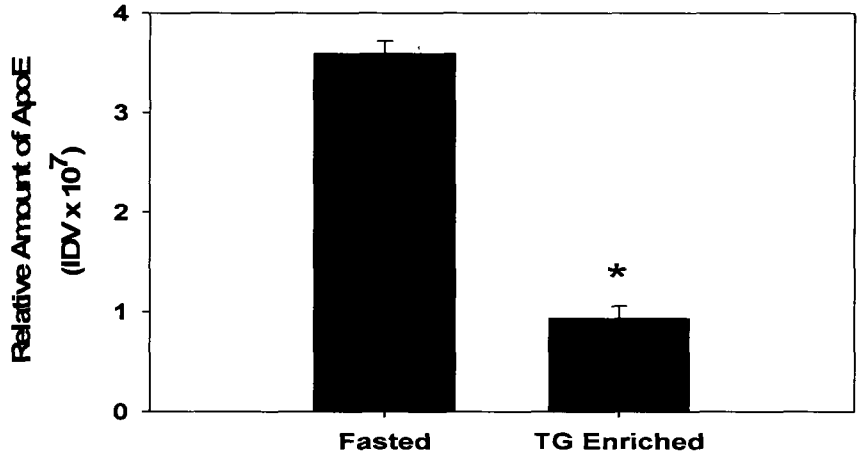


Figure 3.3.1: The effect of enriching HDL with TG on HL displacement. CHO-hHL cells were seeded in 6-well plates and treated with HDL for 45 minutes. Values are the mean ± SD of triplicate determinations from two separate subjects from 3 separate displacement experiments. Significance of difference relative to fasted HDL *$p<0.01$. (Panel A) HDL samples were obtained from fasted normolipidemic subjects and then enriched in TG as described. HL displacement from the cell surface by control and TG-enriched HDL samples was analyzed using Western blots probed for HL and densitometry and is shown relative to HL displacement by apoA-I, normalized to total cell protein. (Panel B) The relative amount of apoE present in control and TG-enriched HDL samples was measured using Western blot analysis.

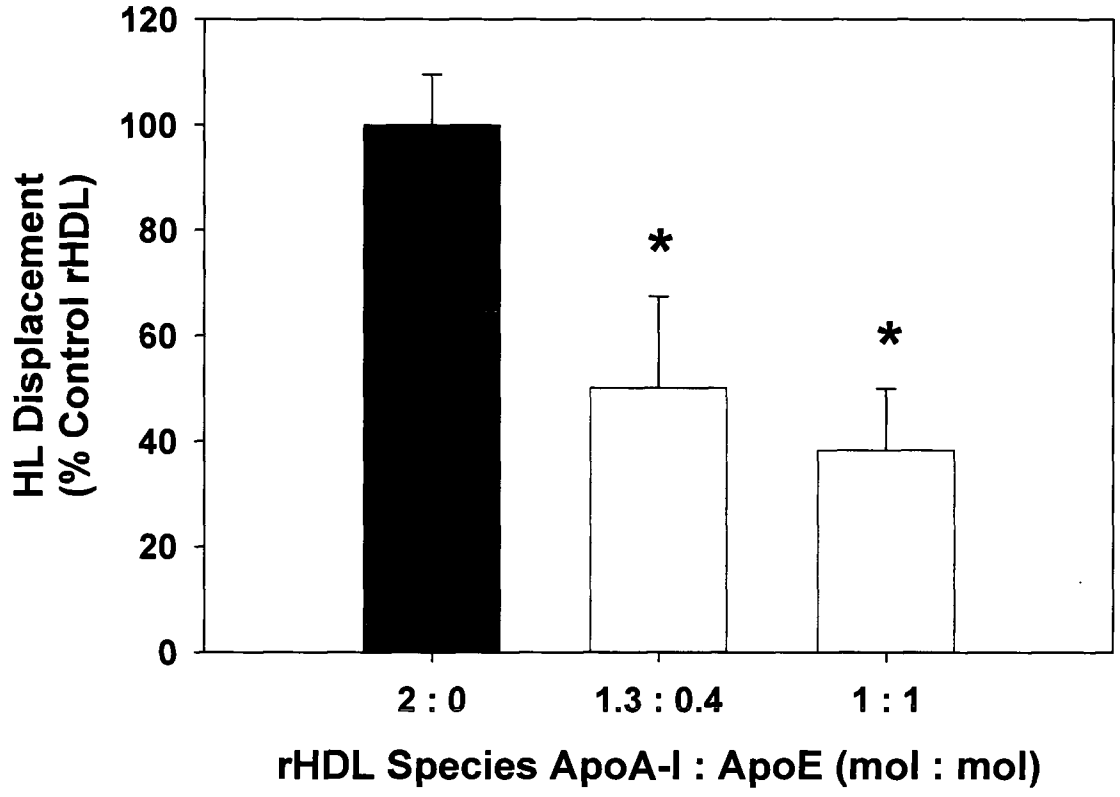


Figure 3.3.2: ApoE inhibits HL displacement by rHDL. Reconstituted HDL particles were prepared using POPC and differing amounts of apoA-I and apoE. CHO-hHL cells were cultured in 12-well plates and treated with the rHDL for 45 minutes. The HL displacement from the cell surface caused by each rHDL species was measured using Western blot analysis and densitometry. The results are shown relative to HL displacement by apoA-I only rHDL, normalized to total cell protein. Values are the mean $\pm$ SD of triplicate determinations and are representative of three separate experiments. Significance relative to control apoA-I rHDL *$p<0.005$.

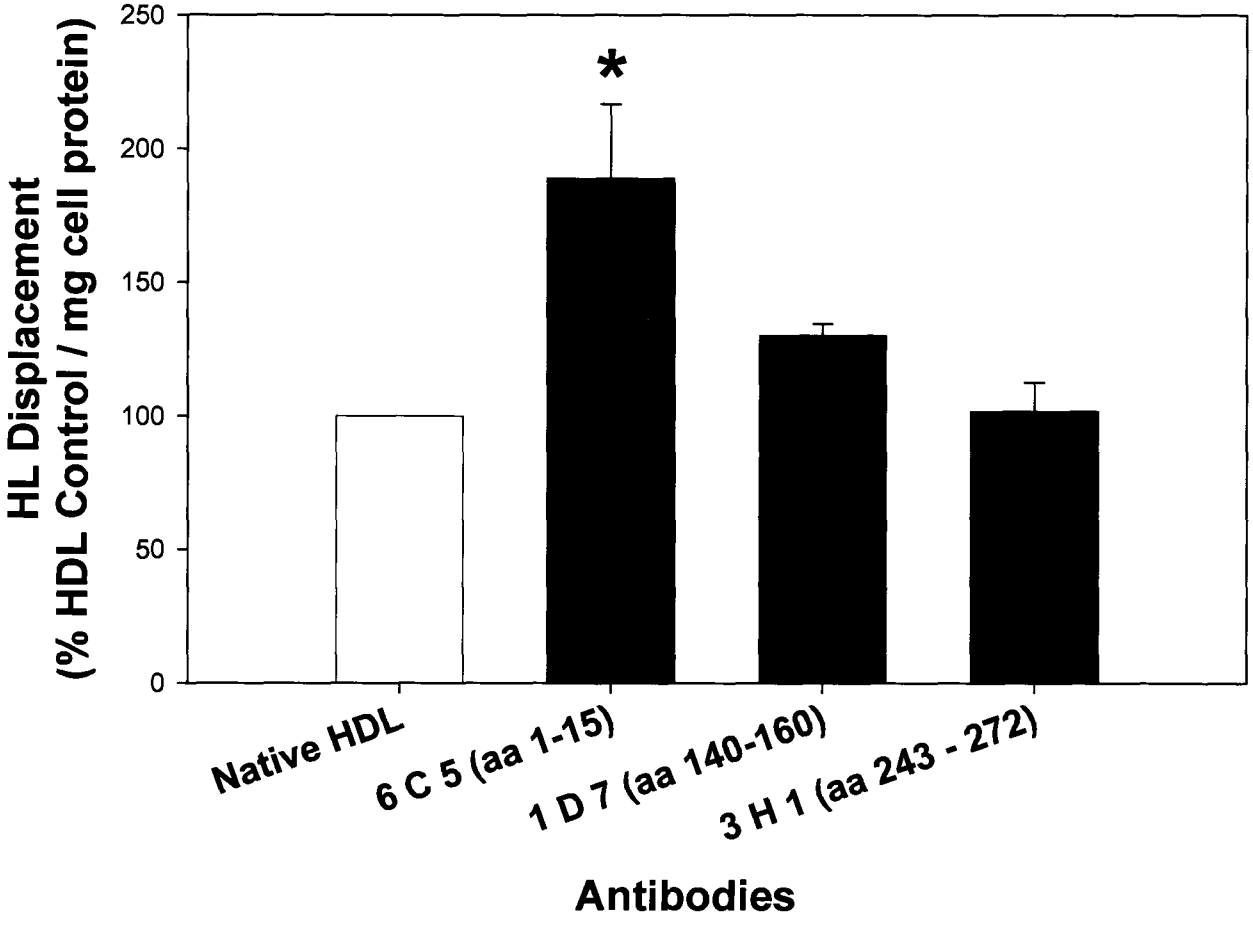


Figure 3.3.3. ApoE antibodies stimulate HL displacement. HDL from 3 different subjects was pooled and incubated with an anti-apoE antibody (6C5, 1D7, or 3H1) for 2 hours in serum free media. CHO-hHL cells were cultured in 6-well plates and treated with HDL ± anti-apoE antibody for 45 minutes. HL displacement from the cell surface was measured by immunochemical analysis and is shown relative to control HDL displacement, normalized to total cell protein. Values are the mean ± SD of triplicate determinations from three displacement experiments. Significance relative to control HDL *$p<0.01$.

3.4 The Effect of Gender on HL Displacement

CHO-hHL cells were treated with increasing concentrations of HDL isolated from male (n=3) and female (n=3) normolipidemic subjects and the media was collected and probed for HL. HDL from both groups reached their maximum displacement at 150 μg protein/mL; however, HDL from women consistently displaced more HL from the CHO-hHL cell surface than male HDL (Figure 3.4.1). Not only does female HDL displace more HL from the surface of CHO-hHL cells, it also contains more endogenous HL than male HDL, as measured by NGGE (Figures 3.4.1 and 3.4.2). HDL from women also contained more HDL_2 compared to HDL from men (Figure 3.4.2). Consistent with what was expected, HL displaced to the media and was associated with multiple different sizes of HDL particles (Figure 3.4.3). ApoA-II, however, was associated with smaller HDL particles in the displacement media samples. ApoE levels in HDL from male and female normolipidemic subjects were quantified immunochemically and shown to be significantly decreased in the female HDL (Figure 3.4.4). There was no significant difference in the cellular uptake of apoA-I (Figure 3.4.5) or apoE (Figure 3.4.6) from male and female HDL samples. To normalize the HDL between subjects for every experiment, each HDL sample was diluted with serum-free medium to a starting concentration of 150μg/mL of total HDL protein prior to analysis.

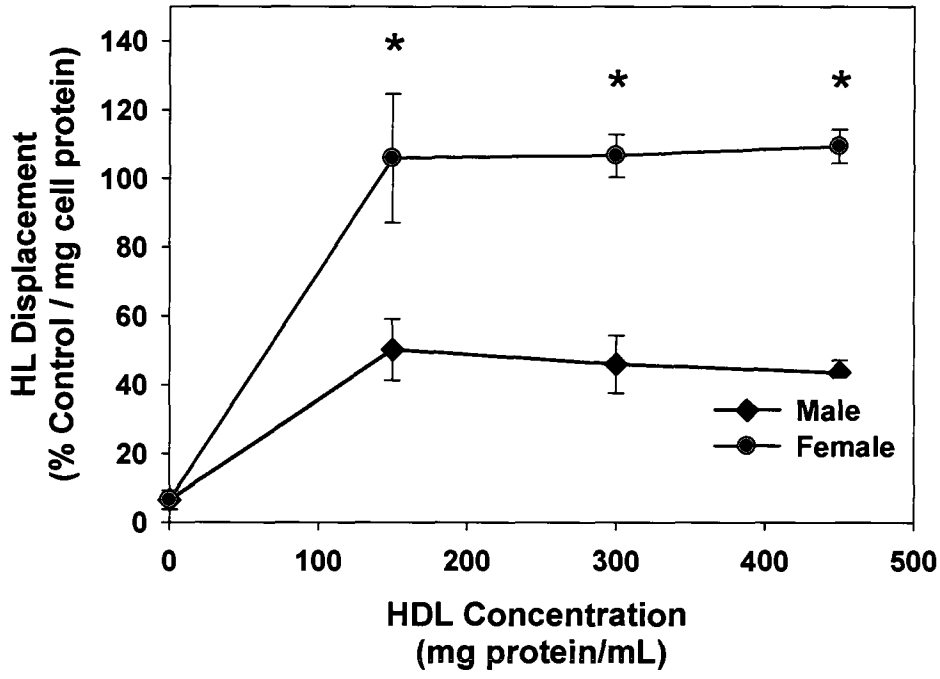


Figure 3.4.1: Gender affects HL displacement. CHO-hHL cells were cultured to confluency and treated with increasing concentrations of HDL from female (n=3) and male (n=3) normolipidemic subjects for 45 minutes. The displacement of HL from the cell surface was measured using Western blot analysis and densitometry. The results are shown relative to apoA-I displacement, normalized to total cell protein. Values are the mean of triplicate determinations ±SD. Significance relative to HL displacement by male HDL *$p<0.05$.

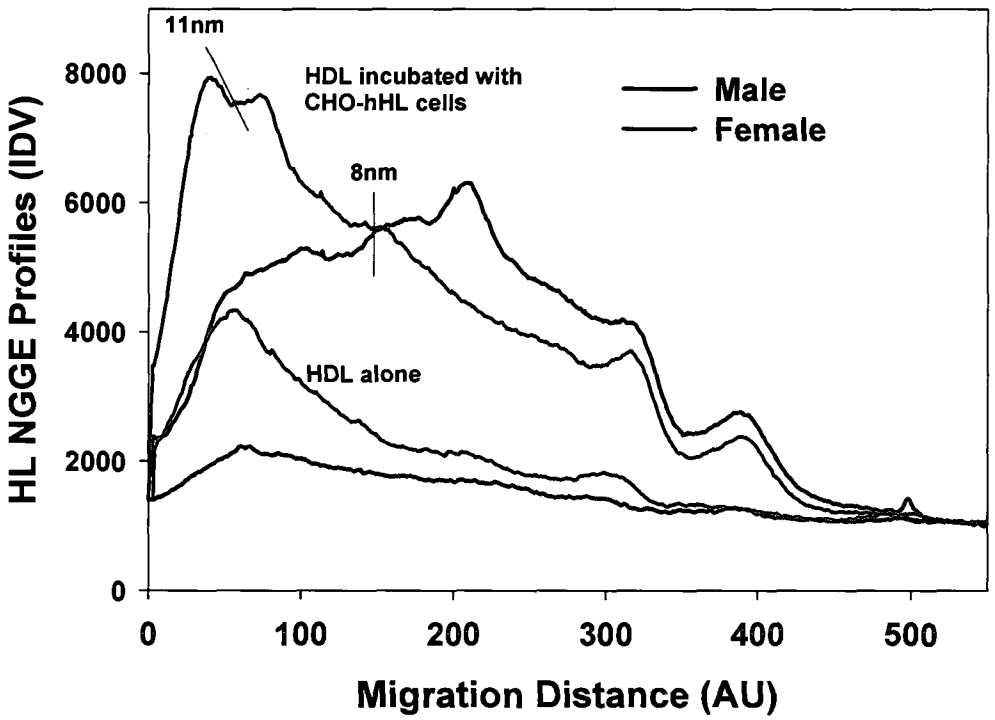


Figure 3.4.2: Gender affects both HL displacement into the media and circulating HDL-bound HL levels. The amount of HL present in media samples from displacement experiments was compared to the amount of HL present in the isolated HDL samples using non-denaturing gradient gel electrophoresis and probed for HL. The HL profiles shown are from one normolipidemic female and one normolipidemic male and are representative of HL profiles seen for other subjects (n=6).

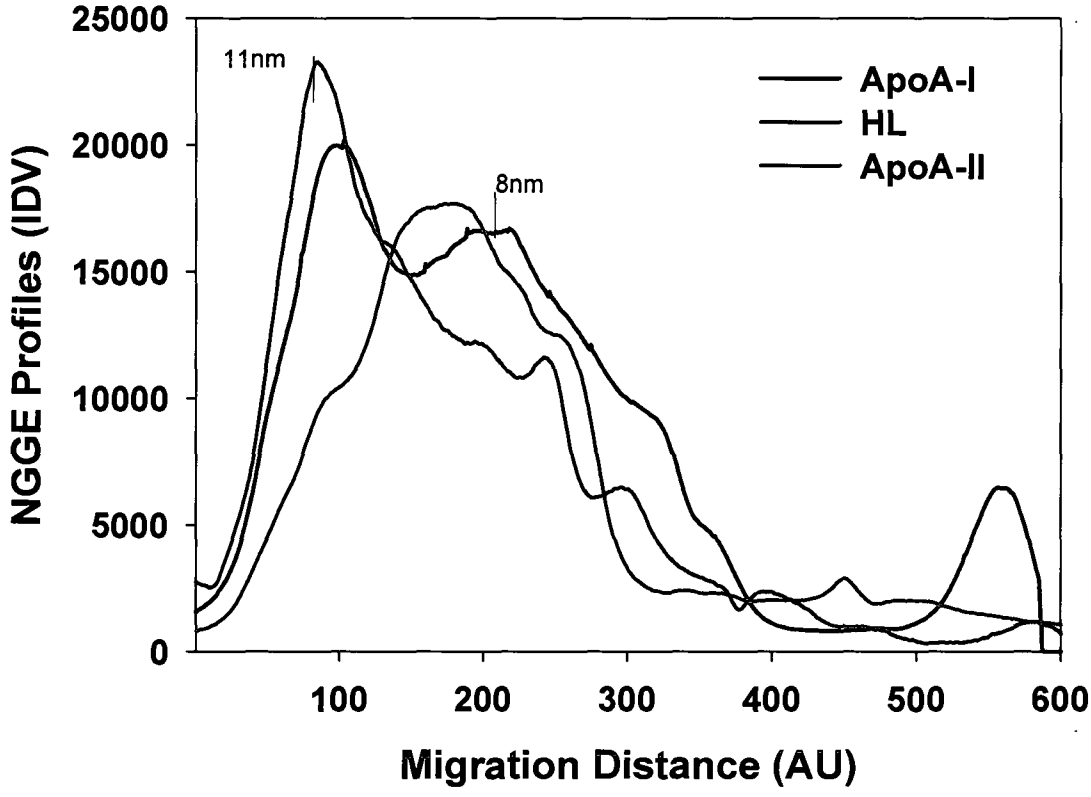


Figure 3.4.3: Both HL and apoA-I are associated with HDL particles. Media obtained from displacement experiments where CHO-hHL cells were treated with human HDL for 45 minutes at a protein concentration of 150 μg/mL, were electrophoresed on a 4-20% polyacrylamide gel under non-denaturing conditions. The gel was then transferred to PVDF and probed by Western blot analysis for apoA-I, apoA-II and HL to determine the association between the three proteins. The profiles shown are from one analysis and are representative of three displacement experiments using HDL from three separate subjects.

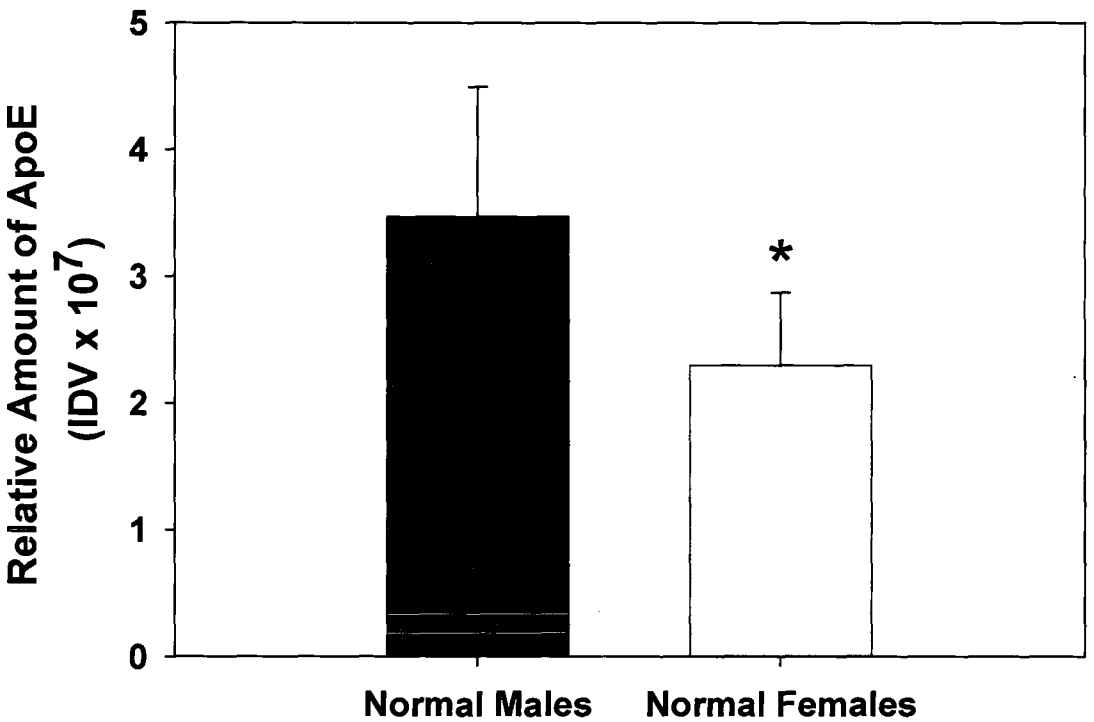


Figure 3.4.4: HDL-apoE levels are elevated in normolipidemic males. The relative amount of apoE present in HDL from fasted normolipidemic males (n=6) and females (n=9) was measured using Western blot analysis and densitometry. Values are the mean of triplicate determinations ± SD. Significance of difference *$p<0.05$.

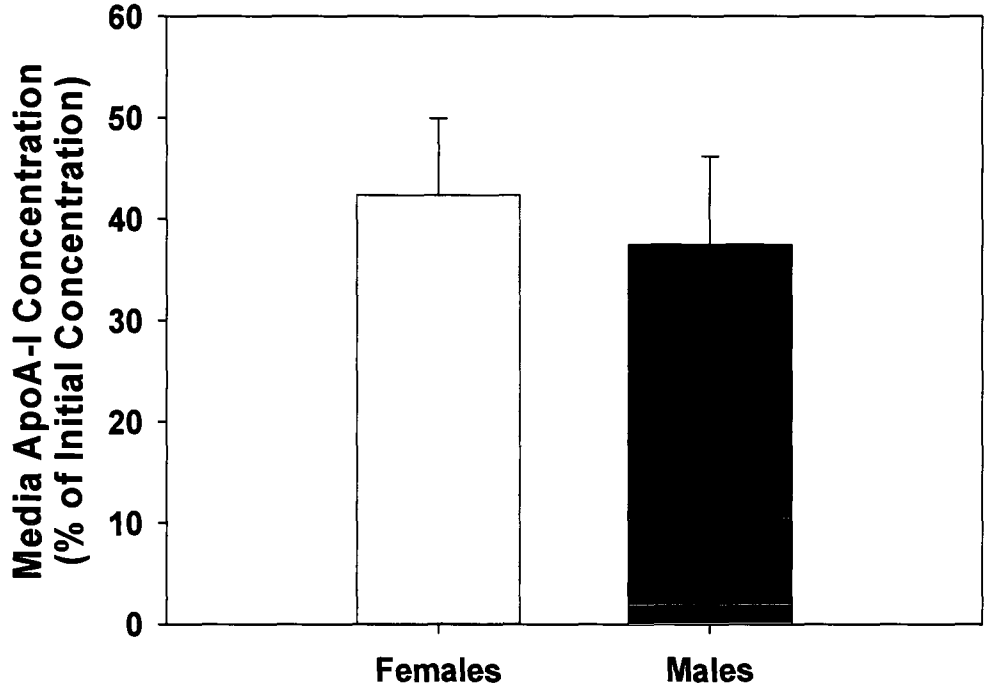


Figure 3.4.5: Cellular HDL-apoA-I uptake is similar for male and female HDL. The amount of apoA-I in the media was measured before and after treatment of CHO-hHL cells with HDL samples. Using an ELISA, the change in the amount of apoA-I present in the media after the displacement experiments was determined and compared for male (n=3) and female (n=3) HDL. The values are the means of triplicate determinations $\pm$ SD.

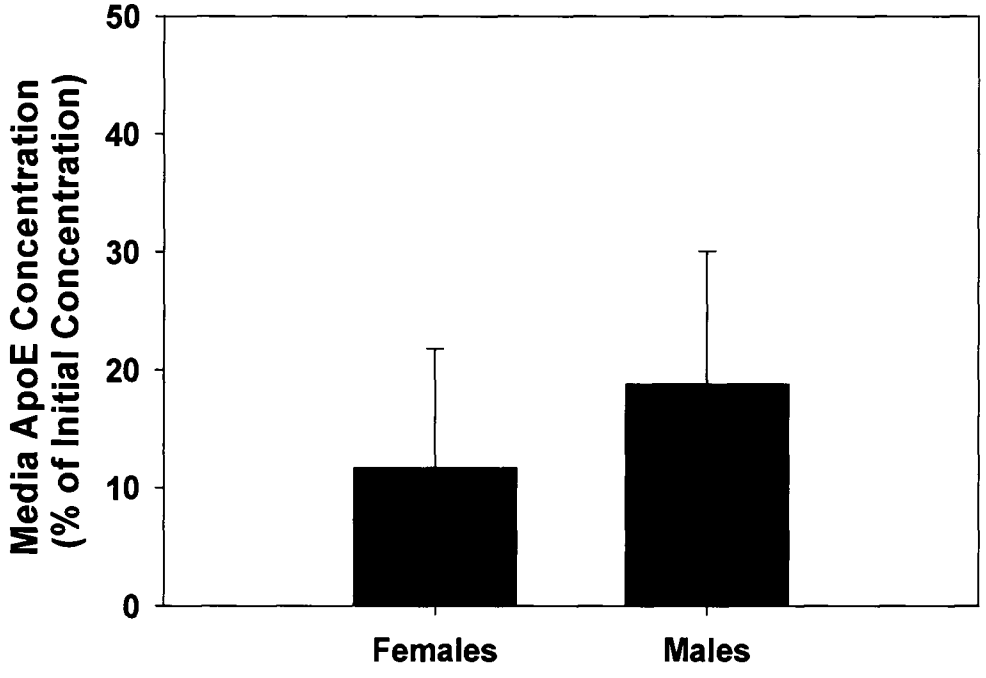


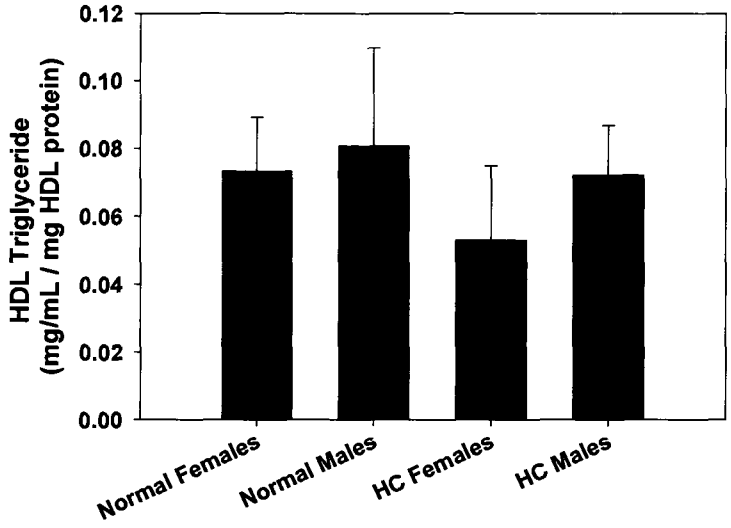
Figure 3.4.6: Cellular HDL-apoE uptake is similar for male and female HDL. The amount of apoE in the media was measured before and after treatment of CHO-hHL cells with HDL samples. The change in the amount of apoE present in the media after the displacement experiments was determined using Western blot analysis and densitometry. The change in apoE present was compared for male (n=3) and female (n=3) HDL. The values are the means of triplicate determinations $\pm$ SD.

3.5 The Effect of Hyperlipidemia on HL Displacement by HDL

3.5.1 Hypercholesterolemia Decreases HL Displacement in Women

HDL was obtained from fasted normolipidemic and dyslipidemic patients. The normolipidemic subjects had fasted cholesterol levels between 3.5 mmol/L and 5.2 mmol/L and TG levels below 2.0 mmol/L. The dyslipidemic patients had cholesterol levels above 5.2 mmol/L. No difference was observed in the TG and cholesterol levels of the HDL from both groups (Figure 3.5.1). The HDL obtained was used in displacement experiments on CHO-hHL cells. These experiments revealed that hyperlipidemia affected the ability of female HDL to displace HL, whereas hyperlipidemia had little effect on HL displacement by HDL from men (Figure 3.5.2). HDL from normolipidemic and hyperlipidemic men displaced, on average, ~60% that of control incubations (Figure 3.5.2B). HDL from normolipidemic females displaced ~100% that of control, while HDL from female hypercholesterolemic patients showed a 50% decrease in HL displacement (Figure 3.5.2A). HDL from combined hyperlipidemic females also showed a trend towards a reduction in HL displacement. HDL from normolipidemic females showed a slight, but not significant, decrease in HDL-apoE content compared to their hyperlipidemic counterparts (Figure 3.5.3). No differences, however, were seen in apoC-III or apoA-II levels between hyperlipidemic and normolipidemic patients (Figures 3.5.4).

A.



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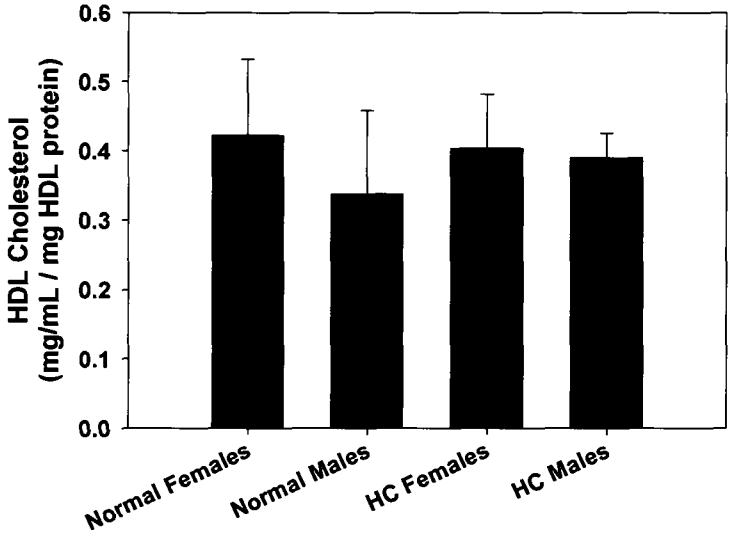
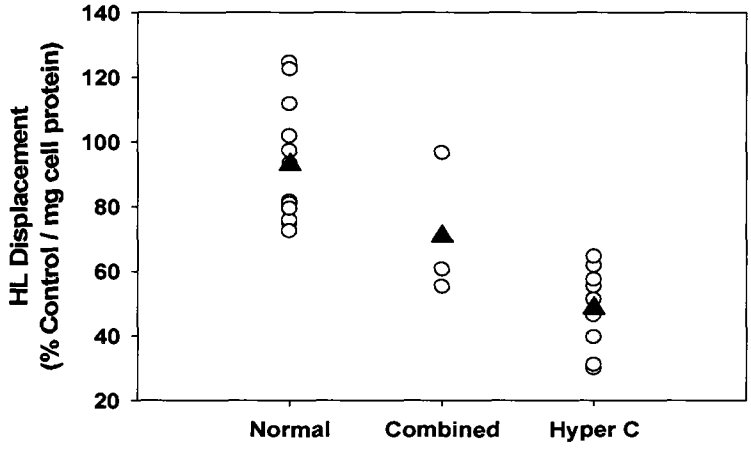


Figure 3.5.1: HDL-TG and cholesterol levels are similar in normolipidemic and hypercholesterolemic subjects. HDL samples were obtained from normal females (n=5), normal males (n=5), hypercholesterolemic (HC) females (n=5) and hypercholesterolemic (HC) males (n=5) and isolated using sequential ultracentrifugation. The HDL samples were analyzed for their TG (Panel A) and cholesterol (Panel B) content using colorimetric assays. All values are the mean of triplicate determinations $\pm$ SD. No statistical significance was observed between groups.

A.



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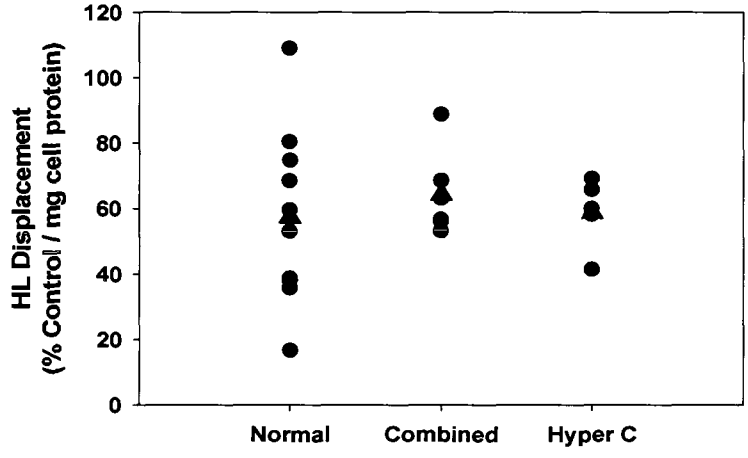


Figure 3.5.2: Dyslipidemia affects HL displacement by HDL. CHO-hHL cells were cultured in 6-well plates and treated with HDL from fasted female (n=10) and male (n=9) normolipidemic subjects as well as HDL from fasted combined hyperlipidemic (n=8) and hypercholesterolemic (n=14) patients for 45 minutes. The displacement of HL from the cell surface was measured using Western blot analysis and densitometry. (Panel A) HL displacement by HDL from female normolipidemic (normal), combined hyperlipidemic (combined) and hypercholesterolemic (hyper C) patients. (Panel B) HL displacement by HDL from male normolipidemic and hyperlipidemic patients. All values were calculated as a percentage of the HL displaced by the apoA-I control and were normalized to total cell protein. The black triangles represent the group mean value and the blue and purple dots show the HL displacement by each subject in the study.

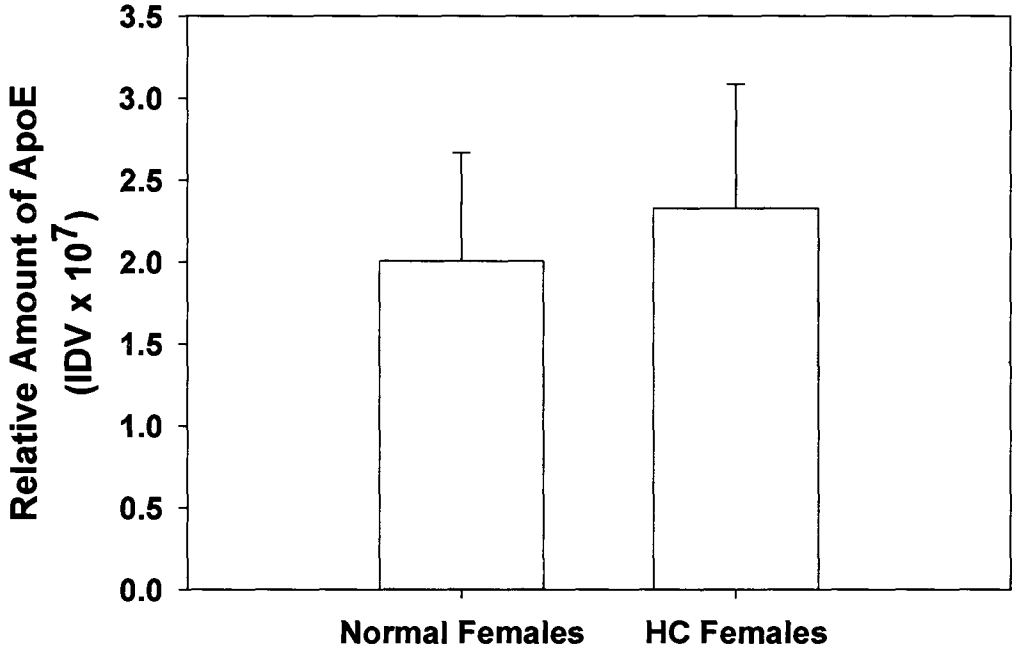
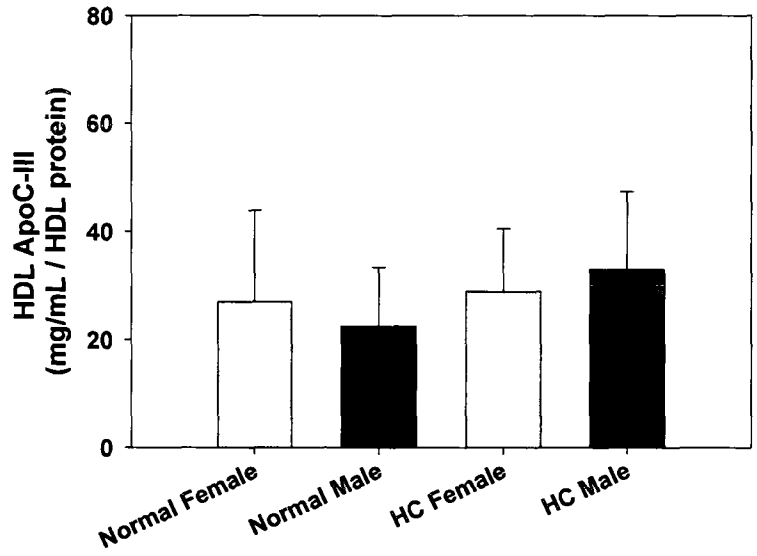


Figure 3.5.3: HDL-apoE levels show a slight, although not significant, trend towards elevation in hypercholesterolemic females compared to normolipidemic females. The relative amount of apoE present in HDL from fasted normolipidemic females (n=3) and hypercholesterolemic females (n=3) was measured using Western blot analysis and densitometry. Values are the mean of triplicate determinations ± SD.

A.



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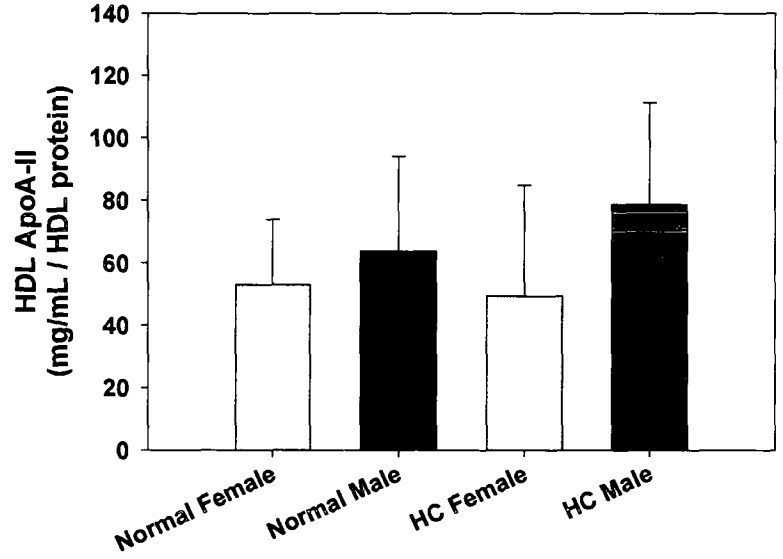
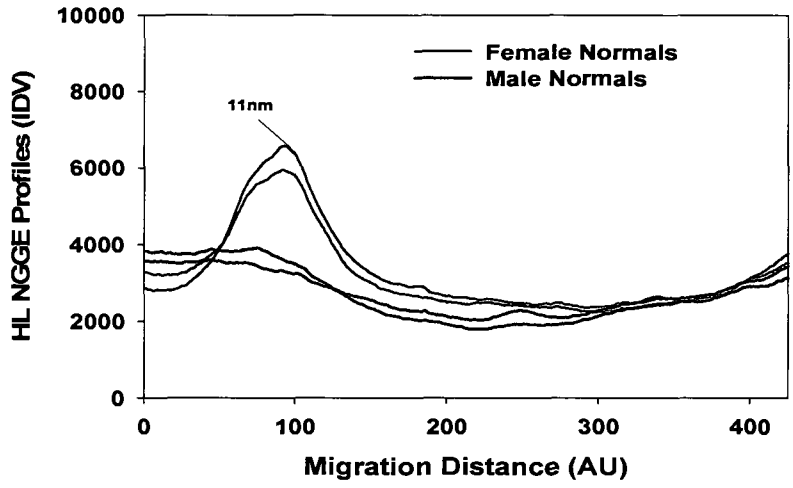


Figure 3.5.4: HDL-apoC-III and HDL-apoA-II levels are similar in normolipidemic and hypercholesterolemic subjects. HDL samples were obtained from normal females (n=5), normal males (n=5), hypercholesterolemic (HC) females (n=5) and hypercholesterolemic (HC) males (n=5) and isolated using sequential ultracentrifugation. The HDL samples were analyzed for their apoC-III (Panel A) and apoA-II (Panel B) content using an ELISA. All values are the mean of triplicate determinations $\pm$ SD. No statistical significance was observed between groups.

3.5.2 Normolipidemic Females Have More HDL-bound HL in their Plasma

Immunoblots of non-denaturing gradient gels were used to determine if HL was associated with isolated HDL samples from normolipidemic and hyperlipidemic patients. As shown in Figure 3.5.5A, female normolipidemic HDL samples contained endogenous HL, which is associated with an 11nm particle similar in size to the HDL_2 class of HDL (Figure 3.5.5A). Only two female profiles are illustrated, but similar profiles were observed for HDL preparations from all other normolipidemic female subjects. Very little HL was detectable in HDL isolated from male or hyperlipidemic subjects (Figures 3.5.5A and 3.5.5B). Figure 3.5.5B shows representative profiles for three female hypercholesterolemic patients. Similar profiles were evident for all other hyperlipidemic patients.

A.



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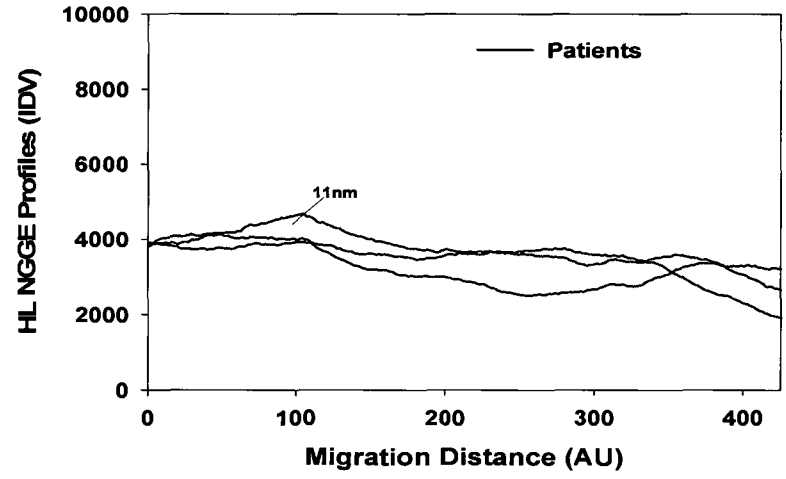

Figure 3.5.5: Hyperlipidemic patients have reduced circulating HDL-bound HL levels. HDL was isolated from blood samples obtained from female normolipidemic, male normolipidemic and female hypercholesterolemic subjects. The HDL samples, isolated using sequential ultracentrifugation, were analyzed using nondenaturing gradient gel electrophoresis and probed for HL. (Panel A) HL densitometry profiles of normolipidemic subjects. Profiles are similar to that observed from experiments with HDL isolated from other normolipidemic subjects (n=6). (Panel B) HL densitometry profiles of female hypercholesterolemic patients. The profiles are from three female patients and are similar to that observed from separate experiments with HDL isolated from other hyperlipidemic male (n=3) and female patients (n=3).

Chapter 4 – Discussion

4.1 Introduction

HL activity is essentially undetectable in fasted plasma and can only be measured after an intravenous injection of heparin. An increased post-heparin HL activity is associated with a pro-atherogenic lipid profile including low HDL levels (*97;192;193*) and small, dense LDL particles (*160;167;194*). *In vitro* experiments have shown that proteoglycan-bound HL is inactive and that the displacement of HL from the cell surface stimulates TG hydrolysis (*126*). An elevated post-heparin activity, therefore, may reflect an increased amount of inactive HL bound to the surface of cells in the liver. Post-heparin HL activity in familial low HDL patients is higher than in normal subjects (*195*). Subjects with high post-heparin HL activity would be expected to exhibit decreased vascular lipolysis and increased plasma TG levels (*196*).

4.2 HL Displacement by HDL and ApoA-I is Similar to Heparin

It has been shown that heparin can displace HL from cell-surface HSPG and that this results in a rapid increase in TG hydrolysis. Ramsamy *et al.* and Rouhani *et al.* also showed that apoA-I and HDL can displace HL from the surface of cells (*162;163*). In this study, it was shown that apoA-I and HDL displace HL similarly to heparin. ApoA-I and HDL reach their maximum HL displacement at a total protein concentration of 150 μg/mL whereas heparin reaches maximum HL displacement at 200 IU/mL (Figures 3.1.1 and 3.1.2).

Not only does HDL displace HL, but the HL remains associated with the HDL after displacement (*163*). Following displacement, HL is bound to HDL particles as shown by

NGGE (Figure 3.4.3). It had previously been shown that HDL_2 has a greater ability to displace cell-surface HL while HDL_3 is poor at liberating HL (*162;163*); however HL was found to be associated with many different sized HDL particles. In the bloodstream, HL seems to mainly be associated with an 11nm HDL particle (Figures 3.4.2 and 3.5.5).

4.3 The Effect of Postprandial Lipemia on HL Displacement by HDL

A postprandial response involves an increase in TG following a meal. TG levels reach their peak approximately 4 hours after the meal. TG levels then decrease back to fasting levels. The magnitude and duration of this response is dependent on the body's ability to hydrolyze and clear the TG. HL and LPL are the enzymes specifically involved in the hydrolysis of TG. An increase in HL and LPL activity would result in an increase in TG clearance during a postprandial response.

ApoE is an exchangeable apolipoprotein that is found on lipoproteins within the HDL density range as well as on the TG-rich lipoproteins. The chylomicrons that are formed following a meal contain apoE. The apoE on the chylomicrons and chylomicron remnants acts as a ligand to bind LDL-R (*71*) and LRP (*86;87*). ApoE mediates the uptake of these TG-rich particles by LDL-R and LRP through its ability to bind to both receptors. During a postprandial response, apoE is shuttled to VLDL and chylomicron remnants (*85*) from HDL. Once the response is over, the apoE is returned to the HDL pool. The more apoE shuttled to chylomicron remnants during a postprandial response, the faster the TG-rich lipoproteins can be cleared from the bloodstream and the shorter the duration of the postprandial peak. Data from this study suggests that the loss of apoE from HDL, during a

postprandial response, can enhance HL displacement by HDL. A greater HL displacement would result in an increase in TG hydrolysis.

Following an 1800-calorie meal, a 40% decrease in HDL-apoE content was observed in normolipidemic subjects (Figure 3.2.3). This decrease corresponded to an almost 50% increase in HL displacement from the surface of CHO-hHL cells (Figure 3.2.2B). Increased HL displacement during a postprandial response therefore may be due to a change in HDL-apoE composition. Postprandial HDL samples also appeared to contain more endogenous HDL-associated HL (Figure 3.2.2A).

4.4 ApoE Affects the Cell Surface Displacement of HL by HDL

Both the lipid and apoprotein components of HDL have been shown to affect its HL displacement ability (*163*). Previous work has demonstrated that HDL apoproteins directly affect HL displacement. ApoA-II has been shown to directly increase the affinity of HL for HDL (*164*) and to increase HL displacement (*163*). ApoA-II has also been shown to inhibit HL activity and to alter the conformation of apoA-I, which may increase the affinity of HL for apoA-I (*164*). ApoC-I can also increase HL displacement by HDL (*163*). Some component in isolated HDL apoproteins was shown to inhibit HL displacement, but the apoprotein was not identified (*163*). Evidence from the present study suggests that this inhibitor is apoE.

To determine the effect of lipid enrichment on HL displacement, Rouhani *et al.* enriched native and reconstituted HDL particles with various lipids and characterized HL displacement. Enrichment with free fatty acids and cholesteryl esters had minimal effects on

HL displacement; however, PL and TG enrichment of reconstituted HDL blocked HL displacement (*163*). TG enrichment of native HDL, however, did not show similar results. When native HDL was enriched with TG, through incubation with Intralipid, an increase in HL displacement was observed (Figure 3.3.1A). Since TG itself has an opposite effect on HL displacement, increased displacement must have been due to some other factor.

The present work suggests that a decrease in HDL-apoE, that occurs when the native HDL is enriched with TG, overcomes the inhibitory effects of TG and stimulates HL displacement. TG-enrichment of native HDL samples had exactly the same effect as a postprandial response. Native HDL enriched 5-fold with TG showed a 3.5-fold decrease in the amount of HDL-apoE (Figure 3.3.1). This compositional change stimulated HL displacement 1.5-fold. To directly evaluate the effect of apoE content on HL displacement, reconstituted particles were created to contain differing amounts of apoE and apoA-I. Increasing apoE content in the synthetic HDL particles significantly decreased HL displacement, by >50% in the complex that was half apoE and half apoA-I (Figure 3.3.2). This data showed that HDL-apoE content directly inhibits HL displacement.

Incubating HDL with the anti-apoE 6C5 antibody resulted in an 85% increase in HL displacement. The antibody 6C5 recognizes an epitope in the N-terminus of apoE consisting of the first 15 amino acids. This epitope is enriched in anionic residues, ie. glutamic acid. Blocking this negatively charged region of apoE appears to inhibit the ability of apoE to block the displacement of HL by HDL. This may suggest that HDL charge can affect the association of HL with the lipoprotein and is consistent with earlier work by *Boucher et al.*, which showed that anionic lipids can decrease HL association with HDL and activate the

enzyme (*148*). In contrast, apoE did not appear to affect HL displacement through its impact on apoE receptor pathways. The monoclonal apoE antibody 1D7 interacts with an epitope specific to amino acids 140-160. This amino acid region is rich in arginine and is known to be responsible for the binding of apoE to LDL-R, LRP, and HSPG (*197;198*). Incubation of HDL with 1D7 had no significant effect on HL displacement.

4.5 The Effect of Gender on the Displacement of HL from the Cell Surface

HDL from normolipidemic females displaced approximately 50% more HL from the cell surface than HDL from male subjects (Figure 3.5.2). HDL from female subjects also contained more endogenous HDL-bound HL than HDL from all other subjects (Figure 3.5.5). NGGE analysis of ultracentrifugally isolated HDL show that HL associates with HDL in the bloodstream in subjects, even without heparin administration. HL has been shown to primarily associate with the larger, 11nm HDL_2 particles when it is displaced from the surface of CHO-hHL cells by HDL (Figures 3.4.3). HDL from normolipidemic females had the highest endogenous HDL-bound HL levels, compared to normolipidemic males and hypercholesterolemic patients (Figures 3.4.2 and 3.5.5) and was the best at displacing HL from CHO-hHL cells. Increased levels of circulating HDL-bound HL in women may partly explain why they usually have lower post-heparin HL activity than men (*199*). Small amounts of HL were shown to be associated with HDL from all patients; however, the lack of HL activity in fasted plasma samples shows that this endogenous HDL-bound HL has no activity in the circulation.

Female normolipidemic subjects in this study were also found to have significantly lower HDL-apoE levels than normolipidemic males. This is consistent with previous

reports, showing that females over the age of 25 have lower plasma apoE levels than males (*200;201*). Reduced HDL-apoE may therefore contribute to the enhanced HL displacement observed for female HDL samples. ApoA-II has previously been shown to increase HL displacement and increased TG to decrease displacement using synthetic HDL particles (*163*); however, no difference was observed in the amount of apoA-II, apoC-III, TG, or cholesterol in the male and female HDL samples.

4.6 Hyperlipidemia has an Effect on HL Displacement by HDL

Hyperlipidemic patients tend to have higher levels of TG as well as increased plasma concentrations of apoE (*89*). Increased TG levels could indicate low levels of LPL and HL activity. Hyperlipidemics have been shown to have high post-heparin HL activities, indicating that they have more inactive HSPG-bound HL compared to normolipidemic subjects (*196*). Also, higher plasma concentrations of apoE, based on previous observations in this study, would be expected to result in less HL displacement.

When comparing HL displacement by HDL from male donors, no difference was observed between normolipidemic and hyperlipidemic subjects. HDL obtained from hypercholesterolemic women, however, showed a 50% decrease in HL displacement ability, when compared to normolipidemic women (Figure 3.5.2A). Hypercholesterolemic females also had less circulating HDL-bound HL in their bloodstream (Figure 3.5.5). This may suggest that less HL is liberated from the liver by the HDL of hypercholesterolemic patients. As mentioned earlier, hypercholesterolemia is commonly associated with elevated post-heparin HL activity (*196*). In contrast to that seen in normolipidemic subjects, there was no significant difference evident in HDL-apoE levels in hyperlipidemic patients. There was

also no difference observed in the HDL-apoA-II, apoC-III, TG or cholesterol levels between the normolipidemic and hyperlipidemic subjects. HDL from female hypercholesterolemic patients showed a slight increase in apoE, compared to the normolipidemic female subjects, but the difference was not significant. Other studies have seen a significant increase in plasma apoE levels in hyperlipidemic patients, as compared to normals (*89;202;203*).

4.7 Mechanism of Action for ApoE Inhibition of HL Displacement

HDL-apoE concentration appears to regulate HL displacement by HDL. Decreases in HDL-apoE content are associated with an increased ability of HDL to displace HL from the cell surface. ApoE may affect HL displacement by regulating the association of HL with HDL, in an opposite manner to that observed with apoA-II (*164*). Both postprandial and female HDL fractions were shown to have reduced apoE levels (Figures 3.2.3 and 3.4.4) and also increased endogenous HL (Figures 3.4.2 and 3.5.5). This appears to confirm that apoE modulates the association of HL with HDL and thereby its ability to bind and displace HL (Figure 4.7.1).

ApoE is thought to bind HDL through hydrophobic interactions and therefore may block HL binding by occupying space on the HDL interface. ApoA-II, however, would also be expected to occupy interfacial regions and yet this apoprotein stimulates HL binding to HDL (*164*). Both HL and apoE contain heparin-binding sites and these domains may play a role in protein-protein interactions on HDL. ApoE has been shown to directly interact with apoA-II (*91;204*) and as such may block apoA-II dependent association of HL with HDL (*164*).

Alternately, apoE may reduce HL release by stimulating an HSPG or receptor-mediated membrane internalization and reducing cell surface HL levels. This may partly explain the lower plateau observed for the male HDL displacement (Figure 3.4.1). Incubations with apoE-rich HDL may cause a reduction in the cellular pool of HL available for displacement. Less HL available to be displaced by male HDL (apoE-rich) would result in a lower maximal HL displacement compared to female HDL (apoE-poor).

ApoE and HL are known to facilitate the binding and uptake of HDL through HSPG and LRP dependent pathways (*205*). ApoE may bind to cell surface HSPG and promote the internalization of HL-enriched membrane domains, lowering the membrane HL available for displacement. This process does not appear to involve the association/uptake of HDL with the CHO-hHL cells, as there was no detectable difference in media HDL levels after incubations with male and female HDL (Figure 3.4.5). Figure 3.4.5 does indicate, however, that some HDL was taken up by the cell. There is approximately a 40% decrease in the amount of apoA-I in the media following a displacement experiment. There is the possibility that apoE may be lost from HDL to membrane HSPG and/or LRP and act to promote HL internalization and reduce membrane HL displacement. There was also no significant difference in the apoE concentration in the media after incubations with male and female HDL. Approximately 20% of the apoE was taken up by the cell during the displacement experiment. Twice as much apoA-I is therefore taken up by the cell compared to the amount of apoE. The increase in apoA-I uptake compared to apoE may suggest that the cell has a greater preference for the uptake of HDL particles that do not contain apoE.

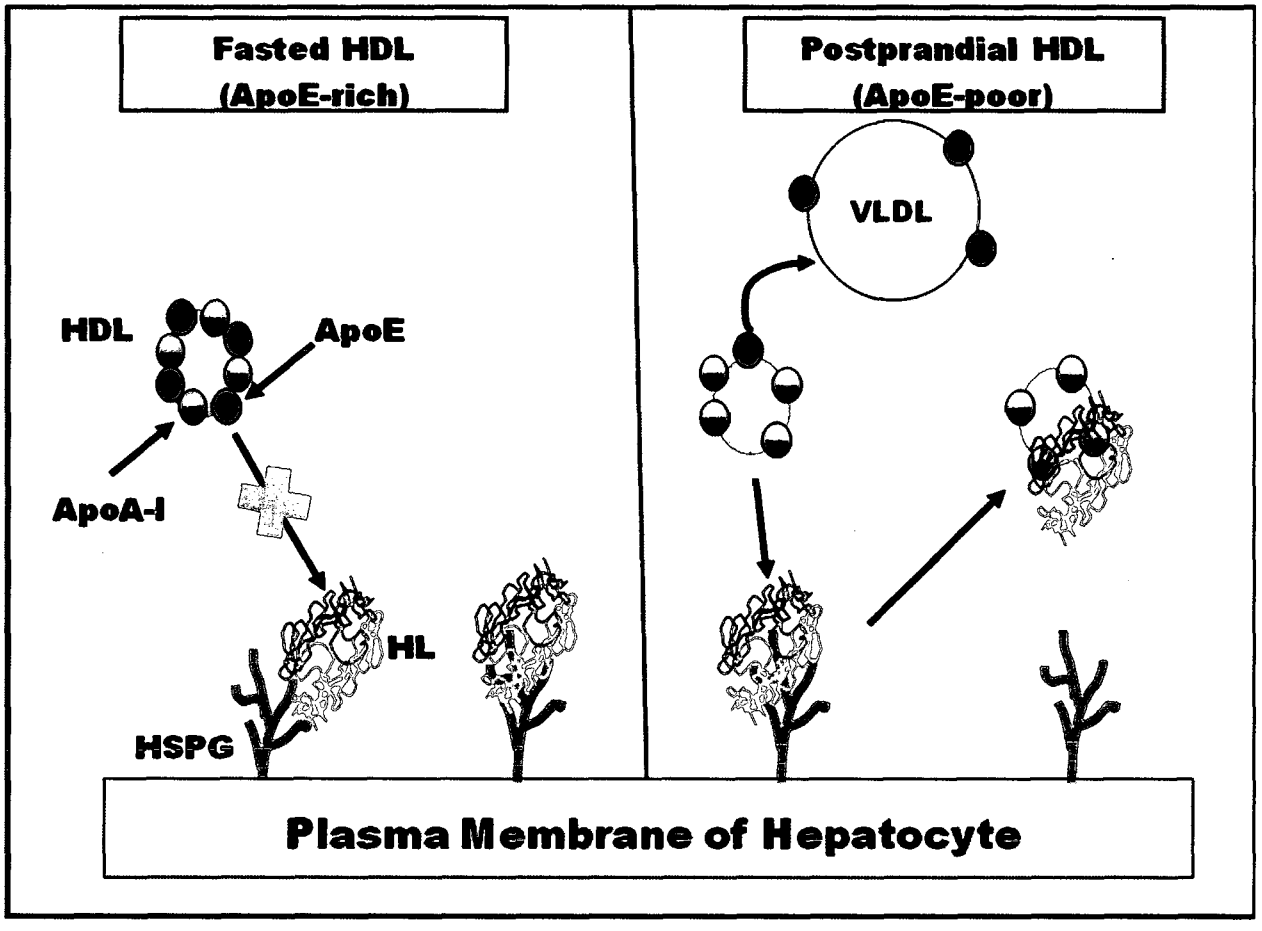

Figure 4.7.1: ApoE inhibits HL displacement by HDL. ApoE-rich HDL is poor at displacing HL from cell surface HSPG. ApoE-poor HDL and HDL that has exchanged apoE with TG-rich lipoproteins are able to displace more HL from the cell surface than apoE-rich HDL.

ApoE isoforms would be expected to impact the internalization of HL and the reduced membrane HL displacement (*206*). While subjects were not all genotyped in this study, HDL from one male subject, with a known apoE 3/2 phenotype, showed an elevated HL displacement, comparable to that seen for females. ApoE2 binds HSPG to a lesser extent than apoE3 and apoE4 (*206*) and as such, may have a lesser effect on the membrane internalization of HL. Displacement experiments using rHDL containing 50% apoE3 and apoA-I showed a 60% decrease in HL displacement compared to the apoA-I only particle. ApoE4 binds to HSPG with a similar affinity to apoE3. When apoE4 is present in rHDL it would be expected to have a similar inhibitory affect to apoE3 on HL displacement. ApoE4, however, has a higher affinity for TG-rich lipoproteins and less apoE4 may be present on native HDL. Less apoE4 on native HDL would result in an increase in HL displacement.

The results from this study indicate that gender, postprandial lipemia, and hyperlipidemia can affect HL displacement. HL displacement by HDL is known to be dependent on HDL composition and these factors may be responsible for altering that composition. One component of HDL, apoE, was shown to inhibit HL displacement. Postprandial HDL and female HDL were both shown to be apoE-poor particles. The low apoE levels in these two types of HDL resulted in an increase in HL displacement compared to fasted and male HDL. Once the HL is displaced from the cell surface it remains bound to the HDL and is inactive. To become active, the HL must be released from the HDL. The release of HL from HDL may be caused by a number of factors. One of these factors may

include an increase in the negative charge in the interfacial environment around the HDL (*148;165*).

4.8 Concluding Remarks and Future Experiments

HDL and apoA-I can displace HL similar to heparin. In this study, it was shown that apoE can inhibit HL displacement by HDL. The shuttling of apoE to TG-rich lipoproteins from HDL during a postprandial response, however, increases HL displacement. Normolipidemic women have less apoE associated with their HDL compared to men. The lower HDL-apoE levels in women results in an increase in HL displacement from the cell surface. Hypercholesterolemia also decreased HL displacement, but only in women. Higher levels of HL were found associated with HDL samples from females and after a postprandial response. The higher level of HDL-bound HL in females and after a postprandial response agrees with the increase in HL displacement seen in the CHO-hHL cell model. Clearly, apoE is inhibiting HL displacement. ApoE, along with other components of HDL, are affecting the liberation of HL by HDL from subjects of different genders, and lipid profiles.

The results from this study have produced many new questions involving HL displacement by HDL. The HDL obtained from hypercholesterolemic females did not have a significantly higher apoE concentration compared to HDL from normolipidemic females. This indicates that other factors may be affecting HL displacement such as other apoproteins and lipids. ApoA-II and apoC-I have been shown to increase HL displacement by HDL (*163*), but the effects of other apoproteins such as apoC-II, apoA-IV, and apoA-V on HL displacement have yet to be tested. It is also crucial to locate the amino acids involved in the apoA-I/HL interaction. Finding the exact location of this interaction would provide a better

understanding of HL displacement by HDL. The location could also provide insight into how the different HDL components are affecting this interaction.

The involvement of apoE in HL displacement creates new questions about how HDL is interacting with the cell surface to liberate HL. ApoE is known to act as a ligand for cell surface receptors. The binding of apoE to HSPG could be affecting the ability of HDL to displace HL. If the binding of apoE to HSPG results in the uptake of HSPG, that would also result in an uptake of HL. This would leave fewer HL particles on the surface of the cell. The uptake of HL caused by apoE-rich HDL would explain why there is a lower plateau for HL displacement by male HDL compared to female. Experiments examining the effect of apoE on the uptake of HL will be performed in the future. Also, the effect of the different apoE phenotypes on HL displacement needs to be examined. Future study participants will be genotyped. To fully understand how HDL is displacing HL, the effect of each HDL component on HL displacement must be understood. Each HDL component could be affecting the affinity of HL for HDL. They could also be interacting with cell surface receptors to deplete the amount of cell-surface HL. Finally, the apoproteins and lipids comprising HDL may be working together to affect the liberation of HL from the cell surface. It is the interactions of all the HDL components together that determine the amount of HL displaced from the surface of liver cells. High post-heparin HL activity is considered a risk factor for heart disease. When more HL is bound to HDL as a result of displacement, however, less HL is bound to the liver, and post-heparin HL activity decreases.

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CURRICULUM VITAE

ELIZABETH YOUNG
Lipoprotein and Atherosclerosis Research Group
University of Ottawa Heart Institute

Education

M.Sc., Biochemistry, ***University of Ottawa,*** University of Ottawa Heart Institute, Ottawa, 2006 – Present (Candidate)

Honours Bachelor of Science Program, ***Wilfrid Laurier University,*** Waterloo, 2002 – 2006
Major: Chemistry with Co-op Option
Minor: Psychology

Ontario Secondary School Diploma, ***Highland Secondary School,*** Dundas, 1997 – 2002

Papers Published

Young, E.K., Chatterjee, C., and Sparks, D.L. (2009) HDL-ApoE Regulates the Cell Surface Displacement of Hepatic Lipase. *American Journal of Pathology* ***(under review).***

Chatterjee, C., **Young, E.K.**, Pussegoda, K.A., Twomey, E.E., Pandey, N.R., and Sparks, D.L. (2009) Hepatic HDL secretion regulates the mobilization of cell-surface hepatic lipase. *Biochemistry* ***(under review).***

Rouhani, N., **Young, E.**, Chatterjee, C., and Sparks, D. L. (2008) HDL Composition Regulates Displacement of Cell Surface-Bound Hepatic Lipase, *Lipids. 43*, 793-804.

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Jelokhani-Niaraki, M., Ivanova, M.V., McIntyre, B.L., Newman, C.L., McSorely, F.R., **Young, E.K.**, and Smith, M.D. (2008) A CD Study of Uncoupling Protein-1 and its Transmembrane and Matrix-Loop Domains, *Biochemical Journal 411(3),* 593-603.

Awards

Natural Sciences and Engineering Research Council Postgraduate Scholarship (PGS-M), for academic achievement, 2007-2008
Ontario Graduate Scholarship, for academic achievement, 2006-2007
Strategic Areas of Development Award, from University of Ottawa for academic achievement, 2006-2007

In-Course Scholarship, for achieving an A average in third year undergraduate, 2005
In-Course Scholarship, for achieving an A- average in second year undergraduate, 2004
In-Course Scholarship, for achieving an A+ average in first year undergraduate, 2003

Research Experience

***Teaching Assistant for Analytical Chemistry,* Wilfrid Laurier University,** Waterloo, Ontario, January 2005 – April 2006

- Assisted students in lab
- Completed the marking of student lab reports efficiently

***Teaching Assistant for Organic Chemistry*, Wilfrid Laurier University,** Waterloo, Ontario, September-
December 2005

- Assisted and supervised students in lab
- Completed marking of student lab reports efficiently

***Summer Research Student,* Wilfrid Laurier University,** Waterloo, Ontario, May-August 2005

- Synthesized and analyzed peptides using solid phase peptide synthesis, HPLC, mass spectrometry, and circular dichroism spectroscopy
- Created new protocols for analysis techniques including amino acid analysis

***Teaching Assistant for Aquatic Environmental Chemistry,* Wilfrid Laurier University,** Waterloo, Ontario, September-December 2004

- Assisted students during experiments in lab
- Completed the marking of student lab reports efficiently

***NSERC Summer Student,* Pulp and Paper Research Institute of Canada,** Pointe-Claire, Quebec, May-August 2004

- Designed and researched an experiment to discover unique compounds present in wood species
- Performed extractions using solid phase extraction and solvent extraction
- Analyzed extracts using GC/FID and GC/MS

Abstracts and Conferences Attended

Young, E.K., Chatterjee, C., and Sparks D.L., (2008) HDL – ApoE Composition Regulates Cell Surface Displacement of Hepatic Lipase. The Canadian Lipoprotein Conference, October 2008.

Young, E.K., Chatterjee, C., and Sparks D.L., (2008) HDL Composition Regulates Cell Surface Displacement of Hepatic Lipase. Atherosclerosis, Thrombosis, and Vascular Biology (ATVB) Conference, April 2008.

Young, E. K., Chatterjee, C., and Sparks, D. L., (2007) HDL Composition Regulates Cell Surface Displacement of Hepatic Lipase. The Canadian Lipoprotein Conference, October 2007.