Chimeras of Lipoprotein Lipase and Hepatic Lipase: Localization of the Apolipoprotein C-II Activation Site of Lipoprotein Lipase

by

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B.Sc., University of Alberta, 1997

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology and Laboratory Medicine)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2003

Department of _Pathology & Laboratory Medicine_

The University of British Columbia
Vancouver, Canada

Date _April 23, 2003_

ABSTRACT

Hepatic lipase (HL) and lipoprotein lipase (LPL) are members of the same lipase gene family, along with pancreatic lipase, the pancreatic lipase-related lipases, endothelial lipase, and phosphatidylserine-specific phospholipase A1. Through their ability to hydrolyze triglycerides and phospholipids in a variety of circulating plasma lipoproteins including chylomicrons, very low and intermediate density lipoproteins (VLDL) and high density lipoproteins, HL and LPL greatly influence lipid metabolism. Unlike HL, however, LPL requires a specific cofactor, apolipoprotein C-II (apo C-II), to hydrolyze triglycerides in chylomicrons and VLDL. The aim of the present study is to identify residues within LPL which enable it to be responsive in the presence of apo C-II. A previous study has identified a segment in the N-terminal domain of LPL (residues 65-86) as having the ability to bind an apo C-II peptide fragment. This segment was found to contain regions of amino acid sequence dissimilarity when compared to the homologous residues in HL. Using site-directed mutagenesis, two sets of chimeras were created in which the two regions of human LPL (LPL residues 65-68 and 73-79) were exchanged with the corresponding human HL sequence. The HL chimeras consisted of a HL backbone with the suspected LPL regions replacing the corresponding HL sequence either individually ($HL_{LPL65-68}$, $HL_{LPL73-79}$) or together (HL_{LPLD}). Similarly, the LPL chimeras were created in which the candidate regions were replaced with the corresponding HL sequence ($LPL_{HL77-80}$, $LPL_{HL85-91}$ and LPL_{HLD}). Using a synthetic triolein substrate, lipase activity of the purified enzymes was measured in the presence and absence of apo C-II. Addition of apo C-II to $HL_{LPL65-68}$ and $HL_{LPL73-79}$ did not

significantly alter their enzyme activity. However, the activity of HL_{LPLD} increased ~5-fold in the presence of apo C-II whereas the activity of native LPL increased ~11-fold. Addition of apo C-II to $LPL_{HL77-80}$ resulted in ~10-fold activation while only ~6-fold and ~4-fold activation in enzyme activity was observed in $LPL_{HL85-91}$ and LPL_{HLD}, respectively. In summary, our results have identified 11 amino acid residues within the amino-terminal domain of LPL (residues 65-68 and 73-79) which appear to act cooperatively to enable substantial activation of human LPL by apo C-II.

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AMINO ACID DESIGNATIONS

Amino Acid	Three Letter Code	Single Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

ABBREVIATIONS

ABCA1	Adenosine triphosphate-binding cassette transporter A1
apo	Apolipoprotein
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesterol ester transfer protein
CRP	C-Reactive protein
Da	Dalton
$diC_{6:0}PC$	Dihexanoylphosphatidylcholine
$diC_{14:0}PC$	Dimyristoylphosphatidylcholine
$diC_{16:0}PC$	Dipalmitoylphosphatidylcholine
$diC_{16:0,18:1}PC$	1-palmitoyl-2-oleoylphosphatidylcholine
$diC_{16:0,18:2}PC$	1-palmitoyl-2-lineoylphosphatidylcholine
$diC_{18:0}PC$	Distearoylphosphatidylcholine
$diC_{18:1}PC$	Dioleoylphosphatidylcholine
$diC_{16:0}PE$	Dipalmitoylphosphatidylethanolamine
DG	Diacylglycerol
dNTP	Deoxynucleotide triphosphate
DOPC	Dioleoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EL	Endothelial lipase
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FC	Free cholesterol

FFA	Free fatty acid
FLLD	Familial lipoprotein lipase deficiency
GF	Growth factor
HBS	Hepes buffered saline
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HL	Hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IDL	Intermediate density lipoprotein
IFN-γ	Interferon gamma
IL	Interleukin
kb	Kilobase
kDa	Kilodalton
LCAT	Lecithin:cholesterol acyl transferase
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDL-R	Low density lipoprotein receptor
Lp	Lipoprotein
LPL	Lipoprotein lipase
LRP	Low density lipoprotein receptor related protein
LXR	Liver X receptor
MI	Myocardial infarction
MW	Molecular weight
oxLDL	Oxidized low density lipoprotein
PAPC	Palmitoylarachidonoylphosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PL	Pancreatic lipase
PLTP	Phospholipid transfer protein

POPC	Palmitoyloleoylphosphatidylcholine
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator responsive element
PS-PLA$_1$	Phosphatidylserine phospholipase A1
PVDF	Polyvinylidene fluoride
RAP	Receptor associated protein
RCT	Reverse cholesterol transport
rHDL	Recombinant high density lipoprotein
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cell
SR-BI	Scavenger receptor, Class B Type I
TC	Total cholesterol
TG	Triglyceride
TNF	Tumour necrosis factor
VLDL	Very low density lipoprotein

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ACKNOWLEDGEMENTS

DEDICATION

I dedicate this thesis to my family, especially my parents, Dale and Julie McIlhargey.

There are no words that adequately express how grateful I am

for their unconditional love, guidance and encouragement throughout

the years. The tremendous support they have provided through

all my endeavours has taught me the joy in pursuing your dreams.

They are the reason I was successful in completing this thesis.

Thank you.

1 INTRODUCTION

1.1 Atherosclerosis

1.1.1 Atherosclerosis Overview

Coronary artery disease (CAD) is defined as the blockage of the arteries supplying the heart with blood and is the manifestation of atherosclerosis or "hardening of the arteries". Atherosclerosis is a progressive disease and is characterized by the deposition of lipids and fibrous elements in the layers of the large arteries, narrowing the area in which blood can flow. Atherosclerosis is the primary cause of heart disease and stroke, and in westernized societies it is the underlying cause of the majority of all deaths and is predicted to remain so for years to come (1).

1.1.2 Risk Factors

Epidemiological studies over the last several decades have revealed a variety of risk factors that influence not only an individual's susceptibility to develop atherosclerosis, but the severity of the disease as well. While several identified risk factors are able to be controlled by an individual's behaviour or medication, others are unable to be manipulated and thus they can be broken down into two groups: modifiable and non-modifiable (2).

1.1.2.1 Modifiable Risk Factors

1.1.2.1.1 Hypercholesterolemia

A large number of studies have demonstrated the influence of plasma lipids and lipoproteins on atherosclerosis development and progression (3-5). Elevated levels of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) represent major risk factors in lesion development (6). Low density lipoprotein (LDL) has been shown to play an important role in atherogenesis because of its effect on the flux of lipids to and from the vessel wall (7). Moreover, LDL-C levels may aid in the formation of thrombi, which is the final step in the majority of acute coronary events.

Fluctuations in the levels of other lipoproteins may confer an additional contribution to atherogenesis. This includes low amounts of high density lipoprotein (HDL) which promotes cholesterol efflux from atherosclerotic lesions and inhibits the oxidation and resulting accumulation of LDL. Other contributing factors are increases in triglyceride-rich lipoproteins such as chylomicrons and very low density lipoproteins (VLDL), increases in small, dense LDL and increases in lipoprotein (a) (Lp(a)) in plasma along with elevated LDL-C.

1.1.2.1.2 Hypertension

High blood pressure alone has been shown to increase overall cardiovascular risk by two to three times (8) and has been described as the most useful single factor in determining CAD risk (9). Hypertension increases the rate of progression of

atherosclerosis by promoting endothelial dysfunction. High blood pressures increase the production of endothelin, which plays an important role in atherogenesis (7), by weakening the response of the vessels to endothelium-derived vasodilators and increasing vascular permeability to various macromolecules including LDL. Hypertension has also been associated with an increase in smooth muscle cell proliferation, response to growth factors and increased leukocyte adherence to the vascular endothelium.

1.1.2.1.3 Type II Diabetes

Type II diabetes not only increases an individual's risk of developing atherosclerosis, it also adversely affects the outcome, with mortality rates significantly higher for individuals with Type II diabetes (10). The typical lipid profile of patients with Type II diabetes consists of elevated total triglycerides and low levels of HDL cholesterol (HDL-C). This can cause abnormal chylomicron and VLDL metabolism, which results in smaller, more dense LDL particles which are known to be markedly atherogenic. In addition, elevated levels of growth factors such as insulin-like growth factor 1 (resulting from hyperinsulinemia), in the presence of hyperglycemia, contributes to the proliferation of the fibromuscular components of the developing atherosclerotic plaque.

1.1.2.1.4 Smoking

Smoking has been linked to significant alterations in whole blood viscosity and platelet reactivity. Studies have also demonstrated the effect of tobacco smoke

on increasing the levels of fibrinogen and carboxyhaemoglobin in the plasma, while at the same time decreasing HDL-C (11) and promoting the oxidation of LDL-C. It is believed that the modifications in the lipid profile are the result of LDL exposure to free radicals present in tobacco smoke.

1.1.2.1.5 Diet, Obesity and Physical Inactivity

When identifying risk factors, diet, obesity and physical inactivity are often discussed in terms of one another since one typically influences the others. Early studies, such as the Keys' Seven Countries Study (12), identified the relationship between a high caloric intake from saturated fat and cholesterol and increased risk of atherosclerosis. Since then, other dietary components and specific foods have been identified that are both positively and negatively associated with the risk of coronary artery disease. These include the source, quantity and composition of dietary fats, proteins, carbohydrates, vitamins, minerals, alcohol and phytochemicals (13). Intake of fatty acids has the ability to either lower or raise LDL-C and HDL-C levels, depending on the level of their saturation, and thus directly contribute a protective or detrimental effect (14-23).

Obesity is closely associated with, and thought to be mediated through, established risk factors such as hypercholesterolemia, hypertension, impaired glucose tolerance and low levels of HDL-C and is thus quite significant as a risk factor (24).

Physical inactivity is detrimental not only in its role of promoting obesity, but also as an independent risk factor for atherosclerosis and has been linked to an increased risk of myocardial infarction (MI) (25). Regular physical activity lowers body fat and blood pressure, increases both pulmonary and cardiovascular capacities and shifts an atherogenic lipid profile towards a more positive profile.

1.1.2.2 Non-Modifiable Risk Factors

1.1.2.2.1 Age and Gender

As all major forms of cardiovascular disease increase with advancing age, age is the dominant risk factor for heart disease (10). Before the age of 60, men develop coronary artery disease at twice the rate of women. The higher level of estrogen found in pre-menopausal women provides a protective effect against atherosclerosis, elevating their HDL. As women reach menopause and their estrogen level decreases, the incidence rate of atherosclerosis rapidly increases to match the rate found in men of a similar age (26).

1.1.2.2.2 Family History and Genetics

The contribution of a positive family history to early-onset atherosclerosis has been known for decades (27-31) and twin studies have given insight to the familial occurrence of coronary artery disease (32,33). The concordance for atherosclerosis is higher in monozygotic than dizygotic twins, suggesting at the very least a genetic component to this increased risk. There is no doubt of the influence of single gene

mutations affecting an individual's risk of developing atherosclerosis. Mutations in any number of genes such as those coding for the adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) or the LDL receptor (LDL-R) have a tremendous impact on lipid metabolism and therefore contribute to the development of atherogenic lipid profiles and the resulting atherogenesis. Over the last several years considerable interest has been generated in identifying gene polymorphisms such as those found in apolipoprotein E (apo E). In the case of apo E, three major isoforms are possible, E2, E3 and E4, resulting in six possible genotypes. In addition to functional differences, apo E2 is found to be associated with lower TC in comparison to apo E3, whereas apo E4 is associated with higher TC (34,35). Such associations have prompted clinical laboratories to routinely test for these polymorphisms in order to assess a patient's atherogenic risk. Interestingly, however, known genetic abnormalities only partially account for the risk predicted by a positive family history for premature coronary artery disease.

1.1.3 Lesion Development

The development and progression of atherosclerotic plaques is a complicated process involving a combination of genetic factors, which defines the limit under which atherosclerosis develops, and environmental influences, which impact a person's risk within that limit (36). These factors set in motion a process described as the "response-to-injury hypothesis" (37). According to this hypothesis, as illustrated in Figure 1, the initial onset of atherosclerosis is preceded by an injury to the vascular endothelium which increases permeability to blood cells, lipoproteins

Figure 1. Response to injury hypothesis of atherogenesis.
A schematic of the steps involved in atherosclerotic plaque formation and subsequent rupture (37).

Arterial wall

Lumen

Endothelium

1. Mechanical or functional insult

T-cell   Monocytes   LDL

2. Increased permeability

3. Platelets aggregate and secrete GFs & chemoattractants

Macrophages

SMC   OxLDL

4. SMC & macrophage proliferation & migration

5. Fibrous cap   Foam cells

6. Uptake of oxLDL to form foam cells

7. Rupture of fibrous cap & thrombus formation

Thrombus
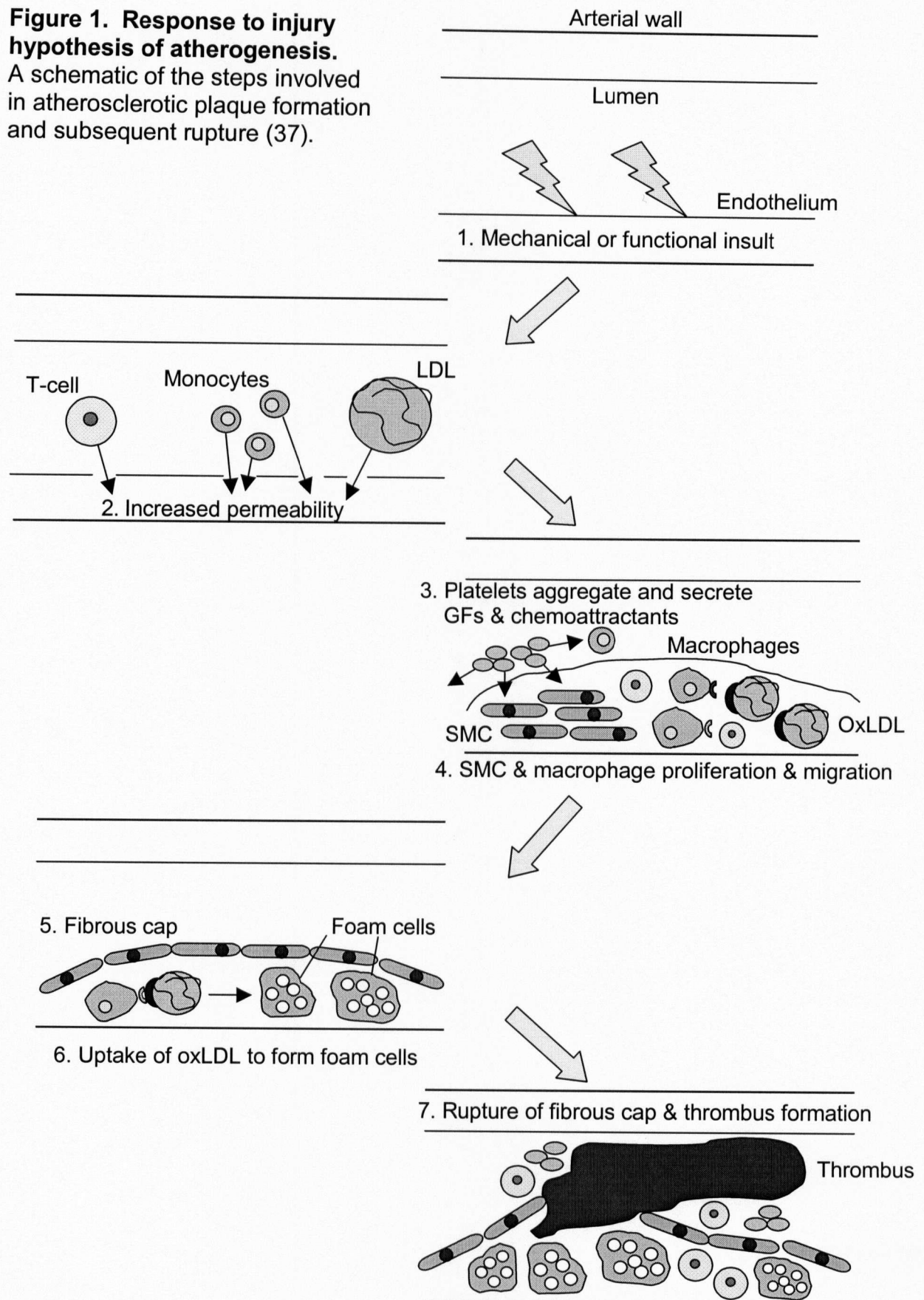
and certain hormones. Platelet aggregation occurs with a subsequent release of chemokines and growth factors. This stimulates smooth muscle cell (SMC) proliferation and their migration, along with macrophages, into the subintima region where the resulting atherosclerotic plaque will develop (38). As this process continues, the plaque matures and the SMCs and extracellular matrix begin to form a fibrous collagen cap that encloses a lipid-rich necrotic core. This core contains macrophages that have endocytosed oxidatively modified LDL through an unregulated scavenger receptor-mediated process, at which point they are called foam cells (36). Sub-endothelial accumulations of these cholesterol-engorged macrophages are found in the early stages of atherosclerotic lesions. These lesions are often referred to as "fatty streaks" and are commonly found in individuals under the age of 30. Initially the fatty streaks are localized to the aorta, but over time they extend to the coronary arteries followed by the cerebral arteries. Although fatty streaks alone are not clinically significant, they are the precursors to more complicated lesions consisting of lipid-rich necrotic debris and SMCs. Over time, lesions can become increasingly complex with calcification, ulceration at the luminal surface and haemorrhage from small vessels that grow into the lesions from the media of the blood vessel wall. The lesion may become large enough to occlude the coronary artery; however, the most significant complication associated with these lesions is an acute occlusion due to the formation of a thrombus or blood clot associated with rupture of the fibrous cap, resulting in unstable angina or non-fatal or fatal MI. Plaques can be classified as either vulnerable or stable, each possessing distinct features (39). Vulnerable plaques have a high lipid content, large numbers

of inflammatory cells, relatively few smooth muscle cells and a thin, fibrous collagen cap (40). Stable plaques, however, tend to have thicker fibrous caps, which protect the blood compartment in the arterial lumen from potentially disastrous contact with the underlying thrombogenic lipid core. The American Heart Association Committee on Vascular Lesions has subdivided atherosclerotic plaque progression into five separate categories, from "fatty streak" to "complicated lesion" (41,42) and distinct morphological types are found within each phase (7).

Analysis of plaque morphology has revealed the presence of macrophages and T-lymphocytes within the lesion, suggesting an inflammatory response underlying the atherogenic process (37). Both clinical (43) and pathological (44) evidence has demonstrated a correlation between the inflammatory response and CAD morbidity and mortality. Great interest is being generated in determining the relationship between inflammatory markers such as C-reactive protein (CRP) and the development of atherosclerosis. Levels of inflammatory markers in plasma have been shown to increase in active coronary syndromes such as unstable angina, thus leading to the theory that inflammation is central in lesion development and progression (44).

1.2 Lipoprotein Metabolism

1.2.1 Lipoproteins

Although the lipoproteins normally present in the blood vary greatly in their size, almost all are found in the form of micelles (45) and are mainly spherical, although one class, pre β-HDL, is in disk form. Lipoproteins consist of a hydrophobic inner core of triglycerides and cholesteryl esters while the surface is a monolayer of phospholipids, free cholesterol and amphipathic proteins called apolipoproteins (apo). There are several main groups of apolipoproteins which can be further broken down into sub-groups. As the size of the lipoprotein increases, the unesterified cholesterol becomes more centralized to the inner core. In this spherical shape, otherwise hydrophobic molecules are able to be freely transported throughout the body.

The density of lipoproteins is inversely related to their size, which reflects the relative contents of high-density surface protein and phospholipid and low-density, non-polar core lipid. Based on their size, density and apolipoprotein content, the lipoproteins can be classified into several groups (Figure 2). The two largest lipoproteins, the chylomicrons and the VLDL, contain mostly triglycerides (TG) in their inner core. Chylomicrons, which are secreted from enterocytes, contain primarily apo B_{48}, while VLDL secreted by hepatocytes contain apo B_{100}. Intermediate density lipoprotein (IDL) is a product of VLDL metabolism and contains similar amounts of triglycerides and cholesteryl ester in its core. LDL is mainly the end product of VLDL metabolism and consists of mostly cholesteryl esters in its

Figure 2. Density versus diameter of the lipoprotein subclasses. Diameter and density increase from the relatively triglyceride poor and cholesteryl ester rich HDL particles to the triglyceride rich and cholesteryl ester poor VLDL and chylomicrons.
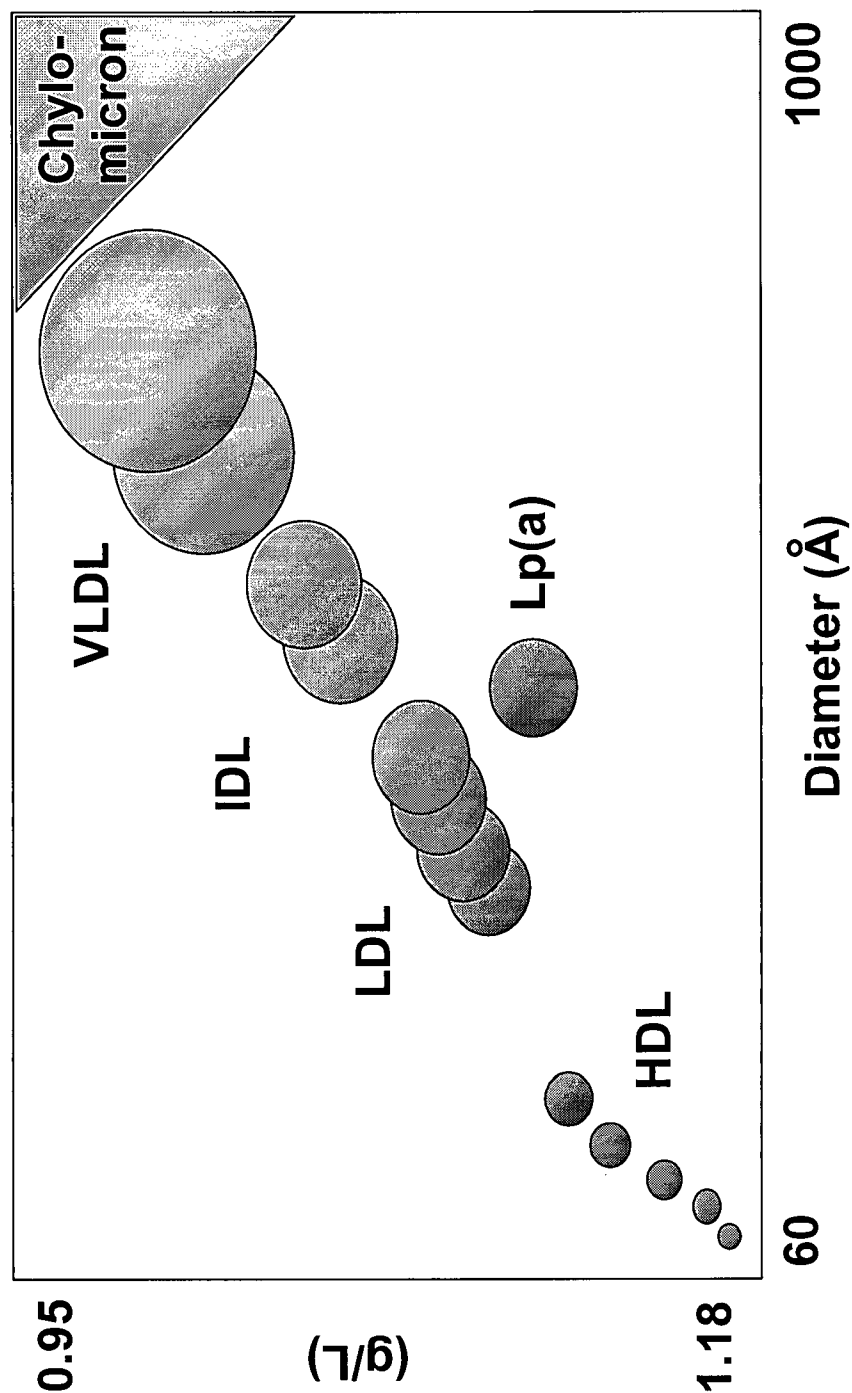
core. High density lipoprotein 2 (HDL_2) and HDL_3, like LDL, contain primarily cholesteryl esters and are produced by metabolic processes within the blood plasma as opposed to being directly secreted by cells. The precursor of mature HDL is known as pre β-HDL (a relatively lipid poor apo A-I particle) and is found in a disc-like shape. As such, it consists of only surface material and has no central, hydrophobic core. Lipoprotein (a) is an LDL particle with a disulfide linkage between apo B_{100} and apo (a) (46) and is present in highly variable amounts in the plasma. The exact compositions of the lipoproteins are shown in Table 1.

	Protein	Apolipoprotein	Triglycerides	Cholesterol	Phospholipids
Chylomicrons	1%	33% Apo A-I 32% Apo C 14% Apo A-IV 10% Apo E 11% Other	90%	5%	4%
VLDL	10%	25% Apo B 55% Apo C 15% Apo E 5% Other	65%	13%	13%
LDL	20%	95% Apo B 5% Other	10%	45%	23%
HDL	50%	65% Apo A-I 25% Apo A-II 10% Other	2%	18%	30%

Table 1. Lipoprotein classes and properties.

Defining the various lipoprotein particles by size is clinically significant with respect to the likelihood of developing atherosclerosis. Higher levels of small, dense LDL confer a more atherogenic effect compared to more buoyant LDL (47). In contrast, higher levels of large, more buoyant HDL appears to be more protective against atherosclerosis than smaller, more dense particles of HDL (48).

Apolipoproteins found on the surface of the lipoproteins possess amphipathic properties due to the presence of both polar and non-polar amino acids. Not only does the composition of apolipoproteins on the lipoprotein surface determine each particle's affinity for lipids and their metabolic fate with respect to specificity of receptor binding, many enzymes involved in lipid metabolism require certain apolipoproteins as cofactors and thus are directed to act towards specific lipoproteins. With the exception of the B apolipoproteins, the apolipoproteins and the free cholesterol are water soluble and have the ability to move freely between lipoproteins. In addition, plasma enzymes participate in these exchanges resulting in a constantly changing system whereby apolipoproteins and other components of lipoproteins are readily exchanged between lipoprotein particles. Lipoprotein size is affected as a result of this constant flux which in turn affects the conformation of the apolipoproteins on the lipoprotein surface and thus their ability to bind to various receptors. All these interactions combine to create a complex and dynamic system in which the turn-over of lipoproteins continuously changes as lipoprotein pools are created and metabolized in the circulation.

Lipoprotein metabolism is a process that is influenced by a broad range of factors including diet, drugs and disease as well as an individual's genetic make-up.

High dietary intake of saturated fats stimulates VLDL synthesis and decrease HDL levels. Thyroid hormones, sex hormones and insulin also modulate lipoprotein synthesis and degradation through transcriptional and post-transcriptional mechanisms. In addition to diseases such as diabetes, which was previously discussed, there are many diseases as a result of genetic abnormalities that also dramatically alter normal lipid metabolism, such as familial hypercholesterolemia and hypertriglyceridemia. The metabolism of lipoproteins is a multi-faceted process that involves two major pathways: the exogenous path and the endogenous path, the latter including reverse cholesterol transport.

1.2.1.1 Exogenous Pathway

The exogenous pathway involves the metabolism of dietary fat (Figure 3). Once ingested, bile acids and phospholipids form micelles in the intestine. The triglycerides in the micelles are hydrolyzed into free fatty acids (FFA) and monoglycerides by pancreatic, stomach and intestinal lipases. The FFA and monoglycerides are absorbed by the intestinal cells along with dietary cholesterol (mainly in the form of cholesteryl esters which are hydrolyzed to free cholesterol and fatty acids by cholesterol esterase). The long chain fatty acids and cholesterol are coupled with apo B_{48} to form chylomicrons (49). Apo B_{48} is a truncated form of apo B, which retains the N-terminal 48% of the protein. This version of apo B arises from editing of the apo B mRNA in the intestine and is not recognized by the major lipoprotein receptors (50,51). The nascent lipoproteins are transported from the endoplasmic reticulum of enterocytes to the Golgi apparatus, packaged into

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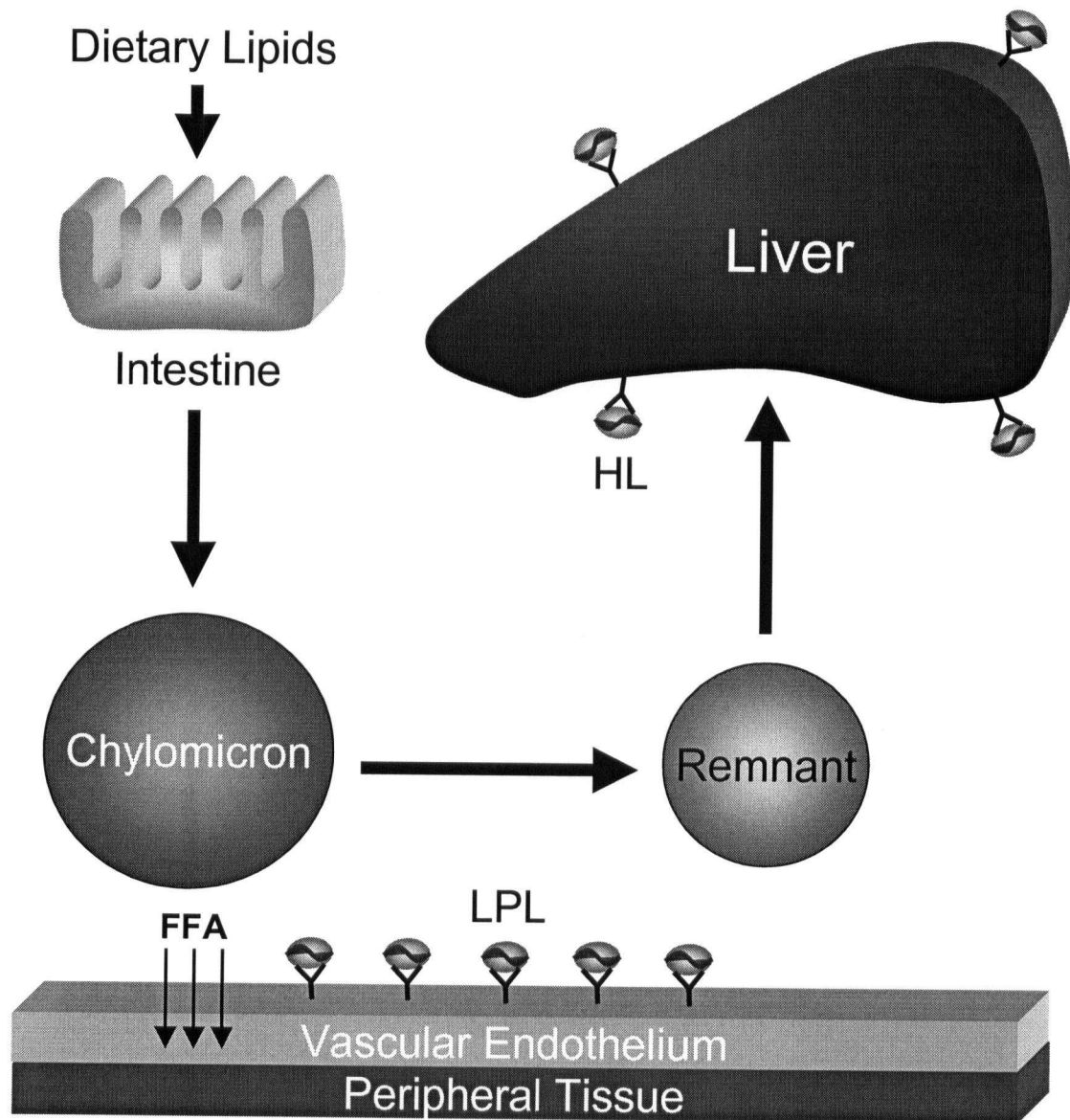
Figure 3. The exogenous pathway. Dietary lipids are absorbed by the enterocyte and packaged with protein into buoyant, TG rich particles called chylomicrons. Chylomicrons are secreted into the plasma where their TG are hydrolyzed by LPL to yield FFA for use or storage by peripheral tissues. Chylomicron remnants are catabolized through the liver.

secretory vesicles and delivered into the extracellular space by exocytosis. Chylomicrons are absorbed by lacteals in the intestinal microvilli where they enter the circulation through the thoracic duct. Triglycerides are responsible for approximately 90% of a chylomicron's total mass whereas only 2% of total mass is composed of protein. Chylomicrons contain several different types of apolipoproteins, namely apo A-I, A-II, A-IV and B_{48} (52). While in the circulation, chylomicrons acquire apo C-I, C-II and C-III along with apo E from HDL (53,54). Apo C-II is bound to the surface of the chylomicron and is a necessary cofactor for the activation of lipoprotein lipase (LPL) (55-58), which hydrolyzes TG and phospholipids. LPL is found throughout the body in tissues such as skeletal muscle, adipose tissue and macrophages, and when chylomicrons come into contact with LPL, and in turn become bound, a large amount of their TG is hydrolyzed. The released fatty acids bind serum albumin and are taken up by adipose and muscle tissues. The chylomicrons now have a smaller inner core since they are now TG poor, and as a result have excess surface material, i.e. phospholipids and apolipoproteins. The excess phospholipids and apo A-I and A-IV are transferred to HDL in exchange for apo E and cholesteryl esters (59,60). As a result of this structural change, the chylomicrons lose their affinity for apo C-I, C-II and C-III, and these too, are transferred to HDL. These new particles, now referred to as chylomicron remnants, are released into the blood and can either be taken up by the liver via the LDL-R or bind to hepatic lipase (HL), also on the liver (61,62). If bound to hepatic lipase, further hydrolysis of the chylomicron remnants' TG and phospholipids occurs along with acquisition of additional apo E. Although this extra

apo E is not required for binding to the LDL-R, it will facilitate the binding to another receptor, the LDL receptor-related protein (LRP). Once the remnant particle is taken-up by the liver, components of it are hydrolyzed in lysosomes. The exogenous path ensures that lipids are delivered to tissues throughout the body.

1.2.1.2 Endogenous Pathway

The production of VLDL provides a mechanism for export of excess triglycerides from hepatocytes. Although this path is a continuous cycle, the synthesis of VLDL by the liver can be considered to be an initial step (Figure 4).

Much like the synthesis of chylomicrons, VLDL requires apo B and microsomal triglyceride transfer protein for assembly. Once synthesized, VLDL is released into the blood through the fenestrae of the hepatic sinusoidal endothelium. Initially containing primarily apo B_{100}, additional apolipoproteins such as apo C-I, C-II, C-III and apo E are added from HDL in exchange for TG through the action of cholesterol ester transfer protein (CETP) (53). VLDL then undergoes hydrolysis by LPL and takes up additional apo E from HDL, resulting in VLDL remnants or IDL. These particles will then be removed from circulation by the liver via the LDL-R or be further catabolized by HL to form LDL. The apo B_{100} found on LDL targets it to the LDL-R, which is found in large concentrations in tissues requiring cholesterol, such as the adrenals and liver. A small percentage of LDL, approximately 10%, is found covalently bound to apo (a) and is, as mentioned previously, referred to as Lp(a). Levels of Lp(a) are genetically determined in part and may vary between individuals by as much as 1000-fold (63). Although a definitive role for Lp(a) has yet to be
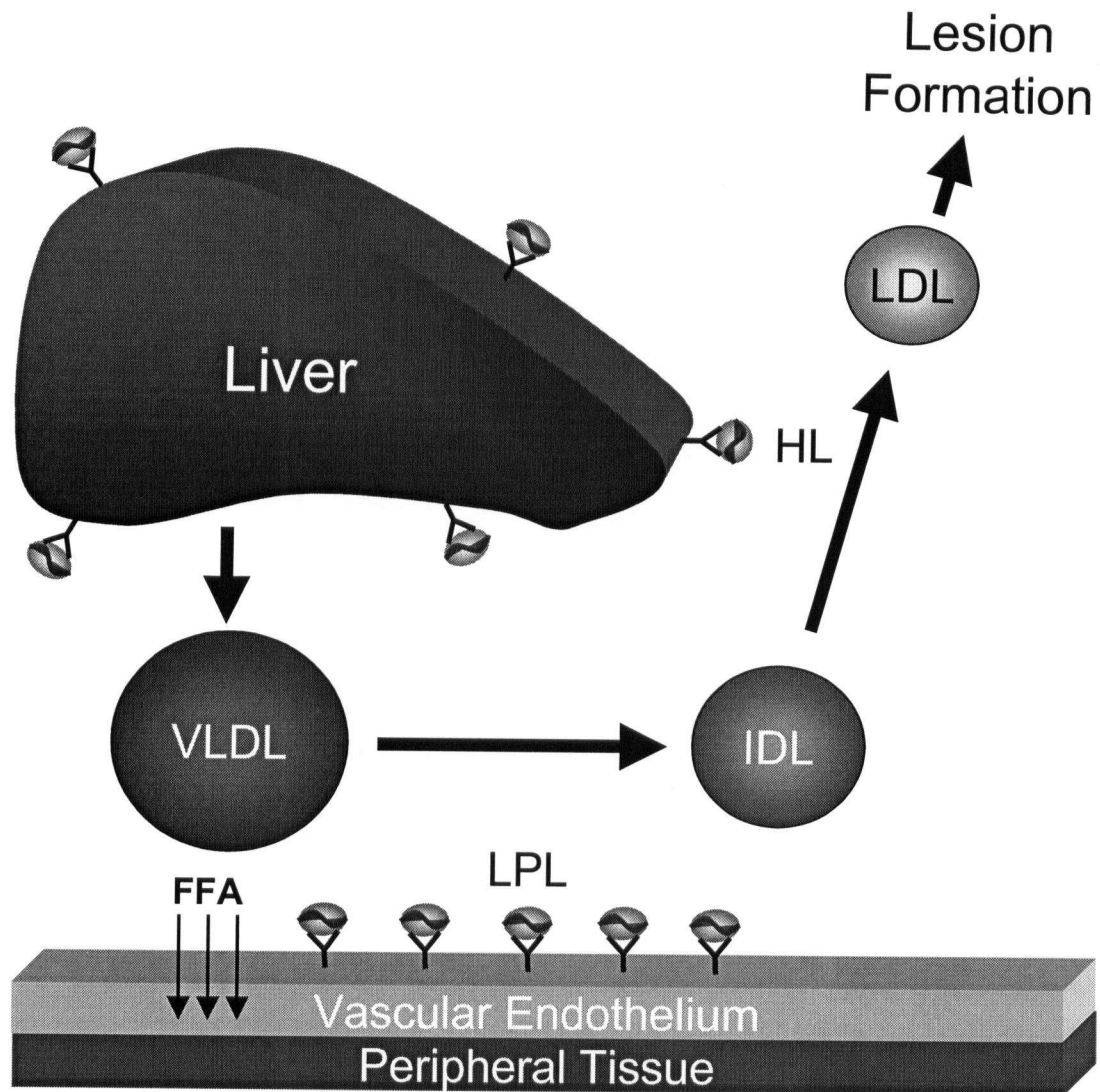
Figure 4. The endogenous pathway. Hepatocytes secrete lipoproteins called VLDL. LPL liberates TG in VLDL resulting in the formation of smaller, more dense IDL. IDL is catabolized through the liver or further hydrolyzed by the action of HL at the surface of the hepatocyte, resulting in the atherogenic lipoprotein species, LDL.

determined, studies have shown that as levels of Lp(a) increase in the plasma, the risk of developing atherosclerosis is strengthened (64,65).

The normal duration for chylomicrons to remain in circulation ranges from five to ten minutes, whereas for VLDL the time is 15 to 60 minutes. This difference can best be explained by the relative sizes of the two types of lipoprotein particles. Chylomicrons, being the larger of the two, are able to bind a larger number of LPL than the smaller VLDL, thus the core triglycerides would be hydrolyzed at a faster rate.

1.2.1.2.1 Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) is the description for the pathway shuttling cholesterol from extrahepatic tissues to the liver for removal from the body. The liver requires cholesterol for the synthesis of VLDL and the primary bile acids, cholic and chenodeoxycholic acids. The bile acids are secreted into the intestine as bile salts where they are converted into the secondary bile acids, deoxycholic and lithocholic acids. The bile acids emulsify fats in the gastrointestinal tract and are either reabsorbed by the gut or excreted in the feces (66,67). This pathway is important since by reducing the accumulation of cholesterol in the wall of arteries, RCT has the potential to prevent atherogenesis.

The primary player in this process is the apo A-I containing lipoprotein, HDL. This process is determined in large part by HDL concentration in the blood, since the plasma concentration of HDL cholesterol and apo A-I correlate negatively with the

incidence of CAD, and the inverse relationship between HDL and CAD has been shown in a variety of epidemiological (68-71) and interventional (72,73) studies. It is widely believed that the relationship between HDL and atherosclerosis is not correlation, but causation, and is explained by the role of HDL in RCT.

The idea of RCT was initially put forth by Glomset (74), and like the endogenous pathway, RCT is a continuous cycle with no defined start and end. The beginning can be considered to be cholesterol efflux from peripheral cells through a transporter in the cell membrane, ABCA1. Small, disc-like, lipid-poor particles known as pre β_1-HDL are the initial acceptors of cellular cholesterol. As more cholesterol is accepted, the particles become larger and are referred to as pre β_2-HDL; a substrate for lecithin:cholesterol acyltransferase (LCAT). Esterification of cholesterol by LCAT and acquisition of additional cholesterol transform the disc-like structures into the spherical lipoprotein, α_3-HDL. These lipoproteins collect more cholesterol from pre β_2-HDL and possibly also from cells (75), thus becoming larger α_2 and α_1-HDL. Subsequently, the cholesteryl esters in HDL can be transferred either directly to target cells via the scavenger receptor, SR-BI, or to apo B containing lipoproteins, such as VLDL and LDL (76), in exchange for TG through the action of CETP (77). In addition, HDL obtains phospholipids by the action of phospholipid transfer protein (PLTP). These large HDL particles are an ideal substrate for HL, which hydrolyzes the TG and phospholipid, resulting in smaller α_3-HDL and lipid-free apo A-I (78,79), the latter rapidly becoming re-lipidated by cellular cholesterol and phospholipid to form pre β_1-HDL, thus the cycle can continue. A schematic of this process is shown in Figure 5.

Efflux of Phospholipid and Free Cholesterol
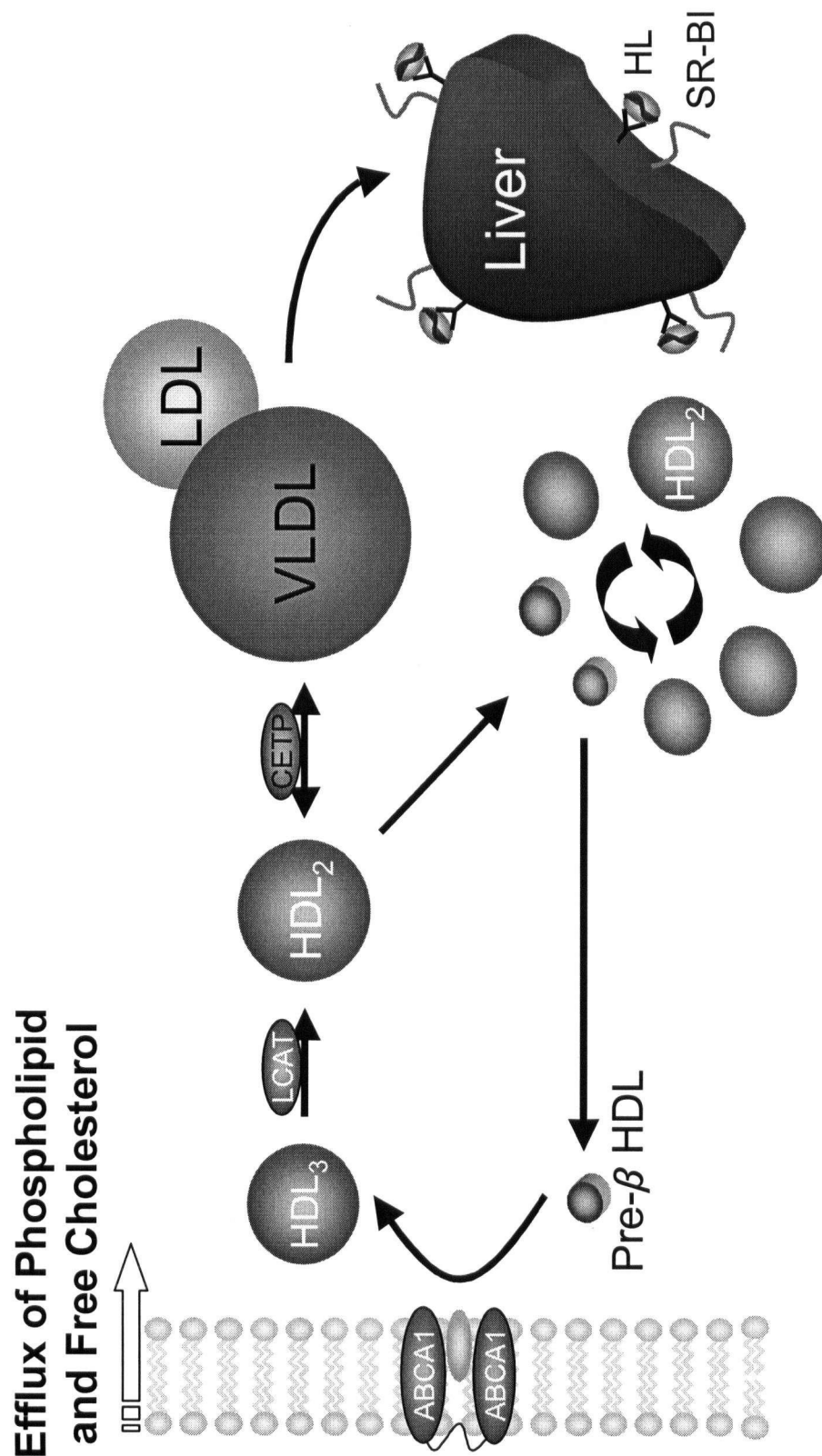
Figure 5. Reverse cholesterol transport. Disc-like pre-β HDL particles absorb FC from the peripheral tissues through the ABCA1 transporter to become larger, HDL_3 particles whereupon the FC is acted on by LCAT to yield cholesteryl ester. CETP exchanges CE for TG from TG rich lipoproteins which are removed from circulation through the liver. The large HDL_2 proceed to the liver where selective uptake of HDL-CE occurs through HL and the SR-BI receptor. Further hydrolysis of HDL_2 by HL can also result in the formation of smaller, lipid poor HDL particles which can participate in uptake of FC from tissues.

The fate of the cholesteryl esters transferred to apo B containing lipoproteins rests with the metabolism of those particles. These lipoproteins have the potential to be taken up by the liver, and in the case of LDL, be endocytosed by cells throughout the body via the LDL-R.

Disruption of the cycle severely impairs RCT and the most striking effect is seen when cholesterol efflux is interrupted, as in the case of the ABCA1 transporter. Patients with a mutation in this gene manifest Tangier disease (75,80-82) and have virtually no lipidation of apo A-I, thus impairing formation of pre β_1-HDL. As a result, the level of HDL dramatically declines and RCT all but stops.

1.3   Lipase Gene Family

The lipase gene family plays a central role in intestinal lipid absorption, energy homeostasis, plasma lipoprotein metabolism and atherosclerosis. Four vertebrate genes derived from a common ancestral gene make up the lipase gene family. This family includes LPL, HL, pancreatic lipase (PL) and endothelial lipase (EL) (83-87). LPL, HL and EL all hydrolyze triglycerides and phospholipids in circulating lipoproteins to varying degrees (88) and these lipases can be considered to be on a spectrum of lipase activities, with LPL having primarily triglyceride lipase activity, EL primarily phospholipase activity and HL falling somewhere in the middle. Recent studies have implicated phosphatidylserine phospholipase A1 (PS-PLA$_1$) to be a closely related member of this family (89,90) and PL-related proteins 1 and 2

have been found to form a subfamily of PLs (91,92). Whereas PL-related protein 1 displays no enzymatic activity, PL-related protein 2 possesses hydrolytic activity against both triglyceride and phospholipid substrates (93). In addition, three *Drosophila* yolk proteins, YP1, YP2 and YP3, also belong to the lipase gene family (94-97). Although they lack lipolytic activity and show no obvious functional similarity to the lipases, a 104 amino acid segment shares sequence similarity to the substrate-binding site of porcine PL (94). Direct sequence comparison, crystal structure data and access to the protein structure database has led to the recognition of a superfamily that includes esterases and thioesterases (98). All members of this superfamily share a characteristic structural feature surrounding the residues of the active site called the α/β hydrolase fold which is responsible for maintaining the position of the conserved residues in the active site pentapeptide (98,99).

Sequence analysis has revealed phylogenetic relationships among the members of the lipase gene family. Based on a shared organization of intron-exon boundaries in addition to high sequence homology, it was proposed that LPL, HL and PL were derived from a common ancestor (84,87,88). The phylogenetic analysis in Figure 6 (88) extends the previous model for the evolution of the lipase gene family (87). The new model demonstrates that PS-PLA$_1$ diverged from pancreatic lipase earlier than LPL, HL and EL, and that LPL and EL are derived from a common ancestor.
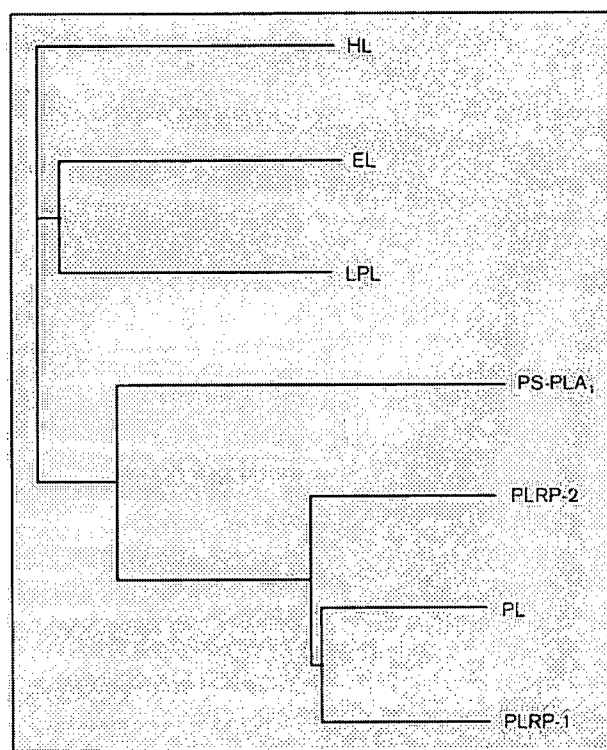
Figure 6. Evolution of the triglyceride lipase gene family (88).
EL, endothelial lipase; HL, hepatic lipase; LPL, lipoprotein lipase; PL, pancreatic lipase; PS-PLA$_1$, phosphatidylserine phospholipase A$_1$; PLRP, pancreatic lipase related protein.

Obtaining detailed structural information on the lipases has proved challenging due to the lack of crystal structure data. Currently, the only crystal structure information on mammalian members of the lipase gene family is available from pancreatic lipase and its sub-family of related proteins (93,100,101). Analysis of this three-dimensional structure has revealed the presence of an amino-terminal domain containing the active site and a carboxy-terminal domain implicated in the binding of colipase (102). Direct sequence comparisons reveal extensive homology between the lipases. LPL demonstrates 53% sequence identity to HL, 45% to EL

and 35% to PL (84,88) and the catalytic triad of serine, aspartate and histidine is completely conserved among LPL, HL, PL and EL. Besides the conservation of the catalytic region, closer inspection of each enzyme's amino acid sequence reveals additional regions of conservation such as cysteine residues, lipid and heparin binding domains and the surface loops and lid. The crystal structure information from pancreatic lipase in combination with the substantial sequence homology and computer modelling (102,103) makes it likely that LPL, HL and EL also possess a similar two-domain structure (104).

1.4   Biochemistry and Genetics of Lipoprotein Lipase

1.4.1   Lipoprotein Lipase Gene

The lipoprotein lipase gene is located on chromosome 8 (8p22) in humans (105). Spanning approximately 30 kb, the gene contains nine introns and ten exons (87,106). Although exons one through nine are relatively small in size (105-276 bp), exon ten specifies the entire 3' noncoding sequence which is 1948 bp in length. The transcription start site has been described to begin 188 nucleotides upstream of the translation initiation codon (106). The 3' untranslated region contains two polyadenylation signals thought to be used alternatively to produce two species of mRNA approximately 3350 and 3750 nucleotides in length (107). The significance of these two transcripts is still not fully understood, however it has been suggested that the longer form is translated more efficiently (108). Although most tissues

express both transcripts, the longer form is the predominant transcript in both cardiac and skeletal muscle (108).

1.4.2 Protein Structure-Function

The translated gene forms a 475 amino acid protein with a 27 amino acid signal peptide which is cleaved, resulting in a mature glycoprotein containing 448 residues with a calculated molecular weight of 50 394 Da (107). An additional 8% for carbohydrates is assumed (109), resulting in a molecular mass of approximately 55 kDa.

LPL has two N-linked glycosylation sites located at Asn 43 and Asn 359. The importance of specific glycosylation on protein expression was first reported by Semenkovich *et al.* who found that mutation of Asn 43 $\prod$ Ala or Gln resulted in an enzymatically inactive protein which accumulated intracellularly and was not secreted into the culture medium (110). It was later shown that this intracellular accumulation occurred within the endoplasmic reticulum (ER) (111). Along with this finding was the discovery that retained LPL in the ER resulted in grossly altered distribution of the ER within the cell and the authors found that intracellular transport of other glycosylated proteins was altered as well, indicating significant implications associated with a mutation of this type. In spite of the considerable effect of no glycosylation at Asn 43, a similar substitution at Asn 359 did not appreciably affect activity, suggesting normal processing and transport. These results were later confirmed by Ben-Zeev *et al.* who also substituted Asn 43 and Asn 359 $\prod$ Gln (112).

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By analogy to PL, LPL appears to be separated into two structurally distinct regions which consist of a large N-terminal domain (residues 1-312) and a smaller C-terminal domain (residues 313-448). Consistent with other lipases in the gene family, LPL has a conserved catalytic triad located at Ser 132, Asp 156 and His 241 (87), which is located in the N-terminus. Ser 132 is part of a Gly-Xaa-Ser-Xaa-Gly consensus sequence present in all serine proteases along with HL, PL and EL and its importance in conservation of catalytic activity was first reported by Faustinella *et al.* by demonstrating that substitution of Ser 132 by either Thr, Ala or Asp resulted in complete inactivation of enzyme activity (113). More detailed analysis of the catalytic triad was performed by Emmerich *et al.* who mutated Asp 156 and His 241 in addition to Ser 132 (114). The authors found that mutation of any one of these residues resulted in total abolishment of catalytic activity, however these substitutions appeared to have no influence on lipid or heparin binding, suggesting alternate residues are responsible for those features of LPL. An interesting finding was that a naturally occurring mutation, Asn 291 ∏ Ser, resulted in approximately half the normal LPL activity (115), suggesting that individual residues outside the catalytic domain can be influential in determining the level of LPL activity.

LPL has the ability to bind to heparin and this function is essential for the interaction of LPL with cell wall glycosaminoglycans and its localization in the endothelial vessel wall. The fact that high ionic strength is required to elute LPL from a heparin matrix (116-118) and decreasing sulfate density in heparan sulfate chains reduces LPL affinity for heparan sulphate proteoglycans (HSPG) (119) suggests that the LPL interaction with HSPG is ionic in nature. As such, heparin

binding consensus sequences, -X-B-B-X-B-X- and -X-B-B-B-X-X-B-X, have been identified where B is a basic residue and X a small, neutral residue (120). LPL residues 279-282 and 292-304 in the N-terminal domain demonstrate homology to these consensus sequences, respectively, and removal of the basic residues in these regions results in a decrease in heparin binding affinity (121,122), more specifically, the identification of five residues (Arg 279, Lys 280, Arg 282, Lys 296 and Arg 297) which were critical determinants of high heparin affinity. More recently, residues located in the C-terminus have been identified as contributing to heparin affinity. Sendak *et al.* demonstrated that mutation of avian LPL residues Lys 321, Arg 405, Arg 407, Lys 409, and Lys 416 resulted in a decrease in affinity for heparin and a triple mutant LPL (R405N, R407N and K409N) possessed almost no high-affinity binding (123). Shortly after Lookene *et al.* found that replacement of Lys 403, Arg 405 and Lys 407 by Ala completely abolished heparin affinity (124) and it has been postulated these residues along with Lys 319, Lys 413 and Lys 414 may form an additional heparin binding cluster (125). An earlier study using a molecular modelling approach based on the PL crystal structure identified a potential heparin binding cluster in the N-terminus comprising Lys 147, Lys 148 and Arg 151 (125) which as of yet has not been shown to bind heparin.

In addition to the mutations in the C-terminus, Sendak *et al.* also mutated the previously identified heparin binding regions of LPL and confirmed a decrease in heparin affinity, although the reduction was considered to be modest (123). Thus, the authors concluded that the candidate residues in the C-terminus represent the major heparin binding domain in LPL (123). The importance of the contribution of

the C-terminal domain in heparin binding was confirmed *in vivo* by Lutz *et al.* who mutated Arg 403, Arg 405 and Lys 407 in the C-terminus (126). The expressed human LPL enzyme was defective in its ability to bind heparin, resulting in an accumulation of inactive LPL in preheparin blood.

Several lipid binding sites within LPL were initially proposed by Winkler *et al.* based on areas of hydrophobicity found in the crystal structure of PL (100), however, the importance of the C-terminus was demonstrated by its removal during proteolytic cleavage which resulted in an N-terminus unable to bind to milk fat globules or chylomicrons (127). Studies of chimeras of LPL and HL also confirmed these results. Wong *et al.* found that after reaction with LPL monoclonal Ab specific to the C-terminus, lipolytic activity was inhibited whereas esterolytic activity was only marginally affected (128,129), indicating that this domain is required for lipolysis, perhaps by promoting the interaction with lipid substrates. Specifically, a cluster of tryptophans at 390, 393 and 394 located in the exposed loop region were identified as directly affecting lipid binding (130,131). In addition, Kobayashi *et al.* described a patient with a mutation in residue 447 of LPL (132,133) which results in premature truncation of the LPL protein and the resulting loss of two amino acids. This mutant LPL is able to hydrolyze water-soluble tributyrin but has reduced ability to hydrolyze triolein, suggesting that the terminal two amino acids are necessary for hydrolysis of long chain fatty acid triglyceride substrates. Analysis of the effect of this mutation on LPL activity has been studied in detail by other authors and the results have been varied. This will be discussed further in the section on LPL polymorphisms.

The lid domain on LPL represents a mobile surface loop that covers the catalytic site and can be rearranged to permit the substrate to have access to the catalytic domain (134). The lid is located between two conserved cysteines (Cys 216 to Cys 239) which form one of the four disulfide bridges in the LPL molecule. Due to its location, it appears as though movement of the lid is necessary to allow substrate access to the catalytic pocket, and similar movement has been demonstrated in a fungal lipase (135,136). Previous studies have suggested that an intact lid is essential for normal lipid binding (137) and more detailed analysis by other groups have confirmed this finding. Dugi et al. found that mutation of the lid to reduce amphiphilicity without changing the predicted secondary structure resulted in abolishment of triglyceride hydrolysis against triolein but not the ability to hydrolyze tributyrin (138). Replacement of the LPL lid with the lid of HL led to expression of an enzyme that retained both triolein and tributyrin hydrolyzing activity, whereas replacement with a short, four amino acid peptide enhanced hydrolysis of short chain fatty acid triglycerides by greater than 2-fold, while the ability to hydrolyze triolein was abolished. Using deletion mutants affecting different parts of the lid, Henderson et al. concludes a requirement for maintenance of charge and periodicity in the proximal and distal segments of the lid must exist to enable normal catalytic functioning (139). Later work demonstrated the importance of the lid in determining substrate specificity. In an LPL chimera with an HL lid, hydrolyzing activity against triolein was reduced to 49% of wild-type whereas its ability to hydrolyze phospholipids increased over 300% (140), demonstrating substrate specificity similar to that of HL. Slightly different results were observed in vivo, however. Although the

LPL chimera with an HL lid demonstrated an 81% reduction in plasma phospholipids compared to 31% in wild-type, no significant alterations in plasma triglyceride concentrations were reported (141). Proper folding of the lid is also important, as shown by Salinelli *et al.* who completely inactivated LPL by substituting Cys 216 and 239 to Ser (142).

In addition to the lid region, another mobile loop has been described in the LPL protein structure that spans from His 53 to Trp 64. It has been proposed that upon opening of the LPL lid, this β-loop may fold back upon the core of the protein. This rearrangement makes the catalytic site more accessible and brings one of the electrophiles of the oxyanion hole into a catalytically competent position (125,134).

Human LPL contains ten conserved cysteine residues, forming four disulfide linkages (Cys 216 and 239, Cys 264 and 275, Cys 278 and 283 and Cys 418 and 438) (125,134). In light of the importance of cysteine residues in the formation and stability of a protein's tertiary structure, it would be expected that substitution of any of these residues or introduction of new cysteines would affect not only the structure of LPL, but also its function. In a naturally occurring LPL mutation in which Cys 216 is replaced by Ser, a complete loss of catalytic activity is observed (143). Replacement of other residues with cysteine such as Ser172Cys (143,144), Arg243Cys (145) and Ser251Cys (146) has also resulted in LPL inactivation in humans.

A feature unique to LPL is its ability to be inactivated in the presence of high salt (1.0 M NaCl). In chimeras created by Davis *et al.*, the authors found that LPL

inhibition by 1.0 M NaCl originates in structural features within the N-terminal domain of LPL (147) and Dichek *et al.* confirmed these results (148). Subdomain chimeras created by Hill *et al.* revealed no apparent contribution to salt sensitivity by the C-terminus of LPL (149). To date, no further localization of this domain has been achieved.

Garfinkel *et al.* were the first to describe the functional unit of LPL as a homodimer (150). By using radiation inactivation, the authors showed that in rat heart and adipose tissue, the smallest functional molecular weight of LPL was approximately 127 kDa, indicating that LPL is functional as a dimer. A later study using sedimentation equilibrium analysis of bovine LPL showed that activity was almost exclusively associated with the dimer fraction (151). Davis *et al.* speculated that the C-terminal domain of one subunit was positioned next to the catalytic site (N-terminal domain) of the adjacent subunit (147), and this was expanded upon by Wong *et al.* who proposed a head-to-tail model where the large N-terminal domain of one monomer is in close proximity to the C-terminal domain of the other monomer (128). A schematic of this model is shown in Figure 7.
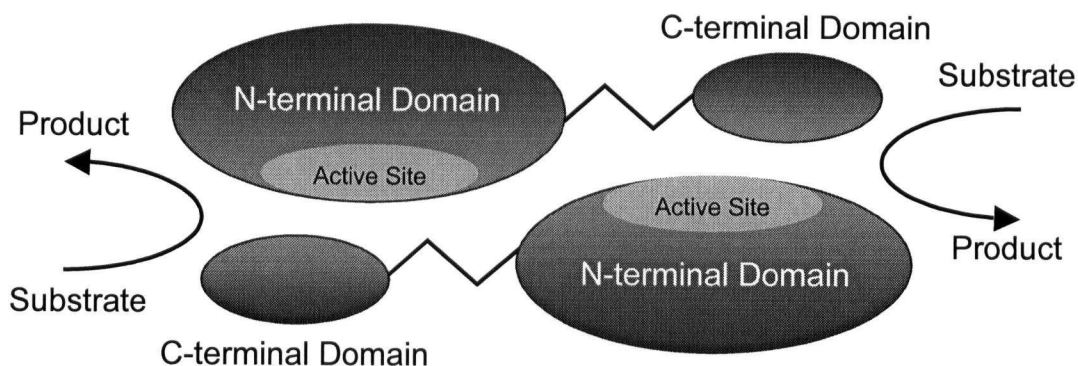
Figure 7. Head-to-tail dimer model. The large, N-terminal domain of one monomer lies in close proximity to the smaller, C-terminal domain of the opposing monomer, such that they work together to catalyze substrate to product.

1.4.3 Biochemistry

Lipoprotein lipase is synthesized by a variety of tissues including skeletal muscle, cardiac muscle and adipose tissue (152) and to a lesser degree in the adrenals, ovaries, kidney, brain, macrophages, lactating mammary cells and certain neuronal cells in the nervous system (153-157). LPL has also been shown to be synthesized in fetal hepatocytes (158), but the production becomes suppressed shortly after birth (159). Unlike most secretory proteins, newly synthesized LPL appears to transiently reside on cell surfaces (160,161). It has been shown that in the case of adipocytes, some of this LPL is re-internalized and then degraded (162), whereas the remainder is dissociated from the cell surface. Pillarisetti *et al.* has suggested that this action is likely due to the action of an endothelial cell heparanase

(163). This newly released LPL is then transferred to the target endothelial cell. Obunike *et al.* has demonstrated that LPL movement from the abluminal side to the luminal side of endothelial cells requires both HSPG and the VLDL receptor (164).

LPL is found bound to the cell surface via HSPG and is released from the vascular bed upon intravenous injection of heparin (165). Lipoprotein lipase in dimer form is a much better ligand for heparin than the monomer, displaying an affinity that is 6000-fold higher (166). LPL also associates with members of the LDL receptor family, such as LRP (167-169), the VLDL receptor (170), glycoprotein 330 (171), as well as regions in the N-terminal domain of apo B (172). In addition to the release from the vascular bed facilitated by heparin, disruption of the endothelium by tumour necrosis factor (173) and exposure to free fatty acids (174) will also release bound LPL into the circulation.

Lipoprotein lipase is primarily a triglyceride lipase, and although its phospholipase activity is thought to be minimal, approximately 1% of its triacylglycerol hydrolase activity (175), one cannot dismiss the importance of its activity on remodelling of lipoproteins. Early studies demonstrated that LPL has phospholipase A1 activity and is able to hydrolyze the primary acyl bond of phosphatidylcholine and phosphatidylethanolamine in triglyceride-rich lipoproteins (176-179), artificial triacylglycerol-phospholipid emulsions (180) and sonicated phospholipid vesicles (181-183). Many of these studies also showed that the phospholipase activity of LPL is stimulated in the presence of apo C-II and inhibited in the presence of apo C-III, regardless of the presence of triglyceride (180-183). Bengtsson *et al.* demonstrated that without a fatty acid acceptor such as albumin,

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the stimulating effect of apo C-II is absent (184) and Hill *et al.* confirmed this finding (149). A later study by Rojas *et al.* found that depending on the form the substrate takes, this does not always hold true (185). With liposomes of phosphatidylcholine and <3% trioleoylglycerol, as opposed to emulsion droplets of the same lipids, albumin was not necessary for continued hydrolysis of triacylglycerols. This was explained by the theory that the resulting fatty acids could be accommodated in the phospholipid bilayer.

Shirai *et al.*, interested in the effect of apo C-II on the catalytic mechanism of phosphatidylcholine hydrolysis, analyzed the rate of hydrolysis in discoidal phospholipid complexes prepared with apo C-III and dipalmitoyl phosphatidylcholine (DPPC) along with guinea pig VLDL, both with varying amounts of apo C-II (186). The authors found that the V_{max} for phospholipid hydrolysis increased in both the complex and VLDL, whereas the apparent K_M decreased regarding triacylglycerol hydrolysis, leaving the authors to suggest that the mechanism of apo C-II regarding catalytic activity differs depending on the composition of the substrate. Further studies on the effect of apo C-II on the phospholipase activity of LPL were performed by Shinomiya *et al.* who were interested in the physical form of the substrate (187). The authors measured the phospholipid hydrolysis rates on Triton X-dimyristoylphosphatidylcholine substrates and found that although the absolute rate of LPL catalysis varied depending on whether the substrate was in a bilayer or micelle form, the activation factor was nearly constant (187), demonstrating that the effect of apo C-II on LPL catalytic activity is independent of the physical form of the substrate.

The importance of a preference by LPL for certain fatty acyl groups appears to be unclear. Several early reports have shown that although apo C-II does indeed stimulate phospholipid hydrolysis, to what extent may be dependent on the fatty acyl chain length. For example, in studies using detergent-solubilized phospholipid, apo C-II did not enhance the rate of LPL hydrolysis of the short chain phospholipid, dihexanoylphosphatidylcholine ($diC_{6:0}PC$) (188), whereas LPL hydrolysis of the long chain dimyristoylphosphatidylcholine ($diC_{14:0}PC$) was enhanced 40-fold in the presence of apo C-II (182,187,189,190). Jackson *et al.* expanded on these studies and measured the rate of phospholipid hydrolysis in monolayers containing sphingomyelin:cholesterol (2:1, molar), apo C-II, and 5 mol % unsaturated or saturated phospholipid of varying chain length (191). At low surface pressures, the authors found the highest level of phospholipid hydrolysis with dioleoylphosphatidylcholine ($diC_{18:1}PC$), followed by 1-palmitoyl-2-lineoylphosphatidylcholine ($C_{16:0,18:2}PC$), 1-palmitoyl-2-oleoylphosphatidylcholine ($C_{16:0,18:1}PC$), dipalmitoylphosphatidylcholine ($diC_{16:0}PC$), $diC_{14:0}PC$ and finally distearoylphosphatidylcholine ($diC_{18:0}PC$), displaying values 5-10 times greater for the monosaturated compared to the disaturated phospholipids. Interestingly, the rate of phospholipid hydrolysis was nearly identical for $diC_{16:0}PC$ and dipalmitoylphosphatidylethanolamine ($diC_{16:0}PE$), suggesting no discernible preference for type of phospholipid. At higher lipid packing densities, the difference in hydrolysis rates between saturated and unsaturated lipids was less apparent, thus the authors concluded that no simple dependency between rate of LPL catalysis and phospholipid fatty acyl chain length and saturation/unsaturation appears to exist

(191). These findings are consistent with a previous study showing no systematic relationship between fatty acyl chain length and the rates of the activation energies for hydrolysis in mixed micelles in the presence or absence of apo C-II (190).

The majority of the catalytic activity of lipoprotein lipase is its triglyceride lipase activity. LPL hydrolyzes triglyceride in both chylomicrons and VLDL (192,193) as well as in lipid emulsions (194,195). Early studies reported that LPL triglyceride lipase activity was directed towards emulsified long chain triacylglycerols at maximal rates in the presence of apo C-II or serum (57,196) and is stimulated in the presence of apo C-II and inhibited in the presence of apo C-III, regardless of the presence of phospholipid (180-183).

A study by Wang $et\ al.$ analyzing the substrate specificity of LPL found that medium chain saturated triacylglycerols were better substrates than long or very short chain saturated triacylglycerols, occurring in the following sequence from fastest hydrolysis to slowest: $C_{8:0}$ (tricaprylin) > $C_{10:0}$ (tricaprin) > $C_{12:0}$ (trilaurin) > $C_{6:0}$ (tricaproin) > $C_{14:0}$ (trimyristin) > $C_{16:0}$ (tripalmitin) > $C_{18:0}$ (tristearin) (197). The release of fatty acids from an equimolar mixture of saturated and unsaturated fatty acids demonstrated an LPL preference for unsaturated fatty acids. The relative order of appearance of fatty acids was $C_{18:1}$ (triolein) > $C_{18:3}$ (trilinolenin) > $C_{18:2}$ (trilinolein) > $C_{14:0}$ > $C_{16:0}$ > $C_{18:0}$. A later study examined the effect of LPL triglyceride hydrolysis on mixtures of short, medium and long chain saturated and unsaturated triglycerides. The authors found that LPL acyl chain preference was as follows: $C_{4:0}$ > $C_{6:0}$ > $C_{8:0}$ > $C_{10:0}$ > $C_{12:0}$ > $C_{18:1}$, regardless of whether or not apo C-II was present (198). The presence of albumin, however, did change this order. The

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authors found that albumin has a tremendous inhibitory effect on shorter chain triacylglycerides in contrast to their activation effect on longer chain triacylglycerides, resulting in the following alteration in the preference order: $C_{8:0} > C_{10:0} > C_{4:0} > C_{12:0} > C_{18:1} > C_{6:0}$. Bengtsson and Olivecrona analyzed the effectiveness of the triglyceride lipase activity of LPL against different physical forms of the substrate (199). The authors found that long-chain triglycerides (triolein) were hydrolyzed 2-6 times faster when presented as an emulsion in gum arabic or Triton X-100 than compared to an emulsion with phosphatidylcholine (PC). The highest rate of triglyceride hydrolysis was with a trioctanoin/Triton X-100 emulsion, however, this rate was only approximately 20% higher than without apo C-II, demonstrating that under certain conditions, high rates of triglyceride hydrolysis can be attained without apo C-II. The authors concluded that the activity of LPL is carefully modulated by the physical chemistry of the lipid substrate (199).

It is well known that apolipoprotein C-III is an inhibitor of LPL catalytic activity, however little is known about this mechanism. *In vitro* studies have shown that apo C-III effectively inhibits the LPL-mediated hydrolysis of VLDL triglycerides (200,201) and a 3-fold molar excess of apo C-III reduced LPL activity by 25% (202). It has been suggested that apo C-III displacement of apo C-II from the lipoprotein surface would result in reduced apo C-II activation of LPL (203) and in addition, apo C-III may also act as a direct noncompetitive inhibitor of LPL and thus would require the presence of an apo C-III binding site on LPL (202). Although regions within the N- and C-terminal domains of apo C-III have been implicated in binding to LPL

(200,204), in particular residues 64 and 65 (205), no localization of this site has been elucidated on LPL.

In addition to apo C-III, it appears as though the phospholipid, sphingomyelin, also has inhibitory properties. Sphingomyelin has been shown to strongly inhibit LPL-mediated hydrolysis in monolayers (206) and emulsion particles (207), and it has been suggested that since LPL needs to bind to the lipid surface to hydrolyze TG, sphingomyelin inhibits LPL activity by affecting the membrane structure (208). This conclusion is supported by the finding that sphingomyelin significantly alters the lipid order and packing in reconstituted HDL surfaces (209,210).

Apolipoprotein E has also been implicated in the inhibition of LPL-mediated hydrolysis. Initial studies have demonstrated an inhibitory effect of apo E on triacylglycerol hydrolysis for both plasma VLDL (211) and artificial triglyceride emulsions containing apo E (212,213). More recently, Jong *et al.* were able to demonstrate that VLDL triacylglycerol hydrolysis by LPL is inhibited by apo E in a dose-dependent manner, suggesting that as VLDL becomes enriched with apo E, its suitability as a substrate for LPL is decreased (214).

1.4.4 Regulation of Lipoprotein Lipase

Lipoprotein lipase synthesis and activity has been shown to be regulated by a number of factors, such as transcriptional and post-transcriptional regulation in addition to a number of physiologic regulators like hormones and nutritional state.

1.4.4.1 Transcriptional Regulation of Lipoprotein Lipase

Some of the most compelling studies regarding the transcriptional regulation of LPL have been conducted *in vitro* using pre-adipocyte cell lines (3T3-L1, 3T3-F442A and Ob17) (215). LPL is one of the earliest genes to be activated in the transition from fibroblast to mature adipocytes (216,217), and an Oct-1/OTF-1 protein factor has been identified which recognizes the sequence motif (ATTTGCAT) in the promoter at -46 relative to the transcription start site (218-220). In transient transfection studies, this sequence has been shown to be necessary for the expression of the reporter gene and introduction of mutations within the octamer sequence decreases expression of the reporter by 5-fold (220). An additional regulatory protein, NF-Y, binds to the CCAAT motif located at -65 relative to the transcription start site (218) and together, these sequence elements are sufficient for basal promoter function (219).

Both positive and negative regulatory elements have been identified within the 5' flanking region of the human LPL promoter. Previato *et al.* have localized a single positive (-368 to -35) and single negative (-724 to -565) element to the promoter region (220), however, more detailed analysis by Enerback *et al.* has led to the suggestion that additional regulatory sites may exist up to -4 kb upstream of the transcription start site (221).

Other studies have suggested that *trans-* and *cis*-acting factors, such as LP-α and LP-β, contribute directly to the regulation of tissue specific LPL expression (221).

Recent evidence has shown that estrogen influences the transcription of LPL. Estrogen markedly decreases LPL mRNA in 3T3-L1 adipocytes expressing the estrogen receptor, which was shown to inhibit basal LPL promoter activity by 7-fold (222). In addition, the TGAATTC sequence located at -1850 to -1856 in the promoter region of LPL was determined to be responsible for the suppression of LPL transcription by estrogen (222).

Another regulator of LPL transcription has recently been identified to be the peroxisome proliferator-activated receptors (PPARs). PPARs function as ligand-dependant transcription factors which bind to a peroxisome proliferator responsive element (PPRE) present in the promoter region of the LPL gene and results in increased LPL expression in a variety of tissues, especially adipose (223,224).

The liver X receptors, LXRα and LXRβ, have also been implicated in transcriptional regulation of LPL. Zhang *et al.* showed that mice fed diets high in cholesterol or an LXR-selective agonist exhibited a significant increase in LPL expression in the liver and macrophages, but not in adipose tissue and muscle (225). Analysis of the gene revealed a functional DR4 LXR response element in the intronic region between exons one and two, which directly binds rexinoid receptor (RXR)/LXR heterodimers. The authors also demonstrated that this binding is sufficient for RXR and LXR agonist-induced transcription of the LPL gene.

In light of these and other studies, it is clear that multiple sites exist for simultaneous transcriptional regulation of LPL expression.

1.4.4.2 Post-Transcriptional Regulation of Lipoprotein Lipase

1.4.4.2.1 Regulation of Lipoprotein Lipase by Glycosylation

Post-transcriptional regulatory mechanisms at the protein level, such as glycosylation, have been shown to affect LPL expression and its activity. The requirements for glycosylation have been discussed in detail in a previous section.

1.4.4.2.2 Regulation of Lipoprotein Lipase by Heparin

Once bound on the cell surface on heparan-sulfate proteoglycans, LPL can either be released into the circulation or internalized and degraded within the lysosomal compartment of the cell. This latter pathway provides a rapid mechanism for modulating the plasma level of LPL (226). *In vitro* studies on guinea pig and avian adipocyte (160,227) and guinea pig cardiac (228) models have demonstrated that the presence of heparin in the medium indirectly decreased both the degradation rate of LPL and its intracellular retention time, thus suggesting that heparin has a stabilizing effect with respect to LPL.

1.4.4.3 Physiological Regulators of Lipoprotein Lipase

1.4.4.3.1 Regulation of Lipoprotein Lipase by Nutritional State

The effect of nutritional state on LPL regulation and activity was the first physiologic regulator to be extensively studied. Hollenberg was the first to determine that the activity of LPL in rat adipocytes was 2-fold greater in fed versus

fasted animals and the activity could be increased by including glucose and insulin in the incubation medium (229). Similar studies demonstrated that carbohydrate feeding increased the activity of LPL in rat adipose tissue over 4-fold and this was due to increased levels of extracellular enzyme (230). In addition, the stimulatory effects of glucose and insulin on LPL activity in adipose tissue in fasted rats was able to be completely blocked either *in vitro* or *in vivo* by protein synthesis inhibitors, such as cycloheximide and puromycin (231,232).

LPL expression in cardiac and skeletal muscle appears to be the inverse to that observed in adipose tissue. In these tissues, the relative LPL activity was lower in carbohydrate-fed rats by a factor of 2 or more when compared to fasted animals (233-235), and unlike adipose tissue, the effects of fasting on cardiac LPL levels are not due to changes in either messenger RNA (mRNA) level or rate of protein synthesis (236).

With respect to human studies, LPL levels in adipose tissue of normal or lean individuals (within 20% of ideal body weight) decreased up to 75% with caloric-restriction compared to control levels determined in the same individuals (237-240). Skeletal muscle levels were shown to be variable, depending on the length of caloric restriction (237,239,240). Upon a glucose/insulin infusion or re-feeding, adipose tissue LPL levels increase while skeletal muscle LPL levels decrease (237,239).

Similar studies have been conducted in obese subjects, who exhibit relatively higher LPL levels per adipocyte, compared to lean controls (241-244). Fasting in these subjects resulted in a decrease in both adipose tissue (241,243) and skeletal

muscle (244) LPL expression. In obese individuals who achieved successful weight reduction, adipose tissue LPL expression while fasting remained elevated, suggesting a possible "metabolic-set point" which primed these subjects for continued weight gain (241,243).

Many additional studies have been conducted to assess the contribution of the fed and fasting state on LPL expression and activity, and although each is evaluating a very specific set of circumstances, it is clear that regardless of the subject, LPL levels will be altered in some fashion.

1.4.4.3.2 Regulation of Lipoprotein Lipase by Hormones

Glucocorticoids have been shown to influence the expression of LPL, although their exact effects remain unclear. Many studies have demonstrated that both hydrocortisone and dexamethasone induce adipogenesis in human primary and murine pre-adipocyte cell lines and this is accompanied by an increase in LPL steady state mRNA levels and enzyme activity, consistent with regulation at the transcriptional level (245-250). In contrast, LPL synthesis and mRNA levels were decreased in primary cultures of isolated rat adipocytes (251), as was LPL activity in murine macrophages and the macrophage-like J774 tumour line (252,253). In human THP1 and primary monocyte-derived macrophages, dexamethasone induces the transcriptional activation of LPL and increases LPL activity 4-fold (254-257).

In rats fed ad-libitum, glucocorticoids greatly increased LPL activity in skeletal and cardiac muscle as well as in the lung (258,259), whereas LPL activity and

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mRNA levels decreased in adipose tissue (251,258,260,261). Interestingly, in rats fasted for 24 hours, administration of glucocorticoids resulted in approximately a 50% increase in LPL activity (262).

Treatment of murine adipose tissue by growth hormone has been shown to induce LPL transcription, mRNA levels and enzyme activity over 5-fold (263). Likewise, overexpression of the growth hormone receptor in these cells increases their LPL mRNA levels and LPL activity (264).

The effect of thyroxine on LPL expression and activity has been studied on thyroidectomized (265-267) and hypothyroid rat models. Relative to controls, LPL activity and rate of protein synthesis in the adipose tissue of hypothyroid rats was increased 4.5- and 2.5-fold, respectively, and in brown adipose tissue, LPL activity was increased over 100-fold (268). These changes occurred in the absence of any change in steady state mRNA levels or transcriptional rate (269). Similar results were observed in slow-twitch skeletal and cardiac muscle which had increased in LPL activity of 10- and 5-fold, respectively, without accompanying changes in protein expression or mRNA levels (270). These findings suggest that thyroid regulation of LPL is mediated at the post-transcriptional level (271).

Catecholamines also appear to influence LPL regulation. Treatment of adipose tissue with adrenaline results in a rapid decrease in enzyme activity (272), while injection of norepinephrine decreased LPL activity in rat white adipose tissue whereas its activity increased in brown adipose tissue and cardiac muscle (273). Additional studies have found that *in vitro* treatment of rat adipocytes with adrenaline

resulted in a decrease in both LPL protein synthesis and degradation, without altering either the transcriptional rate of LPL, its mRNA level or the glycosylation of the intact protein (274). In contrast, exposure of rat cardiac myocytes to adrenaline increases the level of LPL activity, glycosylation and overall synthesis, consistent with both translational and post-translational regulation (275).

1.4.4.3.3 Regulation of Lipoprotein Lipase by Inflammatory Cytokines

A variety of inflammatory cytokines have been shown to influence the regulation LPL, such as tumour necrosis factor (TNF), interferon-γ (IFN-γ), interleukin-1 (IL-1) and interleukin-6 (IL-6).

Administration of human TNF to rats resulted in a decrease in adipose tissue LPL activity (40%) without significantly altering cardiac LPL levels (276). Higher doses of murine TNF in rats led to a 60% decrease in adipose tissue LPL activity in addition to increases in cardiac (>40%), liver (>200%) and lung (>35%) LPL activities (277).

IL-1 has been shown to reduce LPL activity in murine adipocytes up to 80% (250,278-283) with a concomitant decrease in LPL synthesis (280). Studies on primary human monocytes, however, demonstrate that IL-1 does not have any effect on LPL mass or activity unless treatment occurs immediately following plating (284).

In murine adipocytes, IFN-γ inhibits LPL activity up to 80% (279,282) and in human macrophages, IFN-γ decreases LPL activity, synthesis and mRNA levels by up to 50% (284,285).

IL-6 administration *in vivo* to mice resulted in greater than a 50% decrease in adipose tissue LPL activity. Similar results were observed *in vitro* using murine adipocytes (286).

1.5 Biochemistry and Genetics of Hepatic Lipase

1.5.1 Hepatic Lipase Gene

The hepatic lipase gene is located on chromosome 15 (15q21) in humans (95,105). Spanning over 35 kb, the gene contains eight introns and nine exons which account for approximately 1.5 kb (84). Two transcription start sites have been described at 43 and 77 nucleotides upstream of the translation initiation codon (287,288) and the 5'-flanking region of the HL gene spans between nucleotides -1550 and +129 (287,289).

1.5.2 Protein Structure-Function

The mature HL mRNA sequence has been deduced from DNA sequence data to be 1.5 kb and the protein is synthesized as a precursor containing a 22 amino acid signal sequence (290). After processing, the mature glycoprotein contains 476 residues with a calculated molecular weight of 53 431 Da (97,291). With glycosylation, however, HL has a molecular mass of approximately 65 kDa (112,292).

HL has four N-linked glycosylation sites at Asn 20, Asn 56, Asn 340 and Asn 376, two of which (Asn 56 and 376) are conserved in LPL. While Wolle *et al.* found that mutating Asn 56 (homologous to LPL Asn 43) resulted in the production of an inactive enzyme which was not secreted (292), Ben-Zeev *et al.* determined that the glycan chain at Asp 56 was not absolutely required because approximately 25% of the HL molecules lacking this chain were active and secreted (112). Further elimination of both glycosylation sites in the N-terminal domain virtually abolished HL activity and secretion. Regardless of these variances, there is no debate over HL requiring glycosylation at Asn 56 for maximal secretion of a functional enzyme and it has been proposed that glycosylation is required for the formation of the appropriate three-dimensional structure (104,112,292). Like LPL, it is possible that this glycan chain may contribute to the formation of functional dimers. This hypothesis is supported by the fact that pancreatic lipase, which does not have a conserved Asn at this site and thus lacks this N-linked glycan chain, is not known to form a higher ordered structure.

Based on the crystal structure of PL, HL is also believed to have a large N-terminal domain (residues 1-329) and a smaller, C-terminal domain (residues 330-476), and evidence suggests that HL is functional as a homodimer. Hill *et al.* demonstrated by radiation inactivation that the target size of functional HL was calculated to be 109 kDa, whereas the size of the structural unit was determined to be 63 kDa (293), indicating that two HL monomer subunits are required for lipolytic activity, consistent with an HL homodimer. These authors previously proposed that LPL exists in a head-to-tail dimer conformation, and believe the same to be true for

HL. This model predicts that the C-terminal domain of one subunit is juxtaposed to the N-terminal domain of the opposing subunit and that the initial interaction of the enzyme with lipid substrates may be facilitated by contact with the C-terminus of one subunit, enabling the catalytic reaction to occur at the active site found within the neighbouring subunit. A schematic of this model is shown in Figure 7.

There are two hydrophobic segments of ten amino acids involved in interactions with lipids; each containing a serine residue (Ser 145 and Ser 267). In humans, Ser 145 is at the centre of a Gly-Xaa-Ser-Xaa-Gly pentapeptide consensus sequence which is part of a classical Ser-Asp-His catalytic triad found in LPL, PL, EL and other esterases (86,291). In HL, this triad is located at Ser 145, Asp 171 and His 257. Davis et al. demonstrated the importance of the serine residue in catalytic functioning by substituting Ser 147 in rat HL for Gly which resulted in an enzyme with virtually no activity towards triolein or tributyrin (294).

Four heparin binding sites are found throughout the entire enzyme with consensus sequences like -B-B-B-X-X-B- or -B-B-X-B-, where B is a basic residue and X is a small, neutral residue. It is likely that these regions are involved in binding heparan sulfate proteoglycans found on the cell surface (291,295). Previous elution studies from heparin-Sepharose columns suggested that mainly the C-terminal domain is involved in heparin binding (148,149), although a more recent study has identified a positive charge cluster (Lys 297, Lys 298 and Arg 300) in the last 50 residues of the N-terminal domain that has been shown to bind heparin-Sepharose (295). Recent modelling work by Rosenke et al. has identified an additional stretch of nine positive residues spanning both the N- and C-terminal

domains of HL (residues 294-334) which is rich in heparin binding consensus sequences, and demonstrated that a synthetic peptide comprising HL residues 304-323 displayed moderate heparin affinity (296).

Like LPL, HL possesses a lid structure which shields a catalytic pocket. Dugi *et al.* demonstrated that the lid mediates substrate specificity by creating chimeras of HL and LPL in which the lids of each other were switched. HL with an LPL lid was more active against triolein (123% of wild-type) whereas it was less active against phospholipids (0-30% of wild-type) (140). Thus, HL developed a substrate specificity that more closely resembled that of LPL. When a similar study was conducted *in vivo*, slightly different results were obtained. Kobayashi *et al.* found that although HL with an LPL lid hydrolyzed phospholipids at a lower rate compared to wild-type HL (70% vs. 32%, respectively), changes in plasma triglyceride concentration were not readily apparent (141).

In order to determine domain specific features, Wong *et al.* created chimeras of HL and LPL where the N-terminal 329 residues of rat HL and the C-terminal 136 residues of human LPL were combined to form one functional lipase molecule (129). The authors found that this chimera exhibited both esterase and lipase activity with catalytic and kinetic properties closely resembling that of native HL. Antibodies to LPL, however, inhibited the lipase activity, suggesting that the catalytic domain of HL is localized within the N-terminus. Further study on these chimeras by Davis *et al.* revealed that the C-terminal domain appears to modulate substrate specificity. The ratio of phospholipase to neutral lipase activity in both chimeric lipases was enhanced by the presence of the heterologous C-terminus, suggesting that this

domain strongly influences substrate specificity (147). Shortly after, Dichek *at al.* created a chimera with the N-terminal domain of LPL and the C-terminal domain of HL. Treatment with an HL antibody abolished the enzyme's ability to hydrolyze triglyceride emulsion but not tributyrin substrates, indicating a role for the C-terminus of HL in long chain fatty acid hydrolysis (148).

1.5.3   Biochemistry

Like lipoprotein lipase, it is well known that hepatic lipase is found bound to the cell surface via HSPG and is released from the vascular bed upon intravenous injection of heparin (175). Immunolocalizing experiments in rats and rabbits expressing human HL have revealed that HL is concentrated at the surface of hepatic sinusoids, mostly located in the microvilli of parenchymal cells with lesser amounts found at the sinusoid endothelium (297,298). HL activity has been localized in the blood vessels of the adrenals and has been shown to be induced by corticotrophin (299), whereas in the ovary, HL is mostly found in the corpus luteum, while very little activity is detected in pre-ovulatory follicles (300). Despite the presence of HL in the adrenals and ovaries, complete HL mRNA transcripts were never able to be detected in these cells, although a truncated form of HL mRNA, missing the first two exons, has been identified within rat adrenals and ovaries. Accordingly, these tissues synthesize a 45 kDa catalytically inactive HL-like protein that is not exported from the cell (301,302). More recently, however, Gonzalez-Navarro *et al.* has demonstrated that full HL appears to be synthesized in rat and human macrophages as well (303); the first such evidence to show that HL is not

synthesized exclusively within the liver. As a result, the current theory is that HL is primarily synthesized in hepatocytes (298,304) and transported in the circulation where it can accumulate in steroidogenic tissues such as the adrenals or gonads (301,304-306).

Hepatic lipase hydrolyzes a wide range of substrates *in vitro* including monoacylglycerol, diacylglycerol, triacylglycerol and phospholipids (307). *In vitro* studies assessing preferred substrates have been mixed – some demonstrating a higher activity towards phospholipid than triacylglycerol (308,309), whereas others report the opposite (310-314). Regardless of these findings, it is well known that HL possesses both phospholipase and triglyceride lipase activity and like lipoprotein lipase, the two important factors which determine its lipase action are 1) the affinity of the enzyme for the substrate droplets and 2) the ability of the enzyme to hydrolyze the substrate lipid of the emulsion particle (315).

In vivo studies have shown that HL is able to efficiently hydrolyze HDL phospholipids which are mainly composed of phosphatidylcholine (316-318) and can also hydrolyze triacylglycerol from triacylglycerol-rich lipoproteins such as chylomicron remnants and IDL (175,319).

Deckelbaum *et al.* demonstrated differences in substrate preference regarding medium and long fatty acid chains. Hepatic lipase hydrolyzed medium chain triacylglycerols (C_{6-12}) at twice the rate of long chain triacylglycerols (C_{16-20}). In addition, differences in affinity were shown in mixed incubations where increasing amounts of long chain triacylglycerol emulsions resulted in decreased hydrolysis of

medium chain triacylglycerol emulsions whereas increasing medium chain triacylglycerol emulsion concentration had little or no effect on long chain triacylglycerol emulsion hydrolysis (320).

Coffill et $al.$ investigated HL preference with respect to diacylglycerol (DG) and triacylglycerol (321). The authors found that of the total fatty acids liberated from HDL_3, only 1% are from triglycerides, 49% are from diglycerides and the remaining 50% is from phospholipids. In recombinant HDL (rHDL) containing 2 molecules of apo A-I, 120 molecules of phospholipid and 20 molecules of TG, 93% of the fatty acids liberated are from phospholipid. Doubling the TG content of these rHDL particles doubled the rate of fatty acid hydrolysis. Further addition of 10 molecules of DG led to no change in the overall rate of hydrolysis, but affected the substrate specificity, resulting in 61% of the fatty acids liberated originating from DG and the rates of TG and phospholipid hydrolysis was significantly reduced (321). These results suggest that even a small amount of DG can affect the rate of TG and phospholipid hydrolysis by hepatic lipase.

Hepatic lipase possesses phospholipase A1 activity, producing 2-acyl lysophosphatidylcholine and 2-acyl lysophosphatidylethanolamine upon hydrolysis of PC and phosphatidylethanolamine (PE), respectively. It was previously reported that HL prefers PE over PC; demonstrating that clearance of injected HDL-radiolabelled phospholipids in rats is much faster for PE than for PC (322) and another study reported calculated V_{max}/K_M values 30-50 fold higher for PE found in HDL subfractions than PC in these same fractions (323). One study that investigated the hydrolysis of different lipid mixtures by rat HL revealed that the

hydrolysis of phosphatidylcholine was activated by the inclusion of small amounts of phosphatidic acid, phosphatidylethanolamine or phosphatidylserine (324). In fact, the molar ratios of these phospholipids which maximally activated phosphatidylcholine hydrolysis closely correspond to the molar ratios found in the surface lipid film of lipoproteins like HDL. Thus, although PC alone is a poor substrate for HL assays *in vitro*, the presence of small amounts of other phospholipids under physiological conditions may be ideal for HL specificity towards phosphatidylcholine.

While the type and proportion of phospholipid appears to be important in HL activity, so too, does the phospholipid packing and phospholipid acyl composition. Tansey *et al.* demonstrated HL catalyzed hydrolysis of different species of PC (palmitoyloleoyl (POPC), dioleoyl (DOPC) and palmitoylarachidonoyl (PAPC), dipalmitoyl (DPPC)) differed depending on whether the rHDL particle was discoidal or spherical in shape. In discoidal rHDL, the authors found that the amount of phospholipid hydrolyzed occurred in the following order: POPC ≥DOPC = PAPC/DPPC, whereas in spherical rHDL, POPC = DOPC ≥PAPC/DPPC (325).

Several studies have reported inhibition of HL catalyzed triglyceride hydrolysis by apo A-I, C-I, C-II and C-III when using triglyceride emulsions and isolated HDL_2 substrates (326,327) and by apo E when using soluble but not heparin immobilized HL (328). Although HL has a high catalytic activity without apolipoproteins, it appears to be stimulated in the presence of apo A-II or apo E under certain conditions. Jahn *et al.* reported stimulation of HL catalyzed hydrolysis by apo A-II using triglyceride emulsions (329,330) in contrast to previous studies

demonstrating inhibition in similar substrates (327,331), and a more recent study by Thuren *et al.* was unable to show stimulation of HL catalyzed hydrolysis of either triacylglycerol or phosphatidylcholine by apo A-II (332). Thuren *et al.* believes it is possible that differences in apo A-II stimulation of HL activity could be due to the various forms of substrate particles in these studies. It has been suggested that an important determinant of the catalytic activity of hepatic lipase is the physico-chemical state of the substrate, which is dependant upon the properties of the substrate particles themselves (307).

Thuren *et al.* have also demonstrated the ability of apo E to activate triglyceride and phospholipid hydrolysis by hepatic lipase; reporting increases in both triacylglycerol emulsion and phosphatidylcholine micelle hydrolysis (332). The phospholipid findings are consistent with a previous study showing apo E induced stimulation of hydrolysis of phosphatidylethanolamine monolayers by HL at low surface pressures (333).

It appears that although HL does not require any specific apolipoprotein to maintain a high level of catalytic activity, apo E may further stimulate triacylglycerol and phospholipid hydrolysis. As such, HL activity is directed towards apo E containing lipoproteins such as chylomicron remnants, VLDL, IDL and HDL_1, making them the preferred substrates for HL.

1.5.4 Regulation of Hepatic Lipase

Several regulatory elements have been identified within the promoter of rat HL, among them responsive elements for cholesterol (SRE), estrogens (ERE), thyroid hormones (TRE), glucocorticoids (GRE) and for cAMP (334). More recently, another motif possibly involved with glucose and/or insulin responsiveness, the proximal E-box, has been described in humans (335). Botma *et al.* have also identified a binding region for Upstream Stimulatory Factor in the promoter region of human HL (336).

1.5.4.1 Regulation of Hepatic Lipase by Cholesterol

It has been suggested that HL is regulated as a function of cholesterol demand, and in cultured hepatoma cells, an inverse relationship has been described between the cell cholesterol content and the levels of HL mRNA and activity (337). Incubation of these cells with an inhibitor of cholesterol synthesis stimulates both HL and HMG-CoA reductase transcription; an effect which is reversed by mevalonate (338).

Regulation of HL by diet has been poorly investigated and thus little is known. In rats, HL activity has been found to be inhibited by diets rich in saturated fats (339) or enriched fish oil (340). Cholesterol-enriched diets have been shown to reduce HL activity and mRNA expression; in one study by approximately 30% (341). These findings support the theory of a feedback mechanism of regulation of HL by cholesterol.

1.5.4.2 Regulation of Hepatic Lipase by Hormones

The regulation of HL expression by hormones has been well established by several clinical studies. HL activity is suppressed in response to native or alkylated estrogens (342), whereas androgens or anabolic steroids increase its activity (343). Consistent with these results is that HL activity is much lower in pre-menopausal women than in men, but rapidly increases after menopause.

Glucocorticoids have also been implicated in the regulation of HL. In rats, corticotrophin-induced hypercorticism resulted in a decrease of hepatocyte HL activity (344), and similar results are observed in humans, where corticotrophin treatment significantly reduced hepatic HL activity (345).

Catecholamines influence changes in HL expression associated with feeding and fasting. For instance, adrenaline has been shown to reduce HL secretion from hepatocytes as a result of post-translational modifications (346).

HL activity also appears to be influenced by thyroid hormone. It is well documented that hypothyroidism is associated with reduced hepatic lipase activity (347-352), and in patients with progressive coronary artery disease, triiodothyronine (T3) levels and HL activity are reported to be significantly lower than in patients with no progression of disease (353).

Although HL expression is known to be responsive to insulin, this regulation is not clearly understood. HL activity in patients with insulin-dependent diabetes was found to be reduced by 50% (354), while it increased after intraperitoneal

administration of insulin (355). In normoglycemics as well as in Type II diabetes, high insulin levels are associated with high HL activity and low HDL_2-cholesterol (356), consistent with the report of increased HL activity in Type II diabetes (357). In these same cohorts, however, HL activity decreased following provoked hyperinsulinemia (358), making these results difficult to interpret.

1.5.4.3 Regulation of Hepatic Lipase by Heparin

The most dramatic changes in HL expression and activity are observed with heparin, both *in vitro* and *in vivo*. In prolonged incubations, heparin stimulates the recovery of HL activity by 5-8 fold (337). It is speculated that heparin has the ability to stabilize the enzyme, thus enabling escape from endocytosis and secondary degradation. Heparin also stimulates HL transcription and secretion of the mature enzyme (359).

1.6 Biochemistry and Genetics of Apolipoprotein C-II

1.6.1 Apolipoprotein C-II Gene

The gene for apolipoprotein C-II is a member of a 48 kb gene cluster located on chromosome 19 (360,361) that also includes the genes coding for apo C-I and apo E (361-365). Nucleic acid sequence analysis reveals that the size of the gene is 3.3 kb and contains four exons and three introns of 2391, 167 and 298 bases, respectively, (366,367). The first intron is contained within the 5'-untranslated region

of the gene and contains four Alu sequences. The second intron interrupts the codon specifying amino acid -11 of the apo C-II signal sequence whereas the last intron, containing a 38 bp sequence that is repeated six times, interrupts the codon specifying for amino acid +44 of the mature apolipoprotein (367,368).

A 37 bp minisatellite located on the third intron also appears to be present at approximately 60 locations in the genome (366). Upon sequencing, the minisatellite at two additional locations was found to be highly conserved and *in situ* hybridization indicated the loci were clustered in the 19q3.3 band, becoming the first example of a chromosome- and band-specific repetitive element in a mammalian genome (366).

1.6.2 Protein Structure-Function

The human apo C-II amino acid sequence has been deduced both by peptide sequencing (369,370) and by nucleotide sequencing of apo C-II clones (371-374). The mature protein contains 79 amino acids for a molecular weight of 8916 Da and is primarily expressed in the liver and intestine (366,374,375). A noticeable feature of the sequence is the absence of cysteine and histidine residues. Although approximately 30% of the sequence is hydrophobic, plot analysis along the polypeptide chain indicates that there is no exceptionally hydrophobic stretch of sequence (368,376). Although initial model building found a high probability for amphipathic helices between residues 13-22, 29-39 and 44-52 (377,378) thought to be involved in phospholipid binding (379), more recent analysis of apo C-II in the presence of sodium dodecyl sulfate (SDS) by nuclear magnetic resonance has confirmed the presence of three regions of helical conformation (residues 16-36, 50-

56 and 63-77) with the intervening regions demonstrating a more loosely defined helical conformation (380).

The entire apo C-II protein is not required to fully activate LPL. The minimum sequence of apo C-II required for maximal activation was first studied by Kinnunen *et al.* using cyanogen bromide fragments of apo C-II (381). The authors found that the N-terminal fragment (apo C-II$_{1-9}$) and the centre fragment (apo C-II$_{10-60}$) had no effect on the activation of LPL. Only the C-terminal peptide consisting of residues 61-79 resulted in a 4-fold increase in LPL activity whereas native apo C-II increased LPL activity 9-fold. This same C-terminal peptide with the last three residues (Gly-Glu-Glu) removed, decreased its activation ability by greater than 95%. Interestingly, however, an additional peptide comprising apo C-II residues 67-79 demonstrated no ability to activate LPL. A later study substituting a single Lys residue at position 55 by Glu did not lead to the loss of its ability to activate LPL, indicating that this residue is not required for apo C-II interaction with LPL (382). Musliner *et al.*, also studying fragments of apo C-II, demonstrated that the N-terminal 50 amino acids of apo C-II had no ability to activate LPL, whereas the remaining 29 residues in the C-terminus had the same activation properties as native apo C-II (383). More convincing evidence of the importance of the C-terminus of apo C-II in LPL activation was provided by a naturally occurring frame-shift mutation at residue 69 (apo C-II$_{Toronto}$) (384). This mutation resulted in a protein that was unable to act as an LPL activator. In addition, direct involvement of the C-terminus in the interaction with LPL is demonstrated by the finding that the last four amino acids of apo C-II (Lys-Gly-Glu-Glu) competitively inhibit LPL (385), however, this sequence is

not conserved in other species. The canine sequence is Lys-Gly-Asp-Ser (386) and the bovine sequence is Ser-Gly-Lys-Asp (387). These results can be reconciled by noting the similar position of the Lys and Gly residues in all three species, indicating a potential role for these amino acids in LPL activation.

In addition to activation studies, the free energy of binding among various synthetic peptides and native apo C-II has been systematically studied (385). An interesting finding was that although the difference in free energy of binding between apo C-II$_{56-79}$ and apo C-II$_{61-79}$ is only 0.8 kcal, apo C-II$_{56-79}$ is a significantly better activator of LPL than apo C-II$_{61-79}$. This led the authors to the conclusion that even though residues 56-60 contribute little to the overall binding strength in the LPL-apo C-II interaction, this region may be crucial for triggering the conformational change of LPL which enhances its activity (368,385), and although the N-terminus and centre residues do not contribute to LPL activation, the difference in free energy of binding between apo C-II$_{56-79}$ and native apo C-II suggests that these residues contribute to the binding of apo C-II to LPL. Evidence to this effect is supported by MacPhee *et al.* who demonstrated that apo C-II residues 19-39, a putative amphipathic α-helix, bound to small unilamellar vesicles of phosphatidylcholine (388). The authors proposed that the role of this sequence is not only to mediate the binding of protein to a lipid surface, but also to stabilize the lipoprotein complexes by associating with other amphipathic helices within apo C-II and other apolipoproteins.

A lipid binding domain of apo C-II was identified by using the following synthetic fragments: apo C-II$_{56-79}$, apo C-II$_{51-79}$ and apo C-II$_{44-79}$. Although all three peptides have been shown to fully activate LPL, only apo C-II$_{44-79}$ associates with

apo C-II-deficient VLDL. These results taken with the conformational analysis suggest that residues 44-51 represent a lipid binding domain of apo C-II (377). Further study by Olivecrona and Beisiegel revealed that this domain is required for activity of LPL against chylomicrons (389). Another lipid binding domain was recently proposed by Storjohann et al. by analysis of the three-dimensional structure of apo C-II residues 44-79 in the presence of SDS. The authors found the presence of two amphipathic helical domains formed by residues 50-58 and 67-75, separated by a non-helical linker centered at Tyr 63, and concluded that the C-terminal helix, which is better defined and has a larger hydrophobic face than the N-terminal helix, may potentially constitute another lipid binding domain of apo C-II (390). In addition, they propose a new mechanism of LPL activation in which both helices remain lipid bound, while the seven residue linker extends away from the lipid surface in order to project Tyr 63 into the apo C-II binding site of LPL.

The specificity of the lipid binding domain of apo C-II was studied by Dahim et al. who measured the preference of apo C-II residues 13-56 for the substrates and products of lipolysis (diacylphosphatidylcholine, diacylglycerol and fatty acid). They found that the capacity of the surfaces to accommodate the peptide decreased with increasing lipid concentration in the interface, indicating competition between lipid and peptide for the interfacial occupancy and concluded that the distribution of apo C-II among lipoproteins depends on their lipid composition (391).

Seven residues within the α-helix spanning residues 59-75 are fully conserved in apo C-II from ten different animal species (392), suggesting a role in LPL activation. Shen et al. mutated each of these residues individually to determine

the importance of each one. Replacement of Tyr 63, Ile 66, Asp 69 and Gln 70 by Ala lowered the affinity of apo C-II for LPL and LPL's ability to be activated. Although most mutants retained some activation ability, replacement of Tyr 63 by Phe or Trp and Gln 70 by Glu resulted in an almost complete loss of activity. The authors showed that all mutants bound to liposomes with similar affinity as wild-type LPL in the absence of hydrolyzable lipids; however, the inactive mutants did not compete with wild-type apo C-II. In an activation assay most mutants retained some activating ability, thus concluding that the productive LPL-apo C-II complex may be dependent on substrate specificity (393).

1.6.3 Lipoprotein Lipase and Apolipoprotein C-II

Although LPL and HL are both members of the same lipase gene family, important differences exist between these two enzymes. As mentioned previously, a fundamental difference is that LPL requires a specific cofactor, apolipoprotein C-II, to obtain maximal hydrolysis against triglycerides in chylomicrons and VLDL (55-58). It is likely that this cofactor requirement serves to prevent expression of the lipase activity at its site of intracellular synthesis. The importance of apo C-II for LPL function is emphasized by the observation of a significant accumulation of triglycerides in patients who have an inherited defect of the apo C-II gene (394).

Currently, the specific amino acids on LPL responsible for activation by apo C-II have not yet been elucidated; however, several studies have narrowed the possible residues to several candidate regions.

Bruin et al. suggested the importance of Lys 147 and 148 in the activation of LPL by apo C-II. Substitution of these basic amino acids with Ala resulted in a decrease in apo C-II activation by 20% compared to wild-type LPL (395). Domain exchange studies in which the N-terminus of HL was replaced with the N-terminus of LPL (LPL/HL), and vice versa, have localized an apo C-II activation site to the N-terminus of LPL (129,147,148), although activation of the LPL/HL chimera was approximately only half that demonstrated by wild-type LPL in the presence of apo C-II, suggesting a potential contribution by the C-terminal domain of LPL. Evidence to this effect was recently provided by Hill et al. who reported that an apo C-II fragment (residues 44-79) was able to be cross-linked to a subdomain chimera consisting of HL residues 1-414 and LPL residues 389-448 (HL-LPLC2), whereas no cross-linking occurred against wild-type HL or another subdomain chimera consisting of HL residues 1-344, LPL residues 331-388 and HL residues 415-476 (HL-LPLC1) (149). In response to activation by apo C-II, HL-LPLC2 was the only chimera to increase its activity, although approximately only 3-fold compared to wild-type LPL at 7-fold. These findings suggest that the final 60 residues of LPL are involved in not only the binding of apo C-II to LPL, but also the activation response of LPL in the presence of apo C-II, and when compared with the previous chimera studies, it was hypothesized that regions within the N- and C-terminal domains of opposing monomers cooperate to enable maximal activation in response to apo C-II.

1.7 The Role of Lipoprotein Lipase in Lipoprotein Metabolism

1.7.1 Lipoprotein Remodelling

The presence of lipoprotein lipase in plasma was first noted in 1943 by Hahn, who observed the elimination of alimentary lipemia in the plasma of dogs given intravenous injection of heparin (396). Later, this phenomenon was attributed to the presence of a 'clearing factor' (397). It was initially believed that heparin released an unknown surface-active agent which promoted the physical dispersion of lipids. However, subsequent demonstration that post-heparin plasma from humans with severe hyperchylomicronemia (type I hypertriglyceridemia) did not hydrolyze TG from chylomicrons *in vitro* indisputably linked LPL with this disorder (398). Similarly, the observation that apo C-II deficiency, the activator of LPL, also resulted in type I hypertriglyceridemia (399), provided conclusive evidence of the essential role for LPL in initiating chylomicron catabolism (193).

LPL is widely distributed in the heart, skeletal muscle and adipose tissue. It is located on the vascular endothelium where it binds to the triglyceride rich lipoproteins, chylomicrons and VLDL, where its primary enzymatic role is to hydrolyze their core TG, resulting in remnant particles (165).

The function of LPL is to direct the influx of plasma TG in the form of free fatty acids into peripheral tissues. The free fatty acids from the reaction are released into circulation and form water soluble complexes with albumin, which aids in their delivery to tissues for either storage or energy use (400,401). In fed states, LPL in

adipose tissue is upregulated to enable delivery of FFA to adipose tissue for storage, whereas in times of increased energy demand, such as in fasting states, LPL in muscle is upregulated to provide increased FFA to be used for energy (165). As such, LPL has been referred to as a "gatekeeper" (165) and is an important regulatory step in the directing of fatty acid traffic to fulfill the energy requirements of peripheral tissues in a tissue specific manner (368).

Hydrolysis of the core TG by LPL results in the formation of smaller, more dense remnant particles (chylomicron remnants and IDL) with excess apolipoproteins and phospholipid on the surface of the lipoprotein. This excess surface material is able to be transferred into the HDL pool (59,60), whereas the chylomicron remnants and IDL are primarily taken up by the liver via cellular receptors (61,62) such as LDL-R and LRP.

Although LPL does not directly hydrolyze the TG in HDL, LPL activity nonetheless regulates HDL-C levels in a variety of ways (193). First, as previously mentioned, surface lipids and apolipoproteins are transferred to HDL (59,60). Second, the amount of HDL-C that is exchanged for TG is modulated by the amount of VLDL in the circulation. Thus, by decreasing plasma TG, LPL limits CETP-mediated HDL-C reduction (53). Lastly, by altering the lipid composition in the core of an HDL particle, the catabolic rate of HDL apo A-I is altered. For example, if HDL becomes enriched in TG, an ideal substrate for HL, the TG can be more rapidly removed, resulting in smaller, lipid-poor apo A-I which are rapidly removed from circulation (79,402). In fact, increased fractional catabolic rates of apo A-I and low

HDL are observed in individuals with hypertriglyceridemia (403,404) and when LPL

activity is inhibited (405).

1.7.1.1 Non-Catalytic Function of Lipoprotein Lipase

Aside from its enzymatic function, LPL has been shown to act as a receptor

ligand for lipoprotein removal (Figure 8). LPL interacts with both lipoproteins and

cell surface matrix proteoglycans, thus it has the ability to act as a bridge between

these molecules (168,406,407).

Felts et al. were the first to suggest that LPL was a signal for the removal of

chylomicron remnants in the liver following the observation that LPL molecules

remain associated with chylomicrons after hydrolysis (408). In vitro studies have

shown that LPL increases the binding and degradation of lipoproteins to cells

(409,410) and this function has been shown to be independent of the LDL-R

(410,411). Evidence has also been provided that shows LPL is a ligand for the LRP

(167,168,412,413) and the VLDL receptor (170). Beisiegel et al. demonstrated that

β-VLDL binding to LRP is enhanced 40-fold in the presence of LPL and this process

was significantly reduced in cells that were deficient in heparan sulfate

proteoglycans (167). This process, which is independent of catalytic activity, was

later confirmed by other labs along with localization of the binding region to the C-

terminal domain of LPL (169,414-418). Several studies have also demonstrated that

LPL associated with matrix proteoglycans dramatically increases the association of

both LDL and oxidized LDL with subendothelial matrix (419-422) and studies by

Sivaram et al. and Pang et al. showed this interaction appears to involve an
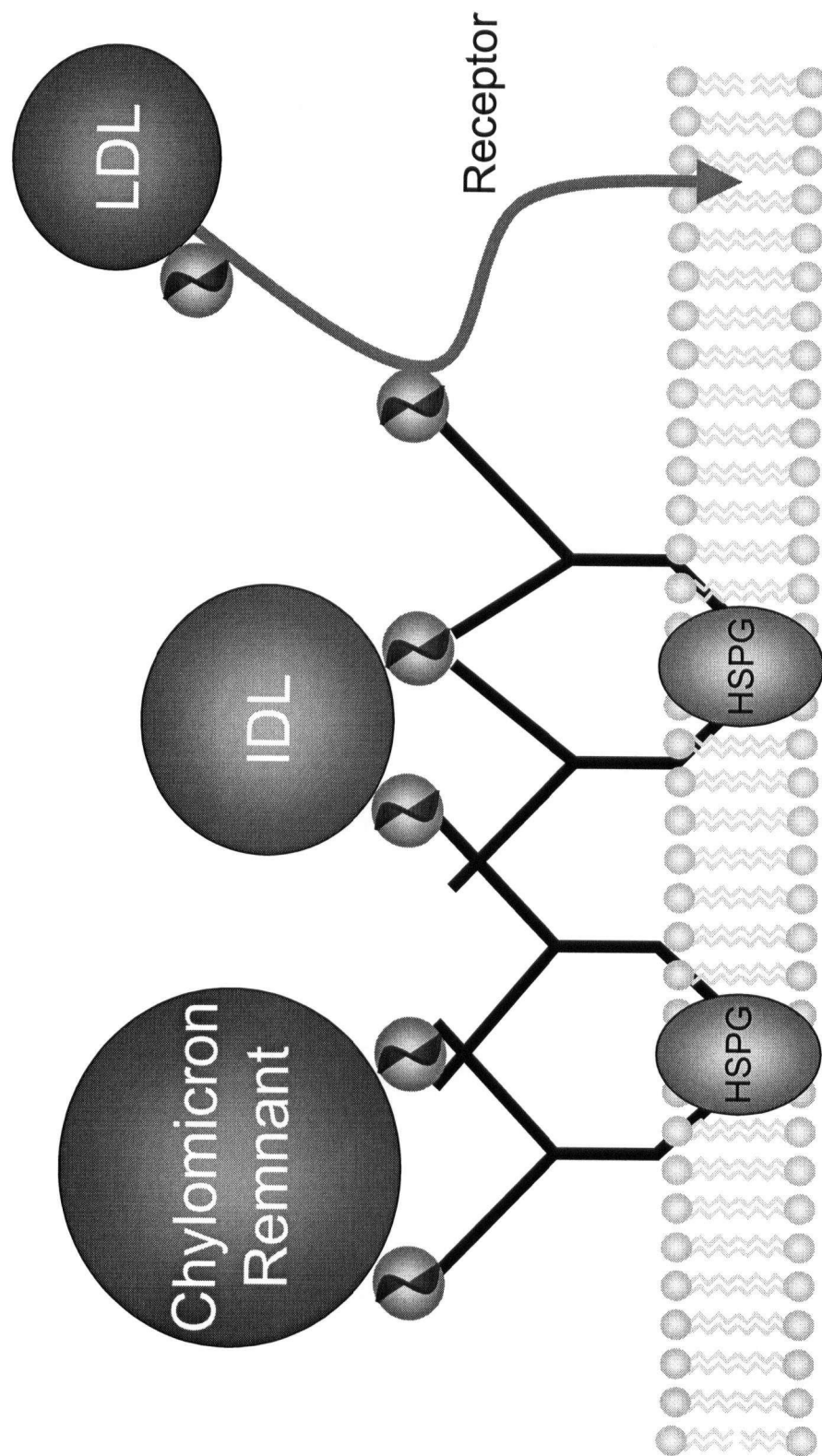
Figure 8. Non-catalytic function of LPL and HL. LPL and HL are believed to influence the catabolism of lipoproteins by two simultaneous processes via its bridging action. The first is receptor mediated endocytosis via a cellular receptor such as the LDL, LRP or VLDL receptors, while the second is believed to involve lipase tethering of the lipoprotein particles to membrane heparan sulfate proteoglycans (HSPG) with concomitant lipid hydrolysis.

association between LPL and the N-terminal 20% in apo B (172,423). In addition, *in vitro* studies on both mouse and human fibroblasts have proposed the direct uptake of lipoproteins through HSPG which may be facilitated through binding of lipoproteins to HSPG via LPL (424,425).

Although the catalytic activity of LPL is not a requirement for its non-catalytic function, Merkel *et al.* have shown that enhanced triglyceride hydrolysis and uptake of whole lipoprotein particles occurs only in the presence of active LPL in the same tissue (426). In addition, the form which LPL takes appears to be important. A study by Pentikainen *et al.* revealed that native LDL bound to LPL monomers whereas oxidized LDL, regardless of the modification, preferentially bound to dimeric LPL (421). Interestingly, however, Krapp *et al.* found that the concentration of monomer in the media correlated negatively with the effect on the uptake of β-VLDL mediated by the dimeric form of LPL (427).

It has now been well established that LPL has three major functions in the metabolism of chylomicrons, VLDL and their remnant particles. First, LPL removes chylomicrons and VLDL from the circulation by tethering the lipoproteins to the vascular endothelium. Second, LPL hydrolyzes the TG in the core of these lipoproteins, and finally, LPL enhances the uptake of the remnant particles by facilitating their binding to various cellular receptors for clearance (428).

1.7.1.2 Selective Uptake of Cholesteryl Ester

Recent evidence has demonstrated an ability of LPL to promote the selective uptake of cholesteryl esters. Panzenboeck *et al.* showed that addition of exogenous LPL to either mouse macrophage control or LPL knockouts resulted in a 2-fold increase in uptake of not only HDL_3 holoparticles, but HDL_3 cholesteryl esters (CE) as well (429). A similar study was conducted by Rinninger *et al.* who found that addition of LPL to human hepatocytes and Hep3B cells enhanced the selective uptake of HDL_3-CE 7-fold (430) and a recent study by Merkel *el al.* found that LPL does not need to be catalytically active to promote CE uptake (426). Rinninger *et al.* also found that HSPG deficiency resulted in a decrease in the LPL-mediated increase in selective CE uptake, whereas LDL-R or LRP deficient cells had no effect. These results suggest that HSPG are important in selective uptake of CE and that this uptake is not mediated through the LDL-R or LRP. Not long after, Seo *et al.* demonstrated *in vitro* and in an *in vivo* mouse model that this selective uptake is not limited to HDL, but also occurs in LDL, independent of SR-BI (431). This finding was confirmed by Rinninger *et al.* who found that although HDL_3-CE selective uptake was increased *in vitro* in the presence of SR-BI, this receptor was not required to mediate this process by LPL (432). At present, the mechanism by which LPL mediates the selective uptake of cholesteryl esters into cells is unknown.

1.7.2 Lipoprotein Lipase Deficiency

Familial LPL deficiency is an autosomal recessive disorder which results in the complete absence of LPL activity in humans. This condition is rare, occurring in

approximately 1 in 1 000 000 individuals (433), however, the rate can be as much as 100-200 times higher (1 in 5000) in certain populations with the founder effect, such as the French-Canadian population in eastern Québec (134,165). When LPL activity is absent, the catabolism of both chylomicrons and VLDL is impaired and massive hypertriglyceridemia occurs (165). Plasma TG levels can vary considerably between individuals. Plasma TG levels are routinely greater than 55 mmol/L (406) and in a French-Canadian patient, TG levels were reported to be as high as 234 mmol/L (433). Hayden *et al.* was the first group to describe mutations in the LPL gene underlying familial chylomicronemia (434). In this condition, there is a dramatic increase in plasma chylomicron levels, resulting in plasma that is milky in appearance (165). Interestingly, chylomicrons do not accumulate indefinitely, but rather reach an eventual equilibrium, indicating the presence of a process which exists for turnover of these particles (134). This mechanism is unclear, but Murthy *et al.* speculate that perhaps the catalytic activity of HL or direct uptake of chylomicrons by the reticulo-endothelial system could play a role (134). The latter part of this suggestion becomes intriguing when considered with additional findings. In individuals with LPL deficiency, LPL mass may be low, normal or even increased, depending on whether the mutation alters LPL structure and production (406). This would suggest that even if LPL catalytic activity is completely abolished, if there is LPL protein present, the non-catalytic function of LPL could still contribute to chylomicron and VLDL uptake.

In addition to the chylomicron elevation, there is a marked decrease in both LDL-C and HDL-C concentrations (165,406,435). Clinical symptoms, which often

present in early childhood, include recurrent abdominal pain, pancreatitis, hepatosplenomegaly, memory loss, lipemia retinalis, eruptive xanthomas, dyspnea and a general failure to thrive (165,406). The expression and degree of severity of these symptoms is somewhat dependent on the TG levels reached (165). At present, the only available treatment for familial LPL deficiency is dietary fat restriction, in particular long-chain fatty acids (134,165).

Heterozygote mutations in the LPL gene result in a partial loss of LPL catalytic activity and are quite common, representing approximately 3-7% in the general population (436-440). A variety of studies have found a 20-62% decrease in post-heparin LPL activity in heterozygotes for LPL mutations (441-447), and individuals with this disorder often display marginally elevated TG levels in the non-stressed state and are frequently asymptomatic (448). Under conditions of stress, however, such as in pregnancy, obesity or diabetes, profound hypertriglyceridemia can be triggered as a result of factors associated with those conditions (144,449). Interestingly, there is significant heterogeneity in the hypertriglyceridemia of carriers, even in subjects carrying the same LPL gene mutation (134). This finding suggests that there are other factors that mediate the effect of the mutation with respect to severity of disease.

Individuals with partial LPL deficiency also have reduced HDL-C and apo A-I levels, indicating a decrease in number of HDL particles. Further analysis by Miesenbock *et al.* and Julien *et al.* revealed that only the particles in the HDL_2 subfraction were affected (446,450). Although many studies report no significant changes in total plasma LDL-C and apo B (441-443,445,451), these authors found

that the LDL particles were more dense in heterozygotes for an LPL mutation (446,447,450).

The lipoprotein alterations found in both homozygotes and heterozygotes for LPL gene mutations are consistent with an increased susceptibility for atherogenesis (436,440). Thus, given the preponderance of LPL mutations in the general population, even a moderate deficiency in LPL activity may represent a significant risk factor for atherogenesis. More detailed analysis regarding LPL activity relating to polymorphisms is covered in the following section.

1.7.3 Lipoprotein Lipase Polymorphisms

More than 70 mutations and functional polymorphisms in the lipoprotein lipase gene coding region have now been described (134,452), mostly causing functional LPL deficiency, and have been identified primarily in patients with familial lipoprotein lipase deficiency (FLLD) (453). The majority of these mutations are rare, although several have a relatively high frequency in specific subpopulations (134,454).

Within the coding region of the LPL gene, three amino acid substitutions have been reported with fairly high allelic frequencies. These are Asp 9 Asn (D9N; G∏A at position 280), Asn 291 Ser (N291S; A∏G at position 1127) and Ser 447 X (S447X; C∏G at position 1595). All of these mutations were identified by single-strand conformation polymorphism and direct sequencing (443,455,456).

1.7.3.1 The D9N LPL Polymorphism

The carrier frequency of the D9N polymorphism has been reported to be approximately 2-7% of the general population, depending on ethnic background (452). Several studies, however, have reported the frequency of this polymorphism to be approximately twice as high in hyperlipidemic patient groups compared to individually matched controls (437,438,443,457). In fact, a recent report on the REGRESS study found that N9 carriers had an increased history of CAD, while in the placebo group, N9 carriers had increased progression of atherosclerosis compared to non-carriers with a calculated risk of 2.16 (437).

The N9 allele appears to be associated with elevated TG. Many studies have demonstrated significantly higher TG levels related to the presence of this allele (443,458-461), although one study with TG levels <4.0 mmol/L did not find an association between the N9 allele and elevated TG (437). This can potentially be explained by TG limits being placed on study participants. Several studies have also found that HDL levels are decreased in the presence of the N9 allele (437,458,460).

LPL activity is also affected by the presence of this polymorphism. Mailly *et al.* found that in a variety of patient groups, seven out of nine carriers, and three out of three control subjects who were carriers, had post-heparin LPL activity below their group mean, representing 15-40% reductions relative to their respective sample means (443). The authors also found that healthy carriers had post-heparin LPL activity and mass reductions by 30% compared to non-carriers. Further analysis revealed no differences in the ratio of monomeric to dimeric LPL and the semi-

purified LPL had similar stabilities and substrate affinities (443), suggesting a reduction in LPL protein expression, and not functionality, as a consequence of this polymorphism.

In vitro studies have confirmed these findings. Zhang et al. found the specific activity of LPL in carrier cells to be the same as for wild-type (462). In addition, stabilities for LPL in carrier cells (443,459,462), along with their affinities for heparin (443,463,464) and cell membrane proteoglycans (462), have been found to be the same as wild-type. A study by Rouis et al. has provided details into the possible mechanism for this finding (463). The authors found that while the cellular mass of LPL in the carrier cells was increased, cellular LPL activity was reduced. This suggests that a larger proportion of LPL may be retained within cells and raises the possibility that LPL in carrier cells is defective in its secretion.

1.7.3.2 The N291S LPL Polymorphism

Although the carrier frequency in the general population for this polymorphism is similar to that for the D9N polymorphism at 2-7% (452), Fisher et al. found no elevation in carrier frequency in either combined hyperlipidemics or other patient groups (455). Reymer et al., however, did find an increase in the S291 frequency in patients with familial combined hyperlipidemia as compared to control, suggesting that S291 may be a predisposing factor for familial combined hyperlipidemia (465). Overall, there appears to be no statistically significant association between the S291 allele and cardiovascular disease. The odds ratio for the association is 1.25,

suggesting only a marginal increase in cardiovascular risk for carriers (439,444,455,466-468).

Like, D9N, the N291S polymorphism appears to be related to elevated plasma TG (455,466) and decreased HDL-C concentrations (457,466), although several other studies have demonstrated no significance between TG (439,457,465,469,470) and HDL-C (455,469,470) levels with the S291 allele.

Both LPL mass and activity *in vivo* has been shown to be significantly reduced (63% and 73%, respectively) in carriers of the polymorphism compared to non-carriers. In addition, the specific activity of S291 was reduced to 76% of wild-type (439).

In vitro studies have found a decrease in dimer and an increase in monomer concentrations (439,462,471), indicating a more rapid dissociation of active LPL dimer to inactive monomer. The half-life of LPL was 145 minutes for wild-type compared to 78 minutes for S291, supporting the hypothesis of an increased dimer dissociation rate (462). When the dimer was measured alone, there was no apparent difference between LPL S291 and wild-type LPL, providing further evidence that the N291S polymorphism affects LPL stability and not functionality.

1.7.3.3 The S447X LPL Polymorphism

While the carrier frequencies of the D9N and N291S polymorphisms are around 2-7%, the frequency for those with S447X is much greater at approximately 20% (452). The C⊓T substitution results in the conversion of a Ser at position 447

to a premature stop codon, which truncates the LPL protein by two amino acids (132). The odds ratio for the association between S447X and cardiovascular disease has been reported to be 0.81 (464,470,472,473), indicating a decrease in CAD risk.

Both total plasma triglyceride levels and VLDL-TG is lower in carriers than controls (464,470,474-476) and it is also associated with lower levels of apolipoprotein-specific subclasses lipoprotein E/B and lipoprotein C-III/B, and apolipoprotein C-III (470).

In vivo data on LPL activity has been limited, but in a study by Peacock *et al.*, no significant association was found between post-heparin LPL activity and carriers of the X447 allele in a group of MI survivors and healthy controls (473). Within the patient group, however, carriers had approximately 25% lower post-heparin LPL activity than non-carriers.

In vitro studies have been mixed, however the trend appears to indicate either unchanged or increased activity and mass in carriers of the polymorphism (132,464,477,478). Measurement of monomeric and dimeric LPL in carrier cells has shown a greater amount of LPL to be in the inactive monomer form, however the specific activity of the dimer is the same as wild-type (464,478). The stability and half-life of dimer LPL are the same in carrier cells as wild-type, indicating that the excess monomer is not due to increased degradation of the LPL dimer, but instead a higher constitutive secretion rate of the monomer (464).

1.7.3.4 The G188E LPL Polymorphism

The G188E LPL polymorphism is due to a transition from a G∏A at position 818 in the LPL gene. Although the frequency of this polymorphism is rare, approximately 0.01-0.3% in control populations, the E188 allele is associated with a significant increase in cardiovascular disease. The odds ratio for this relationship is very large at 5.25, representing greater than a 5-fold increase in risk in individuals expressing this polymorphism (444,479).

The G188E polymorphism is associated with elevated triglycerides (442,445,446,479), lower HDL-C (442,445,446,479) and lower apolipoprotein A-I (479). LPL activity has been shown to be significantly lower in carriers of the E188 allele. In addition, LPL in carriers of this polymorphism has a lower affinity to heparin than wild-type LPL (442).

1.7.3.5 The -93T∏G LPL Polymorphism

In addition to polymorphisms localized to the coding region of LPL, a common mutation has been identified within the promoter of the LPL gene. The -93T∏G transition was first described by Yang et al. (480) in a patient with familial combined hyperlipidemia, and the carrier frequency in healthy Caucasians was subsequently reported to be approximately 1.5-3.5% (481,482), but 18-fold higher in Afro-Caribbeans (482). Caucasians have strong allelic association with the N9 variant, with 83% of individuals expressing one or more of the -93G alleles also carried the N9 allele. This association in Afro-Caribbeans was reduced to only 17% (482).

Triglyceride levels in Caucasians (482) and Afro-Caribbeans (483) expressing -93G/D9 are lower compared to controls, but determining the effect of -93G exclusively on TG has proved difficult due to the high occurrence of this polymorphism with the N9 allele. *In vitro* expression of the -93G allele in rat muscle and human adrenal cells by Hall *et al.* found 24% and 18% increases, respectively, in the activity of the promoter compared to the T allele, which may explain the association of -93G with lower TG levels (482).

Due to the low frequency in Caucasians, the impact of this polymorphism at the population level will not be significant. The case may be different in Afro-Caribbeans who have a frequency that is dramatically higher and most Gs at position -93 occur independently of N9, which may in part explain the lower plasma TG and reduced CAD risk seen in these individuals (484,485).

1.7.4   The Anti- and Pro-Atherogenic Roles of Lipoprotein Lipase

Elucidating the role of lipoprotein lipase in the development and progression of atherosclerosis has presented an interesting paradox. Depending on the level of lipoprotein lipase activity and its site of expression, LPL has the potential to confer either increased or decreased risk towards the development of atherosclerotic lesions.

1.7.4.1 Potential Anti-Atherogenic Effects of Lipoprotein Lipase

In view of the hydrolytic actions of LPL on chylomicrons and VLDL in plasma, LPL is generally considered to be an anti-atherogenic enzyme. The consequence of LPL activity in skeletal and cardiac muscle as well as in adipose tissue is a lipid profile that is driven in a direction that is anti-atherogenic (486-490). *In vivo* studies have demonstrated that LPL overexpression protects wild-type mice against diet-induced hyperlipidemia (486). Clee *et al.* also demonstrated that apo E$^{-/-}$ mice overexpressing LPL only in plasma and not macrophages, had decreased TG and TC as well as significantly decreased lesion size compared to apo E$^{-/-}$ mice with regular LPL expression (491). Overexpression of LPL has also been shown to normalize lipid profiles in apo E and LDL-R knockout mice as well as significantly reduce lesion size (487-489). Heterozygotes for LPL deficiency display a lipid profile that is elevated in TG and decreased in HDL-C levels (436,445,479); a profile that is suggested to be associated with an increased atherogenic risk (492-494). In fact, individuals with LPL polymorphisms that decrease LPL activity have a relative risk of developing CAD that is greater than one (437,444,465,479,495). Conversely, those with the S447X polymorphism, which increases LPL activity, have a relative risk of 0.81, indicating a modest protective effect against CAD (464,470,472,473,496). In addition, Benlian *et al.* recently showed that several LPL deficient patients developed quite severe atherosclerosis (440).

An additional benefit of LPL activity is the production of excess lipoprotein surface material (phospholipids and apolipoproteins) which can be transferred to the HDL pool. Thus, levels of HDL are increased (490), which in turn stimulates reverse

cholesterol transport. HDL has also been shown to inhibit oxidation of LDL by metal ions (497,498).

Finally, the significance of the bridging function of LPL must be taken into consideration. Many studies have demonstrated that LPL associates with lipoproteins and promotes their binding to a variety of receptors as well as HSPG (424,499-502). As a result, there is increased clearance of remnant particles, which have recently been suggested to have atherogenic potential (494,503-506).

1.7.4.2 Potential Atherogenic Effects of Lipoprotein Lipase

The postulate that LPL can increase an individual's atherogenic risk was first put forth by Zilversmit in 1973 (507). He hypothesized that LPL expression in the vessel wall and the resulting hydrolysis of triglycerides in chylomicrons and VLDL would lead to high local concentrations of cholesterol-rich remnant particles. These remnants could then be taken up by the arterial wall and result in cholesterol accumulation.

LPL hydrolysis of triglycerides in chylomicrons and VLDL does result in remnant particles that are smaller in size and rich in cholesteryl ester (505). This may have a detrimental impact considering LPL is expressed by both smooth muscle cells and macrophages, the latter being the primary site of expression in the atherosclerotic lesion (508,509). *In vitro* studies have shown that these remnants are readily taken up by LPL expressing macrophages (510,511) and the free fatty acids released by LPL can be re-esterified by macrophages (512). The culmination

of these actions is the accumulation of cholesteryl esters within the macrophage and their resulting transition to foam cells (510,512,513). In addition, several *in vivo* studies have illustrated the importance of LPL expression in the vessel wall. Semenkovich *et al.* found that heterozygous LPL deficient mice fed an atherogenic diet resulted in a dyslipidemia due to a dramatic increase in non-HDL lipoproteins, however no differences were observed at the site of the lesion (514). This led the authors to suggest that the detrimental effects of dyslipidemia may be influenced by possibly positive effects of decreased LPL expression in the vessel wall. Similarly, Clee *et al.* found that lesions in $LPL^{+/-}$apo $E^{-/-}$ mice were significantly reduced compared to $LPL^{+/+}$apo $E^{-/-}$ mice, despite profound dyslipidemia (491). The importance of macrophages in lesion formation was demonstrated by Renier *et al.* who found that macrophages derived from atherosclerosis susceptible mice has basal LPL activity, mass and mRNA levels two to three times higher than macrophages from atherosclerosis resistant mice (515). A recent study by Babaev *et al.* has also shown the influence of macrophage LPL expression on atherosclerosis (516). The authors found that irradiated mice, fed an atherogenic diet, expressing macrophage $LPL^{-/-}$ had lesions that were 55% smaller in surface area than mice expressing macrophage $LPL^{+/+}$ and 45% smaller compared to heterozygous mice. These results taken together suggest that LPL macrophage expression promotes foam cell formation *in vivo* in the presence of an atherogenic diet (517).

The remnant particles that LPL produces may themselves be atherogenic (504,505). Particles the size of these remnants have been found in the vessel wall

and also in atherosclerotic lesions (518,519). In addition, LPL-mediated hydrolysis of VLDL eventually results in LDL; a major contributor to the development of atherosclerosis (520,521). LDL particles are frequently oxidized in the intimal space by free radicals, which increases their uptake into macrophages (520,521). Furthermore, the interaction of LDL and VLDL with LPL is substantially increased upon mild oxidation (522-524). Oxidized LDL also has the ability to act as a chemoattractant for monocytes (525) and mediate endothelial cytotoxicity (526).

Again, the significance of the bridging function of LPL must be considered. This ability appears to be both anti- and pro-atherogenic; depending on the tissues in which LPL is expressed. Although the bridging in tissues like adipose tissue and muscle is thought to be anti-atherogenic, this function in macrophages and smooth muscle is quite likely detrimental. Not only does LPL enhance the binding of lipoproteins to a variety of receptors, LPL has also been shown to act synergistically with sphingomyelinase in the lesion to enhance the association of LDL with Lp(a) to the vascular wall (527). Thus, the presence of LPL in the vessel wall results in increased retention and accumulation of lipoproteins in the subendothelial matrix (419,420,528,529) and in aortic segments (530,531). These trapped lipoproteins are more susceptible to atherogenic modification, and this, along with the cellular uptake of lipolytic products, would promote the transformation of macrophages to foam cells (193,510,512,513,532,533).

The results of these and many additional studies indicate that LPL activity and protein within the vessel wall may indeed contribute to the development and progression of atherosclerotic lesions.

1.8 The Role of Hepatic Lipase in Lipoprotein Metabolism

1.8.1 Lipoprotein Remodelling

Hepatic lipase hydrolyzes both phospholipids and triglycerides in all classes of lipoproteins. An early study by Nicoll *et al.* analyzing the kinetics of VLDL-apo B removal in patients with non-functional LPL revealed an inverse relationship between HL activity and lipoprotein size (534). Goldberg *et al.* demonstrated that inactivation of HL in the cynomolgus monkey resulted in defective conversion of IDL to LDL (535) and Demant *et al.* found a complete lack of IDL conversion in humans with HL deficiency (536). This was confirmed by Qiu *et al.* who showed a 6-fold decrease in the conversion of IDL to LDL by HL deficient mice (537).

HL has also been shown to be important in the remodelling of LDL by hydrolyzing LDL triglyceride, resulting in smaller, denser LDL particles which are known to be markedly atherogenic (538-541). Auwerx *et al.* analyzed the LDL particles of HL deficient patients and found them to be more buoyant than control subjects (542). In addition, the authors found the peak density and average diameter of the LDL particles positively correlated with HL activity and these particles contained more TG compared to controls. Jansen *et al.* found that in a population of subjects with CAD, HL activity was lower in those with large, buoyant LDL (543). Other studies have found a similar relationship between HL activity and LDL size (544,545). It has also been suggested that HL modulates the physical and compositional properties of LDL. Zambon *et al.* showed that LDL size and buoyancy were inversely associated with HL activity in both CAD positive and normolipidemic

individuals, and the free cholesterol content of LDL and free cholesterol to phospholipid ratio in LDL also correlated with HL activity in both groups (546).

Through its ability to hydrolyze triglycerides and phospholipids, HL is a very important enzyme in the remodelling of HDL particles (547,548), and the hydrolysis of both these components occurs at similar rates (316,317,549). It was first hypothesized by Deckelbaum *et al.* that following depletion of the HDL core TG and CE, HL remodels HDL by increasing the shedding of HDL surface material which is required for the conversion of larger HDL_2 to smaller HDL_3 (550) and discoidal pre β-HDL (551) – an important lipoprotein in reverse cholesterol transport. Recently, studies have shown that overexpression of HL in either wild-type (552) or HL deficient (553) mice greatly reduced HDL-C, phospholipid, TG, apo A-I and apo A-II.

1.8.1.1 Non-Catalytic Function of Hepatic Lipase

Much like lipoprotein lipase, evidence supports a role for hepatic lipase in the metabolism of cellular lipid and lipoproteins, independent of its catalytic activity (Figure 8). Many studies have provided data to show that HL may serve as a ligand for mediating the uptake of lipoproteins by facilitating their interaction with cell surface proteoglycans or receptors (554). HL has been shown to enhance the binding and/or uptake of chylomicrons (555,556), chylomicron remnants (556-559), β-VLDL (555,556,560), LDL (561,562) and HDL (560,561,563) in a variety of cell types.

Both stable and adenovirus-mediated expression studies have provided convincing *in vivo* evidence of the HL-mediated uptake of lipoproteins independent of lipolysis. Dugi *et al.* demonstrated that overexpression of catalytically inactive HL in HL deficient mice enhanced the uptake of HDL (553). Previously, Amar *et al.* conducted a similar study, except the catalytically inactive HL was overexpressed in apo E knockout mice (564). The authors found approximately a 50% reduction in apo B containing lipoproteins, suggesting that HL may facilitate remnant removal that is independent of both lipolysis and apo E, consistent with a previous study by Dichek *et al.* (565). A recent study by Crawford *et al.* of both normal and apo E deficient mice displayed delayed clearance of injected non-HL-lipolyzed chylomicrons while the clearance of HL-lipolyzed chylomicrons was not affected (566). Their observations support previous findings that HL hydrolysis of chylomicrons creates remnants that are rapidly cleared from circulation by the liver. This data also supports the concept that chylomicron remnants can be taken up by the liver by an apolipoprotein E-independent mechanism (566).

The importance of cell surface proteoglycans in the bridging function of HL has been demonstrated by a number of studies. Treatment of HL transfected rat hepatocytes (556,560) as well as primary rat hepatocytes (558) with heparanase abolished most of the HL stimulated uptake of artificial chylomicron remnants (558), β-VLDL (556) and HDL-CE (560). It has been suggested that proteoglycans may facilitate this process by promoting the binding of lipase-lipoprotein complexes with various cellular receptors (554). HL has been shown to directly interact with LRP via its C-terminus (555), and the cellular internalization and degradation of HL mediated

by LRP requires cell surface proteoglycans (567). Ji *et al.* have shown that HDL uptake by rat hepatocytes was decreased in the presence of an LRP inhibitor, but not abolished like that seen when HSPG were removed (560). Thus, the authors concluded that HL may mediate the uptake of HDL by two pathways: an HSPG-dependent LRP pathway and an HSPG-dependent but LRP-independent pathway. In addition, the authors found that HL did not need to be catalytically active to mediate HDL binding and uptake. This finding is consistent with previous studies reporting similar results for other lipoproteins (557,558,561). This study also found that in the presence of HL, addition of apo E did not enhance the uptake of HDL (560). This is consistent with an earlier study by the same group that found that addition of apo E to β-VLDL incubated with rat hepatocytes did not enhance binding (556). The LDL-R has also been implicated in lipoprotein uptake mediated by HL. Choi *et al.* showed that Chinese hamster ovary (CHO) cells overexpressing HL reduced HL-mediated uptake of LDL to the level of control in the presence of anti-LDL-R antibodies, thus suggesting no role for LRP in LDL uptake mediated by HL (562). Conversely, a later study by Krapp *et al.* in LDL-R deficient fibroblasts found no contribution of the LDL-R for the HL-mediated uptake of chylomicrons and β-VLDL (555). A study by Komaromy *et al.* confirmed the results found from both of these groups (561). *In vivo* work by Dichek *et al.* found that overexpression of catalytically inactive HL in LDL-R knockout mice resulted in clearance of apo B containing lipoproteins, suggesting a pathway that is independent of the LDL-R (568). A study by de Faria *et al.* where anti-LDL-R antibodies and receptor-associated protein (RAP; an inhibitor of LRP and other members of the LDL receptor

family) were infused into mice resulted in a decrease in remnant removal by 60% (569). Similarly, Rohlmann *et al.* showed that LRP selectively knocked out in the liver of LDL-R deficient mice resulted in accumulation of cholesterol rich remnant particles, indicating a role for LRP in remnant removal (570). An interesting study by Haudenschild *et al.* demonstrated the specificity of LRP in HL-mediated lipoprotein uptake (571). The authors substituted lysine at position 433 of HL to glutamine which has been shown to inhibit the interaction of HL with LRP *in vitro* and expressed this mutant in HL deficient mice. The results showed impaired clearance of remnant lipoproteins but not HDL, suggesting a different role for LRP in the uptake of these two types of lipoproteins (571).

The findings in the above and additional studies provide strong evidence for a three pronged function in the metabolism of chylomicron and VLDL remnants and HDL. First, HL removes remnant particles and HDL from the circulation by binding to them. Second, HL hydrolyzes the TG and phospholipid in these lipoproteins which alters their composition, and finally, HL enhances the uptake of the remnant particles by facilitating their binding to various cellular receptors or HSPG for clearance.

1.8.1.2 Selective Uptake of Cholesteryl Ester

Many studies have recently proposed a role for HL in the selective uptake of cholesteryl esters. Several *in vitro* studies have demonstrated uptake from apo B-containing lipoproteins (572-576), and more specifically, a direct role for HL in HDL-CE uptake (560,561,563,577-579). It has been suggested that hydrolysis of surface

phospholipids by HL may enhance the interaction of HDL apolipoproteins with membrane sites and thus promote selective uptake (577,580). Acton et al. were the first to show that the selective uptake of HDL-CE is through a putative HDL receptor, the scavenger receptor, SR-BI (581), and Lambert et al. demonstrated that embryonal kidney 293 cells expressing HL and SR-BI increased uptake of HDL-CE by 3-fold compared to cells expressing HL alone (582).

In vivo work by Green et al. in 1991 demonstrated selective uptake of cholesteryl esters from rat LDL in rat adrenals, liver, adipose tissue and lung (575). HL is implicated in the uptake in adrenals and liver since these are the major sites of HL expression. The uptake in the adipose tissue and lung, however, is most likely mediated by LPL, which was discussed earlier. Several years later, Wang et al. showed that SR-BI is upregulated in the adrenals of HL deficient mice, and these mice have significantly decreased cholesteryl ester and free cholesterol adrenal stores (583). Lambert et al. studied the uptake of HDL-CE in HL deficient and wild-type mice and found that CE uptake was decreased in HL deficient mice, suggesting an important role for HL in HDL-CE uptake (584). At the same time, Dugi et al. showed that mice expressing catalytically active or inactive HL enhanced plasma clearance of HDL-CE to approximately the same extent, demonstrating that HL activity is not necessary for this process (553). Similar results were shown by Amar et al. who found that the rate of CE selective uptake from VLDL in apo E-deficient mice was the same, regardless of whether the mice were expressing catalytically active or inactive HL (564).

It is clear that HL has an additional role besides its lipoprotein remodelling and ligand functions. The evidence suggests that HL promotes the uptake of cholesteryl esters from a variety of lipoproteins, and in the case of HDL-CE is mediated, at least in part, through the scavenger receptor, SR-BI.

1.8.2 Hepatic Lipase Deficiency

Hepatic lipase deficiency is an extremely rare disorder that has been reported in only six families worldwide (399,585-589), although it has been suggested that its actual prevalence may be higher due to the difficulty in diagnosing the condition (399). Familial HL deficiency was first reported in 1982 by Breckenridge *et al.* who described two brothers with hyperlipoproteinemia (399). HL deficiency typically mimics the raised cholesterol and TG levels found in type III hyperlipidemia, however individuals with HL deficiency do not display the apo E2/E2 genotype that is characteristic of type III hyperlipidemia (349).

Complete HL deficiency is characterized by elevated levels of plasma cholesterol and triglycerides (590). Specifically, an increase in triglycerides and phospholipids in both the LDL and HDL fractions, resulting in more buoyant lipoproteins (591). Apo A-I levels are also elevated, indicating an increase in the number of HDL particles (399,591,592). In fact, plasma HDL-C levels above the 90th percentile have been observed in several patients (399,592). β-VLDL is also present in the plasma, revealing impaired remnant clearance (591). In addition, patients with this condition have impaired conversion of small VLDL to IDL and an almost complete lack of conversion of IDL to LDL (536).

The profound alterations observed in patients with HL deficiency are consistent with not only the lipolytic role of HL, but also the proposed non-catalytic function.

The impact of HL deficiency on the development and progression of atherosclerosis is not completely understood. Demant *et al.* described a HL deficient patient in generally good health with no clinical evidence of central or peripheral vascular disease (536). Conversely, several compound heterozygotes in the Ontario HL Deficient Kindred, the largest and most studied HL deficient population, developed multi-vessel CAD (593), and a recent study by Brand *et al.* described a new mutation in the first intron of the HL gene which resulted in a non-functional enzyme and premature atherosclerosis (594). An interesting study by Huff *et al.* proposes a potential mechanism for the development of atherosclerosis in those with HL deficiency (595). The authors found that HL deficient β-VLDL readily induced cholesteryl ester accumulation in J774 macrophages, however this was not found in individuals with type III hyperlipidemia (apo E2/E2). This led the authors to conclude that this process was mediated by functional apo E3. The above results suggest that HL deficiency predisposes to premature coronary artery disease, but other factors mediate this effect and likely contribute to the severity.

1.8.3 Hepatic Lipase Polymorphisms

Several groups have recently reported an association between variations in plasma biochemical traits and variation in the promoter of the HL gene (596-600). Although four polymorphisms, all in complete linkage disequilibrium, have been

identified in this region, G-250A, C-514T, T-710C and A-763G, the relationship between the two can be found with a C ∏ T nucleotide change located at position -480 or -514, depending on the nucleotide taken as the transcription start site (290). The -514T allele is relatively common, accounting for 15% in white Americans (601). It has been shown that levels of post-heparin hepatic lipase activity vary greatly depending on the presence of a C or T at this position and this polymorphism alone can explain up to 38% of the variability in HL activity (602). -514C homozygotes have the highest post-heparin HL activity, C/T heterozygotes have HL activity decreased by 25% and -514T homozygotes have the lowest HL activity – a 50% decrease compared to wild-type (597,599). The presence of the T allele, as compared to wild-type, decreases transcription of a promoter/reporter construct in murine hepatoma cells (335) and it has been associated with fasting hyperinsulinemia and insulin resistance (603).

The relationship of this polymorphism with HDL-C and apo A-I levels is not as clear and it has been suggested that the association between the -514T allele and HDL-C is a causal relationship between genetic variation in hepatic lipase activity and plasma HDL-C concentrations (601). In the Finnish participants of the EARS study, -514T homozygotes had plasma HDL-C and apo A-I 10% higher than -514T heterozygotes and -514C homozygotes (598). Similar results were seen in Dutch males who had plasma HDL-C levels 15% higher than -514T heterozygotes and -514C homozygotes (597). In contrast, in Finnish males, -514T homozygotes did not have significantly different plasma HDL-C when compared with subjects of other genotypes (599). However, this study was limited to men with HDL-C in the lower

tertile of the Finnish population, thus the relationship between the -514T allele and elevated HDL-C observed in other studies was not apparent in this cohort. More recent analysis by Ji *et al.* found a connection between the -514T mediated decrease in HL activity and rise in HDL-C and TG in men but not in women (559), and these results were contradicted by Hubacek *et al.* who studied this polymorphism in the Czech population and determined that the -514T allele is associated with higher HDL-C values in women but not men, although males with the -514T allele did display higher total cholesterol than non-carriers. Despite these differences with respect to gender, Hokanson *et al.* found that the -514T genotype is associated with sub-clinical CAD in individuals with Type I diabetes, independent of gender (604).

Consistent with the described function for HL, it appears as though the reported increase in HDL-C materializes within larger HDL particles. Grundy *et al.* determined that the HDL-C increase observed in normolipidemic men with the -514T allele was located in the HDL_2 subfraction while the cholesterol content of the smaller HDL_3 subfraction was actually lower than in those with the -514T allele (605). This finding is in accordance with the proposed role of HL in the remodelling of HDL in the reverse cholesterol transport pathway.

In addition to the effect on HL activity and HDL-C, this genetic polymorphism also appears to influence the metabolism of apo B containing lipoproteins. The -514T allele has been associated with an increased buoyancy of LDL (545). Jansen *et al.* also reported that plasma concentrations of lipoproteins containing both apo

C-III and apo B were 25% higher in -514T homozygotes than in -514C homozygotes (606).

Hypothetically, by influencing the concentration and composition of plasma lipoproteins, this polymorphism may in turn influence an individual's susceptibility to atherosclerosis. Jansen et al. reported that the -514T allele was more common in 782 male patients with angiographically documented coronary artery disease than in 316 asymptomatic controls (597). Tahvanainen et al., however, found no association between the -514T allele and coronary artery disease in Finnish men (599), and neither did Cohen et al., who found no significant increase in coronary artery disease risk as a result of the -514T allele in two groups of case and control individuals (601,607). Statistical analysis of Jansen's cohort by Cohen revealed only a slight increase in -514T allele frequency in patients with coronary artery disease, and thus Cohen concluded that while it may be argued that the data support an association between the -514T allele and coronary artery disease, the magnitude of risk attributable to the -514T allele, if any, appears to be very small (601). Recently, however, more evidence has been put forth implicating the T allele with increased CAD. Anderson et al. found a 1.7-fold higher risk of atherosclerosis in homozygote T allele carriers than in homozygote C allele carriers (608) and Dugi et al. found that the T allele was significantly associated with more severe CAD (609). In addition, Fan et al. described in healthy, hypercholesterolemic T allele carriers, a reduction in adenosine-stimulated coronary blood flow – an early sign of coronary dysfunction (610).

Although allelic variations in the HL promoter appear to result in reduced activity of HL along with a decrease in HDL_2-C, the extent of these reductions is still unclear. A recent study by Ordovas *et al.* on participants in The Framingham Study has provided a possible explanation for large variability between individuals (611). The authors found a consistent and highly significant gene-nutrient interaction showing a strong dose-response effect. For example, the T allele was associated with significantly greater HDL-C concentrations only in subjects consuming <30% of energy from fat. Similar gene-nutrient interactions were also found when the outcome variables were HDL_2-C, large HDL subfraction or HDL size. Interestingly, these interactions were observed for saturated and monounsaturated fat intakes, but not polyunsaturated fat (611). The contribution of genetics in modulating the effect of HL activity has also been proposed. Hirano *et al.* have found that subjects with low HL activity exhibited increased CAD only in the presence of low CETP activity (612). Evidence to support this was provided by Jansen who showed that CAD positive men in the REGRESS study were 7.16 times more often carriers of -514T/T and CETP B2/B2 genotype than controls (613).

Thus, it appears that HL activity is not directly causal in the development and progression of CAD. Instead, the modulation of HL activity by other factors such as diet and genetics is only beginning to be understood.

1.8.4 The Anti- and Pro-Atherogenic Roles of Hepatic Lipase

Much like lipoprotein lipase, the influence of hepatic lipase on the development and progression of atherosclerosis may either be beneficial or

detrimental. Hepatic lipase is known to affect the metabolism of both anti- and pro-atherogenic lipoproteins, but its contribution to the development of CAD is unclear.

1.8.4.1 Potential Anti-Atherogenic Effects of Hepatic Lipase

A number of anti-atherogenic effects result from the action of hepatic lipase. As mentioned previously, it has been well established that HL promotes the selective uptake of HDL cholesteryl esters (580,582,584). In addition, the phospholipase and triglyceride lipase activity of HL remodels the HDL such that the particle is more prone to deliver the cholesteryl ester (577,580), as well as forming pre β-HDL (547,548) which is important in reverse cholesterol transport. HL also stimulates the clearing of chylomicron remnants and IDL (555-560). IDL has been shown to be a major determinant of CAD risk (614), thus low HL activity could lead to an accumulation of these particles, increasing the atherosclerotic risk (613).

A variety of *in vivo* studies have demonstrated the beneficial action of hepatic lipase activity in protecting against the development of atherosclerosis. Overexpression of apo A-II in mice has been shown to inhibit HL activity and raise HDL, but these animals suffer from accelerated atherosclerosis (615,616). Busch *et al.* found that HL overexpression in mice resulted in diminished aortic cholesterol content despite a reduction in HDL levels (617). In the Leiden Intervention Trial, subjects with severe CAD were restricted to a vegetarian diet for two years, after which their HL activity was positively correlated with regression of the lesion size (353). In addition, low HL activity has been reported in patients with clinically overt CAD (353,609,618).

Analysis of the common functional variant in the promoter of the HL gene, the -514C→T polymorphism, has yielded interesting information. This polymorphism is associated with up to a 50% reduction in post-heparin HL activity in humans (597,599). Although a variety of studies have demonstrated somewhat mixed results, recent studies have provided evidence to shown that carriers of the T allele are at an increased risk of developing CAD (608-610). Detailed analysis of this polymorphism was previously discussed.

Again, like lipoprotein lipase, the non-catalytic function of HL must not be forgotten. HL has been shown to act as a ligand for the uptake of remnant lipoproteins (556-563). In light of the fact that these lipoproteins have been shown to be atherogenic (504,505), this role for HL is clearly beneficial.

The above results suggest that modulation of HL activity can potentially benefit an individual's atherogenic risk, however, the type and extent of modulation is unknown.

1.8.4.2 Potential Atherogenic Effects of Hepatic Lipase

The actions of hepatic lipase also contribute to atherogenesis. HL has been shown to reduce LDL size, which is known to be atherogenic (538-541). HL also hydrolyzes phospholipids and triglyceride in HDL, thus converting HDL_2 to HDL_3 and affecting the levels of these particles (317,619). HL activity is typically inversely correlated with HDL-C levels resulting in an increase in CAD risk (613). HL has been suggested to be a factor in the development of familial combined

hyperlipidemia, which is characterized by low HDL-C and the presence of small, dense LDL (620). In human conditions associated with increased atherosclerotic risk, HL activity is often high. For example, men have a higher level of HL activity than premenopausal women and HL activity decreases with exercise (621) and increases with smoking (622).

In vivo studies have demonstrated the contribution of HL to atherogenesis. Mezdour et al. showed that HL deficiency in apo $E^{-/-}$ mice led to a smaller plaque size despite an increase in plasma TC (623). Increased HL activity has also been reported in individuals with familial hypercholesterolemia; a condition that greatly enhances CAD risk (624). In addition, in familial hypercholesterolemia homozygotes, a strong inverse correlation is found between HL activity and extent of coronary calcification (625). Although Barth et al. demonstrated increased HL activity with plaque regression (353), Zambon et al. found that treatment of hyperlipidemic CAD patients in the FATS study resulted in a decrease in HL activity that correlated with a decrease in coronary stenosis (626). Interestingly, analysis of the REGRESS participants revealed that HL activity in normolipidemic or slightly hyperlipidemic males with angiographically proven CAD did not differ from age-matched controls (597). HL deficiency is generally associated with increased atherogenic risk, but the number of affected individuals is too low to accurately assess the contribution of HL to CAD (613).

It is interesting to note that HL expression has recently been detected in macrophages (303). At present, no information is available regarding the contribution of macrophage HL expression to the development and progression of

atherosclerosis. Considering the impact of LPL expression in macrophages, however, it is not unreasonable to assume that macrophage HL will also play an influential role in CAD.

Thus, it appears that although HL activity is related to the development and progression of atherosclerosis in humans, the exact nature of this relationship is not clearly understood. As previously discussed, evidence to support the mediating effect of both diet and genetic factors on HL activity has recently been put forth to help explain the variance seen in these studies (611-613).

1.9 Rationale and Hypothesis

Lipoprotein lipase is an enzyme involved in many aspects of exogenous and endogenous lipid metabolism, including hydrolysis of triglycerides in chylomicrons and VLDL, as well as the release of excess phospholipids and apolipoproteins for entry into the HDL pool. Although the initial role of LPL in the development and progression of atherosclerosis was unclear, as the results of more studies are being reported, it is becoming apparent that depending on the level of LPL activity and its site of expression, LPL may confer either increased or decreased risk for the development of atherosclerosis.

Multiple functional characteristics of LPL and HL have been identified, including the catalytic site, surface loop region, heparin affinity and lipid and receptor binding properties, as well as the requirement of two subunits for lipolytic activity.

However, although these enzymes share a number of structural and functional similarities, specific differences in substrate specificity, cofactor requirements and relative heparin affinity distinguish each enzyme. For example, apolipoprotein C-II present on chylomicrons and VLDL is recognized as an activator of LPL, but no specific apolipoprotein activator exists for HL. Currently, there is evidence to indicate that a specific protein-protein interaction occurs between apo C-II and LPL in 1:1 stoichiometry. Specifically, analyses of chimeric lipases suggest that apo C-II interacts with sequences found within both the N- and C-terminal domains of LPL. At present, detailed information regarding the specific structural and functional mechanisms of LPL has not been obtained and the relationship between the two is not well understood. Since the presence of apo C-II is essential for LPL function, the identification of the specific regions within LPL responsible for its activation will provide much needed insight into the catalytic mechanism of this enzyme.

A previous study aimed at providing more detailed analysis of LPL to further localize the region on the N-terminus of LPL which enables it to be activated in the presence of apo C-II, cross-linked an apo C-II fragment (residues 44-79) to bovine LPL. Following chemical hydrolysis by o-iodosobenzoic acid (IBA) and subsequent peptide separation, the investigators found that the apo C-II preferentially bound and cross-linked to a peptide fragment comprised of LPL amino acids 65-86, effectively narrowing the apo C-II responsive residues in the LPL N-terminus to this region (627).

When the amino acid sequence of the peptide fragment was compared to the corresponding region of HL, amino acids 77-99, two regions of dissimilarity appeared in the amino acid sequence homology. These regions were a four and seven amino acid segment, comprising LPL residues 65-68 and 73-79.

The hypothesis of the present study is that LPL amino acids 65-68 and 73-79 function to enable activation of LPL in response to the presence of apolipoprotein C-II. It is our intention, that by generating chimeras of LPL and HL, we will be able to further localize and analyze the contribution of these residues to the ability of LPL to be activated by apo C-II.

1.10 Specific Aims

1.10.1 Part I: Creation and Analysis of Hepatic Lipase Chimeras

1. Create a series of chimeras (with 6x His-tag) in which two specific amino acid segments ($HL_{LPL65-68}$ and $HL_{LPL73-79}$) contained within the N-terminal domain of HL are replaced with the corresponding LPL sequences.

2. Transfect the newly formed cDNA constructs into CHO cells in order to express and purify the recombinant enzymes.

3. Purify HL and LPL as well as both chimeras from stably transfected cell lines in order to compare their properties with chimeric enzymes.

4. Create a double chimera ($HL_{LPL65-68, LPL73-79}$) with a histidine tag in which both regions of LPL replace the corresponding segments of HL. This will involve:

   a) mutagenesis

   b) cloning

   c) transfection

   d) purification

5. Conduct biochemical analyses of the chimeric enzymes:

   a) assess lipolytic activity and ability to be activated by apo C-II by using synthetic triolein substrate.

   b) measure specific activity

   c) determine kinetic constants

1.10.2 Part II: Creation and Analysis of Lipoprotein Lipase Chimeras

6. Create a series of chimeras (with 6x His-tag) in which two specific amino acid segments ($LPL_{HL77-80}$ and $LPL_{HL85-91}$) contained within the N-terminal domain of LPL are replaced with the corresponding HL sequences.

7. Transfect the newly formed cDNA constructs into CHO cells in order to express and purify the recombinant enzymes.

8. Create a double chimera ($LPL_{HL77-80, HL85-91}$) with a histidine tag in which both regions of HL replace the corresponding segments of LPL.

9. Conduct biochemical analyses of the chimeric enzymes as previously described for the HL chimeras.

2 MATERIALS AND METHODS

2.1 Construction of Apolipoprotein C-II Activation Site Chimeras

Six chimeras were created that focused on the proposed apolipoprotein C-II activation site of human LPL (residues 65-68 and 73-79) (Figure 9). Three of the chimeras had the HL backbone with the suspected regions of LPL replacing the corresponding sections of HL. These enzymes were designated $HL_{LPL65-68}$, $HL_{LPL73-79}$ and HL_{LPLD}. Conversely, the remaining three chimeras consisted of an LPL backbone with the proposed regions exchanged with the corresponding section of HL. These enzymes were designated $LPL_{HL77-80}$, $LPL_{HL85-91}$ and LPL_{HLD}.

2.1.1 Primers and PCR Amplification

To aid in purification of the enzymes, the chimeras and wild-type HL and LPL were constructed with a 6x histidine tag. cDNA for both wild-type HL and LPL had six histidines added on to the carboxyl-terminal end and these were used as templates for their respective chimeras. The histidine tag was added to wild-type HL and wild-type LPL using primers containing histidine codons. The first PCR consisted of the 5' histidine primer and a 3' flanking primer specific for the vector, PCDNA3 (5'-HL/6XHIS, CAT CAT CAT CAT CAT CAT TGA GAT TTA ATG AAG ACC CA; 3'PRIMER/PCDNA3; and 5'LPL/6XHIS, CAT CAT CAT CAT CAT CAT TGA AAC TGG GCG AAT CTA CA; 3'PRIMER/PCDNA3). The second PCR contained the 3' histidine primer and the 5' flanking primer specific to PCDNA3 (3'HL/6HIS, ATG ATG ATG ATG ATG ATG TCT GAT CTT TCG CTT TGA TG;
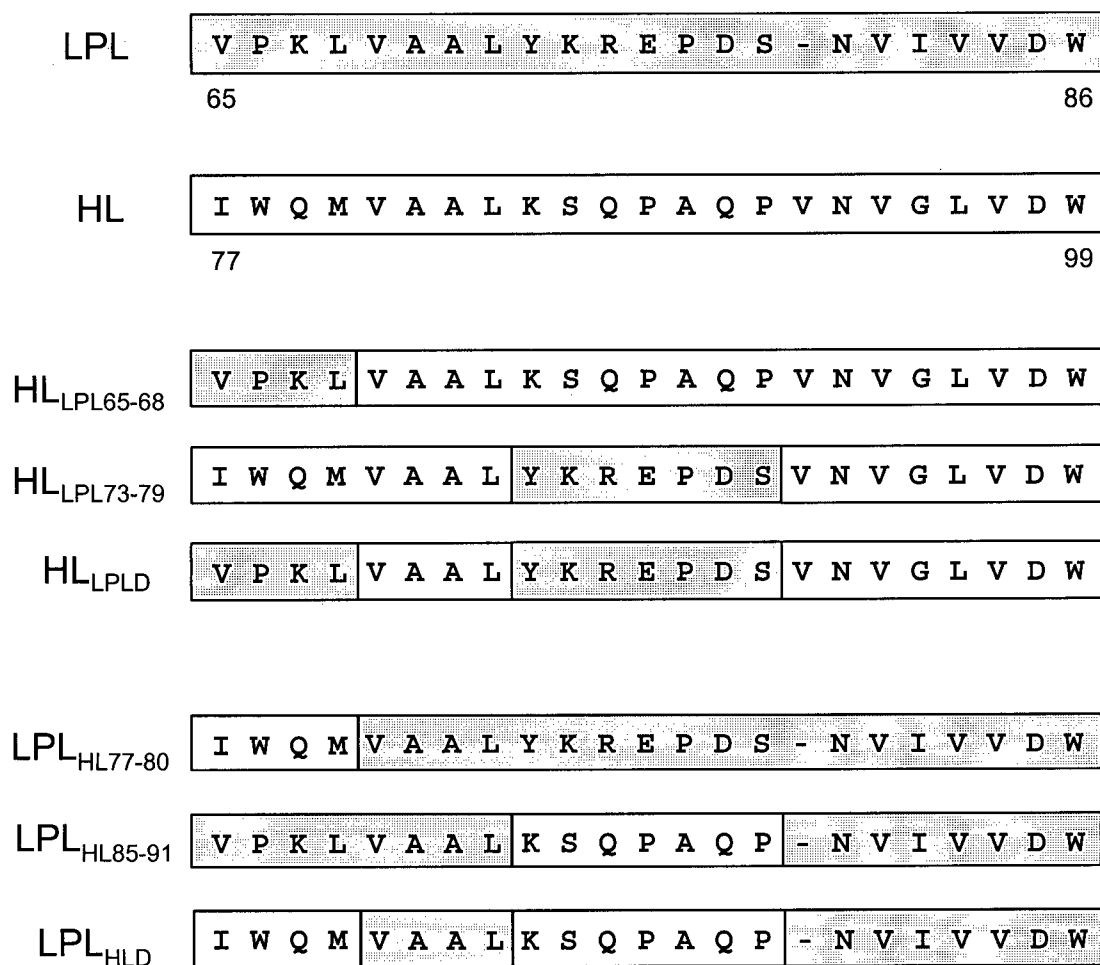
Figure 9. Schematic diagram of HL and LPL constructs identifying LPL candidate regions exchanged with HL sequence. Site-directed mutagenesis was applied to exchange candidate regions of LPL (shaded) with corresponding HL sequence. The newly created constructs are designated $HL_{LPL65-68}$, $HL_{LPL73-79}$ and HL_{LPLD} (HL backbone with LPL regions replacing homologous HL protein sequence) or $LPL_{HL77-80}$, $LPL_{HL85-91}$ and LPL_{HLD} (LPL backbone with suspected regions removed and replaced with homologous HL protein sequence).

5'PRIMER/PCDNA3, AAA TGT CGT AAC AAC TCC GCC; and 3'LPL/6XHIS, ATG ATG ATG ATG ATG ATG GCC TGA CTT CTT ATT CAG AG; 5'PRIMER/PCDNA3). The purified products were joined together in a third and final PCR using the flanking primers 5'PRIMER/PCDNA3 and 3'PRIMER/PCDNA3 for both HL and LPL. For chimeric construction, restriction endonuclease sites were added to primers defining the 5' and 3' termini of the construct to allow for directional cloning. Mutagenic primers (forward and reverse) were designed to span the corresponding boxed coding regions (Figure 9) and overlap with one another so that two PCR products could be combined together in order to form the final full-length cDNA in a third PCR. The primers used for each portion of the chimeras are shown in Table 2.

All PCR reactions contained the same basic reagents using Platinum Pfx polymerase from Invitrogen. Regardless of the chimera being created, the amplification reactions were carried out in final concentrations of 1x Pfx amplification buffer, 1.0 mM MgSO$_4$, 100 ng template DNA, 0.3 mM each dNTP, 0.5 μM each primer and 1.0 unit of Platinum Pfx polymerase made up to a total volume of 50 μl with sterile H$_2$O. The polymerase was added after the sample was heated to a target temperature of 95°C. After this initial heating, the PCR cycle began with the sample being denatured at 95°C for 15 seconds followed by an annealing phase at 55°C for 30 seconds and an extension phase at 72°C. The length of time of the extension phase depended on the length of DNA to be amplified. For the 500 bp segment, the extension time was 1 minute. For the remainder of the DNA, approximately 1200 bp in length, the extension time was 2 minutes. These steps

Name	Sequence
HIND5PKHL	ACT TAA GCT TGC CAC CAT GGA CAC AAG TCC CCT GTG T
1LPLC2HLFOR	AAC TGG GTG CCA AAA CTT GTG GCC GCG CTG AAG
1LPLC2HLREV	GGC CAC AAC TT TGG CAC CCA GTT TTC TAG CAC
BAM3PHL	ACG TGG ATC CAA GGA GTA AGA TTC ATT TAT T
2LPLC2HLFOR	TAC AAG AGA GAA CA GAC TCC GTG ACA GTG GGG CTG
2LPLC2HLREV	CAC GGA GTC TGG TTC TCT CTT GTA CAG CGC GGC CAC CAT
D1LPLC2HLFOR	AAC TGG GTG CCA AAA CTT GTG GCC GCG CTG TAC
D2LPLC2HLREV	CAC GGA GTC TGG TTC TCT CTT GTA CAG CGC GGC CAC AAG
HIND5PKLPL	ACG TAA GCT TGC CAC CAT GGA GAG CAA AGC CCT GCT C
1HLC2LPLFOR	AGT TGG ATC TGG CAG ATG GTG GCC GCC CTG TAC
1HLC2LPLREV	GGC CAC CAT CTG CCA GAT CCA ACT CTC ATA CAT
BAM3PLPL	ACG TGG ATC CGA ATT CAC ATG CCG TTC TTT G
2HLC2LPLFOR	AAG TCT CAG CCG CCC AGC CAA ATG TCA TTG TGG TG
2HLC2LPLREV	ATT TGG CTG GGC CGG CTG AGA CTT CAG GGC GGC CAC AAG
D1HLC2LPLFOR	AGT TGG ATC TGG CAG ATG GTG GCC GCC CTG AAG

Table 2. Primers used for chimera construction.

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were repeated for a total of 30 cycles. A sample picture of these samples on a gel is shown in Figure 10.
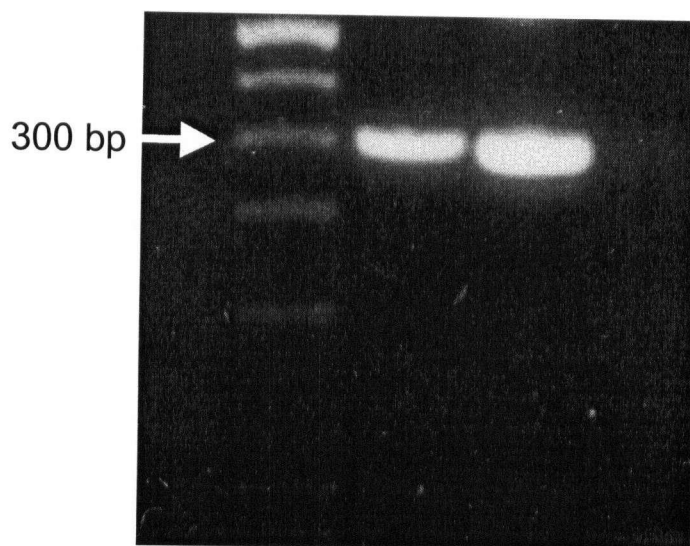
Figure 10. Agarose gel of LPL$_{HL77-80}$ PCR product. Following PCR, the samples were loaded on a 2% agarose gel to determine quantity.

The products were run on a 2% agarose gel, visualized under UV light, excised and purified using the Wizard PCR Preps DNA Purification System (628-630) (Promega).   In this procedure, each agarose slice was transferred to a 1.5 ml microcentrifuge tube and incubated at 70°C until the agarose was completely melted. 1 ml resin was then added and mixed thoroughly. The DNA/resin mix was pipetted onto the Minicolumn and washed with 80% isopropanol. The Minicolumn was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 2 minutes at

10 000 x g. The Minicolumn was transferred to a new tube and 50 μl sterile H_2O was added to the column and incubated for 1 minute. The column was centrifuged for 20 seconds at 10 000 x g to elute the DNA.

The purified products were run on a 2% agarose gel to quantify and were then joined together in a third and final PCR comprised of the above mentioned steps. This product was purified in the same way as the previous samples and again the quantity was determined.

2.1.2 Digestion and Ligation

The final product was digested at the inserted restriction endonuclease sites to enable ligation into a vector. The digestion consisted of the total DNA sample (approximately 50 μl) and final concentrations of 1x *Bam*H1 buffer, 1x BSA, 10.0 units each of *Bam*HI and *Hind*III made up to 60 μl with sterile H_2O incubated at 37°C for 3 hours. The digested sample was run on a 1% agarose gel to determine the quantity and then purified as previously.

The full-length cDNA was then ligated to the pcDNA3 expression vector (Invitrogen, Figure 11) using the Rapid DNA Ligation Kit from Boehringer Mannheim (631-633). The insert DNA was dissolved with the vector, pcDNA3, in a molar ratio of 3:1, in a final concentration of 1x dilution buffer made up to 10 μl with sterile H_2O. 10 μl of 2x T4 DNA ligation buffer was added and mixed, followed by the addition of 1 μl T4 DNA ligase. The solution was mixed and incubated at room temperature for 30 minutes to ensure optimal ligation. The sample was then stored at -20°C.
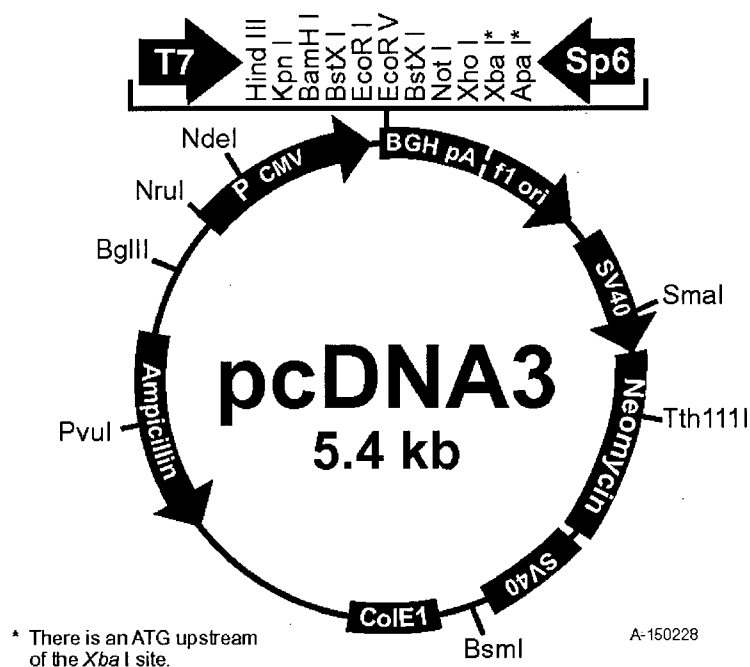
Figure 11. The pcDNA3 vector (634).

2.1.3 Bacterial Transformation and Colony Selection

The procedure used to introduce the plasmids into *Escherichia coli (E. coli)* cells was the One Shot™ TOP10 Competent Cells Kit (Invitrogen). One 50 µl vial of One Shot™ cells was thawed on ice per transformation for a total of eight transformations. 2 µl of 0.5 M β-mercaptoethanol was added to the vial of competent cells and gently mixed. 10 µl of the plasmid mixture was added to the cells and incubated on ice for 30 minutes. The mixture was then incubated for exactly 30 seconds in a 42°C water bath and placed on ice for 2 minutes. 250 µl of

warm SOC medium was added to the vial which was then placed in a shaking incubator at 37°C and 225 rpm for 1 hour. Following incubation, 50 µl of the transformation mixture was plated onto LB agar plates which had been pre-warmed to 37°C and treated with 100 µl of 50 mg/ml ampicillin to enable colony selection. The plates were incubated in a dry incubator in an inverted position at 37°C overnight.

The following day colonies were picked off of the LB agar plates with a pipet tip and smeared on a designated section of a new LB agar plate that had been pre-treated with ampicillin. The tip was then ejected into a culture tube containing 1.5 ml of LB medium and 100 µg/ml ampicillin. This process was repeated for a total of 12 times. The plate was incubated overnight at 37°C whereas the culture tubes were incubated in a shaking incubator at 37°C and 300 rpm.

2.1.4 Plasmid Purification

The plasmids were purified using the protocol supplied by the QIAprep Spin Miniprep Kit (QIAgen). The culture tubes were centrifuged for 10 minutes at 3000 rpm to pellet the bacterial cells. The supernatant was decanted and the pellets were resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. 250 µl Buffer P2 was added and the tubes were gently inverted 4-6 times. The tubes were again gently inverted to mix following the addition of 350 µl Buffer N3. The tubes were then centrifuged for 10 minutes at 10 000 rpm and the supernatant from each was applied to a separate QIAprep column. The columns were centrifuged for 60 seconds at 10 000 rpm and the flow-through was discarded. The columns were

washed by adding 0.5 ml Buffer PB and centrifuging for another 60 seconds after which the flow-through was again discarded. A final wash was performed by applying 0.75 ml Buffer PE to the columns and centrifuging for 60 seconds. The flow-through was discarded and the columns were centrifuged for 60 additional seconds to remove residual wash buffer. The QIAprep columns were transferred to clean 1.5 ml microcentrifuge tubes and the DNA was eluted with 50 µl Buffer EB (10 mM Tris-HCl, pH 8.5).

2.1.5 Selection of Positive Clones

To determine which of the 12 colonies had incorporated the plasmid, the purified plasmids were digested and run on an agarose gel. 8 µl of plasmid DNA was incubated at 37°C for 90 minutes with 10.0 units each of HindIII and BamHI and final concentrations of 1x BamHI buffer and 1x BSA made up to 10 µl with sterile H_2O. The digested plasmids were run on a 0.8% agarose gel to verify the presence of the insert.

2.1.6 Sequencing of Constructs

After verifying the presence of the insert in a particular colony, two different plasmid samples per constructed chimera were sent to the Nucleic Acids Protein Services Unit at the University of British Columbia for sequencing. A report on each sample was returned and the presence of the inserted mutation in the sequence was confirmed for all chimeras. A sample section of the report is shown in Figure 12.
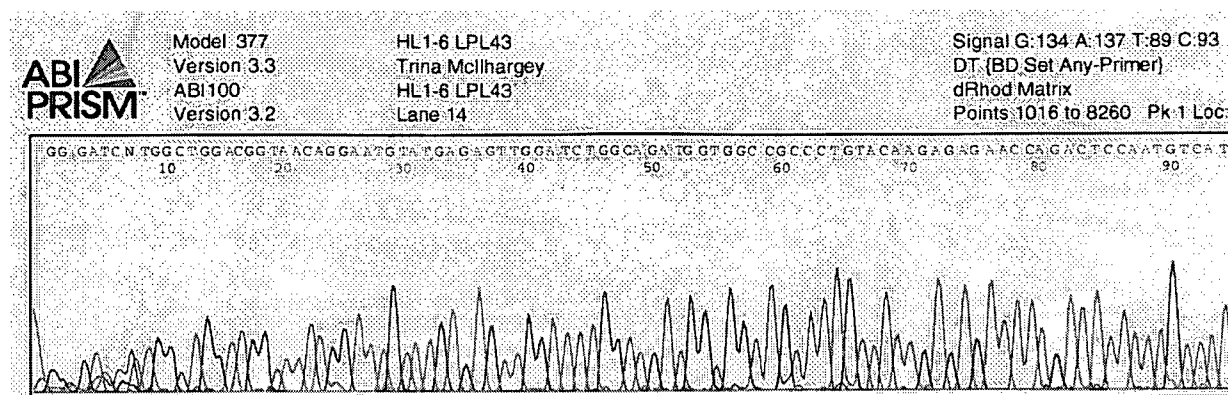
Figure 12. Partial sequencing results of LPL$_{HL77-80}$. Each chimera was sequenced at the Nucleic Acid Protein Services Unit at the University of British Columbia to confirm the inclusion of the mutated region.

2.1.7 Large Scale Plasmid Purification

Upon confirmation of chimera sequence, a significant amount of the plasmid needed to be created for transfection. *E. coli* cells with the confirmed plasmid were removed from the LB agar plate and incubated overnight at 37°C and 300 rpm in a culture tube containing 5 ml LB and 10 µl of 50 mg/ml ampicillin. The following day the cultures were added to 250 ml of LB and again incubated overnight at 37°C and 300 rpm.

The culture was centrifuged for 30 minutes at 3000 rpm and the QIAfilter Maxi Kit (QIAgen) was used to isolate the plasmids. The bacterial pellet was resuspended in 10 ml Buffer P1 followed by the addition of 10 ml Buffer P2. The solution was inverted 4-6 times and incubated at room temperature for 5 minutes.

10 ml Buffer P3 was added to the lysate, mixed and poured onto the QIAfilter cartridge and incubated at room temperature for 10 minutes. The cell lysate was filtered onto a previously equilibrated QIAgen-tip and washed twice with 30 ml Buffer QC. The DNA was eluted with 15 ml Buffer QF which was collected in a 30 ml glass tube and then precipitated by adding 0.7 volumes isopropanol and centrifuging at 15 000 x g for 30 minutes at 4°C. The pellet was washed with 70% ethanol, centrifuged at 15 000 x g for 10 minutes, air-dried and redissolved in 10 mM Tris-HCl, pH 8.5.

2.2   DNA Transfection and Expression

Chinese hamster ovary Pro 5 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics (Gibco-BRL). To mediate the transfection of Chinese hamster ovary cells, a calcium phosphate method was used (635). A transfection mixture was made containing 36 µl of 2 M $CaCl_2$ and 20 µg DNA made to a volume of 300 µl with sterile H_2O. The calcium chloride/DNA mixture was added to 300 µl of 2x Hepes Buffered Saline (HBS) and incubated at room temperature for 30 minutes before being added to a 50% confluent Chinese hamster ovary monolayer. The cells were incubated overnight at 37°C. Stably transfected cells were selected by growth in the presence of geneticin (G418 sulfate; 500 µg/ml) and surviving colonies were selected and expanded. Cell clones expressing maximal quantities of lipase were identified by enzyme activity analysis.

After growth to 85-90% confluency in T-175 flasks, the medium was replaced with OptiMEM (Gibco-BRL) supplemented with 10 units/ml of heparin and 1% antibiotic. The medium was harvested and replaced every 24 h for an eight day period. After centrifugation at 3000 × g for 10 minutes to remove cellular debris, protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma) was added to a final concentration of 0.02 mM and the harvested medium was stored at -80°C.

2.3 Purification of Recombinant Lipases

All purification steps, regardless of the enzyme, were carried out at 4°C. All buffers were degassed prior to use and stored at 4°C.

2.3.1 Purification of Wild-Type Hepatic Lipase & Hepatic Lipase Chimeras

Thawed wild-type HL or HL chimera medium (1 litre) was mixed with NaCl to a final concentration of 0.5 M and applied to an octyl-Sepharose column (2.6 × 25 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.2, containing 0.35 M NaCl. Following a wash with 500 ml of 50 mM Tris-HCL, 0.5 M NaCl, 20% glycerol, protease inhibitor (concentration 0.02mM), pH 7.2 (Buffer A), the lipase was eluted with 500 ml of 50 mM Tris-HCL, 0.35 M NaCl, 20% glycerol, protease inhibitor (concentration 0.02mM), pH 7.2 containing 1.2% Igepal CA-630 (Sigma) onto a heparin-Sepharose column (2.6 × 25 cm). This column was washed with 500 ml of Buffer A prior to elution with 250 ml of 50 mM Tris-HCl, 2 M NaCl, 20% glycerol,

protease inhibitor, pH 7.2 onto a (1 x 10 cm) nickel-nitrilotriacetic acid (Ni-NTA) column (QIAgen). The column was washed with 25 ml of Buffer A before being eluted with 26 ml of 50 mM Tris-HCL, 0.5 M NaCl, 250 mM imidazole, pH 7.2. The eluent was collected in eight fractions, the first one being 5 ml and the rest 3 ml. Each fraction was assayed for activity and the active fractions were concentrated in a Millipore filtration unit (50 kDa MW cut-off) to a final volume of approximately 1 ml and stored at -80°C.

2.3.2 Purification of Wild-Type Lipoprotein Lipase & Lipoprotein Lipase Chimeras

Wild-type LPL and the LPL chimeras were purified in the same manner as the HL chimeras with two exceptions: 1) the octyl-Sepharose step is omitted, therefore the thawed medium is loaded directed onto the heparin-Sepharose column with no NaCl added, and 2) Buffer A had a NaCl concentration of 0.75 M, not 0.5 M.

2.4 Detection of Recombinant Lipases

2.4.1 Electrophoresis

Following each purification, the purified enzyme along with aliquots taken throughout the purification procedure were run on SDS-polyacrylamide (SDS-PAGE) gels for further analysis by either silver stain or western blot.

Samples were mixed with 0.5 volume of buffer containing 2% SDS, 0.1 M Tris-HCl, pH 6.8, 50% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue. The mixture was placed in boiling water for 5 minutes prior to loading onto a 10% acrylamide gel. The gel was run at 30 mA for approximately 80 minutes.

2.4.1.1 Silver Stain

Gels were fixed in 100 ml of 30% ethanol and 10% glacial acetic acid for 30 minutes, and then washed twice with 10% ethanol and three times with deionized water for 5 minutes per wash. The gels were soaked in 50 ml of SilverSNAP™ Stain solution with 1 ml of SilverSNAP™ Enhancer solution (Pierce) for 30 minutes with gentle shaking. The developer was removed and the gels washed with deionized water for 30 seconds. The gels were transferred to 50 ml of SilverSNAP™ Developer with 1 ml of SilverSNAP™ Enhancer for developing until bands appeared. A sample gel is shown on the following page (Figure 13).
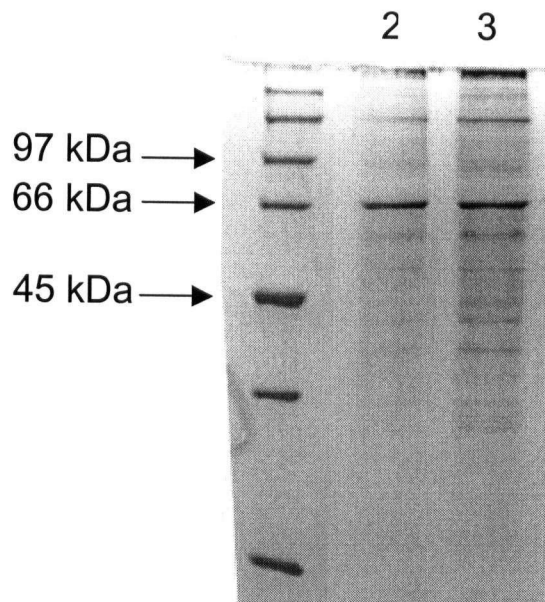
Figure 13. Silver stain analysis of HL$_{LPL65-68}$ and HL$_{LPL73-79}$. All chimeras were separated by SDS-PAGE and silver stained to determine purity. HL$_{LPL65-68}$ is shown in lane 2 and HL$_{LPL73-79}$ in lane 3. The migration pattern of molecular mass standards (in kilodaltons) is indicated on the left.

2.4.1.2 Western Blot

Gels were electroblotted onto a polyvinylidene fluoride (PVDF) hydrophobic membrane (Millipore) which was pre-treated in 100% methanol for 10 seconds. The membrane was placed on filter paper and air dried for 15 minutes. The blot was placed in 15 ml of 1% casein and 0.04% Tween 20 (antibody buffer) containing either a monoclonal antibody specific for human HL (149) or a chicken polyclonal antibody raised against bovine LPL (a kind gift from O. Ben-Zeev) and incubated for 1 h. The blot was then rinsed with PBS (120 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, pH 7.4) and washed for 5 minutes in fresh PBS, then repeated twice. Immunoblotting with the monoclonal or polyclonal antibodies was detected with

either anti-mouse IgG or anti-chicken IgG conjugated to biotin in 15 ml antibody buffer for 20 minutes. After washing, the blot was incubated with streptavidin conjugated to horseradish peroxidase in PBS with 0.1% TX-100 for 10 minutes. The blot was developed with chemiluminescent reagents (Pierce) and exposed to chemiluminescent film (Amersham).

2.4.1.3 Lipoprotein Lipase Enzyme-Linked Immunosorbent Assay (ELISA)

200 µl of 5D2 LPL antibody (a kind gift from Dr. John Brunzell) (441) was added to each well of a Costar High binding EIA/RIA plate at a dilution of 4 µg/ml as previously described (636). The plate was sealed with a mylar plate sealer and incubated at 37°C for 4 hours. The plate was washed 3 times with PBS 0.05% Tween-20. After the third wash 300 µl of PBS/Tween-20 was added per well, the plate was then sealed and left at 4°C overnight. The buffer was removed from the plate and standards (purified bovine LPL (Sigma)) diluted to 0.1 µg/µl in 50% glycerol, 10 mM NaH_2PO_4, pH 7.5, then 4 µl of this solution diluted in 796 µl of 4.56% BSA in PBS to 0.5 ng/µl), controls (heparin-challenge plasma) and samples (LPL, $LPL_{HL65-68}$, $LPL_{HL73-79}$ and LPL_{HLD}) were added to the plate in quadruplicates, 200 µl per well. The plate was sealed and incubated overnight at 4°C. The plate was washed 4 times with PBS/Tween-20, 200 µl per well of 5D2 peroxidase solution (100 ml PBS, 100 µl Tween-20, 50 µl 5D2 peroxidase) was added, the plate was sealed and incubated at room temperature for 4 hours. The plate was washed 5 times with PBS/Tween-20 and in a darkened room, 200 µl per well of OPD substrate (75 ml citrate buffer, pH 5.0, 30 mg OPD tablet (Sigma), 150 µl 3% hydrogen

peroxide) was added, the plate covered and incubated in the dark at room temperature for 10-20 minutes (optical density of highest standard was 0.600-0.800). 50 µl per well of 4 M H_2SO_4 was added to stop color development and optical density read at 492 nm.

2.5   Enzyme Assays

2.5.1   Protein Concentration Assay

Protein concentration was measured by a colorimetric assay developed by Smith *et al.* (637) using a Pierce Micro BCA Protein Assay Reagent Kit (638). Samples were prepared by making up to 1 ml with wash buffer. The BCA Working Reagent (WR) was prepared by combining 25 parts Reagent MA, 24 parts Reagent MB and 1 part Reagent MC. 1 ml WR was added to each sample tube and previously prepared BSA standards. Samples were incubated at 60°C for 60 minutes and their absorbance was measured at 562 nm. A standard curve was prepared to determine the protein concentration in the samples.

2.5.2   Triglyceride Lipase Activity Assay

2.5.2.1 Hepatic Lipase Triglyceride Lipase Activity Assay

Trioleinase activity was measured using a triolein emulsion containing radiolabelled triolein as described by Hill *et al.* (149). The substrate for HL and the

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HL chimeras was made by combining 100 µl 7.5 mg/ml triolein (Sigma), 100 µl 1.0 mg/ml phosphatidylcholine (Sigma) and 50 µCi [^{3}H]triolein (Amersham) which was then dried under nitrogen. 2.1 ml of 0.2 M Tris-HCl, pH 8.8 and 0.4 ml of 1% BSA in 0.2 M Tris-HCl, pH, 8.8 were added and the mixture was sonicated at a 50% pulse for 8 minutes. 0.5 ml of 4% BSA in 0.2 M Tris-HCl, pH 8.8 was added and the substrate was used immediately. Enzyme samples were made up to 100 µl with 50 mM Tris-HCl, pH 8.8 and either 0.15 M or 1.0 M NaCl, pH 8.8, depending on the conditions of the assay. 100 µl of substrate was added to each sample and incubated in a shaking water bath for 60 minutes. The reaction was stopped by the addition of 3.25 ml chloroform:methanol:heptane (1.25:1.41:1) and the phases separated by 1.05 ml of 0.1 M H_3BO_3, 0.1 M K_2CO_3, pH10.5. The tubes were vortexed for 15 seconds and centrifuged for 10 minutes at 1500 x g. A 1 ml aliquot was taken from the upper phase and mixed with 4 ml of ACS scintillation fluid (Amersham) in a scintillation vial and counted to determine radioactivity. Apo C-II dependent lipase activity was determined by performing the assay in the presence of an apo C-II fragment spanning residues 44-79 at a final concentration of 2 µM. This apo C-II fragment has been shown to have the same activating potential as full-length apo C-II (639).

2.5.2.2 Lipoprotein Lipase Triglyceride Lipase Activity Assay

Wild-type LPL and the LPL chimeras were assayed in the same manner as the HL chimeras with two exceptions: 1) the pH of all buffers was 8.2, not 8.8, and 2)

all samples were made up to 100 μl with a buffer of 50 mM Tris-HCl, pH 8.2 and 0.15 M NaCl.

2.6 Kinetic Constants

Kinetic constants were determined using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

2.7 Molecular Modelling

The model of human LPL was generated using as a template the 2.46 Å resolution structure of human pancreatic lipase-colipase complex inhibited by a C11 alkyl phosphonate (640) (PDB access code: 1LPB) which has a 30% homology with human LPL. The model was created using the 3D-JIGSAW algorithm (641) (amino acids 1 through 434 of the mature LPL sequence were modelled) and viewed/analyzed using the Swiss-PdbViewer (642).

3 RESULTS

3.1 Construction of Apolipoprotein C-II Activation Site Chimeras

On comparison of residues 65-86 of LPL with the corresponding region in HL, two regions of dissimilarity were identified (Figure 9). To determine if these sequences were associated with the ability of LPL to be activated by apo C-II, chimeras were created in which the candidate regions of LPL were exchanged with the corresponding HL sequence. The first set of chimeras, designated the HL chimeras, consist of the HL backbone with the suspected LPL regions replacing the corresponding HL sequences. These regions were exchanged individually and together, thus these enzymes are designated HL$_{LPL65-68}$, HL$_{LPL73-79}$ and the double chimeras as HL$_{LPLD}$. The second set of chimeras, the LPL chimeras, is essentially the opposite of the first set. The LPL chimeras have an LPL backbone with the candidate regions of LPL being replaced with the corresponding HL sequences. Again, this was done individually and together, resulting in enzymes with the designation LPL$_{HL77-80}$, LPL$_{HL85-91}$ and LPL$_{HLD}$ (Figure 9).

3.2 Purification and Immunodetection of Chimeras

Following purification of each enzyme, a concentrated sample was run on a polyacrylamide gel (along with samples taken throughout the purification procedure) and silver stained to determine the purity of the sample. In the concentrated sample each HL chimera and wild-type HL showed an intense band at the 65 kDa molecular

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weight standard that was not visible in the starting culture medium. Similar results were seen for the LPL chimeras and wild-type LPL with an intense band that was visible at the 55 kDa standard in the concentrated samples. There was no discernible difference in molecular weight between the chimeras and their respective parental enzymes.

Western blot analysis of all enzymes resulted in a single band at 65 kDa for the HL chimeras and wild-type HL (Figure 14, Panel A) and a single band was visible at the 55 kDa molecular weight marker for the LPL chimeras and wild-type LPL (Figure 14, Panel B). Exchange of the four and/or seven amino acid regions in any of the chimeric lipases appears to have no effect on the molecular weight of the chimeras when compared to the parental enzymes.

3.3 Specific Activity of Recombinant Lipases

3.3.1 Conditioned Medium

The specific activity of all eight enzymes was determined in conditioned medium (Table 3). With the exception of approximately a 10-fold difference between HL and HL_{LPLD}, only modest differences were observed for specific activities among the chimeric enzymes measured within conditioned medium.
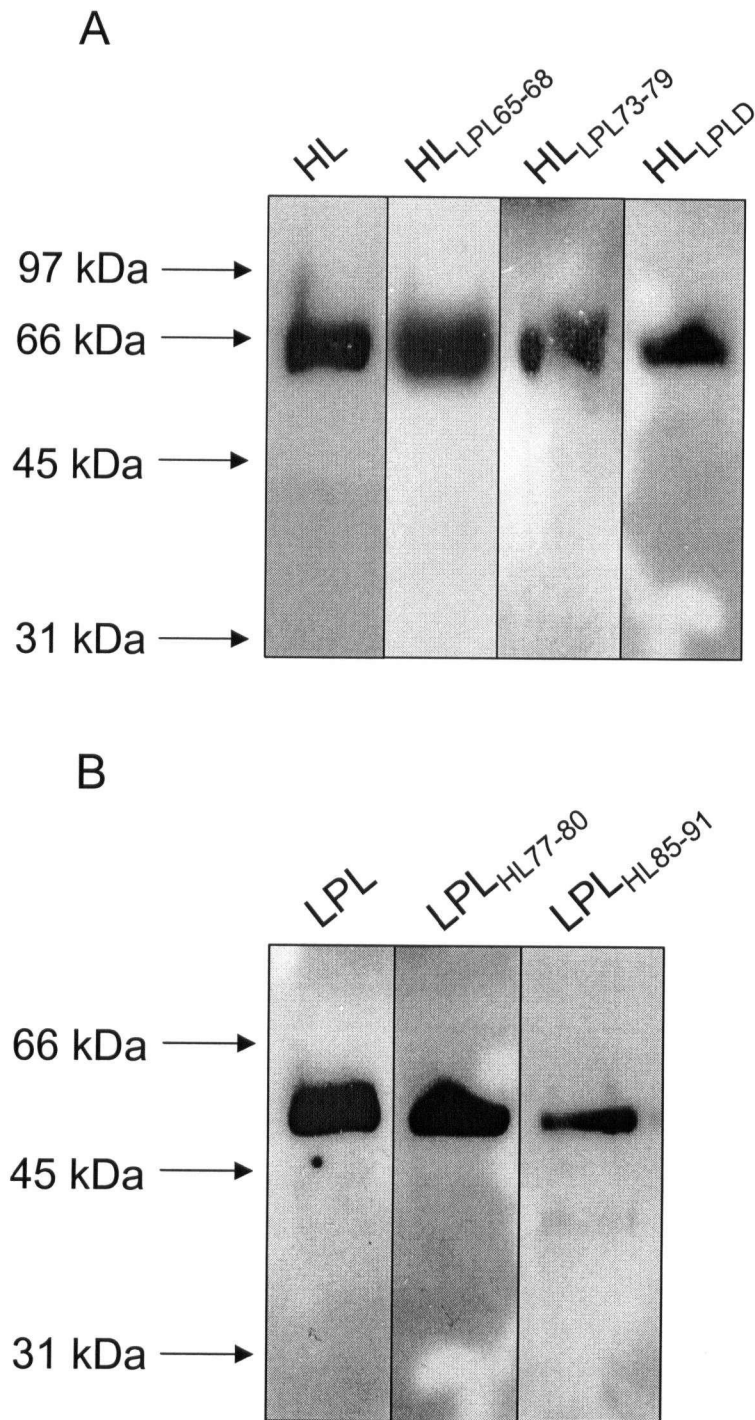
Figure 14. Western blot analysis of wild-type HL, wild-type LPL and chimeric lipases. Wild-type HL and LPL and the chimeras were separated by SDS-PAGE, electroblotted and probed with either an anti-HL monoclonal antibody (wt HL and the HL chimeras, Panel A) or an anti-LPL polyclonal antibody (wt LPL and the LPL chimeras, Panel B). The migration pattern of molecular mass standards (in kilodaltons) is indicated on the left.

	Specific Activity – Medium (nmol/min/mg)	Specific Activity – Purified (nmol/min/μg)	Specific Activity – LPL Mass (nmol/min/μg)
HL	13.37 ± 0.18	4.10 ± 0.03	-
HL$_{LPL65\text{-}68}$	5.15 ± 0.32	0.78 ± 0.21	-
HL$_{LPL73\text{-}79}$	12.11 ± 0.33	0.32 ± 0.16	-
HL$_{LPLD}$	1.60 ± 0.08	0.08 ± 0.01	-
LPL	6.65 ± 0.14	5.81 ± 0.43	35.64 ± 9.20
LPL$_{HL77\text{-}80}$	5.12 ± 0.12	0.41 ± 0.01	7.31 ± 0.92
LPL$_{HL85\text{-}91}$	2.28 ± 0.05	0.01 ± 0.001	23.68 ± 7.23
LPL$_{HLD}$	4.24 ± 0.09	ND	5.99 ± 1.85

ND, not determined

Table 3. Specific activity of chimeras. The specific activity of all eight enzymes was calculated from conditioned media, following protein purification and by measuring LPL mass as described in "Experimental Procedures". Each sample was measured in triplicate and the results are reported as the mean ± S.D.

3.3.2 Purified Enzymes

The specific activity of all wild-type and chimeric enzymes was also determined following their purification (Table 3). In contrast to the relatively mild differences apparent while in the conditioned media, much greater differences were seen following purification. While the specific activity of HL was 4.10 nmol/min/μg, the specific activity of the HL chimeras appeared to decrease as more amino acids were substituted. HL$_{LPL65\text{-}68}$ and HL$_{LPL73\text{-}79}$ had specific activities of 0.78 and 0.32 nmol/min/μg, respectively, whereas the specific activity of HL$_{LPLD}$ was 0.08

nmol/min/µg. The LPL chimeras followed a similar trend where LPL specific activity was 5.81 nmol/min/µg and LPL$_{HL77-80}$ and LPL$_{HL85-91}$ were 0.41 and 0.01 nmol/min/µg, respectively. The specific activity of LPL$_{HLD}$ was unable to be determined due to low protein concentration.

3.3.3 Lipoprotein Lipase Mass

To more accurately determine the specific activity of LPL within culture medium, an ELISA method for LPL was applied (Table 3). The specific activity for wild-type LPL was greatest at 35.64 nmol/min/µg, LPL$_{HL77-80}$, 7.31 nmol/min/µg; LPL$_{HL85-91}$, 23.68 nmol/min/µg and LPL$_{HLD}$, 5.99 nmol/min/µg.

3.4 Time Course Stability of Wild-Type and Chimeric Enzymes

To determine if the differences in specific activity were due to changes in the enzyme's catalytic activity or possible instability, enzyme activity was measured over a 24 h time course. Initially, the HL chimeras had approximately 70% the activity of wild-type HL (Figure 15), but lost activity at a faster rate than wild-type HL with only 8% activity remaining compared to 70% for wild-type HL. This suggests that differences in specific activity following purification may be due to potential instability of the HL chimera structure. By contrast, the LPL chimeras had similar initial activities in comparison to wild-type LPL and were more stable than their HL chimera
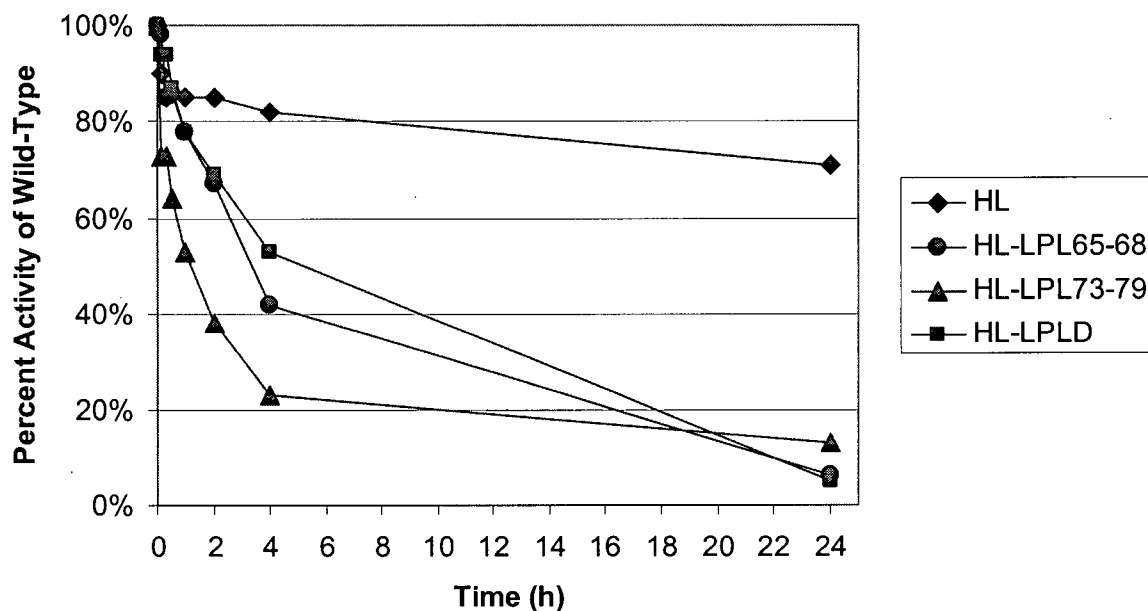
Figure 15. Time course stability of wild-type HL and the HL chimeras.
Conditioned medium from each cell line was collected and samples were taken at 0, 5, 15, 30, 60 min, 2 h, 4 h and 24 h at 4°C. The samples were assayed to determine enzyme activity as described in "Materials and Methods". Each sample was measured in duplicate and the results are reported as the mean.

counterparts with a minimum of 40% activity remaining after 24 hours (Figure 16).

These results suggest that changes in the catalytic potential of the LPL chimeras, in

addition to decreased stability, may have caused the observed reductions in their

specific activity.
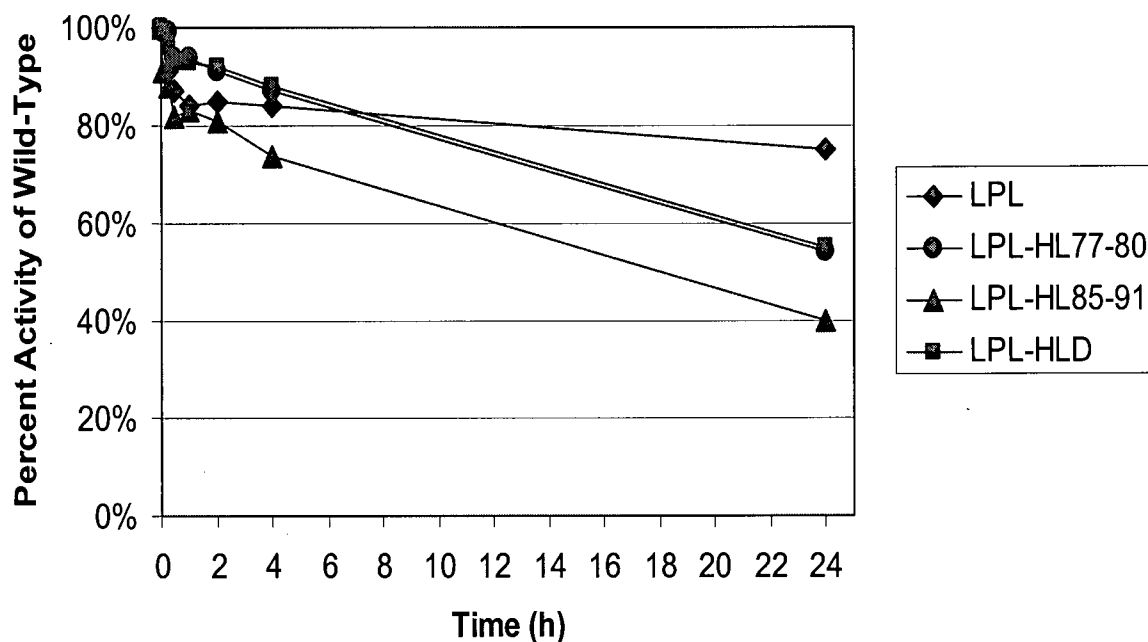
Figure 16. Time course stability of wild-type LPL and the LPL chimeras.
Conditioned medium from each cell line was collected and samples were taken at 0,
5, 15, 30, 60 min, 2 h, 4 h and 24 h at 4°C. The samples were assayed to determine
enzyme activity as described in "Materials and Methods". Each sample was
measured in duplicate and the results are reported as the mean.

3.5 Determination of Kinetic Constants

Due to the decreased stability of the chimeric enzymes, kinetic analyses were
performed using conditioned medium. The kinetic data and apparent K_M and V_{max}
values are shown in Figure 17 and Table 4, respectively. There was very little
variation in the apparent K_M values for both the parental and chimeric enzymes with
values ranging from 176 to 211 nM (Table 4). The low variability in enzyme affinity
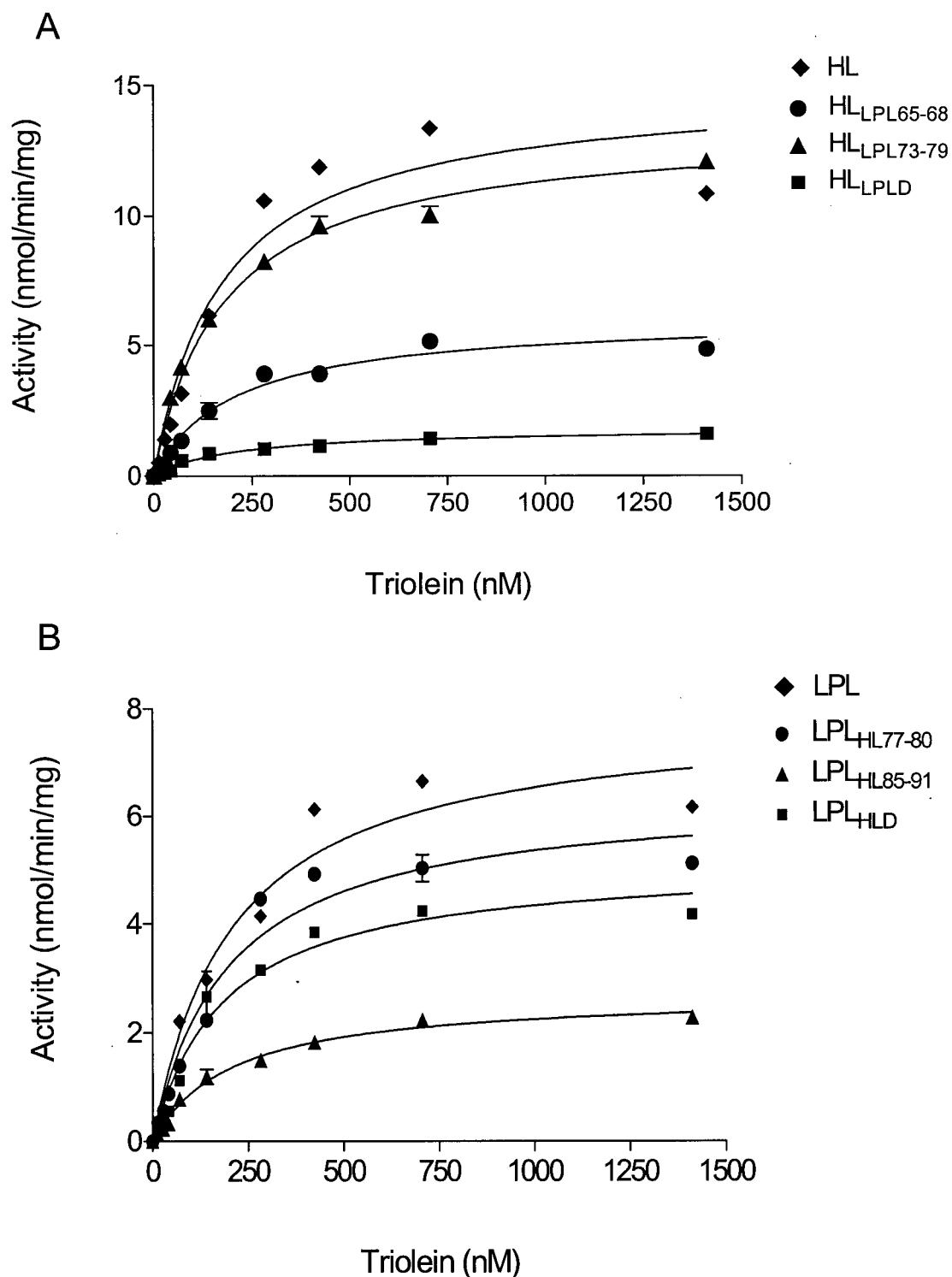
Figure 17. Kinetic analysis of wild-type HL, wild-type LPL and chimeric lipases. Analysis of wild-type and chimeric enzyme kinetics was determined as described in "Materials and Methods". Wild-type HL and the HL chimeras are shown in Panel A and wild-type LPL and the LPL chimeras are shown in Panel B. Each sample was measured in duplicate and the results are reported as the mean.

	K_M (nM)	V_{max} (nmol/min/mg)
HL	176.0	14.91
HL$_{LPL65-68}$	204.3	6.03
HL$_{LPL73-79}$	181.0	13.40
HL$_{LPLD}$	201.9	1.81
LPL	211.7	7.92
LPL$_{HL77-80}$	196.8	6.41
LPL$_{HL85-91}$	203.5	2.70
LPL$_{HLD}$	185.0	5.14

Table 4. Apparent kinetic constants of wild-type HL, wild-type LPL and chimeric lipases. Lipase activity was measured in conditioned media from wild-type and chimeric enzymes using increasing amounts of synthetic triolein, from 0 – 1500 nM, in 0.15 M NaCl and in the presence of apo C-II. Data was analyzed as described in "Materials and Methods". The data are presented as the mean of duplicate measurements.

for substrate suggested minor alteration of key structures involved in substrate binding, despite close proximity to catalytic residues and loop structures (103,125,138,140). By contrast, greater variability was observed in the apparent V_{max} values. The greatest V_{max} value was observed for HL at 14.91 nmol/min/mg but decreased in the HL chimeras as amino acid substitutions were made. V_{max} values for HL$_{LPL65-68}$ and HL$_{LPL73-79}$ were 6.03 nmol/min/mg and 13.40 nmol/min/mg, respectively. The HL$_{LPLD}$ chimera was associated with a substantial decrease in V_{max} of 1.81 nmol/min/mg. As with wild-type HL, wild-type LPL had the largest V_{max} of the LPL enzymes at 7.92 nmol/min/mg. Differences in the V_{max} for the LPL

chimeras were relatively modest with $LPL_{HL77-80}$ at 6.41 nmol/min/mg, $LPL_{HL85-91}$ at 2.70 nmol/min/mg and LPL_{HLD} at 5.14 nmol/min/mg.

3.6   Apolipoprotein C-II Activation of Chimeras

In order to assess the lipolytic activity of the enzymes, lipase assays using synthetic triolein substrate were conducted. Lipase activity was measured under four separate conditions: low salt (0.15 M NaCl) and high salt (1.0 M NaCl) both with and without apo C-II (Table 5). Although chimeras were lower in specific activity compared to wild-type enzymes, it was still possible to determine an activation effect by apo C-II.

3.6.1   Hepatic Lipase Chimeras

Whereas wild-type HL and $HL_{LPL65-68}$ were not activated by apolipoprotein C-II, $HL_{LPL73-79}$ demonstrated a modest increase in activity of approximately 1.7-fold. However, HL_{LPLD} was activated greater than 5-fold in the presence of apo C-II (Table 5).

3.6.2   Lipoprotein Lipase Chimeras

Under the same conditions as above, wild-type LPL had approximately an 11-fold increase in activation in the presence of apolipoprotein C-II and $LPL_{HL77-80}$ was activated nearly to the same extent (Table 5). $LPL_{HL85-91}$, however, was only

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activated approximately 6-fold; about half the activation of its parental enzyme.

Even more compelling data is obtained from LPL_{HLD}. In the presence of apo C-II,

the fold activation of LPL_{HLD} was reduced by two-thirds when compared to wild-type

LPL.

	Low Salt		High Salt	
	-apo C-II	+apo C-II	-apo C-II	+apo C-II
HL	1.0	1.0±0.1	0.8±0.1	0.9±0.2
$HL_{LPL65-68}$	1.0	1.1±0.1	0.7±0.2	0.8±0.2
$HL_{LPL73-79}$	1.0	1.7±1.1	1.0±0.6	1.0±0.6
HL_{LPLD}	**1.0**	**5.0±1.6**	1.4±0.6	2.3±1.7
LPL	1.0	10.9±2.2	0.2±0.2	3.0±2.3
$LPL_{HL77-80}$	1.0	10.1±2.3	0.7±0.1	8.6±3.4
$LPL_{HL85-91}$	**1.0**	**5.7±3.2**	0.5±0.3	1.3±0.8
LPL_{HLD}	**1.0**	**3.6±0.2**	0.5±0.1	1.2±0.1

Table 5. Relative trioleinase activity of wild-type HL, wild-type LPL and chimeric lipases. All enzymes were assayed with synthetic triolein substrate to assess their ability to be activated by apo C-II as described in "Experimental Procedures". Activity was measured under low salt (0.15 M NaCl) and high salt (1.0 M NaCl) conditions, with and without apo C-II (44-79) and is expressed relative to the specific activity of each enzyme in low salt conditions which was assigned a value of 1.0. The data are presented as the mean ± S.D. of three independent measurements in duplicate.

3.7 Molecular Modelling

To understand the relationship of the identified residues with the catalytic triad of LPL, a three-dimensional molecular model was created based on the known structure of human pancreatic lipase (640) (Figure 18). Secondary structure prediction indicated that residues 65-68 and 73-75 (7 of the 11 residues of the apo C-II activation domain) are contained within an α-helix structure (residues 64-75) while residues 76-79 were assigned to random coil. The helix is equivalent to helix α2 in the terminology of pancreatic lipase (100). Tertiary protein modelling places this α-helical region in close proximity to the catalytic pocket (Ser 132, Asp 156, His 241) and its associated loop structures, such as the lid domain (residues 217-238) and the β-5 loop (residues 54-63).
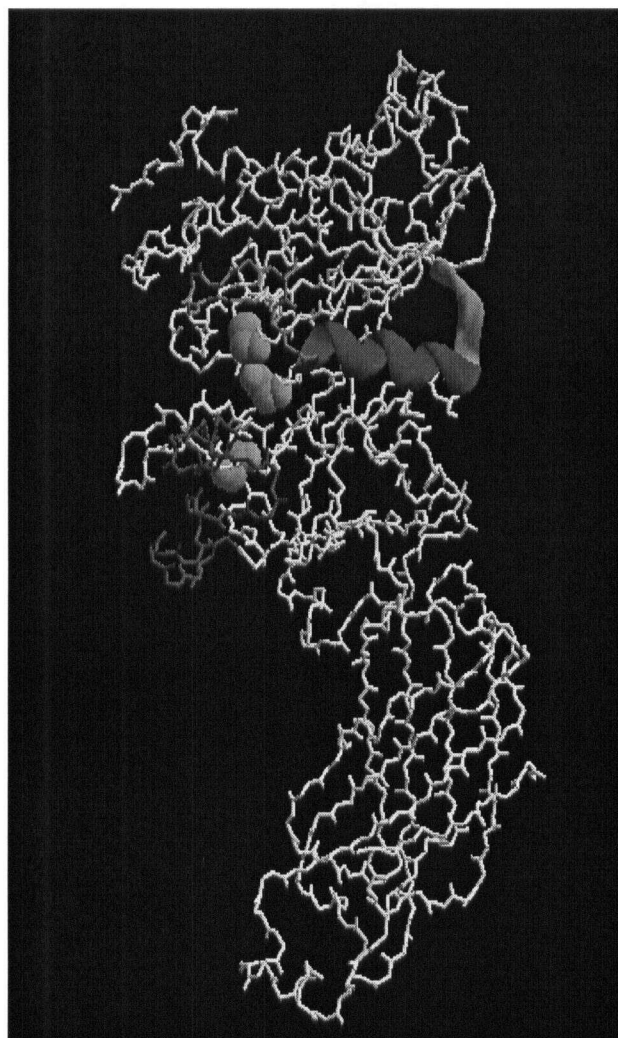
Figure 18. A molecular model of human LPL. Residues 64-79 are displayed in ribbon form with α-helix regions (64-75) displayed in dark gray and random coil (76-79) in light gray. The catalytic triad of LPL (S132, D156 and H241) is space-filled and highlighted in light gray whereas the sequence of loops appear in medium gray (residues 54-63) and dark gray (residues 217-238). The backbone of the remaining residues terminating at 434 is white.

4 DISCUSSION

4.1 Properties of the Recombinant Lipases

4.1.1 Enzyme Stability

It became apparent early in this study that the constructed enzymes were highly unstable and the original purification procedure which was carried-out over four days, and was appropriate for both wild-type LPL and HL, was no longer suitable. As such, the direction of the study was modified by creating all enzymes with the addition of a 6x histidine tag to the terminal end of the C-terminus to aid in the purification protocol.

The introduction of the histidine tagged enzymes enabled a reduction in time with respect to the purification procedure from four days down to two; however the enzyme yield remained close to zero, necessitating the need for other modifications in the protocol. The buffers were degassed and DTT, EDTA, glycerol, Triton X-100 and protease inhibitors were added to reduce oxidation and degradation, while promoting stability of the enzymes. Although these changes had a modest effect on chimera recovery, the final yield was sufficient to enable further biochemical testing and analysis.

The dramatic instability displayed by the chimeras warranted further investigation. The stability of all enzymes was measured in culture medium over a 24 hour period while at a stable temperature of 4°C. Although all enzymes had approximately the same trioleinase activity immediately following removal from the

cells, the chimeras lost their activity at a rapid rate upon sustained removal from the cells compared to their wild-type counterparts, losing at least 50% of their activity in approximately four hours in the case of the HL chimeras (Figure 15). Although the trend in activity loss is different for the two sets of chimeras, each set has a loss of activity pattern that follows that of their respective parental enzyme. For example, the LPL chimeras do not lose activity as rapidly or to the same extent as the HL chimeras – this trend is the same for wild-type LPL as compared to wild-type HL. These findings explain the challenge in obtaining pure and active enzymes over a multi-day purification. In addition, this loss of activity would only be enhanced in a purification scenario where the target enzymes are being separated from other proteins, which are known to be a stabilizing factor.

These results raise the question as to the mechanism which is affecting the enzyme activity. It is known that both LPL and HL are catalytically active in the form of a dimer. Although it is possible that there is an increase in the transition from the dimer to the monomer, the silver stains performed following purification provide another possible explanation. Prior to staining, the purified samples were concentrated in a unit with a 100 kDa molecular weight cut-off, which would have effectively removed all protein below this mass. The silver stains, however, showed bands at molecular weights below this cut-off, indicating that degradation occurred following concentration. In addition, the multiple bands demonstrate that although an increase in the monomer form is possible, it is clear that there is substantial degradation as well. These findings were also confirmed through western blot analysis.

It was initially believed that due to the small size of the candidate regions, substitution of the four and seven amino acid segments would have little, if any, effect on the stability and resulting catalytic activity of the chimeras, however, this does not appear to be the case. When the homology of the substituted residues is analyzed, a sizable difference in charged residues is obvious. LPL amino acids 65-68 contain one charged residue, a positive lysine, at position 67, compared to a neutral glutamine in the corresponding position (residue 79) of HL. The difference in charged residues is even greater in the second candidate region. LPL amino acids 73-79 contain four charged residues, a positive lysine and arginine at positions 74 and 75, respectively, and a negative glutamate and aspartate at positions 76 and 78, respectively, compared to a neutral serine, glutamine, proline and glutamine at the corresponding positions (positions 86, 87, 88 and 90) of HL. As a result, substitution of these regions together either introduces or removes, depending on the chimera, five charged residues in the span of eleven amino acids. Thus, it is not unreasonable to speculate on the alteration of the enzyme's tertiary structure due to the change in charge in a relatively small area. When this observation is taken with the extensive degradation demonstrated in the silver stains and western blots, it seems logical to suggest the exposure and possible introduction of new proteolytic cleavage sites within these chimeras. This charge theory is supported by the specific activity data, which demonstrates a decrease in specific activity in both of the double chimeras than either of their respective single substitution chimeras.

Comparison of the specific activity measurements of the enzymes indicated that the substitution of analogous residues into either lipase resulted in decreased

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activities (Table 3). The lowest activity among the LPL chimeras was associated with the simultaneous substitution of both LPL candidate amino acid regions (~6-fold difference compared to wild-type LPL). A similar result was observed for the HL chimeras. This effect was amplified following purification as the result of an inherent decreased stability associated with the chimeric enzymes, particularly the HL chimeras (Figure 15). Despite reduced chimera specific activities following purification, it was reasoned that apo C-II responsiveness of the remaining active species could be an accurate measure of the part played by the substituted residues.

V_{max} and K_M are the two parameters which define the kinetic behaviour of an enzyme as a function of its substrate. Kinetic analyses of these lipases (Table 4) indicated very little change in apparent K_M, consistent with previous findings indicating that the primary effect of apo C-II is on the apparent V_{max} of the reaction (389). Considering that K_M is an approximate measure of the affinity of the substrate for the enzyme, these results indicate that substitution of the candidate residues in either set of chimeras has little effect on the affinity of the substrate for the chimeras. Conversely, V_{max}, which represents a measure of the velocity of the enzyme at maximal concentrations of substrate, decreases upon the substitution of residues. Thus, the major effect of the substitution of the candidate residues in both sets of chimeras is on the speed of the reaction, resulting in an overall decrease in triglyceride and phospholipid hydrolysis. The greatest difference in apparent V_{max} values was observed between wild-type HL and HL_{LPLD} (~8-fold) suggesting that the presence of LPL residues within this chimera impaired catalysis compared to wild-

type HL. However, the presence of both candidate LPL regions within HL resulted in a loss in activity, which was not due to altered affinity of the enzyme for the substrate, but to other factors, such as stability or the inability to bind substrate productively. Significantly, the level of remaining activity was stimulated 5-fold by apo C-II, the first report of the transference from LPL to HL of cofactor-dependent lipolysis.

4.2 Transfer of Apolipoprotein C-II Responsiveness

The creation of the chimeras was based on one premise – the goal of transferring apo C-II responsiveness.

The development of the domain-exchange strategy (129) has led to a better understanding of lipase structure-function relationships. Typically, domain-specific functions are retained in the chimera, thereby revealing the structural source of the function. This approach has been successfully used to elucidate the structure-function features of numerous members of the lipase gene family. The key to this approach is that independent regions from different enzymes retain their functional conformation due to the structural similarity shared among the lipase family members (643).

The structural and functional similarities between LPL and HL and the fact that HL does not require any specific cofactor to obtain maximal activity, have yielded an ideal environment for the elucidation of the apo C-II activation site on

LPL. Thus, the creation of chimeric lipases had two objectives. The HL chimeras were created with one idea in mind: enabling an enzyme which is normally non-responsive to the presence of apo C-II, to in fact become responsive. The objective for the LPL chimeras was essentially the opposite of the HL chimeras: to create an enzyme which is no longer responsive to the presence of apo C-II.

Measurement of the enzyme activity of the chimeras in the presence and absence of apo C-II indicated that LPL residues 65-68 alone were not able to confer apo C-II reactivity to HL (Table 5). Similarly, the exchange of these same residues for corresponding HL residues within LPL did not appreciably change the fold activation in comparison to wild-type LPL (~10-fold vs. ~11-fold). By contrast, there was an indication that LPL residues 73-79 were involved in apo C-II activation as the presence of this second sequence within HL was associated with a modest increase in activity in comparison to wild-type HL. More convincingly, there was only a ~6-fold activation when these residues were replaced within LPL, a reduction of nearly half the activation observed for native enzyme. However, it was only when both LPL regions (residues 65-68 and 73-79) were replaced that the largest effects in activation were seen. Chimeras with both regions exchanged were associated with a ~5-fold activation in the case of the HL chimera and a 67% reduction in activation for the LPL chimera. These results suggest that LPL amino acids 65-68 and 73-79 appear to act cooperatively in response to activation by apo C-II. Interestingly, the LPL chimera with both candidate regions replaced did not completely abolish activation by apo C-II, nor when inserting these regions into HL did we obtain maximal activation compared to wild-type LPL. These findings indicate that although

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LPL amino acids 65-68 and 73-79 are indeed necessary, they are not sufficient for the complete activation of LPL by apo C-II. It is important to note that this does not dismiss the idea of an apo C-II responsive site within the C-terminal domain of LPL. In fact, this finding is consistent with our previous study indicating that in addition to a region in the amino-terminal domain, an apo C-II responsive region also exists in the carboxyl-terminal domain of LPL (644). These results are more easily interpreted in the context of a head-to-tail dimer model (128,147,645,646), which supports the hypothesis that two apo C-II activation sites within LPL exist – a major site within the N-terminal domain of one subunit and a minor site within the C-terminal domain of the opposing subunit (Figure 19). We speculate that apo C-II interacts simultaneously with both these regions to enable maximum activation of LPL.
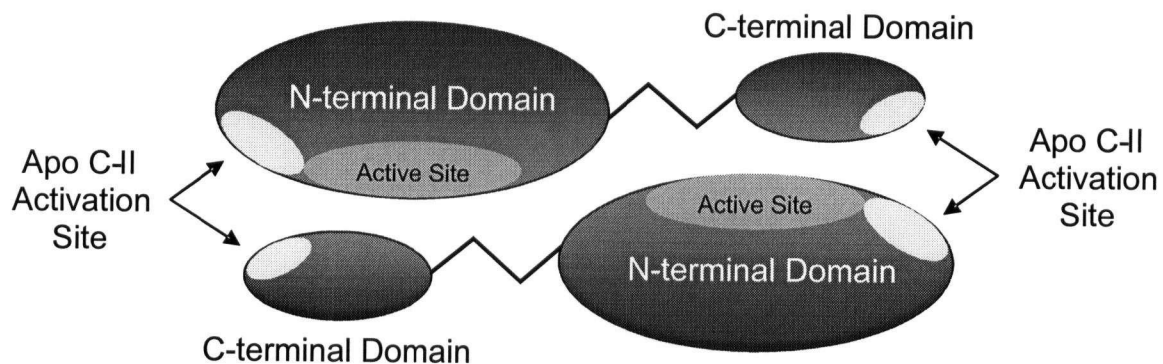

Figure 19. Proposed apolipoprotein C-II activation sites on LPL. Our current hypothesis proposes two apo C-II activation sites exist on lipoprotein lipase. A major site in the N-terminal domain of one monomer and a minor site in the C-terminal domain of the opposing monomer enable maximal activation of LPL by apolipoprotein C-II.

The trioleinase activity of the chimeras was also measured under high salt conditions to determine if the suspected residues were also responsible for the salt sensitivity of LPL. The LPL chimeras did not have the same reduction in activity as wild-type LPL in the presence of 1.0 M NaCl, in particular, LPL$_{HL77-80}$ maintained an activation of approximately 8.5-fold in the presence of high salt compared to wild-type LPL which was only activated 3-fold. Previous studies have localized the salt sensitivity of LPL to the N-terminal domain (147,148), and these results suggest that LPL residues 73-79, and in particular, 65-68, may in part be responsible for this feature of LPL. To more accurately assess the contribution of these regions to salt sensitivity, additional study will be required.

Since LPL binds to heparan sulfate proteoglycans through ionic interactions, it is interesting to speculate on the influence of residues in the candidate regions, in light of the dramatic difference in charged residues in comparison to the corresponding regions of HL, in particular LPL residues 73-79. In fact, heparin affinity was measured on wild-type HL, HL$_{LPL65-68}$, HL$_{LPL73-79}$ and wild-type LPL, without histidine tags (data not shown). Wild-type HL eluted at 0.83 M NaCl and wild-type LPL at 1.31 M NaCl, consistent with previous reports (149). Whereas HL$_{LPL73-79}$ eluted at 0.91 M NaCl, HL$_{LPL65-68}$ eluted at 1.30 M NaCl – virtually identical to that of wild-type LPL. These findings are surprising considering that LPL residues 65-68 contains only one charged amino acid. These results were unable to be duplicated with the histidine-tagged enzymes. In fact, the histidine-tagged LPL molecule eluted at 1.73 M NaCl, approximately 0.4 M NaCl higher than the same enzyme without the histidine tag. This is not unexpected considering that six

residues have been added to the terminal end of these enzymes that have previously been shown to contribute to heparin binding at the same pH (647). Thus, this addition elevates the concentration of NaCl required to elute the enzymes from a heparin-Sepharose column. It is clear that further study of these residues is required to determine their influence on heparin binding.

4.3   Molecular Modelling

Evidence has been provided that apo C-II and LPL participate in a protein-protein interaction which involves two molecules of apo C-II for each LPL dimer (648). The LPL activating domain of apo C-II was initially localized to the C-terminal third of the sequence (649). Furthermore, apo C-II peptide inhibition studies have identified the four terminal amino acids (residues 76-79) as important for the initial binding of apo C-II to LPL, but not directly with activation (650). More specifically, site-directed mutagenesis studies have implicated Tyr 63 as a key residue in the activation mechanism but no single amino acid appears essential for activation (651,652). NMR structural studies of apo C-II have described a number of helical domains thought to associate with lipid (380,390). Two of these domains in apo C-II (residues 50-58 and 66-75) are located in the C-terminal region and the latter is suggested to comprise one of the major lipid binding domains (390). It has been suggested that this helix, together with a helix located in the N-terminal domain (653), may anchor apo C-II to the lipoprotein surface whereas the inter-helical region

formed by residues 59-65 may represent the primary activator domain of apo C-II (390).

The LPL model presented in this study (Figure 18) indicates that residues 65-68 and 73-79 are found in close proximity to the catalytic pocket (Ser 132, Asp 156, His 241) and both loop domains (residues 54-63 and 217-238). In addition, secondary structure prediction indicates that residues 64-75 constitute an α-helical domain that may enable this region to interact with lipid moieties. However, since models of HL also predict α-helix structure in this region[1], secondary structure alone can not explain apo C-II activation. Consequently, helical wheel diagrams of this region for LPL and HL were compared (Figure 20) and differences in the number of charged residues and amphipathicity is readily apparent. The specified LPL region contains Lys 67, Lys 74, Arg 75, Glu 76 and Asp 78 whereas the corresponding HL region contains only a single Lys at position 85. The charged and hydrophobic residues in an LPL helix are arranged in a highly amphipathic manner, whereas the corresponding HL region lacks that character. As a result, we suggest that electrostatic interactions may contribute to the interaction of apo C-II with LPL, permitting substrate access to the active site. We speculate that in LPL this helix (residues 64-75) either directly or indirectly prevents substrate access to the active site in the absence of apo C-II. Furthermore, since HL has obvious compositional differences at this site, this helix in HL normally may not interfere with substrate access, thus obviating the need for cofactor. In summary, we have identified LPL residues 65-68 and 73-79 that appear to act cooperatively to enable substantial

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[1] J.S. Hill, unpublished observations
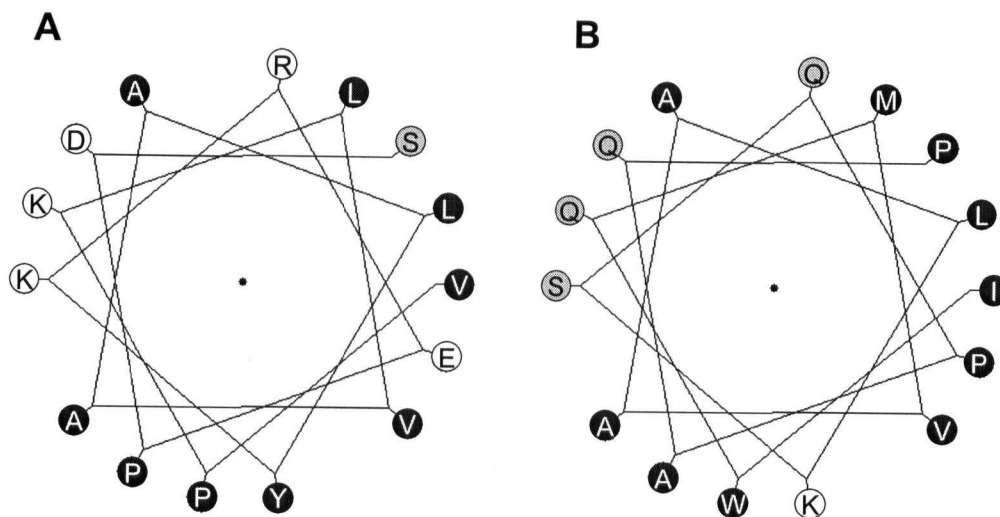
Figure 20. Helical wheel diagrams of LPL and HL. Residues 65-79 of LPL (Panel A) and residues 77-91 of HL (Panel B) are displayed in helical wheel form. Charged residues are indicated in white, hydrophobic in black and hydrophilic in gray.

activation of human LPL by apo C-II; moreover, the responsiveness imparted by these LPL residues can be translocated to HL. These findings suggest these LPL residues are, or are part of, the N-terminal domain cofactor activation site of the enzyme.

It is clear that lipoprotein lipase is a multifunctional protein that is involved in many different aspects of lipid metabolism. As such, it is a very important enzyme with respect to the development and progression of atherosclerosis. As a result,

there are multiple applications for the information obtained from the present study. From the position of the protein biochemist, the identification an apo C-II activation site within LPL is valuable for what it contributes to our understanding of the relationship between the specific structural and functional mechanisms of LPL. From a more clinical standpoint, the information gained brings us one step closer in learning how to effectively modulate the activity of LPL. Considering that the role of LPL in atherosclerosis may be different depending on its site of expression, current therapies aimed at increasing LPL activity on a systemic level no longer appear to be as beneficial as once thought. This has important implications for the development of new treatments; therefore understanding the functions of LPL at all levels, from the biochemical interaction between LPL and apo C-II to the role of LPL in lipid metabolism, is of utmost importance. The identification of residues within lipoprotein lipase that act cooperatively in response to apolipoprotein C-II activation is an important finding that enhances our knowledge of this complex system. Although more work is required to truly understand the interaction of LPL with other components involved in lipoprotein metabolism and the resulting effect on atherogenesis, the present study has provided important information towards the elucidation of this mechanism.

4.4 Future Directions

Although much valuable information has been obtained from the current study, additional questions have been raised. As such, potential avenues for further research are proposed here.

1) More specific analysis of the residues identified in this study would provide insight into their individual involvement in the activation of LPL in response to apo C-II. Thus, creating a series of constructs with specific point-mutations and/or combinations of each charged amino acid, Lys 67, Lys 74, Arg 75, Glu 76 and Asp 78, followed by lipolytic analysis would yield important information regarding which residues are responsible for the cooperative response observed in the present study.

2) Cross-linking studies with apo C-II and existing and future LPL constructs would reveal if there is a direct interaction between apo C-II and the regions of interest, and thus give a better understanding of the mechanism of apo C-II's activation of LPL.

3) A previous study has identified the last 60 amino acids in the C-terminus of LPL as being responsive to activation by apo C-II. A similar study for this region as to that which was just completed in the N-terminal domain would potentially identify more specific sequences in the C-terminus that are responsive to apo C-II. Additional work in this project could involve mutation of residues in both the N- and C-terminal domains to obtain valuable insight into the contribution of both domains and potential mechanisms of activation.

This information could test the hypothesis that the N- and C-terminal domains respond to apo C-II activation simultaneously.

4) The creation of an animal model overexpressing the created constructs would yield important information on the effect of these chimeras in a physiological setting. In addition, in light of the instability of the chimeras following removal from the cells, it would be interesting to see if expression in an *in vivo* model would confer increased stability.

5) The crystallization of LPL would be a tremendous advance in the understanding of the tertiary structure of LPL. Current models are based on the crystal structure of pancreatic lipase; a related enzyme but with significant differences. The challenge lies in the glycosylation of LPL which makes it difficult to obtain a homogenous sample necessary for crystallization.

6) The present study gave a small glimpse of the potential contribution of the charged residues within the identified regions of LPL to heparin binding, especially Lys 67. More detailed analysis of these amino acids by point-mutation in constructs without histidine tags, would yield important information of their individual contribution to the ability of LPL to bind to heparin. To date, several regions in LPL have been studied for their contribution to heparin affinity, however, the highly charged region identified in this study has not been investigated in this regard. In addition, it would be interesting to see the effect of these individual mutations on the stability of the resulting enzymes.

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6   APPENDICES

Appendix A: Sequencing results of HL_{LPL65-68}.
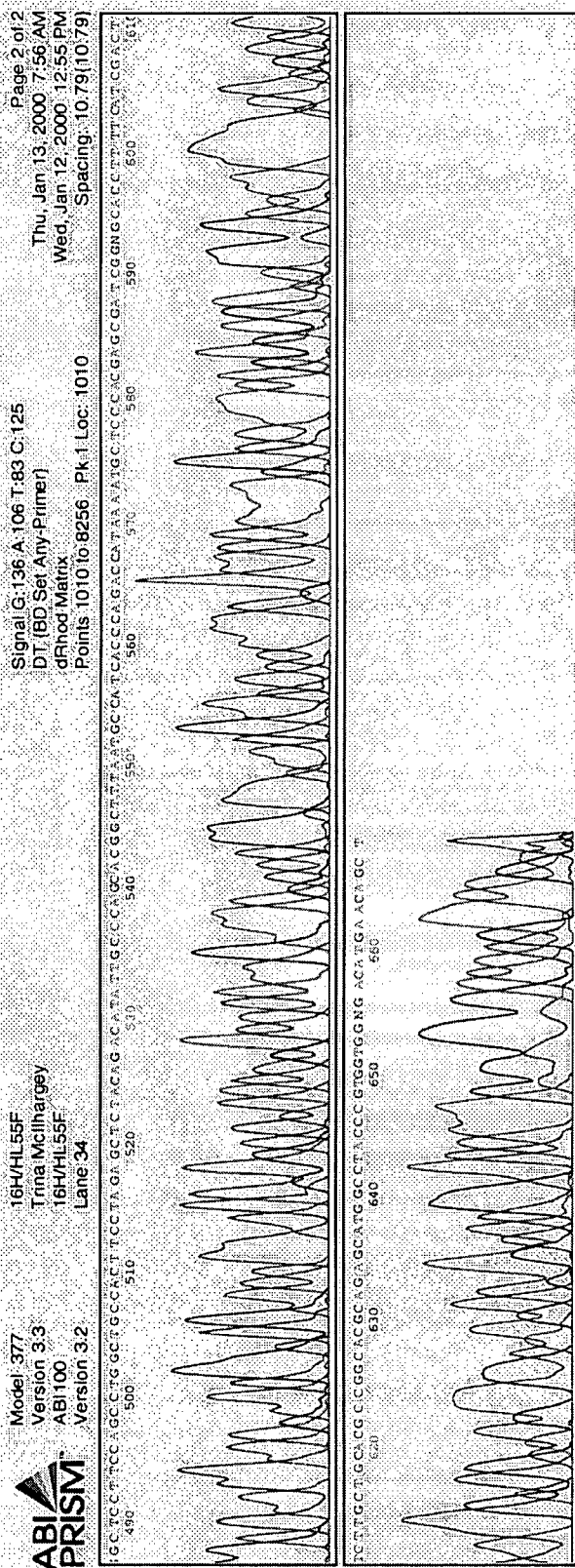
6.2 Appendix B: Sequencing results of HL_{LPL73-79}.
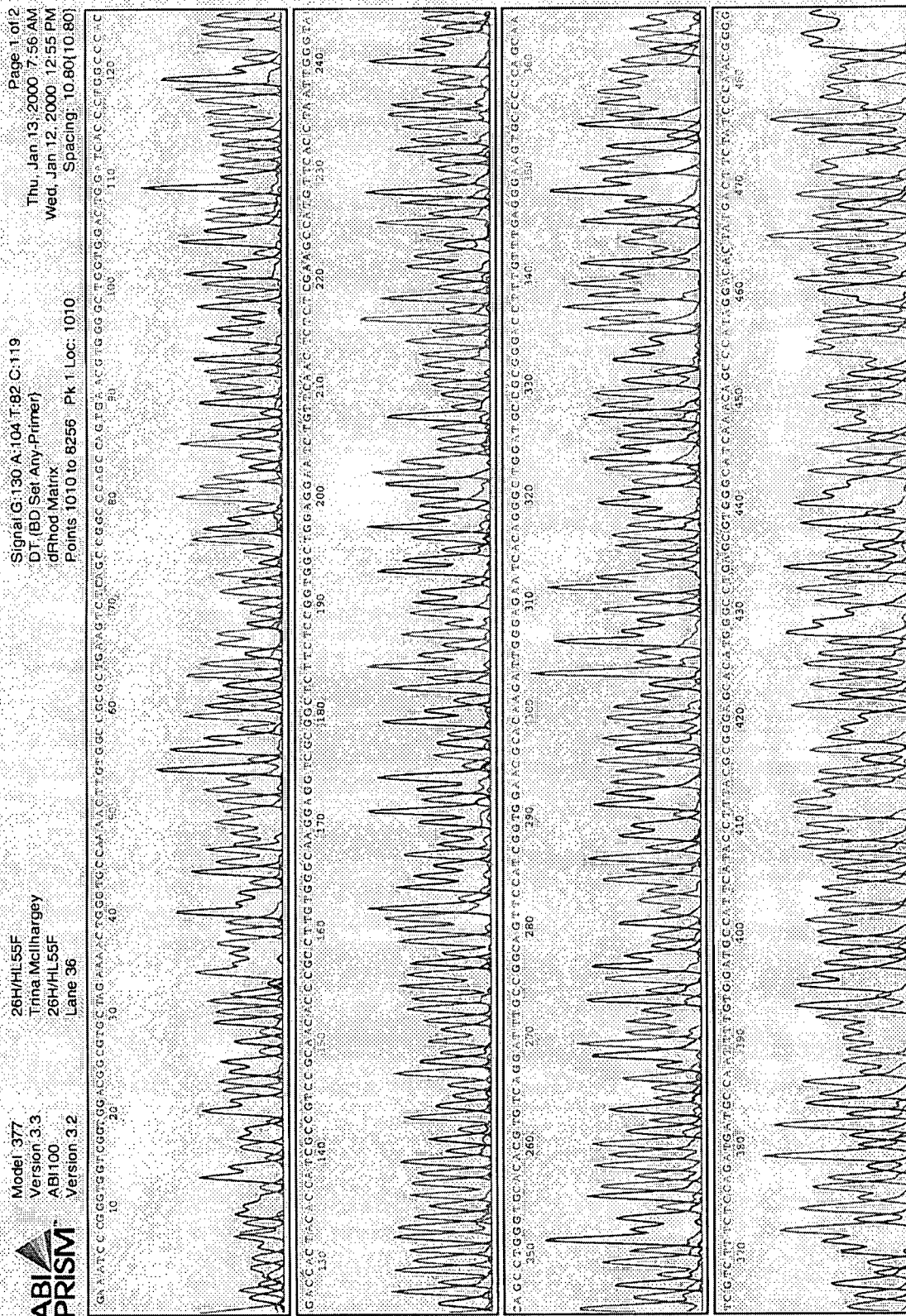
Appendix B: Sequencing results of HL$_{LPL73-79}$.

6.3 Appendix C: Sequencing results of HL$_{LPLD}$.

Appendix C: Sequencing results of HL$_{LPLD}$.

6.4 Appendix D: Sequencing results of LPL$_{HL77-80}$.

6.5 Appendix E: Sequencing results of LPL$_{HL85-91}$.

Appendix E: Sequencing results of LPL$_{HL85-91}$.

6.6   Appendix F: Sequencing results of LPL_{HLD}.

Appendix F: Sequencing results of LPL_{HLD}.