Defining an Intracellular Role of Hepatic Lipase in the Formation of Very Low Density Lipoproteins and High Density Lipoproteins

Michelle Bamji-Mirza

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

ABSTRACT

Hepatic lipase (HL) plays a pivotal role in the catabolism of apolipoprotein (apo) B-containing lipoproteins and high density lipoprotein (HDL) particles through its reported catalytic and non-catalytic extracellular functions. The current study tested the hypothesis that HL expression might impair formation and secretion of hepatic derived very low density lipoproteins (VLDL) and apoA-I (nascent HDL). Stable or transient expression of human HL (hHL) in McA-RH7777 cells resulted in decreased incorporation of [^{3}H]glycerol into cell-associated and secreted (VLDL-associated) ^{3}H-triacylglcyerol (TAG) relative to control cells. Stable expression of catalytically-inactive hHL (hHLSG) also resulted in decreased secretion of VLDL-associated ^{3}H-TAG whereas cell-associated ^{3}H-TAG levels were unchanged. Expression of hHL or hHLSG increased cell-associated ^{35}S-apoB100 with relatively no change in secreted ^{35}S-apoB100. Importantly, hHL or hHLSG expression resulted in reduced ^{3}H-TAG associated with the microsomal lumen lipid droplets (LLD), and increased relative expression of *ApoB* and genes involved in lipogenesis and fatty acyl oxidation. Transient expression of hHL in HL-null primary hepatocytes, mediated by adenoviral gene transfer, resulted in decreased steady-state levels of cell-associated and secreted apoA-I and reduced rates of synthesis and secretion of ^{35}S-apoA-I. HL-null hepatocytes exhibited increased levels of secreted ^{35}S-apoA-I relative to wildtype hepatocytes while cell-associated ^{35}S-apoA-I levels were normal. Transient expression of a hHL chimera (hHLmt), in which the C-terminus of hHL was replaced with mouse HL sequences, exerted an inhibitory effect on apoA-I production similar to that of hHL even though hHLmt was secreted less effectively than hHL with

impaired exit from the endoplasmic reticulum (ER) as compared with hHL. In contrast, stable expression of hHL in McA-RH7777 cells resulted in a dose-dependent increase in cell-associated and secreted ^{35}S-apoA-I levels. These studies demonstrate that hHL has an intracellular (but non-catalytic) role in reducing the content of the LLD and ultimately the buoyancy of secreted VLDL particles, and that the N-terminal sequences of ER-residing hHL directly or indirectly modulates the production and secretion of apoA-I (nascent HDL) from hepatocytes.

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LIST OF ABBREVIATIONS

AADA	arylacetamide deacetylase
ABCA1	ATP-binding cassette transporter, sub-family A, member 1
ANGPTL	angiopoietin-like protein
ANOVA	analysis of variance
apo	apolipoprotein
apobec-1	apoB mRNA editing complex-1
C-terminal	carboxyl-terminal
CAD	coronary artery disease
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
cpm	counts per minute
CPT-1α	carnitine palmitoyl transferase
CT	CTP:phosphocholine cytidylyltransferase
Da	Dalton
DAG	diacylglycerol
DGAT	acyl coenzyme A: diacylglycerol acyltransferase
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EL	endothelial lipase
ER	endoplasmic reticulum
Es-x	esterase-x
FA	fatty acyl
FBS	fetal bovine serum
FED	fish-eye disease
FHBL	familial hypobetalipoproteinemia
FLD	familial LCAT deficiency
G418	geneticin®
GAG	glycosaminoglycan
GPIHBP1	glycosylphosphatidylinositol-anchored HDL-binding protein
HDL	high density lipoprotein
hHL	human HL
HL	hepatic lipase
hHLSG	catalytically-inactive hHL (active site Ser replaced with Gly)
hHLmt	hHL in which the C-terminal residues replaced with mouse sequences
HS	horse serum
HSPG	heparan sulphate proteoglycans
IDL	intermediate density lipoprotein
$iPLA_2$	calcium-independent phospholipase A_2
Kb	kilobase
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoprotein
LDLR	LDL receptor
LLD	luminal lipid droplets

LMF1	lipase maturation factor 1
LPL	lipoprotein lipase
LRP	LDLR-related protein
MAG	monoacylglycerol
mHL	mouse HL
MTP	microsomal triglyceride transfer protein
N-terminal	amino terminal
PAP-1	phosphatidate phosphatase-1
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PEMT	PE *N*-methyltransferase
PFU	plaque forming unit
PL	pancreatic lipase
PLTP	phospholipid transfer protein
PNS	post nuclear supernatant
RCT	reverse cholesterol transport
rHL	rat HL
rpm	revolutions per minute
RT-PCR	real time-polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	short interfering RNA
SLB	sample loading buffer
SR-BI	scavenger receptor class B type I
T1DM	type I diabetes mellitus
TAG	triacylglycerol
TGH	triacylglycerol hydrolase
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)amino methane
USF	upstream stimulatory factor
VLDL	very low density lipoprotein
$VLDL_1$	S_f>100
$VLDL_2$	S_f 20-100
VP	virus particles

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CHAPTER 1. INTRODUCTION

1. I. Overview

Lipoproteins are protein-lipid complexes that transport lipids through the circulation for energy use or storage. Intravascular lipid transport encompasses exogenous (diet-derived) lipoproteins and endogenous (hepatic derived) lipoproteins. There are two major classes of endogenously derived lipoproteins – namely apoB-containing lipoproteins and HDL. As will be described below, abnormalities in the levels of these lipoproteins owing to the under/over production and/or under/over catabolism (due to genetic or environmental factors) is the basis for a number of diseases. Many cell-associated and circulating protein factors and enzymes affect the plasma concentrations and buoyant densities of these macromolecules, and with the aid of genome-wide association studies and follow-up biochemical analyses more factors are being identified. HL has emerged as a genetic determinant of plasma TAG and HDL-cholesterol levels in genome-wide association studies. HL is bound to extracellular surfaces of the liver and can facilitate the binding of apoB-containing lipoproteins or HDL particles to the cell-surface for subsequent selective-lipid or holo-particle uptake. HL also hydrolyzes lipids within these two major lipoprotein classes. Thus, HL reduces the buoyant density and plasma concentrations of HDL particles and apoB-containing lipoproteins. Owing to the fact that the hydrolytic activity of HL has been detected intrahepatically, we studied whether HL also had an intracellular role in the production and secretion of these hepatic derived lipoproteins.

1. II. Hepatic Lipase (HL)

1. II. i. Gene, expression and localization

The HL gene (*LIPC*) is located on chromosome 15 in humans, and it spans over 60 kilobases (kb) with eight introns and nine exons accounting for 1.6 kb [1;2]. The hHL protein is comprised of 499 amino acids including a signal-peptide of 22 amino acids, and the mature secreted glycoprotein contains 477 amino acids (65-kDa) [3].

HL is primarily synthesized and secreted by the liver [4;5], but it has also been found in steroidogenic tissues – rat ovaries and rat adrenal glands [5-7] (where it might promote delivery of lipoprotein cholesterol for steroidogenesis – however, evidence for local synthesis is still controversial), and human and mouse leukocytes, including macrophages [8;9]. In adrenals, HL activity was localized in blood vessels [10], and in ovaries, HL was mostly found in the corpora lutea [11]. Once secreted, HL is bound, via heparan sulphate proteoglycans (HSPG), to the external surfaces of hepatic sinusoids and hepatocyte microvilli, and in interhepatocyte spaces [4].

1. II. ii. Member of the mammalian triacylglycerol lipase gene family

HL is a member of the mammalian TAG lipase gene family that includes pancreatic lipase (PL), lipoprotein lipase (LPL) and endothelial lipase (EL). All are secreted N-linked glycoproteins that hydrolyze two principal lipid substrates, TAG and phospholipids, but with varying efficiencies [12]. PL and LPL are largely TAG lipases, EL is principally a phospholipase, and HL can hydrolyze both substrates with similar efficiency [12]. LPL, HL and EL are extracellular lipolytic enzymes of the vascular compartment involved in plasma lipid metabolism [13], whereas PL is secreted into the intestinal lumen and hydrolyzes dietary TAG.

Lipases have been found in several organisms, ranging from plants to mammals [14]. They are water-soluble enzymes that hydrolyze water-insoluble substrates including acylglycerols, phospholipids or cholesteryl esters (CE). Members of the mammalian TAG lipase gene family cleave their substrates (acylglycerols and phospholipids) primarily at the *sn*-1 position [12;15]. HL has been described to also hydrolyze diacylglycerol (DAG) and monoacylglycerol (MAG) with varying efficiencies, with higher efficiency towards DAG [16;17].

PL is secreted by pancreatic acinar cells, and acts in the intestinal lumen to hydrolyze bile-emulsified TAG. The released fatty acyl groups (FA) are taken up by intestinal enterocytes. LPL is synthesized by a variety of cell types including adipocytes and myocytes from skeletal muscle and heart [18]. LPL is bound to the surface of the capillary endothelium by HSPG and the recently identified glycosylphosphatidylinositol-anchored HDL-binding protein (GPIHBP1) [19]. TAG within apoB-containing lipoproteins is hydrolyzed by LPL into FA which are accessible to the underlying tissues. The remnant apoB-containing particles are further hydrolyzed by HL at the liver. Lipoprotein particles can also be taken up by the liver via receptor-mediated endocytosis. This process is also assisted by LPL and HL; independent of their catalytic activity, where these proteins act as ligands that facilitate uptake of remnant particles [20;21].

Along with their roles in the metabolism of apoB-containing lipoproteins, the lipases also have important functions in the formation and metabolism of HDL. The hydrolysis of TAG-rich apoB-containing lipoproteins by LPL causes the core of the lipoproteins to shrink, resulting in the shedding of surface lipids, which can associate with apoA-I and form nascent HDL particles. HL and EL play important roles in HDL remodeling by hydrolyzing the phospholipid and TAG associated with these lipoproteins. All these lipases emerge as

important genetic determinants of circulating lipid levels in human populations [22-24] and contribute to a number of pathogenic states including atherosclerosis, inflammation, and obesity.

1. II. iii. Structure and functional domains

Owing to the conservation of all disulfide bonds, the 3-dimensional structure of the mammalian TAG lipase gene family members are modeled from the x-ray crystallographic structure of PL [25], and considered a two domain structure; a globular amino (N)-terminal domain, and a small carboxyl (C)-terminal domain (**Fig. 1.1**). LPL, EL and HL share a number of functional domains including the Ser-Asp-His catalytic triad, a lipid-binding surface loop (lid domain) shielding the catalytic pocket, and in the case of LPL, EL and HL, HSPG binding domains [26;27]. The primary function of the N-terminal domain is catalysis, while the C-terminal domain provides supporting functions aiding catalysis such as the binding of lipid substrates and enzyme cofactors, and also providing binding sites for HSPGs and GPIHBP1 (in the case of LPL) [19]. Among the lipase family members, only PL functions as a monomer [28], while LPL, HL and EL are active as non-covalent head-to-tail homodimers [29-31]. Additionally, both PL and LPL require a co-factor for activity. The co-factor for PL activity (colipase) binds to the C-terminal domain of the enzyme [32], while the co-factor for LPL (apoC-II) may bind to both the N- and C-terminal domains of LPL [33]. The three main functional domains of HL, namely the catalytic site, and the lipid-binding, and HSPG-binding domains will be described below.

1. II. iii. a. Catalytic site

The catalytic site within the N-terminal domain of these lipases (Fig. 1.1) is similar to that of serine proteases, where a Ser-Asp-His triad provides a charge-relay mechanism that catalyzes the hydrolytic events [34]. The serine of the catalytic triad is part of an amino acid consensus sequence Gly-X-Ser-X-Gly (where X denotes an amino acid) that is shared by all lipases [13;35]. Point mutation of this Ser amino acid into Gly results in a catalytically-inactive lipase.

1. II. iii. b. Lipid binding domain

Lipid binding regions are present within both the N- and C-terminal regions of mammalian TAG lipase family members [36]. Within the N-terminal domain there exists a 22-amino acid surface loop (also called a 'lid domain') that covers the catalytic site of these enzymes at resting state thereby restricting substrate access (Fig. 1.1). Studies of chimeric LPL and HL proteins, in which their lid domains were switched, show that this lipid-binding region mediates substrate specificity [37]. The C-terminal domain of LPL and HL contains two highly conserved hydrophobic stretches [38], and studies with LPL and HL chimeras suggest that this region may also be involved in lipid substrate interaction and stability of the lipase homodimer [36].

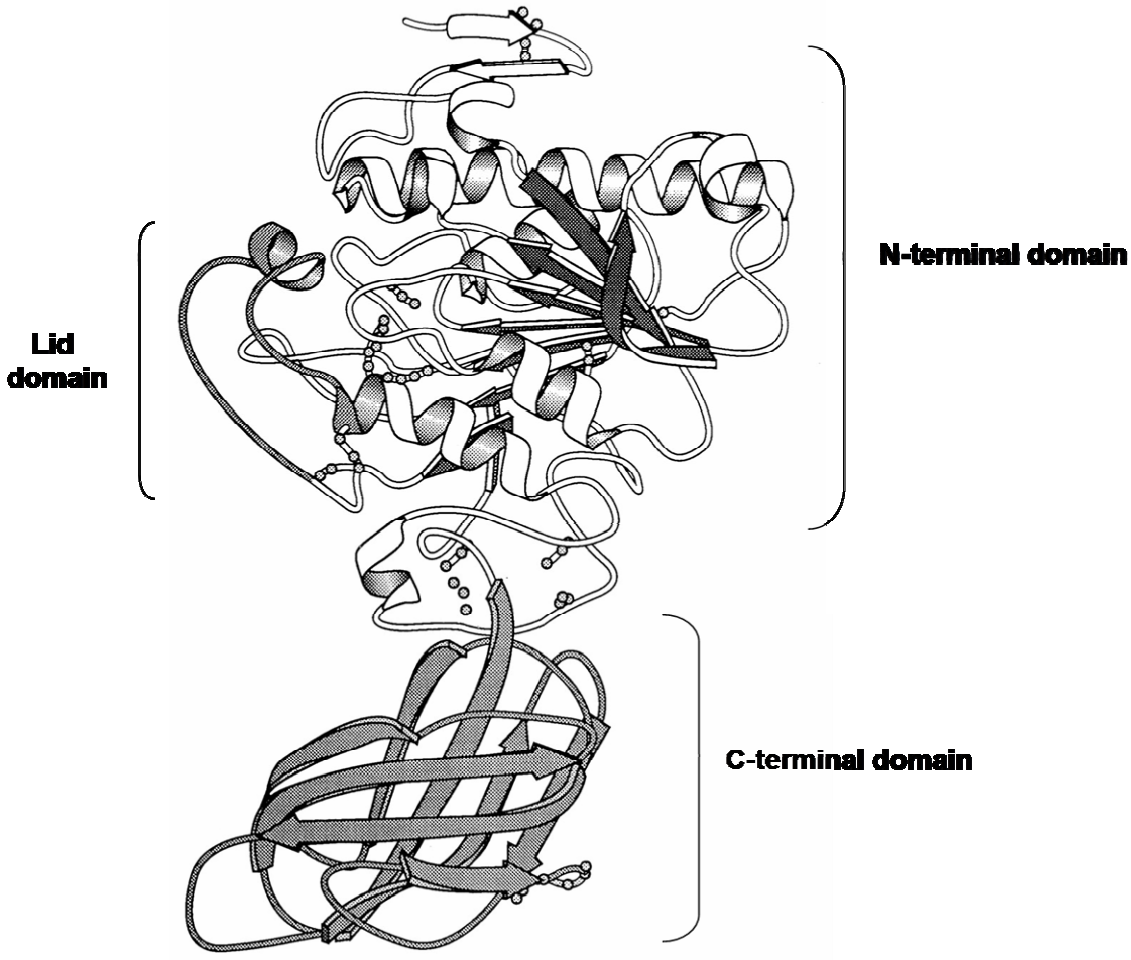
1. II. iii. c. HSPG binding domain

HSPG are expressed by endothelial cells that line hepatic sinusoids, hepatocytes, and space of Disse and form part of the extracellular matrix. Proteoglycans are composed of a core protein onto which glycosaminoglycan (GAG) side chains are covalently attached.

Fig. 1.1. Tertiary structure of the mammalian TAG lipase gene family members.

Schematic representation of the structure of the α-carbon backbone of horse pancreatic lipase as depicted in Dugi *et al.* [34]. The N- and C- terminal domains are indicated. Solid arrows represent β-sheet structures, and amino acids of the catalytic triad and the disulfide bridges are represented as ball-and-sticks. The lid domain covering the catalytic pocket is also highlighted.

Fig. 1.1



GAG are linear polysaccharides consisting of a repeating disaccharide unit, generally of an acetylated amino sugar alternating with uronic acid, and the polysaccharide chains are modified at various positions by sulfation and N-acetylation [39]. Units of N-acetylglucosamine and glucuronic⁄iduronic acid form heparan sulfate. HSPG are highly negatively charged (owing to the sulfation sites) and act as receptors binding positively charged domains on numerous proteins, including apoE-, and apoB-containing lipoproteins, LPL and HL [40]. Heparin, another GAG, consists mainly of 2-O-sulfated iduronic acid and 6-O-sulfated N-sulfated glucosamine units, and is able to release HL (and other HSPG-binding proteins) from cell surface by competing for binding sites on HSPG [40]. Findings from another study suggest that heparin-stimulated release of HL activity from primary rat hepatocytes was associated with tyrosine kinase activity [41]. Common consensus sequences for HSPG binding were identified, xBBxBx and xBBBxxBx, where B and X denote basic and hydropathic (neutral and hydrophobic) amino acids, respectively, and they exist in α-helix or β-sheet secondary structures [42]. Studies of chimeric lipases containing portions of HL and either LPL or mouse HL showed that regions in the C-terminal domain of HL were mainly responsible for HSPG binding [33;43-45]. More recently, systematic analysis of the human HL sequence revealed the presence of two putative HSPG binding domains; one in the N-terminal domain and the other at the C-terminal end of the protein [26].

We have previously shown that the different HSPG binding activity between hHL (mainly cell surface bound) and mHL (mainly blood-borne) is attributable to sequence divergence at the carboxyl termini of the two proteins [45]. A chimeric hHL protein (designated hHLmt), containing the carboxyl-terminal sequence of mHL (**Fig. 1.2**), exhibited reduced HSPG binding activity yet retained catalytic activity [45]. To determine the functions of the C-terminal HSPG-binding domains of hHL *in vivo*, hHL and the HSPG-binding deficient

chimera (hHLmt) were expressed in C57BL/6 mice by adenoviral-mediated gene transfer. Pre-heparin plasma levels of apoA-I and HDL-associated cholesterol and phospholipid were profoundly decreased 7 days post-infection in hHLmt-expressing mice relative to hHL-expressing mice [46]. This was not surprising considering the expression of a more blood-borne hHL would theoretically result in more interactions of hHLmt with its HDL substrate in the plasma than the more cell-surface bound hHL. Most interesting was the observation that adenovirus infection (*ex vivo*) of C57BL/6 primary hepatocytes with hHLmt resulted in decreased levels of secreted apoA-I, but comparable levels of cell-associated apoA-I, relative to hHL- or control-adenovirus infection [46]. One of the major aims of the PhD thesis investigation was to determine if (and the mechanism by which) hHLmt expression impaired apoA-I secretion.

1. II. iv. Functions

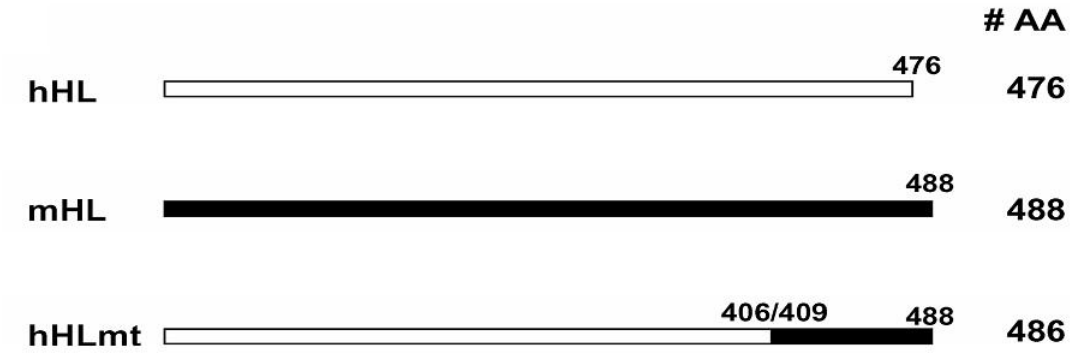
Commonly occurring variation in HL [22;23;47;48] as well as rare forms of HL deficiency [49;50] have been linked to altered levels of plasma TAG and cholesterol. Four linked polymorphisms have been identified in the promoter region of the HL gene: G-250A, C-514T (also referred to as C-480T [51]), T-710C, and A-736G [52;53]. The G-250A polymorphism is associated with lower HL activity and increased HDL-cholesterol levels [54]. The C-514T polymorphism has also been associated with increased HDL-cholesterol levels [54].

HL deficiency is a rare autosomal recessive disorder. To date, five missense mutations associated with diminished (T383M, L334F, and A144T) or complete (S267F and R186H) deficiency of HL activity have been identified [49;50;55]. Thr383Met results in impaired secretion while Ser267Phe results in an inactive lipase [56]. Patients with HL-deficiency mainly exhibit hypercholesterolemia and/or hypertriglyceridemia and accumulation of TAG-

Fig. 1.2. Schematic representations of hHL, mHL, and the HSPG-binding deficient hHLmt.

The protein diagrams of human hepatic lipase (hHL), mouse HL (mHL) and hHL with the mouse C-terminal tail (hHLmt). The numbers indicate the amino acid positions of the corresponding proteins. The amino acid length of each protein is indicated under '# AA.' Modified from Brown *et al.* [43].

Fig. 1.2



rich HDL and apoB-containing lipoproteins [49;55;57-59]. In some cases, HL deficiency increased prevalence of premature atherosclerosis [60-62]; while in others, increased HL was described in coronary artery disease (CAD) patients [63].

In mice, HL deficiency resulted in mild dyslipidemia as compared to wildtype littermates [64;65]. Introducing HL deficiency into apoE-null or low density lipoprotein (LDL) receptor-null background resulted in increased plasma concentrations of apoB-containing lipoproteins, TAG, phospholipids and cholesterol [66;67]. Conversely, overexpression of HL in HL-null mice was associated with a 50-80% decrease in cholesterol, TAG, phospholipids, HDL-cholesterol and apoA-I [21;68]. To elucidate the separate contributions of the lipolytic versus ligand-binding (non-catalytic) functions of HL to lipoprotein metabolism, researchers created a catalytically-inactive form of recombinant HL (namely HLSG). Expression of HLSG in HL-null mice resulted in significantly decreased levels of cholesterol, phospholipid and HDL-cholesterol, but no changes in apoA-I [21].

Overexpression of HL in a HL-null/LDLR-null background resulted in ~60-70% reduction of plasma VLDL-associated TAG, phospholipid, and cholesterol under chow or Western diet [69;70]. Liver-specific expression of HL in HL-null/apoE-null mice also resulted in ~40% reduction in plasma levels of TAG, phospholipid, and cholesterol [71]. The expression of catalytically inactive HLSG, in a background lacking endogenous HL and LDLR, reduced the levels of plasma cholesterol, non-HDL-cholesterol and apoB, but not to the same extent as with expression of catalytically-active HL [70]. Furthermore, liver-specific expression of either hHL or hHLSG on an apoE- and HL-null background exhibited decreased levels of plasma VLDL-associated TAG [71].

Thus, two major functions have been ascribed to HL, namely (i) a lipolytic enzyme that catalyzes hydrolysis of TAG and phospholipids present in apoB-containing lipoproteins and HDL [72-75], and (ii) a ligand that facilitates binding and uptake of lipoproteins by cell surface receptors or proteoglycans, as shown *in vitro* [72;74], animal [20;21;76], and human [77] studies. Additional *in vitro* and cell culture studies have suggested that HL is catalytically-inactive when associated with HSPG, and HDL can displace HL from cell surface HSPG [78;79]. Both functions of HL contribute to the catabolism and clearance of lipids and lipoproteins from circulation; however it remains unclear whether HL action increases or decreases the risk of developing atherosclerosis [80]. Whether high HL expression is pro- or anti-atherogenic is probably context-dependent and may depend on other genetic or metabolic factors [81;82].

1. II. v. Intracellular activity

Although the majority of the literature describes the extracellular roles of HL in the catabolism and clearance of circulating lipoproteins, some studies have described HL activity intracellularly [83-85]. The intracellular activity of HL has been detected within the ER/Golgi apparatus secretory pathway from primary rat hepatocytes [83], HL-transfected Chinese hamster ovary cells [84], and lipase maturation factor 1 (LMF1)-transfected fibroblasts from combined lipase-deficient mice [85].

1. II. vi. Maturation

The intracellular maturation of HL involves the conversion of catalytically-inactive monomers into functional dimers prior to secretion [84]. This is apparently a slow process that occurs in the ER and takes hours to accomplish [86]. HL undergoes a series of co- and post-

translational maturation steps within the ER, including glycosylation, glycan processing, and protein folding prior to dimer assembly. Lipase maturation employs the two general chaperone systems of the ER [86] as well as a recently identified lipase-specific chaperone termed LMF1 [85] (**Fig. 1.3**). Once maturation is complete, the lipases exit the ER and are secreted to extracellular sites. Of note, hHL contain four glycosylation sites (at positions 20, 56, 340, and 375) of which Asn-56 appears to be crucial for the secretion process, rather than for the acquisition of catalytic activity [87;88].

LMF1 is a membrane-bound protein located in the ER that is essential for the folding and maturation of LPL, HL and EL [85;89]. Recent studies identified mutations in LMF1 that cause combined lipase deficiency and hypertriglyceridemia in humans. These mutations resulted in the truncation of an evolutionarily conserved domain (of unknown function) within LMF1 named DUF1222, which is essential for interaction with lipases and their attainment of enzymatic activity [90]. As very little is known about this protein, future direction in lipase maturation research is directed towards understanding LMF1 function, expression and regulation.

1. II. vii. Regulation of HL

HL is regulated at the transcriptional level by several factors, including cholesterol (reduces HL mRNA and activity), estrogens (increases HL activity), thyroid hormones (increases HL activity), insulin (stimulates HL) and glucocorticoids (decreases HL mRNA and activity) [91]. Interestingly, the common HL gene variant C-514T renders a promoter sequence ineffective in upstream stimulatory factor (USF) binding [92]. USF1 is a transcription factor and a key regulator of lipid homeostasis and glucose metabolism. The C-514T polymorphism thus impairs the stimulatory regulation exerted by insulin through USF1 and

lowers HL expression and HL activity in plasma. Heparin also stimulates HL activity; it removes HL from cell surface and has been reported to stimulate HL transcription [93]. FA such as oleate also stimulate the activity of the proximal HL promoter and the secretion of HL from HepG2 cells [92]. The LMF1 regulates HL levels post-translationally, and extracellular HL activity is regulated by angiopoietin-like proteins (ANGPTLs), a family of secreted glycoproteins [94].

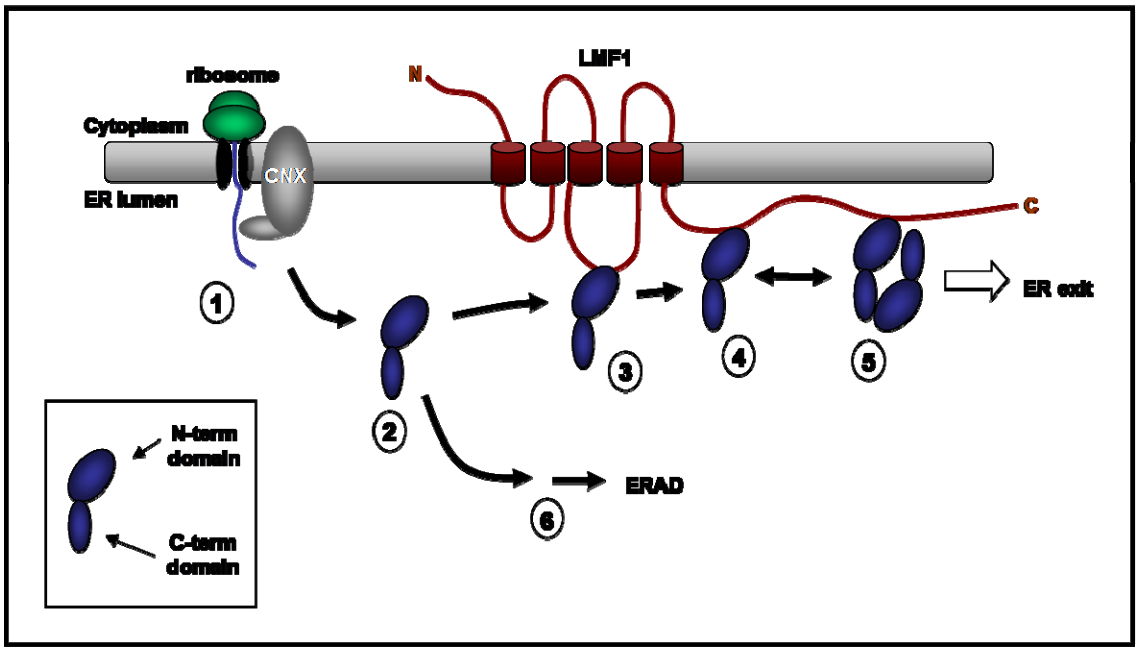
1. II. viii. Internalization and degradation of HL

Rat HL (rHL) behaves similarly to hHL in terms of its affinity to HSPG; little rHL circulates in the blood and most is present at the extracellular surface of the liver. Studies in human hepatoma cells and more recently in Sprague-Dawley rats showed that the internalization and degradation of HL was linked to its high affinity binding to LDLR related protein (LRP1) [95]. In rat hepatocytes, HL was enriched in hepatic endosome fractions in a pattern characteristic of ligands and receptors destined for lysosomal degradation [95]. These data thus suggest that HL internalization and subsequent lysosomal degradation are mediated by LRP1. The association of HL with HSPG on the cell surface is obviously a prerequisite for internalization. As such, heparin incubation may be considered to stabilize HL which escapes from internalization and degradation [96].

Fig. 1.3. Proposed mechanism of HL maturation and the role of LMF1.

The CNX chaperone of the ER aids the folding of the translating lipase (1). All partially folded lipase species are inactive and retained in the ER (2). With the aid of LMF1 association (3), the lipase monomer is properly folded (4) and assembled into head-to-tail dimers (5) that exit the ER. Terminally misfolded forms are destined for ER-associated degradation (ERAD) (6). The inset illustrates the N-terminal and C-terminal domains comprising the lipase monomer. CNX, calnexin; LMF1, Lipase maturation factor 1. Modified from Doolittle *et al.* [86].

Fig. 1.3


ribosome
Cytoplasm
ER lumen
CNX
N
LMF1
C
ER exit
ERAD
1
2
3
4
5
6
N-term domain
C-term domain

1. III. Very low density lipoproteins (VLDL)

1. III. i. Structure

Each VLDL (size: 30-80 nm; d = 0.95 – 1.006 g/mL [97]) particle is composed of one molecule of apoB-100, multiple copies of exchangeable apolipoproteins (such as apoE and apoC), along with varied amounts of TAG, phospholipid and CE, depending upon the size of the particles [98].

1. III. ii. Overproduction/Impairement

Hepatic VLDL overproduction and impairment of the catabolism or clearance of TAG-rich lipoproteins from the circulation represent the two major contributors to hypertriglyceridemia. Many patients with hypertriglyceridemia manifest elevated plasma TAG, accumulation of small dense LDL particles and reduced HDL-cholesterol levels, all of which are closely associated with cardiovascular diseases. Genome-wide association studies identified factors influencing plasma TAG concentrations [22-24], and in two of these recent studies, the HL gene *LIPC* is strongly associated with plasma levels of TAG.

Impaired secretion of hepatic VLDL is frequently associated with massive accumulation of TAG in the liver (termed hepatosteatosis), which is common in nonalcoholic fatty liver diseases [99;100]. Hypobetalipoproteinemia is a condition characterized by very low plasma levels of LDL-cholesterol and apoB, and may be caused by mutations in the gene encoding apoB [101]. Another condition, abetalipoproteinemia, characterized by practically no detected apoB in plasma, is caused by mutations in the gene encoding the microsomal triglyceride transfer protein (MTP) [102].

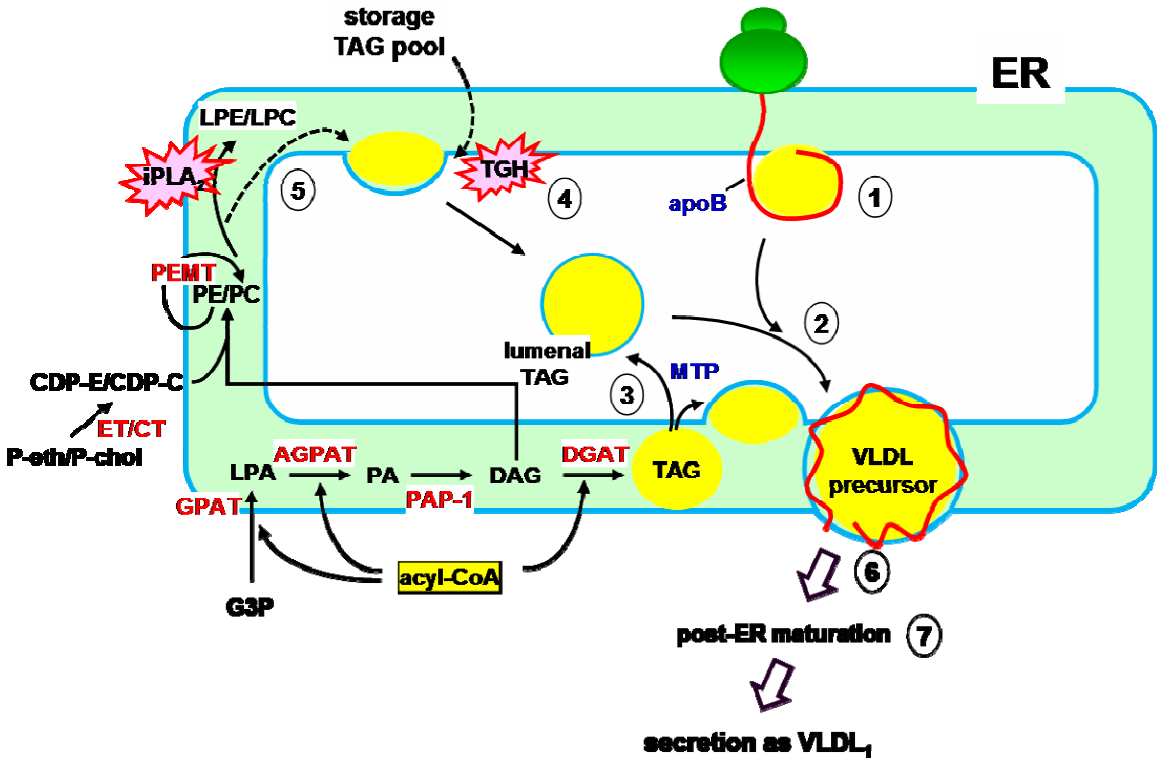
1. III. iii Assembly

VLDL assembly occurs in hepatic microsomes and involves the association of apoB-100 with lipids (mainly TAG and phospholipids), in which MTP plays a crucial role (**Fig. 1.4**). Major factors affecting VLDL assembly/secretion include the integrity of apoB, lipid substrate availability, and the activity of MTP [98]. Factors that affect VLDL assembly/secretion will ultimately affect hepatic and plasma levels of TAG and cholesterol. There is a debate in the literature about the microsomal location and mode of assembly of VLDL particles. Several studies demonstrate that VLDL assembly is completed in the ER before the particle enters the Golgi apparatus [103;104]. However, experimental evidence also suggests that VLDL maturation occurs in post-ER or in the Golgi compartment [105-108]. Following fractionation of McA-RH7777 cells treated with oleic acid, VLDL particles were found within the Golgi but not the ER [105], and treatment with brefeldin A, which interferes with ER-to-Golgi trafficking [109], has been found to inhibit VLDL formation but not the production of pre-VLDL [110]. Furthermore, pulse-chase experiments using HepG2 or McA-RH7777 cells demonstrated that inhibition of new TAG synthesis or MTP activity after a 60 minute chase did not affect the density of secreted apoB-containing lipoproteins [111]. Thus, although it is not clear how the lipid is added to apoB, it appears that lipidation of apoB takes place in two distinct steps occurring in separate organelles by different mechanisms. The formation of pre-VLDL particles takes place within the ER and is dependent on MTP, whereas lipidation of the pre-VLDL particles with TAG occurs post-ER and does not require MTP.

Fig. 1.4. Proposed model of VLDL assembly.

The apoB polypeptide initiates lipid recruitment during translation and translocation (1); this process may or may not require the activity of MTP. The nascent apoB-lipid particle acquires additional TAG from the LLD in a step-wise fashion (2). The activity of MTP is required for partitioning of TAG into the lumen or the membranes of ER microsomes for VLDL assembly (3). In addition to that synthesized from the de novo pathway (catalyzed by GPAT, AGPAT, PAP-1, and DGAT), the TAG substrate utilized for VLDL assembly is also derived from esterification of fatty acyl chains originated from TGH-mediated hydrolysis of existing cytosolic and lumenal TAG storage pools (4) or from phospholipid turnover catalyzed by $iPLA_2$ (5). The resulting VLDL precursor exits the ER (6), and maturation of $VLDL_1$ is achieved through ER/Golgi trafficking (7). AGPAT, acylglycerol-3-phosphate acyltransferase; CDP-C, CDP-choline; CDP-E, CDP-ethanolamine; CT, CTP:phosphocholine cytidyltransferase; DAG, diacylglycerol; DGAT, acyl-CoA:diacylglycerol acyltransferase; ET, CTP:phosphoethanolamine cytidyltransferase; G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; $iPLA_2$, calcium independent phospholipase A_2; LPA, lysophosphatidate; LPC, lysophophatidylcholine; LPE, lysophosphatidylethanolamine; MTP, microsomal triglyceride-transfer protein; PAP-1, phosphatidate phosphatase-1; PC, phosphatidylcholine; P-cho, phosphocholine; PE, phosphatidylethanolamine; P-eth, phosphoethanolamine; TAG, triacylglycerol; TGH, TAG hydrolase; VLDL, very low density lipoproteins. Modified from Sundaram *et al.* [92].

Fig. 1.4


storage
TAG pool
ER
LPE/LPC
iPLA2
TGH
5
4
apoB
1
PEMT
PE/PC
2
lumenal
TAG
MTP
3
CDP-E/CDP-C
ET/CT
P-eth/P-chol
AGPAT
LPA
PA
DAG
DGAT
TAG
VLDL
precursor
GPAT
PAP-1
acyl-CoA
G3P
6
post-ER maturation
7
secretion as VLDL1

1. III. iii. a. ApoB

ApoB is a member of a gene family (large lipid transfer protein) that spans insects and invertebrates [112]. Members of this family undergo lipid loading prior to secretion, and may experience similar disulfide bond formation, glycosylation, and folding [113]. In humans, the *APOB* gene covers 43 kb of chromosome 2, consists of 28 introns and 29 exons, and is expressed by hepatocytes and enterocytes. In the small intestine of all mammals, a 27-kDa enzyme called apoB mRNA editing complex-1 (apobec-1) binds to an 11-nucleotide-long AU-rich sequence 5 bases downstream of cytidine-6666 in the apoB mRNA. Apobec-1 then deaminates cytidine-6666, converting it to uridine, and forming a UAA stop codon in the process [114;115]. As a result, a truncated protein consisting of the N-terminal 48% of apoB is produced, known as apoB-48, and is the obligatory apolipoprotein for chylomicrons (size > 100 nm; $d < 0.95$ g/mL [97]). The full-length apoB-100 protein is 4536 amino acids, whereas apoB-48 is 2152 amino acids. In mice and rats, apobec-1 is expressed in both the liver and intestine, resulting in hepatic production of both apoB-100 and apoB-48 [116]. In humans, apobec-1 is not expressed in the liver. Thus in humans, apoB is synthesized by the liver and intestine, and secreted by the liver in the form of (apoB-100-containing) VLDL, and from the intestine as (apoB-48-containing) chylomicron.

The 3-D structure of apoB-100 or apoB-48 has not been solved at the atomic level due to their enormous size and extreme hydrophobicity. However, based on modeling algorithms and homology of N-terminal apoB to lamprey vitellogenin (another member of the large lipid transfer protein family), structural models of various apoB domains have been suggested. ApoB-100 is proposed to have a pentapartite structure consisting of 5 domains: $\beta\alpha_1$, β_1, α_2, β_2, α_3, named because of their α-helical or β-sheet structure [117]. ApoB-48 consists of the $\beta\alpha_1$ and β_1 domains and part of the α_2 domain. The $\beta\alpha_1$ domain consists of

hydrophobic regions that form strong, irreversible associations with TAG [118] and has been proposed to form a lipid-binding pocket able to recruit phospholipids from the ER membrane and initiate lipoprotein assembly [119]. The amphipathic α-helices of the α_2 and α_3 domains are proposed to form flexible regions that associate with lipid in a reversible fashion [120]. Similarly to the β1 domain, the amphipathic β-sheets of the β2 domain likely recruit lipid as well [121]. Truncation studies with apoB-60, apoB-72, apoB-80, apoB-88, apoB-94 (recombinant apoB proteins contained the N-terminal 60%, 72% 80%, 88% and 94%, respectively, of the full-length apoB-100) or apoB-100 in McA cells revealed a linear correlation between the length of the apoB molecule and the lipoprotein diameter, and an inverse relationship between the length of the apoB molecule and the peak lipoprotein density [121].

1. III. iii. a. 1. Modifications

Hepatic apoB-100 is regulated primarily at a post-translational level [122]. Proper folding of apoB-100 and its association with lipids are assisted by interaction with the chaperone-like protein termed MTP and by post-translational modification of the apoB-100 polypeptide. Human apoB-100 undergoes several post-translational modifications including disulfide bond formation, N-linked glycosylation, and cysteine-linked palmitoylation [123]. The importance of disulfide bonds within the N-terminal domain of the protein was revealed by the drastic inhibition of secretion of apoB-17, apoB-29, and apoB-41 (recombinant apoB proteins contained the N-terminal 17%, 29% and 41%, respectively, of the full-length apoB-100) upon co- or post-translational treatment with dithiothreitol, which reduces disulfide bonds [124;125]. Mutational analyses showed that N-linked glycosylation sites are important for apoB stability and secretion of TAG-rich lipoproteins [126]. Furthermore, apoB palmitoylation,

where FA palmitate is covalently linked to cysteine amino acids via thioester bonds, within the N-terminal region of apoB is not required for the ability of apoB-48 to assemble lipoproteins [123].

1. III. iii. a. 2. Degradation

Apparently, a large amount of apoB is degraded either co- or post-translationally. ApoB that undergoes terminal misfolding co-translationally will likely be degraded by proteasomes [127], whereas apoB-100 that misfolds post-translationally may be degraded by autophagy [128]. Due to the hydrophobic nature of apoB-100, its association with lipid occurs co-translationally. If there is too much apoB synthesized for the amount of lipid available, the excess apoB that cannot fold correctly will be ubiquitinated and delivered to cytosolic proteasomes for degradation [129-131]. In contrast to proteasomal degradation of apoB, which takes place when lipids are limiting, degradation of apoB through autophagy occurs when TAG availability is normal. Autophagy acts on apoB that has exited the ER, been extensively damaged by oxidative stress and formed large aggregates [128;132;133].

1. III. iii. a. 3. Mutations

Familial hypobetalipoproteinemia (FHBL) is an autosomal co-dominant disorder characterized by low plasma levels of LDL-cholesterol and apoB [101]. The genetic basis of the disease is heterogeneous and mainly associated with mutations within the *APOB* gene. The majority of *APOB* mutations in FHBL are nonsense, frameshift, or splicing variants that lead to truncated apoB proteins [134]. Nonsynonymous, nontruncating mutations have also been reported to occur in FHBL. Transfection studies have shown that, unlike truncation mutants that are often secreted normally, the nontruncation mutants are impaired in their secretion [135].

1. III. iii. a. 4. Regulation

ApoB expression is regulated at the post-transcriptional level. Evidence suggests that regulation of apoB translation is mediated by 5' and 3' untranslated regions (UTR) of the apoB mRNA. Results demonstrated that the 5' UTR likely improves apoB translation, while 3' UTR negatively affects apoB translation, perhaps by decreasing stability of the apoB mRNA [136]. It has been demonstrated that insulin decreases apoB mRNA translation while protein kinase C isoforms may activate apoB mRNA translation [137].

1. III. iii. b. Lipids

TAG utilized for VLDL assembly and secretion originates from multiple biosynthesis pathways. FA chains used for TAG synthesis are either synthesized from the *de novo* pathway, derived from esterification of FA chains originated from hydrolysis of existing storage TAG pools, or from phospholipid turnover catalyzed by calcium-independent phospholipase A_2 ($iPLA_2$) (Fig. 1.4) [138-140]. Phosphatidate phosphatase-1 (PAP-1) converts phosphatidate to DAG which can then be utilized for the synthesis of TAG or phospholipids such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) (Fig. 1.4) [141]. In mammals, PAP-1 is encoded by the lipin gene family consisting of lipin-1, -2 and -3 [142]. Expression of lipin-1 in McA-RH7777 cells increased the assembly and secretion of TAG-rich VLDL [143]. The final step in the *de novo* synthesis of TAG is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) (Fig. 1.4). Two *DGAT* genes encode the respective DGAT1 and DGAT2, which are structurally unrelated and show hepatic expression [144;145]. Overexpression of human DGAT1 or DGAT2 in McA-RH7777 cells resulted in increased production and secretion of VLDL [146]. The major phospholipid of

VLDL is PC. Two pathways are involved in hepatic PC synthesis; the CDP-choline pathway contributes approximately 70% of total PC synthesis, whereas the remainder 30% is synthesized through the PE methylation pathway (Fig. 1.4) [147]. CTP:phosphocholine cytidylyltransferase (CT) catalyzes the rate limiting step in the CDP-choline pathway, and mice lacking the CTα isoform displayed reduced plasma levels of both HDL and VLDL [148;149]. The PE methylation pathway is catalyzed by PE *N*-methyltransferase (PEMT), a product of the *Pemt2* gene [147]. Transfection of pemt2-cDNA into hepatoma cells has been shown to inhibit cell proliferation and induce apoptosis [150].

Hepatic TAG synthesis occurs at the ER, and the resulting TAG is stored (in the form of lipid droplets) in the cytosol or in association with the ER/Golgi within the membrane or lumen (referred to as LLD) [151-153] (Fig. 1.4). It is believed that the cytosolic TAG pool is metabolically connected to the ER/Golgi reservoir via a process termed 'hydrolysis/re-esterification' through which the stored TAG is utilized as substrate for VLDL assembly [152;154;155]. Existing TAG is presumably catalyzed by TAG hydrolase (TGH) (Fig. 1.4) [138], and the resulting FA can also be used for phospholipid biosynthesis. The lumenal TAG substrate for VLDL assembly exists in association with non-apoB apolipoproteins, and formation of these LLD appears to require the activity of the MTP [153;156;157]. Recent experimental evidence has suggested that formation of LLD (TAG-rich IDL/LDL-like particles in the microsomal lumen) under lipid-rich conditions may require apoC-III [158;159]. However, how LLD-TAG is incorporated into nascent VLDL precursor and the lipid and protein factors that regulate the formation of LLD is not known.

Mechanisms by which certain FA species preferentially promote VLDL secretion remain to be defined. Certain FA species may affect transcription of genes involved in TAG synthesis, which in turn may affect VLDL assembly and secretion. Studies with McA-

RH7777 cells have shown that TAG derived from oleic acid (18:1 n-9) was partitioned into microsomes and was effectively secreted as VLDL, whereas TAG derived from eicosapentaenoic acid (20:5 n-3) was stored in the cytosol and was poorly secreted [140]. As such, increased hepatic TAG may be compartmentalized and thus not necessarily available for VLDL assembly and secretion.

In addition to hydrolysis/re-esterification of TAG, hydrolysis of phospholipids also contributes FA substrates for TAG synthesis during VLDL assembly/secretion [139;160]. The process of phospholipid-hydrolysis/TAG-re-esterification in hepatic cells may also be compartmentalized, and FA chains derived from PC or PE are utilized differently for VLDL assembly/secretion [140]. Because phospholipid synthesis not only supplies lipid substrates for VLDL but also contributes to the biogenesis and maintenance of ER/Golgi membranes, it impacts both the cargo and trafficking machinery for VLDL assembly and secretion.

1. III. iii. c. Microsomal triglyceride transfer protein (MTP)

MTP is a heterodimer consisting of a 97-kDa catalytic subunit and protein disulfide isomerase (PDI) [161]. The MTP heterodimer is retained in the ER via its association with PDI, which has an ER retention KDEL sequence [162]. Individuals with mutations in the *MTTP* gene have abetalipoproteinemia characterized by practically no detectable apoB in plasma [102]. The precise role of MTP in VLDL assembly is not known. MTP has been proposed to increase translocation of apoB across the ER membrane [163], transfer lipids such as phospholipids cotranslationally onto apoB [164], and participate in movement (partitioning) of TAG across the ER membrane [153] (Fig. 1.4). MTP appears to associate with apoB within the $\beta\alpha_1$ region, forming an ionic interaction [146;165;166]. The use of MTP inhibitors has demonstrated that although MTP associates with the $\beta\alpha_1$ domain, and may play a role in forming a lipid-

binding pocket for initial phospholipid recruitment, it does not appear to have an enzymatic role in the synthesis, lipidation, or secretion of the $\beta\alpha_1$ domain [167]. MTP-inhibition in primary mouse hepatocytes resulted in reduced levels of non-apoB-associated TAG pool within the microsomal lumen [157]. Since MTP inactivation did not inhibit TAG synthesis, this study and others suggested that the activity of MTP is required for the partitioning and accumulation of TAG within the ER/Golgi lumen [153;156;157].

1. III. iv. Factors that affect assembly

There is a multitude of lipid and protein factors that affect the assembly and secretion of VLDL which has recently been elegantly reviewed [98;168]. Secreted apolipoproteins apoE, apoC-III and apoA-V have been implicated. Protein factors involved in intracellular vesicular trafficking also play profound roles in VLDL assembly and secretion. Furthermore, proteins associated with cytosolic lipid droplets as well as lipoprotein receptors (such as the LDLR [169]) have also been shown to impact this process.

A handful of microsome-resident protein factors have been identified that play a role in the mobilization and utilization of TAG for VLDL assembly and secretion [170-172]. Expression of these microsome-resident enzymes has been shown to exert different impacts on the metabolism of hepatic TAG and apoB-100 associated with VLDL. For instance, cells expressing TGH exhibited increased depletion of stored TAG (with little effect on ^{3}H-TAG synthesis) and accompanied increase in secreted VLDL-TAG mass and apoB-100 [170]. It was thus suggested that TGH expression promoted TAG-rich VLDL secretion through increased TAG substrate availability, which was probably achieved via the process termed hydrolysis/re-esterification. Evidence was obtained for another ER-associated protein, arylacetamide deacetylase (AADA), whose expression resulted in decreased ^{3}H-TAG in the

cell and an accompanying decrease in ^{3}H-TAG and apoB-100 secretion [171]. Expression of yet another ER-resident enzyme, esterase-X (Es-x) had no effect on ^{3}H-TAG or apoB-100 secretion, even though TAG synthesis was impaired [172].

1. III. v. Catabolism of apoB-containing lipoproteins

VLDL is catabolized in the vascular compartment to IDL and LDL particles (size: 28 nm; d = 1.006 – 1.063 g/mL [97]) by LPL and HL. The resulting particles can be cleared from the plasma by peripheral (and hepatic) tissues via the LDLR (which bind the ligands apoB-100 and apoE). Importantly, apoB-48 of intestinally secreted chylomicrons lacks the apoB-100 LDLR-binding region. Thus, chylomicrons are catabolized by LPL and HL, and the resulting remnant chylomicron particles can be removed from the circulation by either the LDLR or LRP1 in the liver through interactions with chylomicron-associated apoE [173]. As such, apoE is the critical ligand responsible for remnant lipoprotein clearance by binding LDLR, LRP and HSPG [174]. Importantly, heparinase treatment resulted in decreased remnant lipoprotein binding and uptake by 80-90% in fibroblasts and hepatocytes showing the importance of HSPG in these processes [175]. Additionally, rapid reuptake of newly secreted lipoproteins ('secretion-recapture' process) also regulates the net output of apoB by cultured liver cells considering many cells secrete products for which they possess receptors [176].

In the binding and uptake of remnant lipoproteins, the HSPG are involved directly as a receptor [177]. The remnant lipoprotein particles are captured by interaction of apoE with HSPG. LPL and HL may contribute by assisting the binding of particles to HSPG. Sequestration of these apoB-containing lipoproteins at the cell surface via associations with HSPG can facilitate receptor-mediated lipoprotein uptake (discussed above) or HSPG can internalize the remnants directly. The mechanisms of direct HSPG-mediated lipoprotein

internalization are unknown [174]. Importantly, studies showing that HL was implicated in cell surface lipoprotein sequestration used exogenously supplied labeled lipoproteins to HL-expressing cells [177;178]. One of the discoveries of the current PhD thesis investigation was direct evidence for HL-mediated cell-surface sequestration of newly secreted apoB-containing particles.

1. IV. High density lipoproteins (HDL)

1. IV. i. Structure and function

HDL particles (size: 8-12 nm; d = 1.063 – 1.21 g/mL [97]) comprise a pool of lipoproteins that are heterogeneous in size, density, protein and lipid content. Plasma levels of HDL are positively correlated with plasma levels of apoA-I [179] and apoA-I deficiency is characterized by the complete absence of HDL-associated cholesterol [180;181], suggesting that apoA-I is the major protein component of this class of lipoproteins. The lipid components of HDL can include, but are not limited to, phospholipid, cholesterol, CE and TAG. As will be described below, HDL particles can form within the hepatocytes prior to secretion and can also form in plasma from a variety of sources. Unlike apoB-containing lipoproteins where the synthesis and secretion of the particles are unidirectional, these very processes in HDL production and catabolism are multidirectional.

Plasma HDL-cholesterol levels are inversely associated with CAD risk in large epidemiologic studies [182-184]. The best characterized protective action of HDL and apoA-I is their ability to stimulate the removal of cholesterol from cultured cells and tissues – the so called reverse cholesterol transport (RCT) pathway [185-187]. The 'reverse' refers to the ability of HDL to carry excess cholesterol, which cannot be catabolized in most cells, from non-hepatic tissues back to the liver for excretion in bile. Proof that specifically raising HDL-

cholesterol results in reduced rates of CAD is lacking in humans; however, the best evidence comes from animal models that are transgenic for human apoA-I. Overexpression of human apoA-I in C57BL/6 mice fed atherogenic diets led to a greater than 2-fold increase in plasma HDL-cholesterol and marked protection against arterial fatty streak development [188].

The *APOA1* gene is found on chromosome 11. ApoA-I is synthesized primarily in the liver as a 267 amino acid prepropeptide [189], which is cotranslationally cleaved at the N-terminus resulting in a 249 amino acid proapoA-I. The proapoA-I is secreted [189], and rapidly cleaved at the N-terminus to form the mature 243 amino acid (28-kDa) apoA-I protein [190]. Thus, the existence of protease activity, which has been demonstrated in human serum and on the surface of human endothelial and hepatoma cells [191], is necessary to cleave the hexapeptide and generate mature apoA-I.

ApoA-I contains characteristic 11- and 22-amino acid repeats of amphipathic α-helices, which confer strong lipid-binding properties [192]. It has been demonstrated that the 3-dimensional structure of apoA-I consists of a two domain structure, comprising an N-terminal α-helix bundle spanning amino acids 1-187 and a separate less organized C-terminal region spanning the remainder of the molecule [193]. The links between the two-domain structure and function of apoA-I remain to be established [193].

1. IV. ii. Biogenesis

In the traditional model of reverse cholesterol transport [194], apoA-I is secreted as a lipid-free protein from the hepatocyte or enterocyte, acquires cholesterol from peripheral tissues to form mature HDL particles (with the aid of ATP-binding cassette transporter A1 (ABCA1) and lecithin:cholesterol acyl transferase (LCAT)), and the HDL particles return to the liver for excretion of cholesterol into bile. More recent studies have challenged this view

showing that 1) the major organ for the ABCA1-mediated production of HDL is the liver [195], 2) most of the apoA-I secreted by the liver appear in nascent HDL particles that contain phospholipid and cholesterol [196;197], and 3) a significant portion of the newly synthesized apoA-I is lipidated intracellularly [198-200].

Although apoA-I exists primarily in a lipid-bound state on HDL particles in plasma, lipid-free apoA-I is known to be an acceptor of cell derived cholesterol and phospholipids via ABCA1 [185;201]. ABCA1 is a membrane transporter that mediates the transport of cellular cholesterol and phospholipids to lipid-poor apolipoproteins [185;202-205]. ABCA1 is highly expressed in the liver and tissue macrophages [206]. Identification of this transporter was facilitated by studies of the severe hypoalphalipoproteinemia condition Tangier disease. In these patients, the synthesis of apoA-I was normal, but the lipidation of apoA-I with cellular phospholipids and cholesterol by skin fibroblasts was defective [207]. In 1999, several groups reported that the mutations in Tangier disease leading to impaired HDL formation was within *ABCA1* [185;201;203;204].

The interaction of apoA-I with ABCA1 at the cell surface results in the lipidation of apoA-I and the formation of nascent HDL particles (**Fig. 1.5**) [208]. Data suggest that a functional ABCA1 transporter leads to the formation of a cell surface lipid-containing site for the binding and lipidation of apoA-I at the plasma membrane [208]. Other data suggest that apoA-I is lipidated intracellularly and secreted as nascent HDL (Fig. 1.5) [198;209]. Kinetic studies have demonstrated that apoA-I undergoes ABCA1-independent co-/post-translational phospholipidation in the ER, followed by significant ABCA1-dependent phospholipidation and cholesterol transfer at the Golgi (Fig. 1.5) [209-211]. It is also believed that lipidation of apoA-I occurs through a retroendocytosis pathway in which ABCA1-mediated cellular cholesterol efflux involves the endocytosis and resecretion of apoA-I (Fig. 1.5) [197;212].

Furthermore, shedding of cholesterol, phospholipid, and apolipoproteins from chylomicrons and VLDL during LPL-mediated lipolysis is potentially another source of nascent HDL particles.

Nascent HDL particles can acquire additional cholesterol and phospholipids from hepatic and/or extrahepatic tissues via ABCA1-mediated efflux, progressively generating particles that are more cholesterol enriched (Fig. 1.5). Nascent HDL-associated cholesterol can be esterified by the HDL-associated enzyme LCAT [213;214]. The CE thus moves into the core of the particle, converting nascent HDL into a spherical particle with a monolayer of phospholipids and proteins on its surface (Fig. 1.5). Additionally, the ATP-binding cassette transporter GI and SR-BI mediate cholesterol transport from cells to mature HDL particles [215;216].

1. IV. iii. Remodeling

HDL undergoes structural and compositional changes (remodeling) in the vascular compartment by the action of lipases, and by the exchange of apolipoproteins and lipids between circulating lipoprotein particles. EL, LPL and HL play a role in the remodeling of HDL. EL binds to the endothelial surface and primarily hydrolyzes phospholipids on circulating HDL particles, resulting in accelerated apoA-I catabolism [12;217]. HDL particles are modified by TAG-rich lipoprotein surface lipids and proteins during the hydrolysis of these lipoproteins by LPL. HL hydrolyzes HDL-associated TAG and phospholipids, generating smaller HDL particles and lipid-free or lipid-poor apoA-I that can then recirculate in the RCT pathway. Transfer of neutral lipids between HDL and apoB- containing lipoproteins can also occur in the vascular compartment. CE associated with HDL can be transferred to the apoB-containing lipoprotein fraction by the lipid transporter cholesteryl

ester transfer protein (CETP) in exchange for other neutral lipids including TAG [218;219]. The action of phospholipid transfer protein (PLTP) promotes the transfer of phospholipids from VLDL and chylomicrons onto HDL and contributes to the remodeling of HDL by creating larger and smaller HDL particles [220]. Thus, plasma levels of HDL represent a balance between the rates of production and rates of clearance from plasma.

1. IV. iv. Catabolism

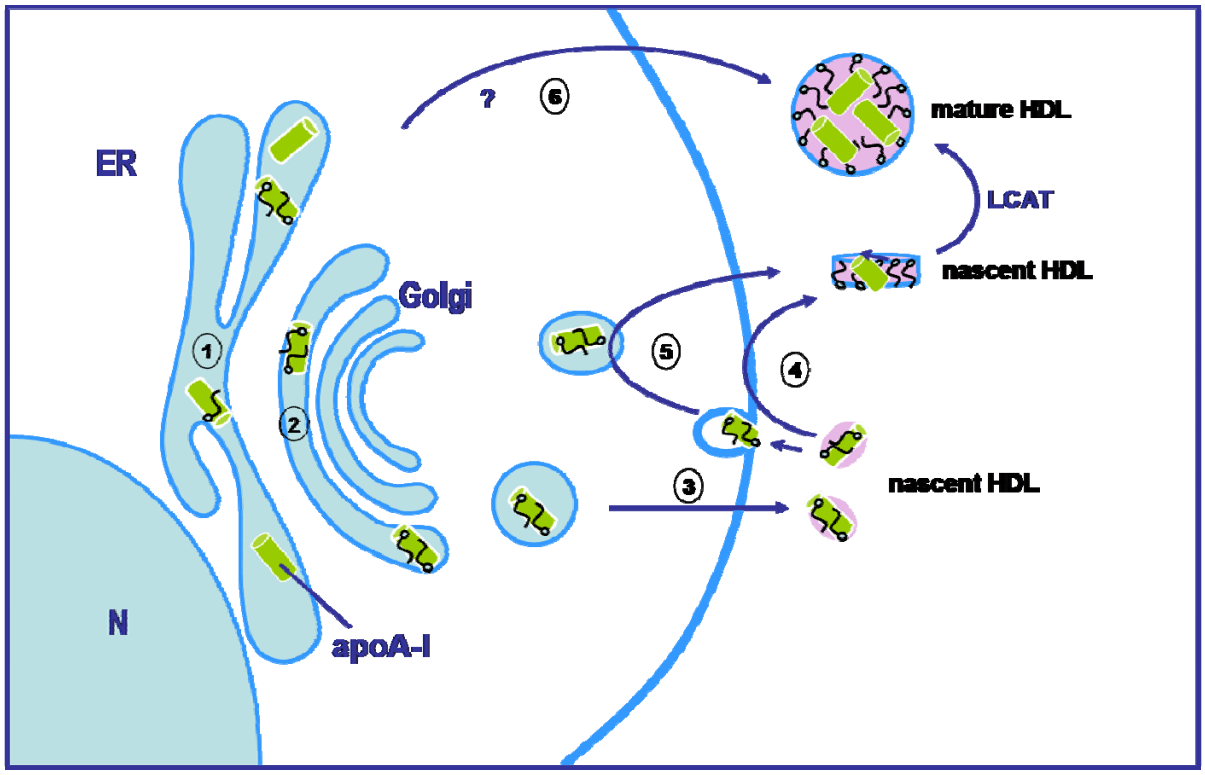
Completion of the putative RCT pathway involves the delivery of HDL-associated cholesterol back to the liver. This 'selective uptake' process is mediated by SR-BI [221], and HL has also been implicated in this process [222]. The exact cellular mechanisms for selective uptake of HDL-associated CE are largely unknown but involve the selective transfer of CE to the cell without the net internalization and degradation of the lipoprotein itself [223]. Cholesterol delivered to the liver on HDL, unlike that on apoB-containing lipoproteins via the LDL receptor and LRP1, appears to be preferentially trafficked for excretion into bile [221]. The actions of SR-BI are therefore critical to the final steps of RCT and to the generation of lipid-free or lipid-poor apoA-I that can then potentially re-circulate in the putative RCT pathway [224].

Lipid-poor apoA-I endocytosis and lysosomal degradation are believed to occur in both liver and kidney by receptor-mediated processes. Although the receptor-mediated processes for apoB-containing lipoprotein capture and internalization are largely understood, the HDL/apoA-I receptors are elusive. A handful of proteins have been proposed as candidate HDL receptors, namely HDL binding protein 1 and 2 (in the liver) and

Fig. 1.5. Proposed mechanism for hepatic production and secretion of apoA-I/HDL.

ApoA-I is associated with phospholipid in the ER (1) and Golgi (2), via ABCA1-independent and –dependent mechanisms, and can be secreted as a 'lipid poor' particle (3). ApoA-I can acquire lipids at the cell surface through an ABCA1-dependent fashion to form nascent HDL (4). ApoA-I lipidation can also occur through retro-endocytosis of apoA-I (also requiring active ABCA1) (5). It is unclear as to whether mature HDL can be secreted directly from the cell (6). LCAT esterifies apoA-I associated cholesterol forming mature HDL particles. Squiggly lines depict phospholipidation; pink colour represents cholesterol (or cholesteryl ester) association. ApoA-I, apolipoprotein A-I; ER, endoplasmic reticulum; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; and N, nucleus. From Yao lab.

Fig. 1.5


ER
Golgi
N
apoA-I
mature HDL
LCAT
nascent HDL
nascent HDL
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6
1
2
3
4
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cubulin/megalin (in the kidneys), but there are insufficient data to firmly establish these proteins as HDL receptors capable of internalizing these lipoproteins [225].

1. IV. v. Hypo- or hyperalphalipoproteinemia

Mutations in apoA-I, ABCA1 and LCAT have all been shown to underlie familial hypoalphalipoproteinemia. ApoA-I deficiency, due to mutations in the apoA-I gene, is characterized by the complete absence of HDL-associated cholesterol [180;181]. The loss of function of ABCA1, due to mutations in its gene, underlies Tangier disease, which is characterized by low levels of HDL-associated cholesterol [185;201;203]. Mutations in the LCAT gene either cause familial LCAT deficiency (FLD) or fish-eye disease (FED) [226]. Both conditions are characterized by 5-10% of normal HDL-associated cholesterol and corneal opacification. Differences in clinical phenotypes are related to the fact that in FLD plasma LCAT activity is virtually absent, whereas in FED patients, LCAT is partly active (mainly on apoB-containing lipoproteins). In contrast, complete loss of CETP activity, due to mutations in the CETP gene, can result in up to 5 times the normal HDL-cholesterol levels, called hyperalphalipoproteinemia [227].

1. IV. vi. Regulation

Genome-wide association studies showed that genes associated with HDL-cholesterol levels include LPL, HL, EL, apoA-I-ApoC3-ApoA4-ApoA5 gene locus, ABCA1, PLTP, CETP, LCAT and ANGPTL4 [23]. The regulation of the apoA-I expression occurs primarily at the transcriptional level, mediated by cis-acting DNA sequences in the proximal promoter of the apoA-I gene [228], and partly at the post-transcriptional level by increasing the stability of the partially spliced and unspliced nuclear apoA-I mRNA [229]. Gel retardation and DNase I

foot printing revealed that the apoA-I promoter contains three distinct regions to which proteins can bind, denoted as sites A, B and C [229]. Binding of transcription factors to all 3 sites is required for the expression of apoA-I gene, suggesting that a combination of different transcription factors determine the activity of the apoA-I gene enhancer in the hepatocytes [230;231]. The transcriptional induction of the apoA-I gene is influenced by several nutritional, hormonal, and pharmacological factors [230;231], including thryroid hormone, glucocorticoids, estrogen, and peroxisome proliferator-activated receptor-α agonists.

Up to quite recently, there were no therapeutic agents available that had a major effect on plasma HDL-cholesterol levels, other than the fibrate medication, through manipulation of transcriptional regulation of apoA-I [232]. A novel compound RVX-208 increases apoA-I mRNA and protein levels in cultured hepatoma cells and increases plasma apoA-I and HDL-cholesterol in an animal model by mechanisms that are not yet clear [232]. This compound is currently under investigation in human CAD patients.

1. V. Rationale & hypothesis

In vitro, animal, patient, and genome-wide association studies have shown that HL plays a very important role in the catabolism and clearance of apoB-containing lipoproteins as well as HDL particles. Additionally, *in vivo* and *ex vivo* studies using hHL and the HSPG-binding deficient hHLmt suggested that hepatic expression of hHLmt may impair the secretion of apoA-I and contribute to the observed hypoalphalipoproteinemia phenotype. Based on the facts that the machinery of production for VLDL or HDL particles exists within the hepatocyte, and that HL acquires its catalytic activity during maturation within the hepatocyte, it was hypothesized that HL played an important role in the production/secretion of these lipoprotein particles. This Ph.D. investigation thus has two aims; 1. to determine

whether the expression of HL negatively impacted the intracellular or secreted levels of (VLDL-associated) TAG/apoB-100 (and its underlying mechanism), and 2. to determine whether the expression of hHLmt (or hHL) impaired the secretion of apoA-I from the hepatocyte (and its underlying mechanism).

CHAPTER 2. MATERIALS AND METHODS

2. I. Materials

Dulbecco's modified Eagle's medium (DMEM), met/cys-free DMEM, Williams Medium E, Hepatozyme medium, fetal bovine serum (FBS), horse serum (HS), Lipofectamine, Geneticin® (G418), antibiotic/antimycotic, and TriZol® Reagent were purchased from Invitrogen Canada (Burlington, ON). Heparin, tributyrin, fibronectin, non-conjugated oleate and fumed silica were purchased from Sigma-Aldrich (Oakville, ON). The Primaria dishes were from BD Biosciences (Mississauga, ON). The [^{35}S]met/cys was obtained from MP Biochemicals (Solon, OH), and [1-^{14}C]-tributyrin and [2-^{3}H]-glycerol were obtained from American Radiolabeled Chemicals (St. Louis, MO), respectively. Protease inhibitor cocktail and chemiluminescent substrates were purchased from Roche Diagnostics (Laval, PQ). For immunoblot analysis, the antibodies to detect apoE and apoA-I were purchased from BioDesign International (Saco, ME), and the antibodies to detect calnexin and giantin were obtained from Stressgen Bioreagents (Ann Arbor, MI) and Abcam (Cambridge, MA), respectively. Antibodies against hHL and actin for immunoblot analysis were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Millipore (Temecula, CA), respectively. The antibodies to detect rat apoB-100 and apoA-I (by immunoprecipitation) were generated in our laboratory [159]. The anti-hHL antibody used for immunoprecipitation of HL was a kind gift from Dr. Ann White (University of Texas Southwestern Medical Center). The QuantiTect Reverse Transcription Kit and the iQ SYBR Green Supermix were purchased from Qiagen (Mississauga, Ontario) and BioRad (Mississauga, ON), respectively.

2. II. Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA), and HL-null mice were obtained from Jackson Labs (Bar Harbor, ME) and backcrossed onto a C57BL/6 background. The mice were maintained on a normal chow diet and a 12-h light/12-h dark cycle. The chow diet (Teklad Global 18% Protein Rodent Diet) from Harlan Laboratories (Indianapolis, IN) contained 18.6, 6.2, 44.2, 3.5, 14.7, and 5.3% of crude protein, fat (ether extract), carbohydrate (available), crude fiber, neutral detergent fiber, and ash, respectively. Female C57BL/6 or HL-null mice aged 8-14 weeks were used for hepatocyte isolation.

Primary hepatocyte isolation

Primary hepatocytes were isolated from mice by collagenase liver perfusion as previously described [233;234], and plated at a density of 1 x 10^6/well on fibronectin-coated 6-well Primaria dishes in Williams medium E containing 10% FBS and 100 U/mL antibiotic/antimycotic (Williams complete media). The viability of the cell preparation was at least 85% (as determined by trypan blue exclusion method prior to plating cells). To ensure healthy vibrant cells, the monolayer was washed vigorously with plain Williams medium E two hours after plating, and was then replaced with Williams complete media. The primary cells were harvested or infected with adenovirus 4 h after plating.

Construction of expression plasmids

The pCMV5-hHL expression plasmid was generated as previously described [45]. To generate pCMV5-hHLSG, mutagenesis of the active site Ser-145 into Gly was performed

with pCMV5-hHL as a template using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The sense oligonucleotide to generate the mutant was 5'-CCTAATTGGGTACAGCCTGGGTGC-ACACGTGTCAGG-3'

Generation and maintenance of McA-RH7777 cells stably expressing hHL or hHLSG

The McA-RH7777 cell line was obtained from the American Type Culture Collection and cultured in DMEM containing 10% FBS and 10% HS. McA-RH7777 cells stably expressing hHL or hHLSG, were obtained by co-transfection with pSV2neo and either pCMV5-hHL or -hHLSG (by Lipofectamine [following manufacturer's instructions] or calcium phosphate transfection method, respectively). Clones were selected in G418 (400 μg/mL) and after screening for hHL expression, the cells were maintained in G418 (200 μg/mL). McA-RH7777 cells were subcultured in maintenance medium 48 h prior to plating cells for experimentation, and were plated at a density of 1.8×10^6 cells per 60 mm dish (or 7-8 million per 100 mm dish) 16 h prior to experimentation in DMEM supplemented with 20% FBS.

Adenovirus generation and cell infection

The adenoviruses encoding hHL, hHLmt and enhanced green fluorescent protein (EGFP) were generated at the Viral Vector Core Facility, Canadian Stroke Network, Ottawa Hospital Research Institute at the University of Ottawa using the AdEasy adenoviral system (Q-Biogene, Carlsbad, CA) according to manufacturer instructions. The adenovirus encoding luciferase was generated as previously described [46]. The 'empty-vector' adenovirus was a kind gift from Dr. Robin Parks, Ottawa Hospital Research Institute. McA-RH7777 cells or primary mouse hepatocytes were incubated with adenovirus in a minimal amount of serum-

free DMEM (eg. for a 60 mm dish, used 1 mL media) for 1 h, culture media was added, and the cells were used for experimentation after 36 hours.

Immunoblot analysis

McA-RH7777 cells were incubated with serum-free DMEM medium, or primary cells were incubated with serum-free Hepatozyme medium ± heparin (100 U/mL) for 4 h. The cells were harvested in sample loading buffer (SLB: 10 mM Tris, pH 8.0, 8 M urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and bromophenol blue), and the secreted hHL, apoA-I, and apoE proteins were adsorbed onto hydrated fumed silica (Cab-O-Sil) as previously described [235], and eluted in SLB. For a 1 mL medium sample, 100 μL of fumed silica/water slurry (250 mg/mL) was incubated for 2 h and the proteins were eluted in SLB at 85°C for 15 minutes. The cell and medium protein samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane for immunoblot analysis with appropriate antibodies against HL, apoA-I, actin, and apoE. The densitometry of the bands was quantified using Quantity One software from BioRad.

Tributyrin in-vitro enzyme assay

The McA-RH7777 cells stably expressing hHL or hHLSG were incubated for 4 h with serum-free DMEM supplemented with 100 U/mL heparin. The conditioned media was harvested and centrifuged at low speed (750 rpm) to remove cell debris. Equal volume of the conditioned medium from each cell type was then used as an enzyme source in an *in vitro* [^{14}C]tributyrin-labeled activity assay as described [236]. The reaction buffer (50 mM Tris-HCl, pH 8.3, 1 M NaCl and 2% bovine serum albumin), [^{14}C]tributyrin substrate, and the

conditioned media were incubated for 1 h at 37 °C in a shaking water bath. A 0.1 M NaOH solution and a chloroform:methanol:heptane (10:9:7; by volume) solution were used to stop the reaction and separate the aqueous phase, respectively. A fraction of the aqueous phase was counted for ^{14}C by scintillation counting, converted to nmol butyrate released/h/mL medium and plotted. This assay measures esterase activity.

Metabolic labeling of lipids

McA-RH7777 cells were labeled for the indicated times with [^{3}H]glycerol (5 μCi/mL) in DMEM containing 20% FBS and 0.4 mM oleate (unless otherwise indicated) and either with or without 100 U/mL heparin. Total lipids were extracted from cells and media with chloroform/methanol/acetic acid/saturated NaCl/H_2O (4:2:0.01:1:2; by volume) and resolved by thin layer chromatography (TLC) using Silica Gel 60 plates as previously described [139]. The solvent system chloroform/methanol/acetic acid/formic acid/H_2O (70:30:12:4:2; by volume) was used for the separation of PC, and hexane/diethyl ether/acetic acid (70: 30:1; by volume) was used for the separation of TAG. The radioactivity associated with ^{3}H-TAG, and -PC was excised from the TLC plates, quantified by scintillation counting and normalized to cell protein levels.

Metabolic labeling of proteins

Cells were pretreated with met/cys-free (Met^-/Cys^-) medium for 30 min and then labeled with [^{35}S]met/cys (100 μCi/mL) in Met^-/Cys^- DMEM for indicated times. Pretreatment and labeling media were supplemented with ± heparin (100 U/mL) and FBS (10% in the case of primary cells and 20% in the case of McA-RH7777 cells). The respective ^{35}S-labeled proteins were immunoprecipitated from the cells and media and resolved by

SDS-PAGE (5% gel for apoB and 12% gel for apoA-I, apoE and albumin). Radioactivity associated with the ^{35}S-labeled proteins was excised from the SDS-PAGE gel, quantified by scintillation counting and normalized to cell protein levels.

Subcellular Fractionation

McA-RH7777 cells were labeled with [^{3}H]glycerol for the indicated times and harvested, or primary hepatocytes were harvested 36 h post hHL- or hHLmt-adenovirus infection, in homogenization buffer (25 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA), homogenized by ball bearing homogenizer, and fractionated as previously described [106]. The cell lysate was centrifuged at 700 rpm for 5 min to remove non-homogenized cells, and subjected to 11,870 x*g* for 10 min at 4°C to separate the post-nuclear supernatant (PNS) from the pellet that contained the mitochondria and nuclei. The PNS was subjected to 165,000 ×*g* for 30 min at 4°C to isolate the microsomal pellet from the cytosolic fraction. In the case of lipid labeling in McA-RH7777 cells, the membranous and lumenal fractions of the microsomes were separated as previously described [106]. Briefly, an equal volume of 0.2 M Na_2CO_3 was gently mixed with the resuspended microsomal pellet for 30 min, and the membranes were pelleted by centrifugation (100,000 rpm, 16 min, TLA 100.3 rotor). Lipids were extracted from all the fractions and analyzed by TLC as described above. In the case of primary hepatocytes infected with hHL or hHLmt, the microsomal pellet was subjected to Nycodenz gradient as previously described [106] to further separate the microsome into ER and Golgi fractions. In short, the resuspended microsomal pellet was layered atop a nonlinear Nycodenz gradient created from centrifuging a step gradient (10, 14.66, 19.33 and 24%) of Nycodenz solutions. After centrifugation with the sample, an aliquot of each fraction was

resolved by SDS-PAGE and immunoblot analysis on hHL, hHLmt, apoE, calnexin and giantin was performed as described above.

Density fractionation of lipoproteins by cumulative rate flotation ultracentrifugation

Cells were labeled with [^{3}H]glycerol or [^{35}S]met/cys for the indicated times as described above. Lipoproteins, either present in the media or within the microsomal lumen, from metabolically labeled cells were fractionated into $VLDL_1$ (S_f>100), $VLDL_2$ (S_f 20-100) and other lipoproteins as described previously [156]. The sample was adjusted to d=1.10 g/ml with KBr, and added to the bottom of a Beckman SW40 centrifuge tube. The sample was the overlaid with 3 ml of d=1.065 g/ml NaBr, 3 ml of d=1.02 g/ml NaBr, and 3 ml of d=1.006 g/ml NaCl. After ultracentrifugation (40,000 rpm, 20 °C, 148 min), $VLDL_1$ was collected from the top 920 μL of the gradient, and after an additional ultracentrifugation step (37,000 rpm, 15 °C, 18 h), $VLDL_2$ and other lipoproteins fractions (3 – 13) were collected from top to bottom. To ensure that the first fraction (and all subsequent fractions) was properly isolated and removed from the remaining fractions, the pipette tip was placed just above the meniscus when unloading fractions from the centrifuge tube. The lipids were extracted from each fraction and analyzed by TLC as described above.

Real time-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TriZol reagent following the manufacturer's instructions. To ensure optimal RNA isolation, RNase inhibitors were used on all surfaces and the resulting RNA was stored at -80 °C. Reverse transcription was performed using QuantiTect Reverse Transcription Kit according to manufacturer's directions. PCR was performed on the iCycler (BioRad) using iQ SYBR Green Supermix, following

manufacturer's instructions. Primer sequences for PCR are listed in Table 2.1. The cycle threshold values were normalized to cyclophilin A.

Statistical analysis

Values were expressed as mean ± SD. The significance of differences among control and hHL-expressing cells was analyzed using Student's t-test. $P < 0.05$ was considered significant. The significance of differences among control, hHL, and hHLSG or hHLmt was analyzed by ANOVA followed by Dunnett's post-hoc tests. $P < 0.05$ was considered significant.

Table 2.1. Nucleotide sequences of the RT-PCR primers

Gene	Accession No.	Forward	Reverse
Apob	NM_019287	TCCTGCTTCTGTTCTTGGACACCA	ACGTACTTCCGGAGGTGCTTGAAT
CPT1α	NM_031559	AGACCGTGAGGAACTCAAACCCAT	CACAACAATGTGCCTGCTGTCCTT
Dgat1	NM_053437	GGCATTCACAGCAATGATGGCTCA	CCACACAGCTGCATTGCCATAGTT
Dgat2	NM_001012345	TGTCACCTGGCTCAACAGATCCAA	TATCAGCCAGCAGTCAGTGCAGAAs
Mttp	NM_001107727	AAGGCCAATATGGACATCCAGGGT	TGGTTATTACCACAGCCACCCGAT
Ppia	NM_017101	GTCCATGGCAAATGCTGGACCAAA	CAAAGACCACATGCTTGCCATCCA

CHAPTER 3. RESULTS

3.1 Human hepatic lipase impairs hepatic VLDL assembly and secretion

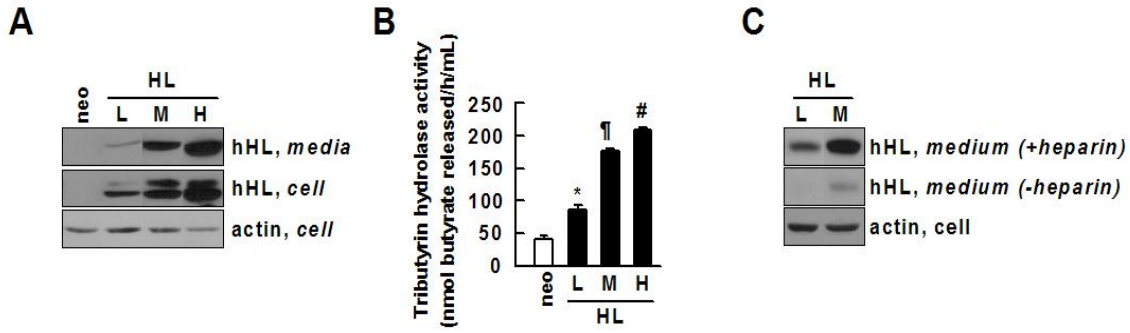
3.1.1 Expression of Human HL resulted in decreased cell-associated and secreted levels of ^{3}H-TAG, and increased synthesis of ^{35}S-apoB-100, while the levels of secreted ^{35}S-apoB-100 were unchanged

In the current study, the potential role of hHL on hepatic VLDL assembly and secretion was determined by stable or transient expression of hHL in McA-RH7777 cells under lipid rich conditions (20% FBS and 0.4 mM oleate). Studies with McA-RH7777 cells have shown that TAG derived from exogenously added oleic acid (18:1 n-9) was partitioned into microsomes (in the form of LLD [158;159]) and TAG-rich VLDL secretion was many-fold higher compared to no oleate supplementation [156]. Among the stable clones that expressed varying levels of hHL (**Fig. 3.1.1A**), the TAG hydrolysis activity (using tributyrin as a substrate) was readily detectable in heparin-treated media, confirming that the recombinant enzymes were catalytically active [heparin competes for HSPG binding sites along HL thereby releasing HL into the media] (Fig. 3.1.1B). Also, little hHL was detectable in conditioned media containing no heparin (Fig. 3.1.1C), suggesting that the recombinant hHL, as expected, was mainly bound to the extracellular matrix [45]. To determine the result of hHL expression on the levels of newly synthesized and secreted TAG, the stably expressing hHL cells and neomycin resistant control cells (neo) were labeled with [^{3}H]glycerol for up to 2h. With increasing levels of hHL expression in the McA-RH7777 cells, the levels of cell-associated and secreted ^{3}H-TAG were decreased (**Fig. 3.1.2A**). The levels of cell-associated ^{3}H-PC were slightly (but not significantly) increased relative to neo, while the secretion of ^{3}H-PC was not affected by hHL expression (Fig. 3.1.2B), indicating that the reduced levels of cell-associated ^{3}H-TAG in hHL-expressing cells were not due to impaired uptake of [^{3}H]glycerol by the cells. Furthermore, the cell-associated decrease in ^{3}H-TAG and increase

Fig. 3.1.1. Expression level of hHL and tributyrin hydrolase activity of post-heparin media from McA-RH7777 cells stably expressing hHL.

A. Steady-state levels of cell-associated and secreted hHL (from 4 h serum-free DMEM containing 100 U/mL heparin) were determined by immunoblot analysis from McA-RH7777 cells stably expressing varying levels of hHL or neomycin resistant control cells (neo). Cell-associated levels of actin were shown as a loading control. The molecular masses of the two cell-associated hHL bands are 53 and 66 kDa, and the molecular mass of secreted hHL is 66 kDa. *B.* The 4 h post-heparin conditioned media were used as an HL enzyme source in a [^{14}C]tributyrin-labeled *in vitro* enzyme assay. The data were presented as nmol of butyrate released/h/mL of media. Error bars indicate mean ± SD of triplicate determinations from one assay and is representative of at least two independent assays. *C.* Steady-state levels of secreted hHL from 4 h conditioned media using serum-free DMEM only (- heparin) or serum-free DMEM supplemented with 100 U/mL heparin (+ heparin). Cell-associated levels of actin were shown as a loading control. Statistical significance: ¶ $P < 0.001$; # $P < 0.01$; * $P < 0.05$ (Student's *t* -test of hHL versus neo control). HL-L, -M and -H denote McA-hHL cells exhibiting relatively low, medium and high levels of hHL expression.

Fig. 3.1.1


A
neo
HL
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hHL, media
hHL, cell
actin, cell
B
Tributyrin hydrolase activity
(nmol butyrate released/h/mL)
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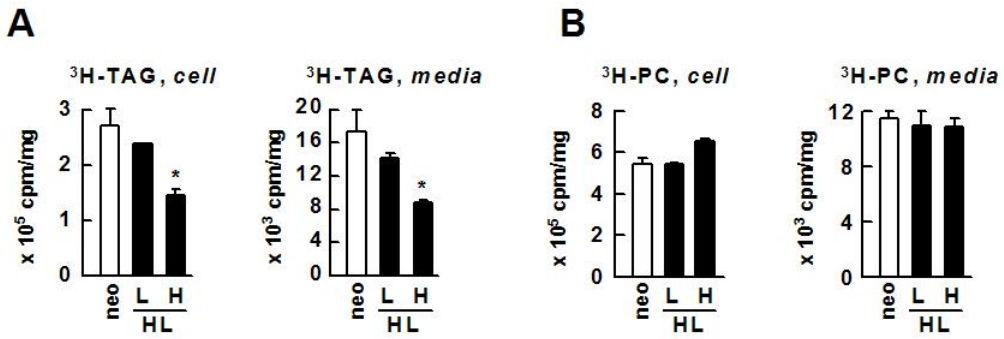
HL
C
HL
L
M
hHL, medium (+heparin)
hHL, medium (-heparin)
actin, cell

Fig. 3.1.2. Culture of McA-hHL expressing cells under lipid-rich conditions resulted in decreased cell-associated and secreted levels of [^{3}H]TAG.

McA-hHL clones or neo control cells were labeled with [^{3}H]glycerol for 2 h in DMEM containing 20% FBS and 0.4 mM oleate. At the end of the labeling period, lipids were extracted from cells and media and separated by TLC. Radioactivity associated with [^{3}H]TAG (*A*) and [^{3}H]PC (*B*) in the cell (*left panels*) and media (*right panels*) was quantified by scintillation counting. Results were expressed as cpm/mg cell protein. L and H correspond to relatively 'low' and 'high' levels of hHL-expression in McA-RH7777 cells. Error bars indicate mean ± SD of triplicate determinations from one experiment and represent at least 2 independent experiments. Statistical significance: * $P < 0.05$ (Student's *t* -test of hHL versus neo control).

Fig. 3.1.2


A
^{3}H-TAG, *cell*
x 10^5 cpm/mg
3
2
1
0
*
neo
L
H
HL
^{3}H-TAG, *media*
x 10^3 cpm/mg
20
16
12
8
4
0
*
neo
L
H
HL
B
^{3}H-PC, *cell*
x 10^5 cpm/mg
8
6
4
2
0
neo
L
H
HL
^{3}H-PC, *media*
x 10^3 cpm/mg
12
8
4
0
neo
L
H
HL

in ^{3}H-PC were also observed when the cells were labeled in the absence of oleate (**Fig. 3.1.3**). Under these conditions, the source of FAs for TAG synthesis was obtained from the hydrolysis/re-esterification of existing TAG or phospholipid stores, or from *de novo* synthesis of FA. As such, a small pool of FAs was available for TAG synthesis which was exhausted fairly quickly (30 min) by the [^{3}H]glycerol. These data indicate that hHL can negatively impact intracellular TAG levels in cells undergoing basal TAG synthesis as well as in conditions of enhanced lipogenesis. Moreover, short time labeling (15 and 30 min) of McA-RH7777 cells stably expressing hHL and neo control cells also revealed a decrease in the levels of cell-associated and secreted ^{3}H-TAG (**Fig. 3.1.4A**), and a slight but significant increase in the levels of cell-associated ^{3}H-PC (Fig 3.1.4B). Decreased incorporation of [^{3}H]glycerol into cell-associated and secreted ^{3}H-TAG was also observed in McA-RH7777 cells transiently expressing hHL via adenovirus-mediated gene transfer (**Fig. 3.1.5**), suggesting that the observed decrease in ^{3}H-TAG in stable McA-hHL clones was not due to an adaptation of chronic hHL expression.

The effect of hHL expression on VLDL metabolism was determined further by the analysis of ^{35}S-apoB-100 synthesis and secretion. Metabolic labeling experiments with [^{35}S]met/cys, showed that hHL expression did not impair but rather increased the rate of ^{35}S-apoB-100 synthesis, relative to control, while the rate of ^{35}S-apoB-100 secretion was relatively unchanged (**Fig. 3.1.6A**). The rates of synthesis and secretion of ^{35}S-albumin were unaffected in hHL-expressing cells relative to control (Fig. 3.1.6B). Although rat hepatocytes synthesize and secrete apoB-48 [116], the levels of cell-associated or secreted ^{35}S-apoB-48 were undetectable in McA-hHL, -hHLSG or neo control cells.

3.1.2 hHL expression resulted in decreased secretion of VLDL-associated ^{3}H-TAG, while the levels of secreted VLDL-associated ^{35}S-apoB-100 were unchanged

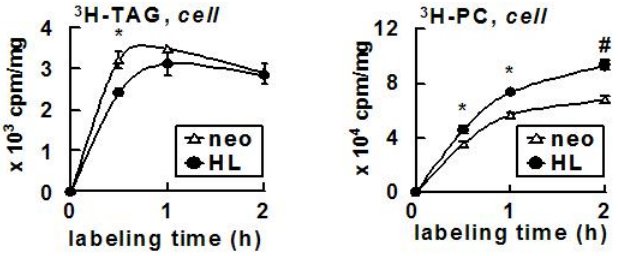
The effect of hHL expression on VLDL secretion was determined by [^{3}H]glycerol labeling followed by fractionation of the medium lipoproteins into $VLDL_1$, $VLDL_2$, IDL/LDL, and HDL fractions. Fractionation of the 2 h conditioned media revealed that secreted ^{3}H-TAG associated with $VLDL_1$ and buoyant $VLDL_2$ (fraction 2) was decreased by at most 40% from hHL-expressing cells compared to neo control (**Fig. 3.1.7A**). Secretion of ^{3}H-PC associated with $VLDL_1$ or $VLDL_2$ fractions was unaltered by hHL expression (Fig. 3.1.7B).

The effect of hHL expression on VLDL secretion was determined further by analysis of ^{35}S-apoB-100-containing lipoproteins. Fractionation of medium lipoproteins following [^{35}S]met/cys labeling revealed that although the overall ^{35}S-apoB-100 distribution among various lipoprotein fractions was similar between hHL-expressing and neo control cells, there was a slight increase in ^{35}S-apoB-100 associated with dense $VLDL_2$ (fraction 3) and IDL/LDL (fractions 4 and 5), which was more evident in the images than in the plot (**Fig. 3.1.8A**). Levels of secreted ^{35}S-albumin were unaltered by hHL expression (Fig. 3.1.8B). The point trying to be made is that were denser apoB-100-containing lipoproteins assembled and secreted in hHL-expressing cells. To show these differences, the fractional results (% of total) for the density distribution were shown.

Fig. 3.1.3. Stable expression of hHL in McA-RH7777 cells resulted in decreased levels of cell-associated [^{3}H]TAG in the absence of oleate.

McA-hHL or neo control cells were labeled with [^{3}H]glycerol for up to 2 h in DMEM containing 20% FBS. At the end of the labeling periods, lipids from the cells were extracted and separated by TLC. Radioactivity associated with [^{3}H]TAG and [^{3}H]PC was quantified by scintillation counting and expressed as cpm/mg cell protein. Error bars indicate mean ± SD of triplicate determinations from one experiment and represent 2 independent experiments. Statistical significance: # $P < 0.01$; * $P < 0.05$ (Student's *t* -test of hHL versus neo control).

Fig. 3.1.3


^{3}H-TAG, *cell*
x 10^3 cpm/mg
4
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1
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labeling time (h)
^{3}H-PC, *cell*
x 10^4 cpm/mg
12
8
4
0
*
*
#
neo
HL
0
1
2
labeling time (h)

Fig. 3.1.4. Culture of McA-hHL cells in lipid rich conditions for short durations also resulted in decreased synthesis and secretion of [^{3}H]TAG.

McA-hHL or neo control cells were metabolically labeled with [^{3}H]glycerol in DMEM containing 20% FBS and 0.4 mM oleate for up to 30 min and then the lipids were extracted from cells (*left panels*) and media (*right panels*) and separated by TLC. The radioactivity associated with [^{3}H]TAG (*A*) and [^{3}H]PC (*B*) was quantified by scintillation counting. Results were expressed as cpm/mg cell protein. Error bars indicate mean ± SD of triplicate determinations from one experiment and is representative of at least two independent experiments. Statistical significance: # $P < 0.01$; * $P < 0.05$ (Student's *t* -test of hHL versus neo control).

Fig. 3.1.4

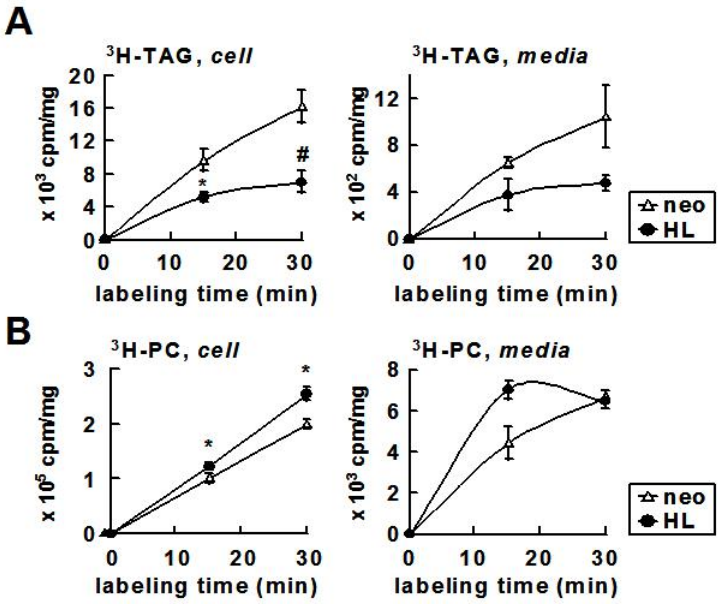

A
^{3}H-TAG, *cell*
x 10^3 cpm/mg
labeling time (min)
^{3}H-TAG, *media*
x 10^2 cpm/mg
labeling time (min)
neo
HL
B
^{3}H-PC, *cell*
x 10^5 cpm/mg
labeling time (min)
^{3}H-PC, *media*
x 10^3 cpm/mg
labeling time (min)
neo
HL

Fig. 3.1.5. Transient expression of hHL in McA-RH7777 cells resulted in decreased levels of cell-associated and secreted [^{3}H]TAG.

McA-RH7777 cells were infected with either hHL-encoding (HL) or empty vector control (ctrl) adenovirus particles at a titre of 40 pfu/cell. *A.* Steady-state levels of secreted hHL (from 4 h serum-free DMEM containing 100 U/mL heparin) were determined by immunoblot analysis and compared to McA-hHL stable cells (S). Cell-associated levels of actin were used as loading control. *B & C*. McA-RH7777 cells transiently expressing hHL or infected with the control virus were metabolically labeled with [^{3}H]glycerol for up to 60 min in DMEM containing 20% FBS and 0.4 mM oleate. At the end of the labeling periods, lipids were extracted from the cells (*left panel*) and media (*right panel*) and separated by TLC. The radioactivity associated with [^{3}H]TAG (*B*) and [^{3}H]PC (*C*) was quantified by scintillation counting. Results were expressed as cpm/mg cell protein. Error bars indicate mean ± SD of triplicate determinations. Statistical significance: # $P < 0.01$; * $P < 0.05$ (Student's *t* -test of hHL versus control).

Fig. 3.1.5

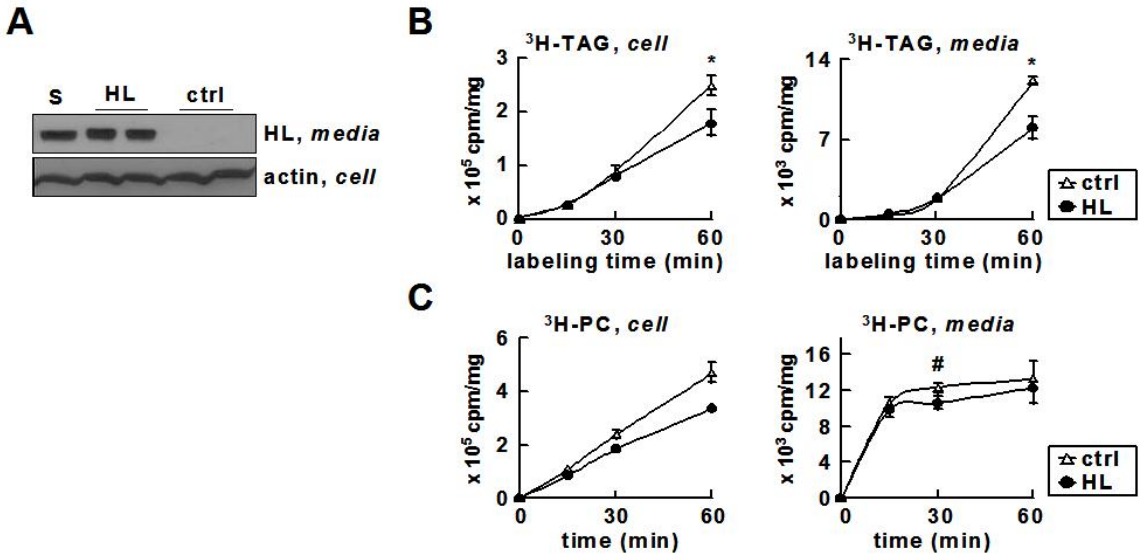

A
S
HL
ctrl
HL, media
actin, cell
B
3H-TAG, cell
x 10^5 cpm/mg
labeling time (min)
3H-TAG, media
x 10^3 cpm/mg
labeling time (min)
ctrl
HL
C
3H-PC, cell
x 10^5 cpm/mg
time (min)
3H-PC, media
x 10^3 cpm/mg
time (min)

Fig. 3.1.6. Culture of McA-hHL cells under lipid rich conditions increased the synthesis but not the secretion of [^{35}S]apoB-100.

McA-hHL or neo control cells were metabolically labeled with [^{35}S]met/cys for up to 2 h in met-/cys- DMEM containing 20% FBS and 0.4 mM oleate. [^{35}S]apoB-100 (*A*) and -albumin (*B*) were recovered by immunoprecipitation from the cell (*left panels*) and media (*right panels*) after the indicated labeling times, resolved by SDS-PAGE, exposed to film (*top panels*), quantified by densitometry analysis and normalized by cell protein levels. To combine duplicate determinations and obtain standard deviations, results were expressed relative to the highest value in each plot, set to 100% (*bottom panels*). Error bars indicate mean ± SD of duplicate determination from one experiment and is representative of two independent experiments.

Fig. 3.1.6

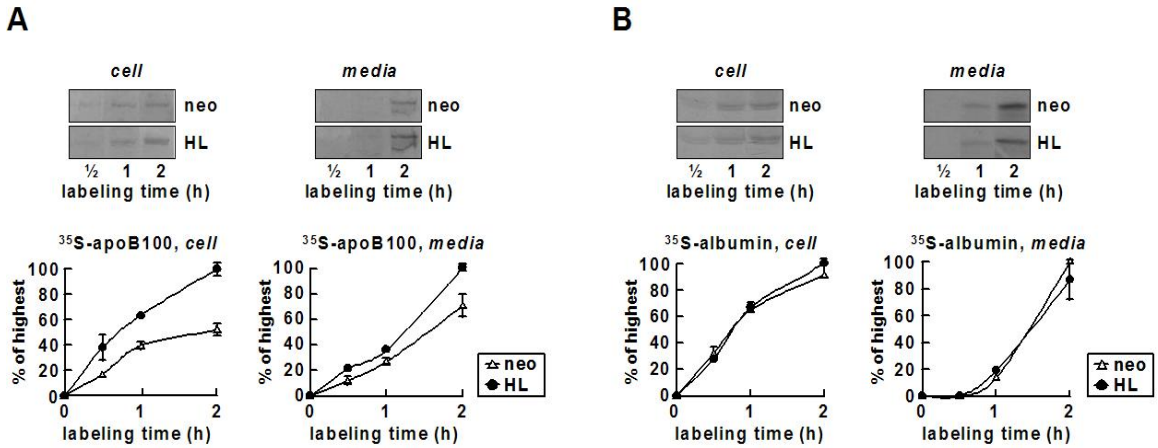

A
cell
media
neo
HL
½ 1 2
labeling time (h)
35S-apoB100, cell
35S-apoB100, media
% of highest
100
80
60
40
20
0
0 1 2
labeling time (h)
neo
HL
B
cell
media
neo
HL
½ 1 2
labeling time (h)
35S-albumin, cell
35S-albumin, media
% of highest
labeling time (h)
neo
HL

Fig. 3.1.7. Culture of hHL expressing cells under lipid-rich conditions resulted in decreased levels of secreted VLDL-associated [^{3}H]TAG.

McA-hHL or neo control cells were labeled with [^{3}H]glycerol for up to 2 h in DMEM containing 20% FBS and 0.4 mM oleate. At the end of the labeling period, the conditioned media were subjected to cumulative rate floatation ultracentrifugation. The radioactivity associated with TAG (*A*) and PC (*B*) in each fraction was quantified by scintillation counting and expressed as cpm/mg cell protein. Values are representative of two independent experiments.

Fig. 3.1.7

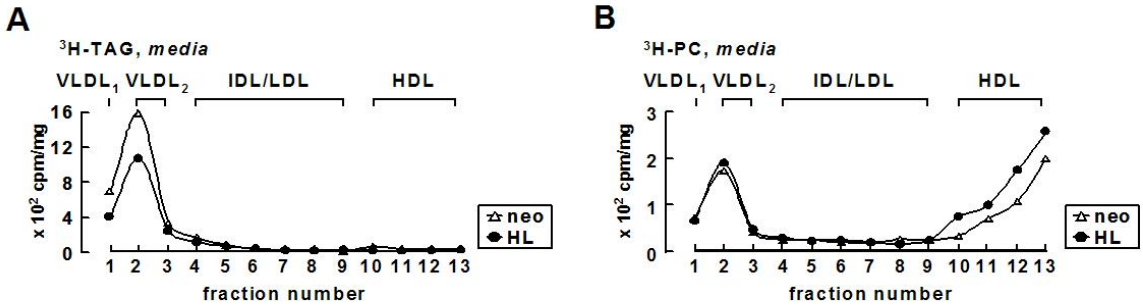
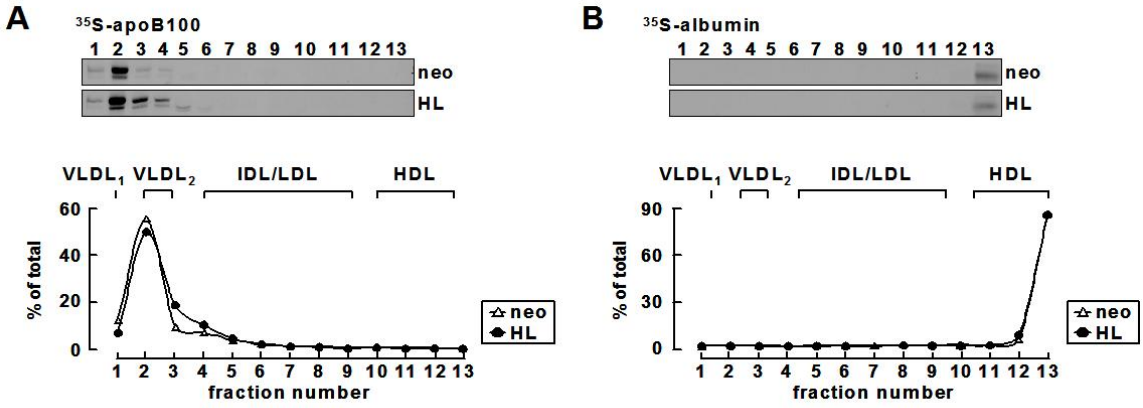

A
3H-TAG, media
VLDL1 VLDL2 IDL/LDL HDL
x 10² cpm/mg
0 4 8 12 16
1 2 3 4 5 6 7 8 9 10 11 12 13
fraction number
neo
HL
B
3H-PC, media
VLDL1 VLDL2 IDL/LDL HDL
x 10² cpm/mg
0 1 2 3
1 2 3 4 5 6 7 8 9 10 11 12 13
fraction number
neo
HL

Fig. 3.1.8. Expression of hHL did not impair [^{35}S]apoB-100 secretion.

McA-hHL or neo control cells were metabolically labeled with [^{35}S]met/cys for 2 h in DMEM containing 20% FBS and 0.4 mM oleate followed by fractionation of the media lipoproteins by cumulative rate floatation ultracentrifugation. The [^{35}S]-labeled apoB-100 (*A*) and -albumin (*B*) were recovered from the 13 fractions by immunoprecipitation, resolved by SDS-PAGE, exposed to film (*upper panels*), and quantified by scintillation counting. The radioactivity in each fraction is presented as a percent of total radioactivity in the media (*bottom panels*). Values are representative of two independent experiments.

Fig. 3.1.8


A
35S-apoB100
1 2 3 4 5 6 7 8 9 10 11 12 13
neo
HL
VLDL1 VLDL2 IDL/LDL HDL
% of total
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13
fraction number
neo
HL
B
35S-albumin
1 2 3 4 5 6 7 8 9 10 11 12 13
neo
HL
VLDL1 VLDL2 IDL/LDL HDL
% of total
90
60
30
0
1 2 3 4 5 6 7 8 9 10 11 12 13
fraction number
neo
HL

3.1.3 The catalytic function of hHL was not required for decreased levels of secreted ^{3}H-TAG.

To determine whether the catalytic activity of hHL was required for the observed phenotypes of TAG and apoB-100 in hHL-expressing cells, we generated stable cell lines expressing a mutant hHL in which Ser-145 at the catalytic site of the enzyme was substituted with Gly (designated hHLSG) (**Fig. 3.1.9A**). As expected, the mutant hHLSG lost TAG hydrolysis activity (Fig. 3.1.9B) [please note that Ser-145 to Gly mutation also abolishes phospholipase activity [237]]. Metabolic labeling experiments with [^{3}H]glycerol showed that unlike hHL expression that exhibited decreased cell-associated levels of ^{3}H-TAG, stable expression of hHLSG in McA-RH7777 resulted in [^{3}H]glycerol incorporation that was comparable to neo control cells (**Fig. 3.1.10A,** left panel). Conditioned media from hHLSG cells 2 h after [^{3}H]glycerol labeling displayed ^{3}H-TAG levels that were decreased by ~60% relative to neo control (Fig. 3.1.10A, right panel). The levels of cell-associated and secreted ^{3}H-PC from hHLSG cells were comparable to neo control (Fig. 3.1.10B). These data show that the catalytic function of hHL was not required for the reduction in secreted VLDL-associated ^{3}H-TAG, whereas it was important in reducing the levels of [^{3}H]glycerol-labeled newly synthesized cell-associated TAG.

Following 2-h [^{35}S]met/cys labeling, McA-hHLSG cells exhibited increased levels of cell-associated ^{35}S-apoB-100, while the levels of secreted ^{35}S-apoB-100 were comparable to neo control (**Fig. 3.1.11A**). The cell-associated and secreted levels of ^{35}S-albumin were relatively comparable among the three cell lines (Fig. 3.1.11B). These data show that the catalytic activity of hHL was not required for the increase in cell-associated ^{35}S-apoB-100.

3.1.4 hHL- and hHLSG-expression impaired the assembly of ^{3}H-TAG-rich VLDL within the microsomal lumen

To determine the impact of the catalytic function of hHL on intracellular TAG distribution, we contrasted subcellular localization of ^{3}H-TAG between hHL- and hHLSG-expressing cells. Whereas the cytosolic ^{3}H-TAG levels were comparable between the hHL, hHLSG and neo cell lines at the end of 1 h [^{3}H]glycerol labeling (**Fig 3.1.12A**, left panel), the microsome-associated ^{3}H-TAG was 50% of control in hHL-expressing cells and comparable to neo in hHLSG cells (Fig. 3.1.12A, right panel). Further separation of microsomes into membrane and lumenal fractions revealed a significant 60% decrease of ^{3}H-TAG associated with the microsomal membrane (Fig. 3.1.12B, left panel), and a slight (but not significant by ANOVA) 30% decrease of ^{3}H-TAG associated with the microsomal lumen of hHL-expressing cells relative to neo control (Fig. 3.1.12B, right panel). As we are determining the difference of two means, *t*-test analysis revealed that the difference between hHL-expressing and neo control was statistically significant [238]. In McA-hHLSG cells, the microsomal membrane-associated ^{3}H-TAG levels were comparable to neo control cells (Fig. 3.1.12B, left panel), whereas the levels of ^{3}H-TAG associated with the microsomal lumen were decreased (although not significantly) by 30% relative to control (Fig. 3.1.12B, right panel). The ^{3}H-PC associated with the cytosol and total microsome fractions were comparable amongst the three cell lines (Fig. 3.1.12C). The microsomal lumen-associated ^{3}H-PC was increased by ~40% in hHL-expressing cells relative to neo control (Fig. 3.1.12D, right panel), whereas the level of ^{3}H-PC associated with the microsomal membrane was unchanged between cell lines (Fig. 3.1.12D, left panel). These data show that although the catalytic activity of hHL may decrease the microsomal membrane-associated levels of newly

Fig. 3.1.9. Expression level of hHLSG and tributyrin hydrolase activity of post-heparin media from McA-RH7777 cells stably expressing hHLSG.

A. Steady-state levels of secreted HL (4 h serum-free DMEM containing 100 U/mL heparin) were determined by immunoblot analysis from McA-hHLSG cells relative to McA-hHL (HL). Cell-associated levels of actin were used as loading control. *B.* The 4 h post-heparin conditioned media were used as an enzyme source in a [^{14}C]-labeled tributyrin *in vitro* enzyme assay. The data were presented as nmol of butyrate released/h/mL of media. Error bars indicate mean ± SD of triplicate determinations. Statistical significance: ¶ $P < 0.001$; * $P < 0.05$ (Student's *t* -test of hHL versus neo control, or hHLSG versus hHL).SG-L, -M and -H denote McA-hHLSG cells exhibiting relatively low, medium, and high levels of hHLSG expression.

Fig. 3.1.9

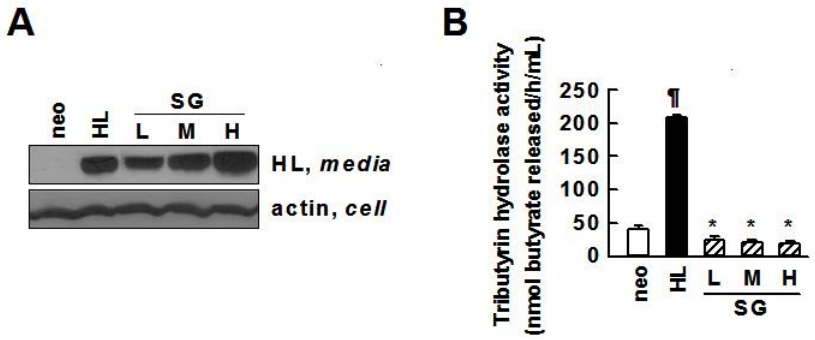

A
neo
HL
SG
L
M
H
HL, media
actin, cell
B
Tributyrin hydrolase activity
(nmol butyrate released/h/mL)
250
200
150
100
50
0
¶
*
*
*
neo
HL
L
M
H
SG

Fig. 3.1.10. McA-hHLSG stables cultured in lipid-rich conditions exhibited reduced levels of secreted [^{3}H]TAG and comparable levels of cell-associated [^{3}H]TAG relative to neo control.

McA-hHL, -hHLSG or neo control cells were labeled with [^{3}H]glycerol for 2 h in DMEM containing 20% FBS and 0.4 mM oleate. Lipids were extracted from the cells (*left panels*) and media (*right panels*) and separated by TLC. Radioactivity associated with [^{3}H]TAG (*A*) and –PC (*B*) was quantified by scintillation counting and normalized by cell protein levels. Results were expressed relative to neo, set to 1. Error bars indicate ± SD of triplicate determinations from at least two independent experiments. Statistical significance: * $P <$ 0.05 (One-way ANOVA of hHL or hHLSG versus neo control).

Fig. 3.1.10

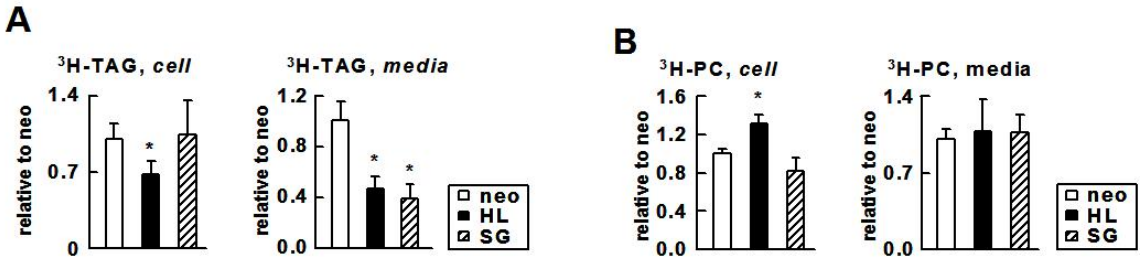

A
^{3}H-TAG, *cell*
relative to neo
1.4
0.7
0
*
^{3}H-TAG, *media*
relative to neo
1.2
0.8
0.4
0.0
*
*
neo
HL
SG
B
^{3}H-PC, *cell*
relative to neo
1.6
1.2
0.8
0.4
0.0
*
^{3}H-PC, media
relative to neo
1.4
0.7
0.0
neo
HL
SG

Fig. 3.1.11. Culture of McA-hHLSG under lipid rich conditions resulted in increased levels of cell-associated [^{35}S]apoB-100 but comparable levels of secreted [^{35}S]apoB-100 relative to neo control.

McA-hHL, -hHLSG or neo cells were metabolically labeled with [^{35}S]met/cys for 2 h in met$^-$ /cys$^-$ DMEM containing 20% FBS and 0.4 mM oleate. The ^{35}S-apoB-100 (*A*) and –albumin (*B*) were recovered by immunoprecipitation from the cell (*left panels*) media (*right panels*), resolved by SDS-PAGE, exposed to film, quantified by densitometry analysis and normalized by cell protein levels. Results were expressed relative to neo, set to 1. Error bars indicate ± SD of duplicate determinations from at least two independent experiments. Statistical significance: * $P < 0.05$ (One-way ANOVA of hHL or hHLSG versus neo control).

Fig. 3.1.11

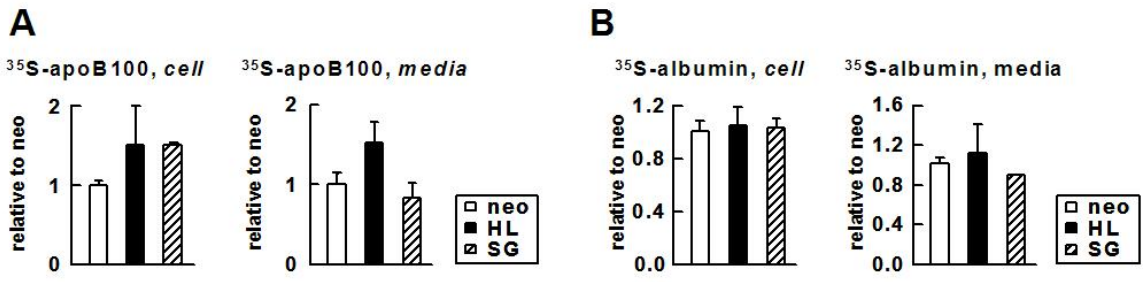
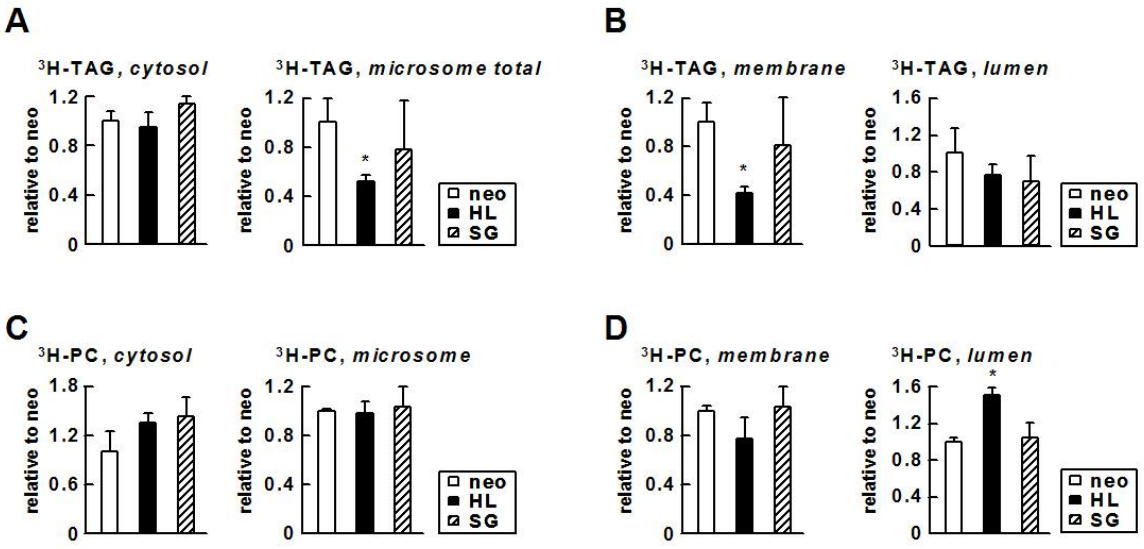

A
35S-apoB100, cell
35S-apoB100, media
relative to neo
2
1
0
neo
HL
SG
B
35S-albumin, cell
35S-albumin, media
1.2
0.8
0.4
0.0
1.6

Fig. 3.1.12. Catalytic function of hHL is not required to decrease the microsomal lumen-associated [^{3}H]TAG.

McA-hHL, -hHLSG or neo control cells were metabolically labeled for 60 min in DMEM containing 20% FBS and 0.4 mM oleate. The cells were homogenized and the cytosolic and microsomal (*A & C*) fractions were isolated. The microsomal fraction was further separated into membrane and lumen (*B & D*). Lipids were extracted from the subcellular fractions and resolved by TLC. The radioactivity associated with [^{3}H]TAG (*A & B*), and [^{3}H]PC (C & *D*) was measured by scintillation counting. The data were converted to cpm/mg of cell protein and plotted relative to neo, set to 1. Error bars indicate ± SD of duplicate determinations from two independent experiments. Statistical significance: * $P < 0.05$ (One-way ANOVA of hHL or hHLSG versus neo control). These are established laboratory protocols; the purity of the cytosol and microsomal fractions was determined by calnexin western blot analysis.

Fig. 3.1.12


A
^{3}H-TAG, *cytosol*
^{3}H-TAG, *microsome total*
relative to neo
1.2
0.8
0.4
0
*
neo
HL
SG
B
^{3}H-TAG, *membrane*
^{3}H-TAG, *lumen*
1.6
C
^{3}H-PC, *cytosol*
^{3}H-PC, *microsome*
1.8
0.6
D
^{3}H-PC, *membrane*
^{3}H-PC, *lumen*

synthesized ^{3}H-TAG, the reduction of microsomal lumen-associated ^{3}H-TAG is not dependent on catalytic activity. As such, the intracellular catalytic activity of hHL did not contribute to the reduced VLDL-associated ^{3}H-TAG secretion (Fig. 3.1.7A) from hHL-expressing cells. Interestingly, the relative distribution of [^{3}H]TAG in the cytosol seemed slightly increased in hHL- or hHLSG-expressing cells relative to control (**Fig. 3.1.13**). [Please note, because the nuclear/mitochondrial fraction (see Materials and Methods section) was not counted for radioactivity, the cytosolic and microsomal fractions amount to total cell]. These data suggest that hHL or hHLSG expression may inhibit partitioning TAG into the microsomal lumen. Although an over-interpretation, with increased number of independent experiments these observations can potentially become statistically significant sound conclusions.

To ascertain if the decreased VLDL-associated TAG from hHL- and hHLSG-expressing cells was attributable to attenuated VLDL lipidation, we determined the density distribution of newly synthesized ^{3}H-TAG within the microsomal lumen. After 1-h [^{3}H]glycerol labeling, the association of ^{3}H-TAG with IDL/LDL fractions was reduced by ~40% in both hHL- and hHLSG-expressing cells relative to control cells (**Fig. 3.1.14A**). The association of ^{3}H-PC with IDL/LDL fractions within the microsomal lumen was increased by ~20% in hHL-expressing cells (Fig. 3.1.14B), whereas the distribution of ^{3}H-PC within the microsomal lumen was not affected by hHLSG expression relative to neo (Fig. 3.1.14B). These data show that the accumulation of TAG-rich precursor lipid pools, which have been suggested to be used for bulk lipidation of VLDL [158], was impaired in hHL- and hHLSG-expressing cells.

3.1.5 HL-mediated cell-surface association of VLDL particles partially accounted for reduced levels of ^{3}H-TAG in the media of HL-expressing cells.

Human HL is mainly associated with the cell-surface (via HSPG), and has been implicated to sequester apoB-containing lipoprotein particles at the cell surface via its ligand-binding property [177;239]. In an effort to determine if the observed decrease of ^{3}H-TAG in the conditioned media was attributable to the bridging function of HL (hHL and hHLSG) sequestering TAG-containing lipoproteins at the cell surface, the labeling medium was supplemented with 100 U/mL heparin. The cell-associated levels of ^{3}H-TAG and -PC in either of the cell lines did not change with the addition of heparin (compare **Fig. 3.1.15A** to Fig 3.1.10A, left panels). The levels of secreted ^{3}H-TAG from hHL- or hHLSG cells were decreased (but not significantly by ANOVA) by 30% in the presence of heparin (Fig. 3.1.15A, right panel). By *t*-test analysis, the level of ^{3}H-TAG in the conditioned media from hHL- or hHLSG-expressing cells was significantly decreased by 30% relative to neo control [238]. Secreted VLDL-associated ^{3}H-TAG levels were decreased by 25% from hHL- and hHLSG-expressing cells relative to control after 2 h labeling in the presence of heparin (Fig. 3.1.15C). In the presence of heparin, the levels of ^{3}H-TAG in the conditioned media from McA-hHL or -hHLSG cells were significantly higher than in the absence of heparin (**Table 3.1**). The levels of ^{3}H-TAG in the conditioned media from neo control cells were unchanged ± heparin (Table 3.1). The levels of cell-associated and secreted ^{3}H-PC in the 3 cell lines were relatively unaltered with the addition of heparin (compare Fig 3.1.15B and 3.1.10B). In the presence of heparin, secretion of ^{3}H-PC associated with $VLDL_1$ or $VLDL_2$ fractions was unaltered by hHL or hHLSG expression (Fig. 3.1.15D). The findings arising from the addition of heparin to the labeling media suggest that HL-mediated sequestration of

Fig. 3.1.13. Relative distribution of [^{3}H]TAG was increased in McA-hHL and –hHLSG cells relative to control.

McA-RH7777 cells expressing hHL, hHLSG, or neo control cells were metabolically labeled for 1 h in DMEM containing 20% FBS and 0.4 mM oleate. The cells were homogenized and the cytosolic and microsomal fractions were isolated. Lipids were extracted from the subcellular fractions and resolved by TLC. The radioactivity associated with [^{3}H]TAG was measured by scintillation counting, and the data were converted to cpm/mg of cell protein. Results show distribution of [^{3}H]TAG between cytosol and total microsomes. Data are presented as percent of total [^{3}H]TAG.

Fig. 3.1.13

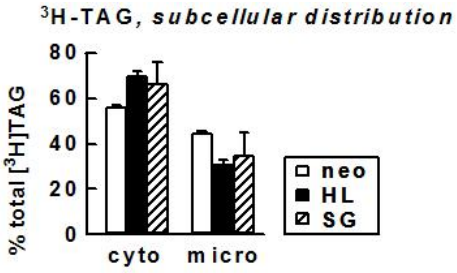

^{3}H-TAG, *subcellular distribution*
% total [^{3}H]TAG
80
60
40
20
0
cyto
micro
neo
HL
SG

Fig. 3.1.14. McA-hHL and -hHLSG stables exhibited decreased levels of [^{3}H]TAG-associated with IDL/LDL within the microsomal lumen.

McA-hHL, -hHLSG and neo cells were metabolically labeled with [^{3}H]glycerol for 60 min in DMEM containing 20% FBS and 0.4 mM oleate followed by cell homogenization and subcellular fractionation to obtain the lumenal fraction of microsome. The lumenal contents were subjected to cumulative rate flotation ultracentrifugation, and the lipids were extracted from each fraction and separated by TLC. The radioactivity associated with [^{3}H]TAG (*A*) and [^{3}H]PC (*B*) in each fraction was quantified by scintillation counting. Results were expressed as cpm/mg cell protein. Values are from combined duplicate determinations and are representative of two independent experiments.

Fig. 3.1.14

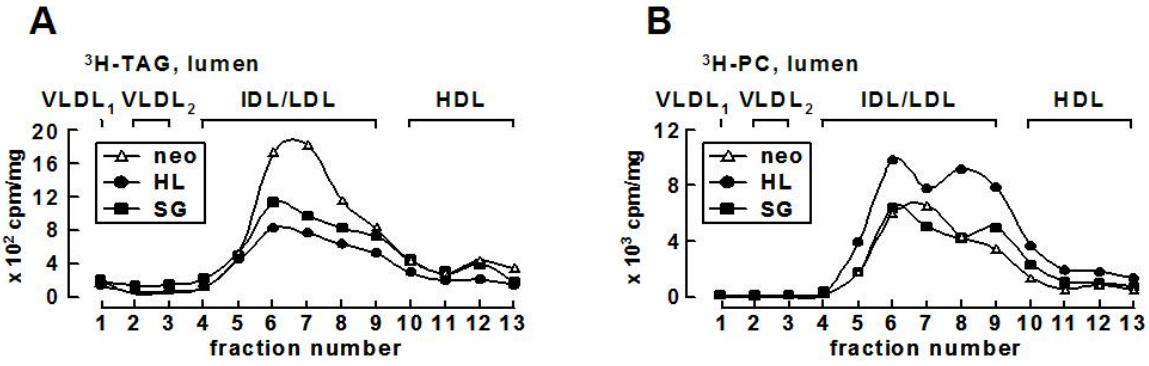
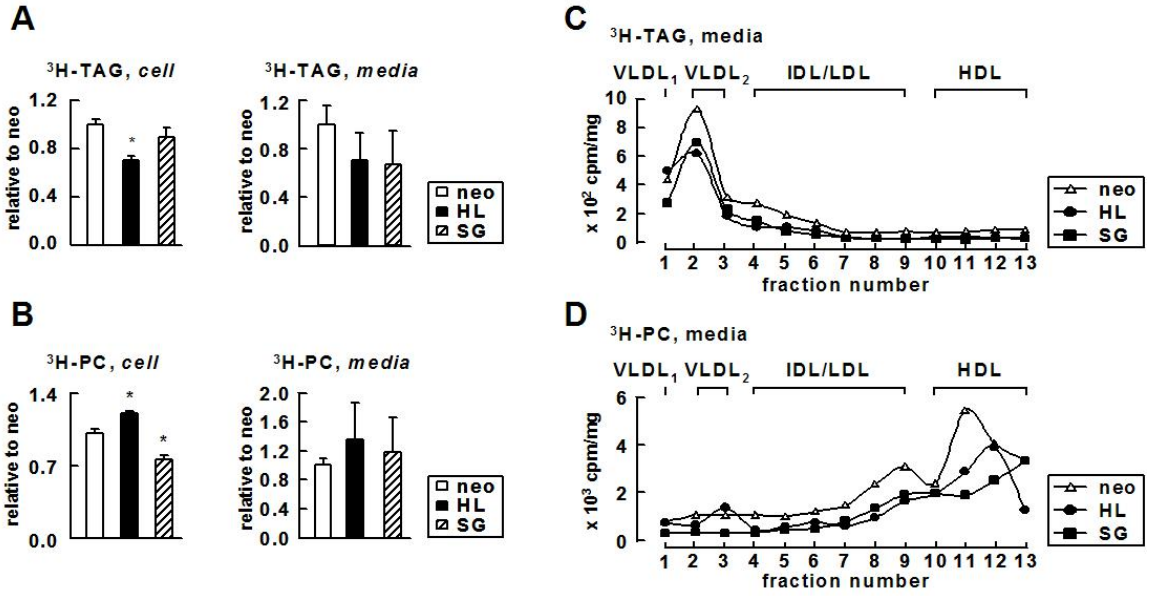

A
^{3}H-TAG, lumen
VLDL$_1$
VLDL$_2$
IDL/LDL
HDL
neo
HL
SG
x 10^2 cpm/mg
fraction number
B
^{3}H-PC, lumen
VLDL$_1$
VLDL$_2$
IDL/LDL
HDL
neo
HL
SG
x 10^3 cpm/mg
fraction number

Fig. 3.1.15. McA-hHL or -hHLSG cultured in the presence of heparin and under lipid-rich conditions also secreted reduced levels of VLDL-associated ^{3}H-TAG relative to control.

McA-hHL, -hHLSG or neo cells were labeled with [^{3}H]glycerol for 2 h in DMEM containing 20% FBS, 0.4 mM oleate and 100 U/mL heparin. Lipids were extracted from the cells and media (*A & B*), or conditioned media were subjected to cumulative rate flotation ultracentrifugation (*C & D*). Lipids were extracted from the cells, media or the 13 fractions and separated by TLC. Radioactivity associated with [^{3}H]TAG (*A & C*) or [^{3}H]PC (*B & D*) was quantified by scintillation counting and normalized by mg cell protein. In *A* and *B*, values were presented relative to neo, set to 1. Error bars indicate mean ± SD of triplicate determinations from at least two independent experiments.

Fig. 3.1.15


A
3H-TAG, cell
3H-TAG, media
relative to neo
1.2
0.8
0.4
0.0
neo
HL
SG
B
3H-PC, cell
3H-PC, media
1.4
0.7
2.0
1.6
1.2
C
3H-TAG, media
VLDL1 VLDL2 IDL/LDL HDL
x 10² cpm/mg
fraction number
D
3H-PC, media
x 10³ cpm/mg

lipoproteins at the cell surface partially accounts for the reduction in ^{3}H-TAG levels observed in conditioned media from hHL- or –hHLSG expressing cells relative to control (in the absence of heparin). A 40% decrease in ^{3}H-TAG associated with the IDL/LDL fraction of the lumen, and a 25% reduction of secreted ^{3}H-TAG associated with the VLDL fraction in the presence of heparin suggested that the HL protein impaired the formation and secretion of TAG-rich VLDL particles.

Similar to the results obtained in the absence of heparin, [^{35}S]met/cys labeling in the presence of heparin, also showed a tendency (or trend) of increased cell-associated ^{35}S-apoB-100 from hHL- or hHLSG-expressing cells relative to neo control (**Fig. 3.1.16A**). Although purely observational, the level of ^{35}S-apoB-100 in the conditioned media seemed to be increased from McA-hHL or –hHLSG cells in the presence relative to the absence of heparin (**Table 3.2**). Fractionation of the secreted ^{35}S-apoB-100-associated particles revealed that the hHL- and hHLSG-expressing cells presented with a slightly increased ^{35}S-apoB-100 association with the buoyant $VLDL_2$ fraction (fraction 2) than neo control cells (Fig. 3.1.16B). These data suggested that a considerable amount of apoB-associated lipoproteins of VLDL density was associated to the cell surface of HL (either hHL or hHLSG) expressing cells.

3.1.6 Altered expression of apoB and lipogenesis genes in McA-RH7777 cells expressing hHL or hHLSG.

To determine whether the increase in cell-associated ^{35}S-apoB-100 in hHL- or hHLSG-expressing cells was due to increased expression of the *ApoB* gene, RT-PCR analysis was performed. Interestingly, RT-PCR analysis of the *ApoB* mRNA showed that the relative expression of *ApoB* was upregulated by ~4-fold or ~8-fold relative to control in McA-RH7777 cells stably expressing hHL or hHLSG, respectively (**Fig. 3.1.17A**). In an effort to determine if the observed decrease in the levels of microsome lumen-associated ^{3}H-TAG in hHL- and hHL-SG cells was due to reduced levels of MTP, we measured the relative expression of *Mttp* in these cells. RT-PCR analysis revealed that Mttp expression was not decreased but rather increased by ~4-fold in hHL- or –hHLSG cells relative to neo (Fig 3.1.17A). To determine if the reduction in cell-associated ^{3}H-TAG was due to impaired TAG synthesis or enhanced FA oxidation, gene expression of critical enzymes in these processes was analyzed. RT-PCR analysis was performed on the genes encoding DGAT1 and DGAT2, enzymes that catalyze the final step in the biosynthesis of TAG [145]. The relative gene expression of *Dgat1* was unchanged and that of *Dgat2* was increased in hHL- or hHLSG-expressing cells relative to neo (Fig. 3.1.17B). The relative expression level of the gene encoding carnitine palmitoyl transferase (CPT-1α), the key enzyme controlling the rate of mitochondrial FA oxidation was also increased significantly in hHL and hHLSG relative to neo control cells (Fig. 3.1.17B). The RT-PCR data suggest that hHL or hHLSG expression in McA-RH7777 did not compromise the TAG biosynthesis pathway and may promote mitochondrial β-oxidation.

Table 3.1. ^{3}H-TAG levels in conditioned media from hHL, hHLSG, or neo control cells after [^{3}H]glycerol labeling in the presence or absence of heparin.

McA-hHL, -hHLSG or neo control cells were labeled with [^{3}H]glycerol for 2 h in DMEM containing 20% FBS, 0.4 mM oleate, and ± 100 U/mL heparin. Lipids were extracted from the media and separated by TLC. Radioactivity associated with ^{3}H-TAG was quantified by scintillation counting and normalized by cell protein levels. Error bars indicate ± SD of triplicate determinations, and results are representative of two independent experiments.

	^{3}H-TAG (cpm/mg)		
± heparin	neo	HL	SG
-	3776 ± 515	1403 ± 271	1181 ± 340
+	3542 ± 593	2676 ± 867*	3069 ± 1109*

Statistical significance: * $P < 0.05$ (Student's t -test of + vs. - heparin).

Table 3.2. ^{35}S-apoB-100 levels in conditioned media from hHL, hHLSG, or neo control cells after [^{35}S]met/cys labeling in the presence or absence of heparin.

McA-hHL, -hHLSG or neo control cells were labeled with [^{35}S]met/cys for 2 h in met-/cys-DMEM containing 20% FBS, 0.4 mM oleate, and ± 100 U/mL heparin. ^{35}S-apoB-100 was recovered by immunoprecipitation from the media, resolved by SDS-PAGE, exposed to film, quantified by scintillation counting, and normalized by cell protein levels. Error bars indicate mean ± SD of duplicate determinations.

	^{35}S-apoB-100 (cpm/mg)		
± heparin	neo	HL	SG
-	404 ± 3	619 ± 231	332 ± 79
+	295 ± 13	767 ± 196	1026 ± 119

Fig 3.1.16. McA-hHL or -hHLSG cultured in the presence of heparin and under lipid-rich conditions did not impair ^{35}S-apoB-100 secretion.

McA-hHL, -hHLSG or neo cells were labeled with [^{35}S]met/cys for 2 h in DMEM containing 20% FBS, 0.4 mM oleate and 100 U/mL heparin. ^{35}S-apoB-100 and –albumin were recovered from the cells (*A*), or ^{35}S-apoB-100 was recovered from the 13 fractions after conditioned media were subjected to cumulative rate flotation ultracentrifugation (*B*). The ^{35}S-apoB-100 and –albumin were resolved by SDS-PAGE and exposed to film (*top panels*). In *A*, radioactivity was quantified by densitometry, normalized by mg cell protein, and values were presented relative to neo, set to 1 (*bottom panels*). In *B*, radioactivity associated with ^{35}S-apoB-100 in each fraction was quantified by scintillation counting, normalized by mg cell protein, and presented as a percent of total ^{35}S-apoB-100 radioactivity in the media (*bottom panels*)

Fig. 3.1.16

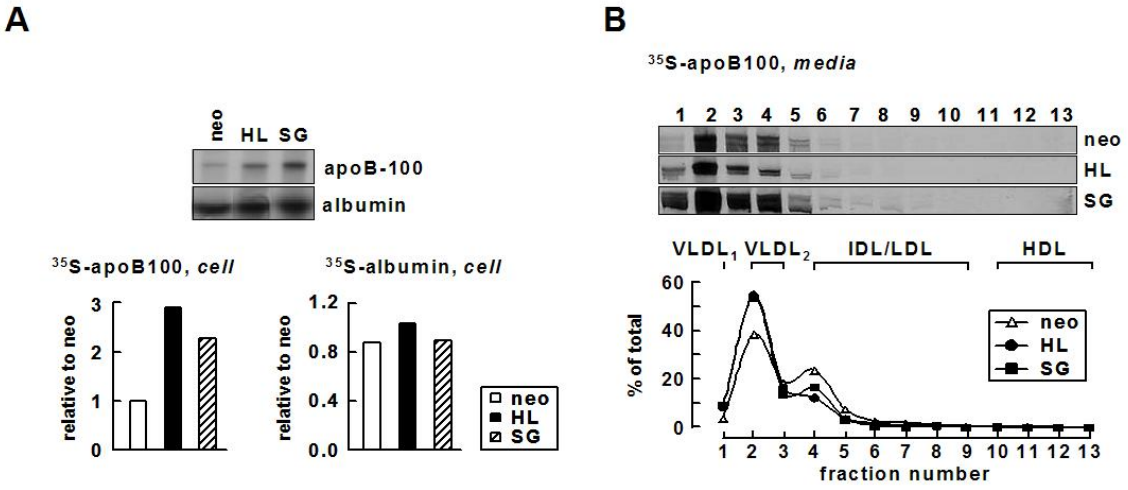
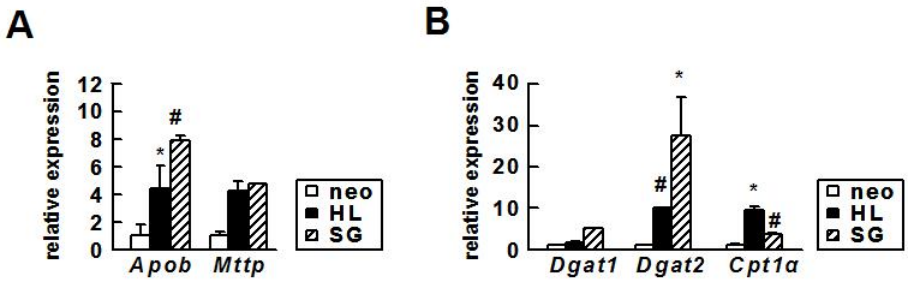

A
neo
HL
SG
apoB-100
albumin
35S-apoB100, cell
relative to neo
3
2
1
0
35S-albumin, cell
relative to neo
1.2
0.8
0.4
0.0
neo
HL
SG
B
35S-apoB100, media
1 2 3 4 5 6 7 8 9 10 11 12 13
neo
HL
SG
VLDL1
VLDL2
IDL/LDL
HDL
% of total
60
40
20
0
neo
HL
SG
1 2 3 4 5 6 7 8 9 10 11 12 13
fraction number

Fig. 3.1.17. hHL and hHLSG cells exhibit increased expression of genes involved in lipogenesis.

Total RNAs were extracted from McA-hHL, -hHLSG, and neo cells and quantitative reverse-transcriptase PCR analysis was performed on genes encoding apoB-100 (*ApoB)* and MTP (*Mttp*) (*A*), and DGAT1, DGAT2 (*Dgat1* and *Dgat2*) and CPT1α (*Cpt1α*) (*B*). The values were normalized to cyclophilin A (*Ppia*) and plotted relative to neo control cells (set as 1). Error bars indicate mean ± SD of at least duplicate determinations from at least three independent experiments. Statistical significance: # $P < 0.01$; * $P < 0.05$ (Student's *t* -test of hHL or hHLSG versus neo control).

Fig. 3.1.17


A
relative expression
12
10
8
6
4
2
0
*
#
Apob
Mttp
neo
HL
SG
B
relative expression
40
30
20
10
0
*
#
*
#
Dgat1
Dgat2
Cpt1α
neo
HL
SG

3.2 Human hepatic lipase impacts hepatic apoA-I production and secretion

3.2.1 Transient expression of hHL or hHLmt resulted in decreased steady-state levels of cell-associated and secreted apoA-I

In the current study, the potential role of hHL or hHLmt (the HSPG-binding deficient hHL chimera) on the hepatic production and secretion of apoA-I was determined by transient expression of HL in primary hepatocytes isolated from HL-null mice. Immunoblot analysis showed that the hHL and hHLmt proteins were robustly expressed and secreted from primary cells after transient expression via adenoviral-mediated gene transfer (**Fig. 3.2.1A**). The level of secreted hHL was ~3-fold higher in the presence rather than in the absence of heparin, whereas the level of secreted hHLmt was increased by 2-fold in the presence rather than in the absence of heparin. (Fig. 3.2.1A, right panel). This confirms what has previously been shown, that secreted hHL is mainly cell surface bound and can be displaced by heparin into the media [240], whereas secreted hHLmt, which has been shown to mimic cell surface binding of mHL [45] is mainly found in the media [241]. Of note, the level of secreted hHLmt was ~25 % of secreted hHL under heparin conditions although the cell-associated levels of hHL and hHLmt were comparable (Fig. 3.2.1A). The cell-associated and secreted levels of apoE were comparable in hHL- and hHLmt-expressing cells and no-infection control in the presence or absence of heparin (Fig. 3.2.1B).

To determine if hHL or hHLmt expression affected the levels of apoA-I, we performed immunoblot analysis of apoA-I from primary hepatocytes infected with either control-, hHL- or hHLmt-adenovirus for 36 h. The steady-state levels of cell-associated and secreted apoA-I were markedly decreased from hHL- and hHLmt-expressing cells relative to control at high doses of adenovirus (**Fig. 3.2.2A**). The steady-state levels of secreted apoE from hHL- and hHLmt-expressing cells were decreased by almost half relative to control

adenovirus infected cells at high doses (Fig. 3.2.2B). It was previously reported that endogenous HL is dissociated from the cell surface by exogenous apoA-I via a high-affinity association [242]. If endogenously secreted apoA-I could also associate with or displace cell-surface bound HL, we incubated the cells with heparin to remove potential HL-apoA-I complexes from the cell-surface. In the presence of heparin, steady-state levels of cell-associated apoA-I were slightly but not significantly decreased in hHL- and hHLmt-expressing cells relative to control cells at high doses of adenovirus (**Fig. 3.2.3A**, left panel), and the steady-state levels of secreted apoA-I were decreased from hHL- and hHLmt-expressing cells relative to control-adv expressing cells (Fig. 3.2.3A, right panel). The levels of cell-associated or secreted apoE were relatively unchanged in hHL-, hHLmt-expressing and cells infected with control adenovirus, at each dose (Fig. 3.2.3B). These data showed that transient expression of either hHL or hHLmt resulted in decreased steady-state levels of secreted apoA-I in the presence or absence of heparin relative to cells infected with control adenovirus. Further, the data show that the effect of hHLmt expression on the levels of secreted apoA-I was comparable to that of hHL. This was in stark contrast to what was previously reported [46]. The discrepancy could be due to the differences in the way the levels of hHL and hHLmt were normalized. Specifically, in the 2004 study, the amount of virus particles/cell of hHL and hHLmt was normalized based on similar post-heparin plasma enzymatic activity [46]; whereas, in the present study, the amount of plaque forming units (pfu)/cell of hHL and hHLmt was normalized based on similar hepatic protein levels (see Discussion section for further elaboration).

Fig. 3.2.1. hHL and hHLmt were synthesized and secreted from primary hepatocytes infected with adenovirus encoding either hHL or hHLmt.

HL-null primary hepatocytes were infected with 5 pfu/cell of either hHL- or hHLmt-adenovirus. Thirty-six hours post-infection, the cells were incubated with serum-free Hepatozyme media containing ± 100 U/mL heparin for 4 h. HL (*A*) and apoE (*B*) associated with the cells (*left panels*) and media (*right panels*) were resolved by SDS-PAGE, detected by immunoblot analysis (*upper panels*), and quantified by densitometry analysis (*bottom panels*). Error bars indicate mean ± SD of at least three independent experiments.

Fig. 3.2.1

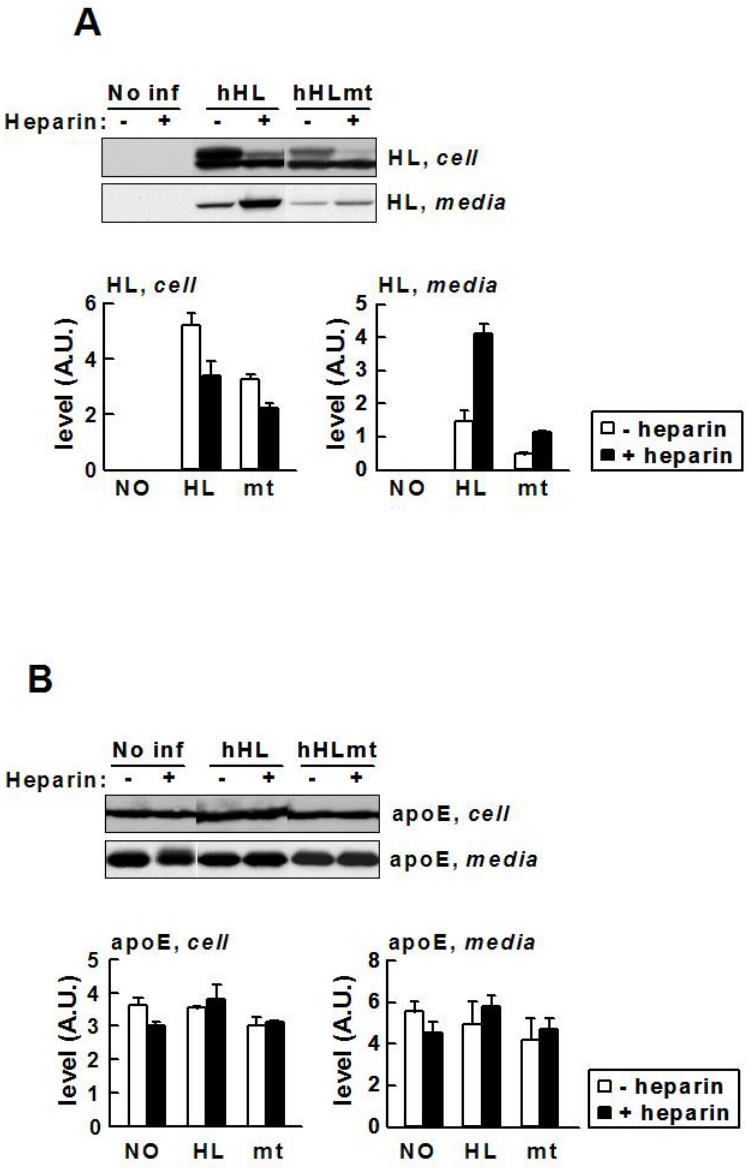

A
No inf
hHL
hHLmt
Heparin: - + - + - +
HL, cell
HL, media
HL, cell
level (A.U.)
NO
HL
mt
HL, media
level (A.U.)
NO
HL
mt
- heparin
+ heparin
B
No inf
hHL
hHLmt
Heparin: - + - + - +
apoE, cell
apoE, media
apoE, cell
level (A.U.)
NO
HL
mt
apoE, media
level (A.U.)
NO
HL
mt
- heparin
+ heparin

Fig. 3.2.2. Transient expression of hHL or hHLmt in primary hepatocytes decreased the steady state levels of cell-associated and secreted apoA-I.

HL-null primary hepatocytes were infected with control-, hHL- or hHLmt-encoding adenovirus at 10 and 20 pfu/cell. Thirty-six hours post-infection, the cells were incubated with serum-free Hepatozyme media for 4 h. ApoA-I (*A*) and apoE (*B*) were recovered from the cells (*left panels*) and media (*right panels*), resolved by SDS-PAGE, and detected by immunoblot analysis (*top panels*). The bands were quantified by densitometry and expressed relative to no infection (0) control, set to 1 (*bottom panels*). Error bars indicate mean ± SD of at least three independent experiments. Statistical significance: * $P < 0.05$ (One-way ANOVA analysis of hHLmt or hHL vs. control-adv). Control adenoviruses used encoded either EGFP or luciferase.

Fig. 3.2.2

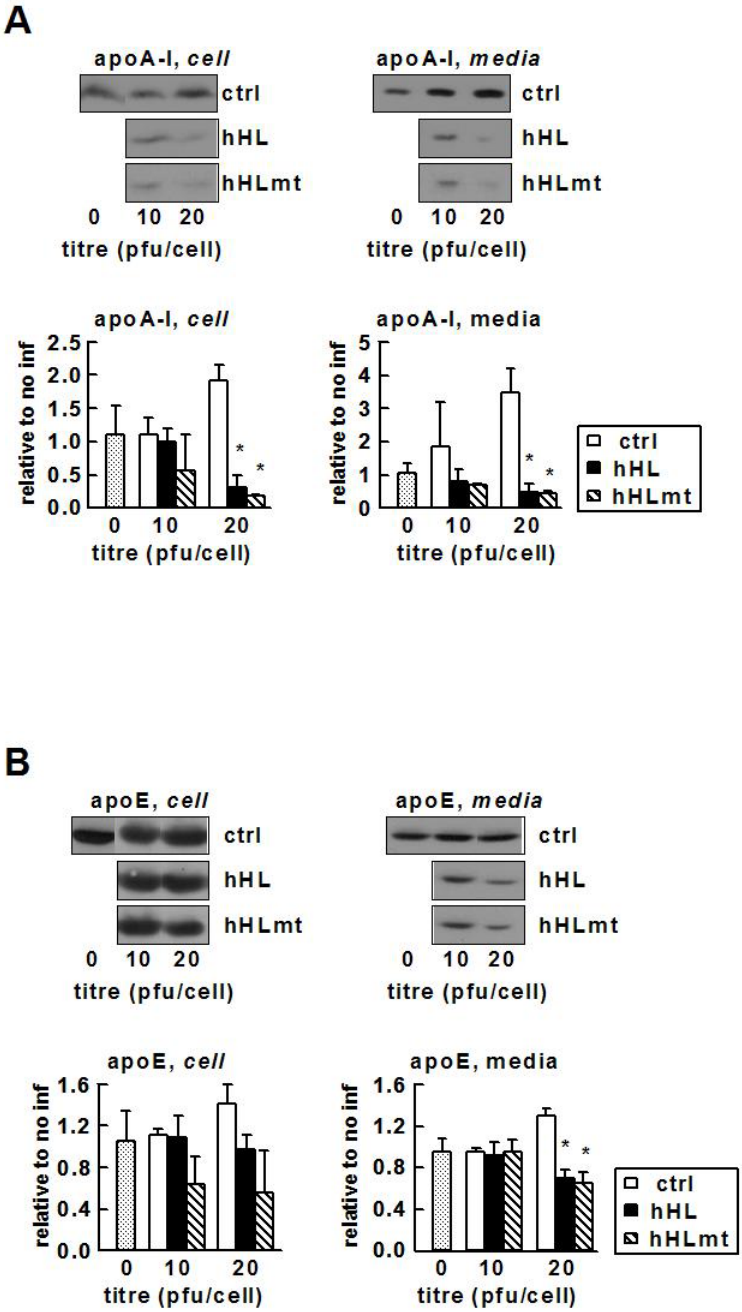
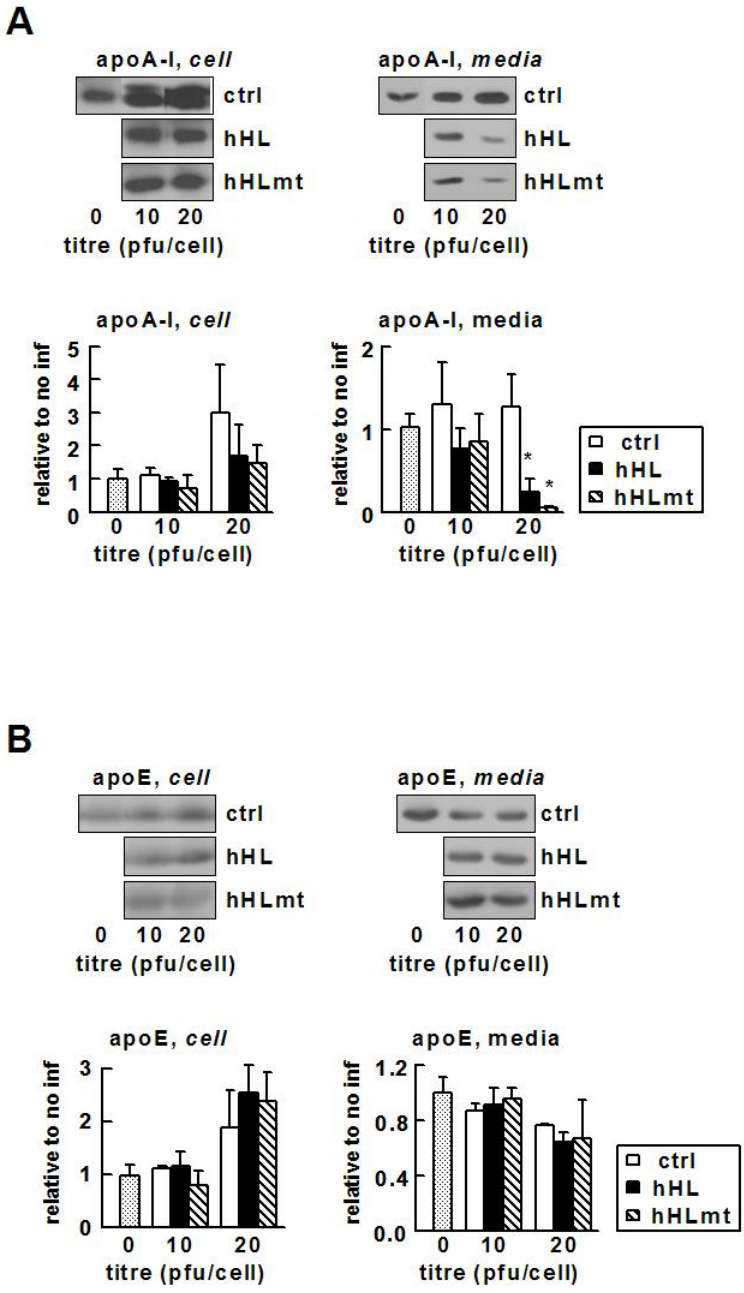

A
apoA-I, cell
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoA-I, media
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoA-I, cell
relative to no inf
2.5
2.0
1.5
1.0
0.5
0.0
0 10 20
titre (pfu/cell)
apoA-I, media
relative to no inf
5
4
3
2
1
0
0 10 20
titre (pfu/cell)
ctrl
hHL
hHLmt
B
apoE, cell
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoE, media
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoE, cell
relative to no inf
1.6
1.2
0.8
0.4
0.0
0 10 20
titre (pfu/cell)
apoE, media
relative to no inf
1.6
1.2
0.8
0.4
0.0
0 10 20
titre (pfu/cell)
ctrl
hHL
hHLmt

Fig. 3.2.3. Transient expression of hHL or hHLmt in primary hepatocytes decreased the steady state levels of secreted apoA-I in the presence of heparin.

HL-null primary hepatocytes were infected with control-, hHL- or hHLmt-encoding adenovirus at 10 and 20 pfu/cell. Thirty-six hours post-infection, the cells were incubated with serum-free Hepatozyme media containing 100 U/mL heparin for 4 h. ApoA-I (*A*) and apoE (*B*) were recovered from the cells (*left panels*) and media (*right panels*), resolved by SDS-PAGE, and detected by immunoblot analysis (*top panels*). The bands were quantified by densitometry and expressed relative to no infection (0) control, set to 1 (*bottom panels*). Error bars indicate mean ± SD of at least three independent experiments. Statistical significance: * $P < 0.05$ (One-way ANOVA analysis of hHLmt or hHL vs. control-adv). Control adenoviruses used encoded either EGFP or luciferase.

Fig. 3.2.3


A
apoA-I, cell
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoA-I, media
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoA-I, cell
relative to no inf
0 1 2 3 4 5
0 10 20
titre (pfu/cell)
apoA-I, media
relative to no inf
0 1 2
0 10 20
titre (pfu/cell)
ctrl
hHL
hHLmt
B
apoE, cell
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoE, media
ctrl
hHL
hHLmt
0 10 20
titre (pfu/cell)
apoE, cell
relative to no inf
0 1 2 3
0 10 20
titre (pfu/cell)
apoE, media
relative to no inf
0.0 0.4 0.8 1.2
0 10 20
titre (pfu/cell)
ctrl
hHL
hHLmt

3.2.2 Transient expression of hHL or hHLmt resulted in decreased rates of synthesis and secretion of apoA-I.

To determine if hHL- and hHLmt-expression decreased the rate of synthesis or secretion of apoA-I, we performed metabolic labeling experiments with [^{35}S]met/cys. Incorporation of [^{35}S]met/cys into cell-associated and secreted apoA-I was decreased by up to 50% in hHL- or hHLmt-expressing cells relative to EGFP-expressing cells (**Fig. 3.2.4A**). Incorporation of [^{35}S]met/cys into cell-associated and secreted apoE were comparable between hHL-, hHLmt-expressing or control cells (Fig. 3.2.4B). Interestingly, although the rates of synthesis of hHL and hHLmt were comparable, the rate of hHLmt secretion from the primary cells (performed in the presence of heparin to most accurately quantify the secreted HL proteins) was less than half of hHL (Fig. 3.2.4C). These data corroborate the reduced steady-state levels of secreted HL observed from hHLmt-expressing cells (Fig 3.2.1A). The reduced rate of secretion might be due to an inherent protein folding problem of the hHLmt-expressing cells due to the overexpression of the chimera protein. However, since some hHLmt is secreted at the expected size and since secretion of apoE from both cell types was comparable, the data suggest that the reduced rate of secretion of hHLmt, relative to hHL, may be due to amino acid sequence differences between hHL and hHLmt (please refer to section 4 for further clarification).

Thus, although the initial purpose of the study was to determine the mechanisms underlying the observed decrease of secreted apoA-I from hHLmt- relative to hHL-expressing cells, our findings show that the expression of either hHL or hHLmt in primary mouse hepatocytes resulted in decreased levels of cell-associated and secreted apoA-I. The potential of wildtype hHL expression affecting the levels of cell-associated or secreted apolipoproteins was novel and warranted further investigation. Accordingly, primary

hepatocytes from HL-null and wildtype mice were isolated and metabolically labeled with [^{35}S]met/cys for 2h. Interestingly, primary hepatocytes from HL-null mice exhibited a slight but significant 20% increase in the levels of secreted ^{35}S-apoA-I relative to wildtype primary hepatocytes (**Fig 3.2.5A**). Cell-associated and secreted levels of ^{35}S-apoE were comparable between the two cell types (Fig. 3.2.5B). These data demonstrate that HL expression affects apoA-I levels *ex vivo*.

3.2.3 HL impairs the production of apoA-I at the ER.

In an effort to determine the mechanism(s) whereby hHL reduces the cell-associated and secreted levels of apoA-I in primary cells, we contrasted the subcellular distribution of apoA-I and HL within hHL-, hHLmt-expressing and control cells. Subcellular fractionation revealed that there was an increased association of apoA-I with the microsomal fraction in HL-expressing cells relative to no infection control even though the cell-associated levels of apoA-I were comparable in hHL or hHLmt-expressing cells relative to no infection control (**Fig. 3.2.6A**). The levels of cell-associated or microsome-associated apoE were unaltered in hHL- and hHLmt-expressing cells relative to no infection control (Fig. 3.2.6B). Further separation of the microsomal fraction into Golgi and ER fractions revealed that hHL distributed in the Golgi apparatus and ER whereas hHLmt was mainly in the ER (Fig. 3.2.6C). Unfortunately, endogenous levels of apoA-I were undetectable in the microsomal fractions, but the distribution of apoE was unchanged between hHL and hHLmt expressing cells (Fig. 3.2.6C). We have shown that the association of apoA-I with the microsomal fraction is increased in either hHL- or hHLmt-expressing cells, and that the hHLmt protein is mainly distributed in the ER fraction.

Fig. 3.2.4. Transient expression of hHL or hHLmt in primary hepatocytes resulted in decreased rates of synthesis and secretion of [^{35}S]apoA-I, and the rate of secretion of hHLmt was 40% of hHL.

HL-null primary hepatocytes were infected with 5 pfu/cell of either EGFP-, hHL- or hHLmt-encoding adenovirus. Thirty-six hours post-infection, the cells were labeled with [^{35}S]met/cys for up to 2 (or 4) h in DMEM supplemented with 10% FBS and 100 U/mL heparin. The [^{35}S]apoA-I (*A*), [^{35}S]apoE (*B*), and [^{35}S]HL (*C*) were recovered from the cells (*left panels*) and media (*right panels*), resolved by SDS-PAGE, exposed to film (*upper panels*), and quantified by scintillation counting (*bottom panels*). In *C*, cells were infected with 20 pfu/cell of each adenovirus, labeled for 4 h, and the radioactivity expressed relative to the highest point in each plot, set at 100 %. Error bars indicate mean ± SD from two independent experiments.

Fig. 3.2.4

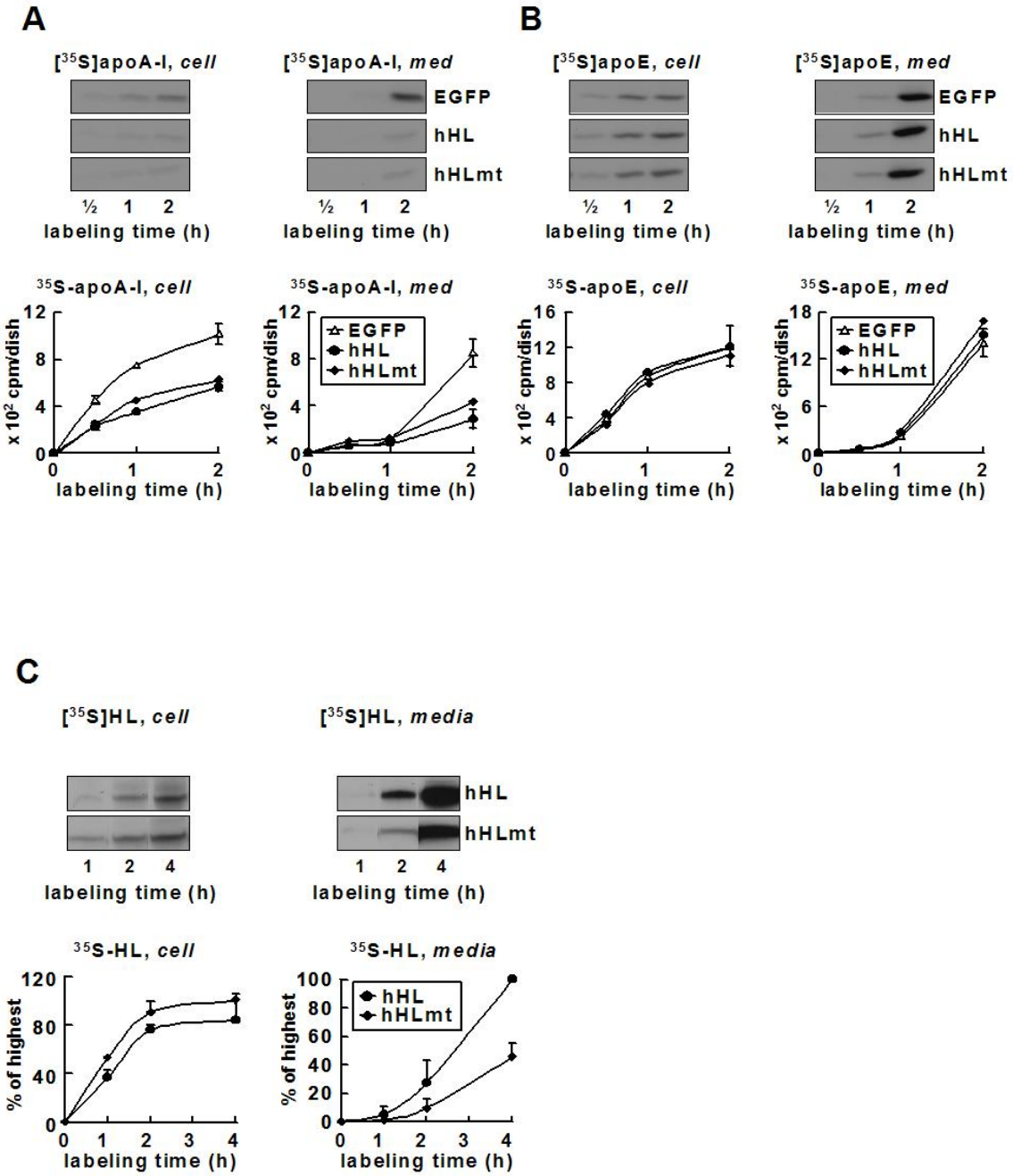

A
B
[35S]apoA-I, cell
[35S]apoA-I, med
[35S]apoE, cell
[35S]apoE, med
EGFP
hHL
hHLmt
½ 1 2
labeling time (h)
35S-apoA-I, cell
35S-apoA-I, med
35S-apoE, cell
35S-apoE, med
x 10² cpm/dish
C
[35S]HL, cell
[35S]HL, media
1 2 4
35S-HL, cell
35S-HL, media
% of highest

Fig. 3.2.5. HL-null primary hepatocytes secrete an increased level of [^{35}S]apoA-I relative to wildtype hepatocytes.

Primary hepatocytes from HL-null (KO) or wildtype (WT) mice were labeled with [^{35}S]met/cys for 4 h in DMEM supplemented with 10% FBS and 100 U/mL heparin. The [^{35}S]apoA-I (*A*) and [^{35}S]albumin (*B*) were recovered from cells (*left panels*) and media (*right panels*), resolved by SDS-PAGE, visualized on film (*insets*) and quantified by scintillation counting. Results were expressed relative to wildtype, set to 1. Error bars indicate mean ± SD of at least three independent experiments. Statistical significance: # $P <$ 0.01 (Student's *t*-test of KO vs. WT).

Fig. 3.2.5

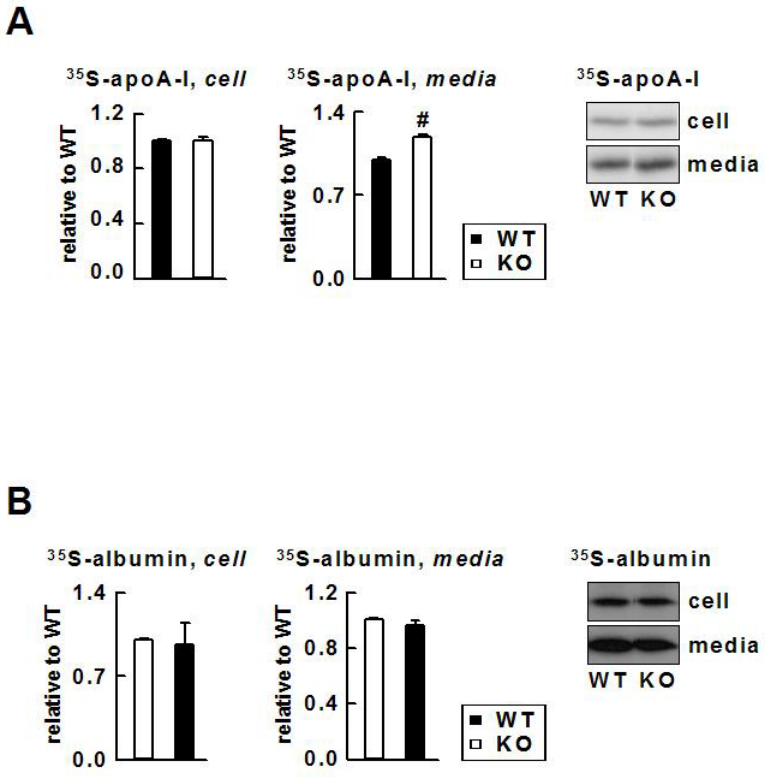
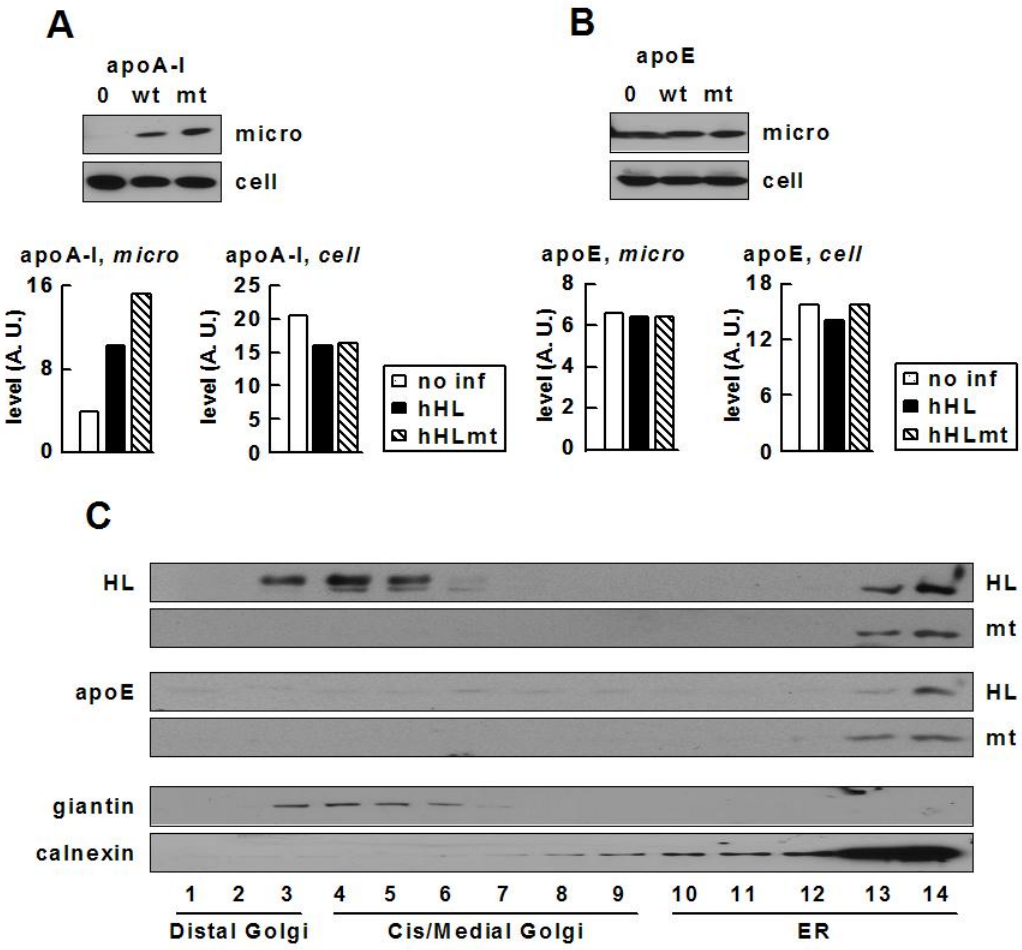

A
35S-apoA-I, cell
relative to WT
1.2
0.8
0.4
0.0
35S-apoA-I, media
relative to WT
1.4
0.7
0.0
#
WT
KO
35S-apoA-I
cell
media
WT KO
B
35S-albumin, cell
relative to WT
1.4
0.7
0.0
35S-albumin, media
relative to WT
1.2
0.8
0.4
0.0
WT
KO
35S-albumin
cell
media
WT KO

Fig. 3.2.6. hHL- or hHLmt-expressing cells exhibited increased levels of microsome-associated apoA-I relative to no infection control, and hHL and hHLmt were differentially distributed intracellularly.

A & B. HL-null primary hepatocytes were infected with 5 pfu/cell of either hHL- or hHLmt-adenovirus. Thirty-six hours post-infection, the hepatocytes were homogenized and the microsomal fractions were isolated by sequential centrifugation steps. ApoA-I (*A*) and apoE (*B*) were harvested from the microsomal fraction (*left panels*) and cell homogenate (*right panels*), resolved by SDS-PAGE, visualized by immunoblot analysis (*top panels*) and quantified by densitometry (*bottom panels*). Results are representative of two independent experiments. *C.* After hHL and hHLmt infection, the microsome was separated into Golgi and ER fractions. The steady-state distribution of HL (*upper 2 panels*), apoE (*middle 2 panels*), and giantin and calnexin (*bottom 2 panels*) were determined by immunoblot analysis. Results are representative of at least three independent experiments.

Fig. 3.2.6


A
apoA-I
0 wt mt
micro
cell
apoA-I, micro
level (A. U.)
16
8
0
apoA-I, cell
25
20
15
10
5
0
no inf
hHL
hHLmt
B
apoE
0 wt mt
micro
cell
apoE, micro
8
6
4
2
0
apoE, cell
18
12
6
0
C
HL
mt
apoE
giantin
calnexin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Distal Golgi
Cis/Medial Golgi
ER

Since the expression of either hHL or hHLmt negatively impacts the levels of cell-associated apoA-I, it is attractive to speculate that the HL (temporarily) residing in the ER is the site at which apoA-I impairment occurs.

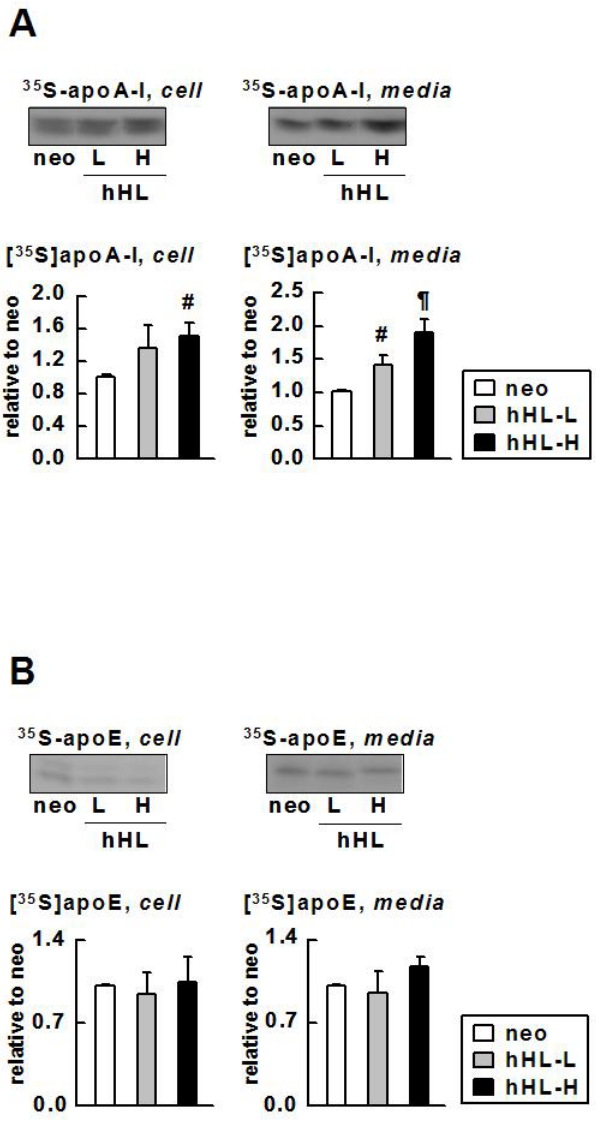
3.2.4 Stable expression of hHL resulted in increased cell-associated and secreted levels of apoA-I.

Stable expression of hHL in McA-RH7777 cells however, resulted in a dose-dependent increase in the levels of cell-associated and secreted ^{35}S-apoA-I (**Fig. 3.2.7A**). The cell-associated and secreted levels of apoE were comparable between the 3 cell lines (Fig. 3.2.7B). The reason for the discrepancy between transient and stable expression of hHL within two different cell-types is uncertain. Although stable expression of hHL in McA-RH7777 resulted in contrasting phenotypes to what was observed by transient expression of hHL (or hHLmt) in HL-null hepatocytes, the McA-RH7777 data further demonstrates that HL expression affects the protein levels of apoA-I.

Fig. 3.2.7. Stable expression of hHL in McA cells resulted in increased rates of synthesis and secretion of apoA-I relative to neo control cells.

McA cells stably expressing hHL or neo control cells were labeled with [^{35}S]met/cys for up to 4 h in DMEM containing 20% FBS. The [^{35}S]apoA-I (A) and [^{35}S]apoE (*B*) proteins were recovered from cells (*left panels*) and media (*right panels*) by immunoprecipitation, resolved by SDS-PAGE, visualized on film (*top panels*), and quantified by scintillation counting (*bottom panels*). Results were expressed relative to neo control, set to 1. L and H correspond to relatively 'low' and 'high' levels of hHL-expression in McA-RH7777 cells. Error bars indicate mean ± SD from at least three independent experiments. Statistical significance: # $P < 0.01$; ¶ $P < 0.001$ (Student's *t*-test of hHL-L or H vs. neo).

Fig. 3.2.7


A
35S-apoA-I, cell
neo L H
hHL
35S-apoA-I, media
neo L H
hHL
[35S]apoA-I, cell
relative to neo
2.0
1.6
1.2
0.8
0.4
0.0
#
[35S]apoA-I, media
relative to neo
2.5
2.0
1.5
1.0
0.5
0.0
#
¶
neo
hHL-L
hHL-H
B
35S-apoE, cell
neo L H
hHL
35S-apoE, media
neo L H
hHL
[35S]apoE, cell
relative to neo
1.4
0.7
0.0
[35S]apoE, media
relative to neo
1.4
0.7
0.0
neo
hHL-L
hHL-H

CHAPTER 4. DISCUSSION

4.1 Human hepatic lipase impairs hepatic VLDL assembly and secretion

Data obtained from the present transfection studies suggested that stable expression of HL, regardless of its catalytic activity, exerted a negative impact on the formation of the LLD and consequently the assembly and secretion of TAG-rich lipoproteins under lipid-rich conditions. This study is the first demonstration that HL, a secretory lipase, negatively impacts TAG-rich VLDL-assembly/secretion by attenuating the availability of TAG substrates without lowering apoB-100 secretion. *In vivo* and cell culture studies have shown that along with its catalytic activity in hydrolyzing triglycerides and phospholipids within circulating lipoproteins, HL also possess a non-catalytic role (also called the ligand-binding function) in which cell-surface bound HL enhances the sequestration and subsequent internalization of circulating lipoproteins. Our findings indicate that there also exists an intracellular and non-catalytic role of HL in the assembly and secretion of VLDL particles. Two additional important findings from this study are that i) hHL may play a role in the modulation of cellular lipid homeostasis via mechanisms that do not require its catalytic activity; and ii) it presents first-hand evidence that HL sequesters newly secreted VLDL particles at the cell surface. Notably, although the catalytic activity of HL was not directly measured in the cell, we extrapolated that intracellular HL activity would behave similarly to enzyme activity of secreted HL proteins (considering HL acquires catalytic activity during maturation prior to secretion [83;84]).

Stable or transient expression of hHL in McA-RH7777 cells resulted in decreased cell-associated levels of ^{3}H-TAG. It is unclear whether this decrease in cell-associated ^{3}H-

TAG was attributable to decreased TAG synthesis, rapid turnover, or both. RT-PCR analysis revealed that the expression of genes involved in TAG synthesis was not decreased in hHL expressing McA-RH7777 cells suggesting that TAG synthesis was not impaired. Reduced incorporation of [^{3}H]glycerol into TAG was observed as early as after 15 min labeling suggesting that the decrease of cell-associated ^{3}H-TAG may not be due to rapid turnover of TAG. However, a decrease in cell-associated ^{3}H-TAG was not observed in McA-RH7777 cells stably expressing the catalytically-inactive hHL suggesting that the hydrolytic activity of hHL was important for the reduced incorporation of [^{3}H]glycerol into TAG. Moreover, RT-PCR analysis revealed that the expression of CPT-1α was increased in both hHL- or hHLSG-expressing cells suggesting the decrease in cell-associated ^{3}H-TAG could be due at least partially to enhanced β-oxidation. It was unclear as to whether the partitioning of newly synthesized TAG was impaired, or if the FA (oleate) was shuttled to β-oxidation in the presence of HL. McA-RH7777 cells stably transfected with Es-x accumulated less TAG and had increased production of acid-soluble metabolites (an indicator of β-oxidation) during incubations with 0.4 mM oleic acid when compared to control [172]. Reduction of cellular TAG persisted in the presence of an esterase inhibitor E600 indicating that Es-x-mediated TAG lowering can be largely explained by reduced partitioning of exogenous FA to TAG and increased redirection to β-oxidation, rather than by increased TAG turnover [172].

In vitro studies have shown that HL hydrolyzes TAG, DAG, and MAG [16;17]. As such, if the decrease in cell-associated ^{3}H-TAG was due, at least in part, to the hydrolytic activity of hHL, the enzyme would be capable of hydrolyzing newly synthesized TAG and several of its biosynthetic precursors. Although a potential intracellular catalytic role of hHL is exciting, this concept should be regarded with caution. Intracellular lipases are tightly regulated, especially lipases that possess phospholipase activity which could potentially

hydrolyze intracellular membranes. Lipotoxicity in the cell, owing to increased generation of FA from the hydrolysis of glycerolipids by HL, is another concern. However, there is indirect evidence that ANGPTLs, a family of secreted glycoproteins, can inhibit HL activity [94]. If there exist intracellular proprotein convertases that can liberate the active N-terminus of ANGPTLs within the cell, ANGPTLS could potentially regulate the intracellular activity of HL.

HL expression resulting in decreased levels of cell-associated ^{3}H-TAG was in contrast to another HL study. The livers of HL-null mice were shown to contain lower liver TAG content than wildtype mice on both chow or high fat diet [243]. This discrepancy may be explained by the measurement of steady-state levels versus newly synthesized levels of lipids. Also, HL-null mice may have compensated for the loss of HL and this contributed to the observed phenotype; whereas in our study, the effect of HL on TAG levels was observed with transient expression of hHL in McA-RH7777 cells. On the other hand, our studies were performed *in vitro* and with the overexpression of hHL, whereas their study was conducted *in vivo* where physiological regulation by hormones and other plasma factors play a role in generating the phenotype.

The decrease in the media levels of VLDL-associated ^{3}H-TAG observed from McA-hHL cells would classically be interpreted as enhanced VLDL catabolism from the reported extracellular functions of hHL. In our study however, the decrease in media ^{3}H-TAG was observed in conditioned media even under conditions where the lipolytic and the ligand-binding properties of hHL were effectively blocked. These findings suggest that hHL has an intracellular (but non-catalytic) role in impairing the assembly and/or secretion of these TAG-containing VLDL particles. Studies with McA-RH7777 cells have shown that TAG derived from oleic acid was partitioned into microsomes and was secreted as VLDL [140].

Studies on MTP-null mice revealed the absence of lipid bodies within the ER/Golgi lumen, whereas in wildtype mice the lipid particles were observed [153]. These results and subsequent studies showed that MTP activity was required for mobilization and partitioning of TAG substrates into the microsomal lumen for VLDL assembly. In our study, subcellular fractionation showed that hHL- or hHLSG-expressing cells exhibited decreased levels of the microsomal lumen associated ^{3}H-TAG relative to neo control. These data suggested that HL expression (irrespective of catalytic activity) impaired the partitioning of newly synthesized TAG into the microsomal lumen.

Cell culture studies have suggested that the LLD-associated TAG is required for the bulk lipidation stage of VLDL assembly [152;153;158;159]. In our study, floatation of the microsomal luminal contents revealed that HL expression (irrespective of catalytic activity) decreased the levels of IDL/LDL-associated ^{3}H-TAG. Altogether, subcellular fractionation and floatation of the microsomal lumenal contents suggested that the expression of HL impairs the partitioning of newly synthesized TAG into the lumen preventing the formation of the LLD and resulting in the assembly and secretion of relatively poorly lipidated VLDL with little change in the number of VLDL particles.

That HL expression resulted in decreased levels of secreted [^{3}H]TAG was in contrast to one study, where the authors claimed that the rate of TAG secretion into the plasma was decreased in HL-null mice as compared to wildtype animals [244]. This was determined by Triton WR1339-mediated inhibition of lipase activity followed by the subsequent measurement of plasma TAG levels by mass analysis. Whereas our study observed the levels of newly synthesized [^{3}H]TAG, their study observed the mass of TAG from all pools. In addition to the inhibition of hydrolysis, Triton has properties that adversely influence lipoprotein metabolism [245].

Increased incorporation of [^{3}H]glycerol into cell-associated PC, but not secreted PC, was observed in McA-RH7777 cells stably expressing hHL expressing cells even after short time labeling of 15 minutes, in the absence of oleate (basal conditions), or in the presence of 100 U/mL heparin. However, an increase in cell-associated ^{3}H-PC was not observed in McA-RH7777 cells transiently expressing hHL or in McA-RH7777 cells stably expressing hHLSG, suggesting that this increase in newly synthesized PC occurred from chronic exposure to catalytically-active hHL. DAG is at the crossroads of CDP-choline derived PC and TAG biosynthesis; by some unknown process that requires its catalytic function, HL may preferentially favour the biosynthesis of PC over TAG. The finding that McA-hHL cells increased cell-associated ^{3}H-PC was surprising considering HL, along with being a TAG hydrolase, also possesses phospholipase activity. Interestingly, although the levels of ^{3}H-PC associated with all other subcellular fractions were unchanged, that in the microsomal lumen was increased by 40%, and this increase in ^{3}H-PC was associated with the IDL/LDL fraction. The increase of ^{3}H-PC and a decrease in ^{3}H-TAG associated with the LLD could be indicative of an increase in the number of newly synthesized phospholipid-enclosed TAG-containing lipid droplets within the microsomal lumen of hHL expressing cells.

Stable expression of hHL or hHLSG in McA-RH7777 cells did not decrease but rather increased the levels of cell-associated ^{35}S-apoB-100. The reason for enhanced synthesis of ^{35}S-apoB-100 in hHL- or hHLSG-expressing McA-RH7777 cells is unclear. RT-PCR analysis showed that McA-hHL or –hHLSG cells exhibited increased levels of *apoB* expression relative to neo control cells. This suggested that the increase in the rate of synthesis of apoB-100 in McA-hHL cells occurred, at least partially, at the transcriptional level and is independent of HL catalytic activity. As apoB expression is regulated at the post-transcriptional level, presumably via the stability of the apoB mRNA [136], it is possible that

HL expression somehow increases the stability of apoB mRNA thereby increasing its translation. The potential role of hHL in affecting the transcription of apoB-100 is novel. Due to its large size and hydrophobicity, apoB requires the help of a number of ER chaperones to assist its proper folding [246;247]. The overexpression of HL in McA-RH7777 cells could have potentially acted as another apoB chaperone thereby increasing the levels of newly synthesized cell-associated apoB.

Taken together, a decrease in the levels of secreted VLDL-associated ^{3}H-TAG and a slight increase in the levels of VLDL-associated ^{35}S-apoB-100 suggest that hHL- or hHLSG-expressing McA cells secrete lipid-poor particles. Alternatively, the decrease in VLDL-associated ^{3}H-TAG and slight increase in the levels of VLDL-associated ^{35}S-apoB-100 may suggest that HL expression promoted the use of pre-formed (unlabeled) TAG as a lipidation source in the assembly and secretion of VLDL. Follow up experiments, similar to what was performed in Lehner et al. [170], need to be performed to determine the TAG source for VLDL assembly/secretion in HL-expressing cells.

The mechanism by which HL potentially impaired partitioning of TAG into the microsome is unclear. HL is a secretory protein and thus is temporarily localized within the microsomal lumen during its maturation along its secretory pathway. Interestingly, cellular HL has a distinctive sedimentation profile, where the inactive mass overlaps the region containing active dimeric HL [84]. Furthermore, its maturation to catalytically active HL takes hours rather than minutes [86]. The microsomal lumen residence time of HL appears sufficient to support the putative function of reducing the lipid partitioning from the microsomal membrane into the lumen for which the catalytic function seems to be not required as suggested by our data. In an elegant study, Doolittle et al. [86] isolated interacting cellular factors of HL in an effort to understand its maturation process. The authors isolated several

other proteins that were not involved in HL maturation; however, none was involved in lipid binding or partitioning. Additional studies on the maturation of HL showed that the fully functional homodimer of HL associated with the microsomal membrane protein LMF1 [90]. Furthermore, owing to its function as lipolytic enzyme, HL is said to have lipid-binding regions [36]. Although purely speculative, these findings show that HL could potentially associate with the microsomal membrane, interact with newly synthesized TAG and impair the partitioning into the lumen (most probably mediated by other protein factors) (**Fig. 4.1**). It is important to note however, that reconstitution of vesicle budding using microsomes isolated from rat hepatoma McA-RH7777 cells revealed that vesicles containing apoB-100 were distinct from 'typical' protein/cargo trafficking vesicles [105;108]. Thus, HL maturation and VLDL assembly may occur in distinct cellular compartments, and as such HL may not influence VLDL assembly from within the same microsomal compartment.

Cell culture studies have been conducted to better understand which factors are involved in the partitioning and mobilization of intracellular TAG, and how this TAG distribution affects its utilization and ultimately the size of the secreted particles [170-172]. This is the first report that hHL, a secreted lipase, negatively impacts the levels and partitioning of newly-synthesized cell-associated TAG. For example, TGH-transfected cells showed a 2-fold increase in the rate of depletion of prelabeled TAG stores as revealed by lipid pulse chase analysis, whereas the level of newly synthesized [^{3}H]TAG was comparable to control cells [170]. TGH transfected cells also secreted 25% greater mass of TAG into the medium and had increased levels of apoB-100 in the VLDL compared with control cells [170]. TGH has been implicated to play a role in the hydrolysis of stored TAG pools thereby providing FA to the microsomes for the synthesis of TAG required for the assembly of VLDL [154;170]. The expression of AADA, another ER-associated protein, resulted in decreased levels of cell-

associated and secreted [^{3}H]glycerol labeled TAG [171], similar to what we have observed in this study with hHL expression. However, AADA expressing cells exhibited decreased apoB-100 secretion as evidenced by mass analysis via immunoblot analysis. Es-X expressing cells exhibited reduced levels of cell-associated [^{3}H]glycerol labeled TAG, but the secretion of [^{3}H]TAG and apoB-100 (as determined by immunoblot analysis) was unchanged [172]. Decreased secretion of TAG with no concomitant decrease in apoB-100 has also been observed *in vivo* in animal models such as liver specific inactivation of the transcription factor XBP1 in mice [248].

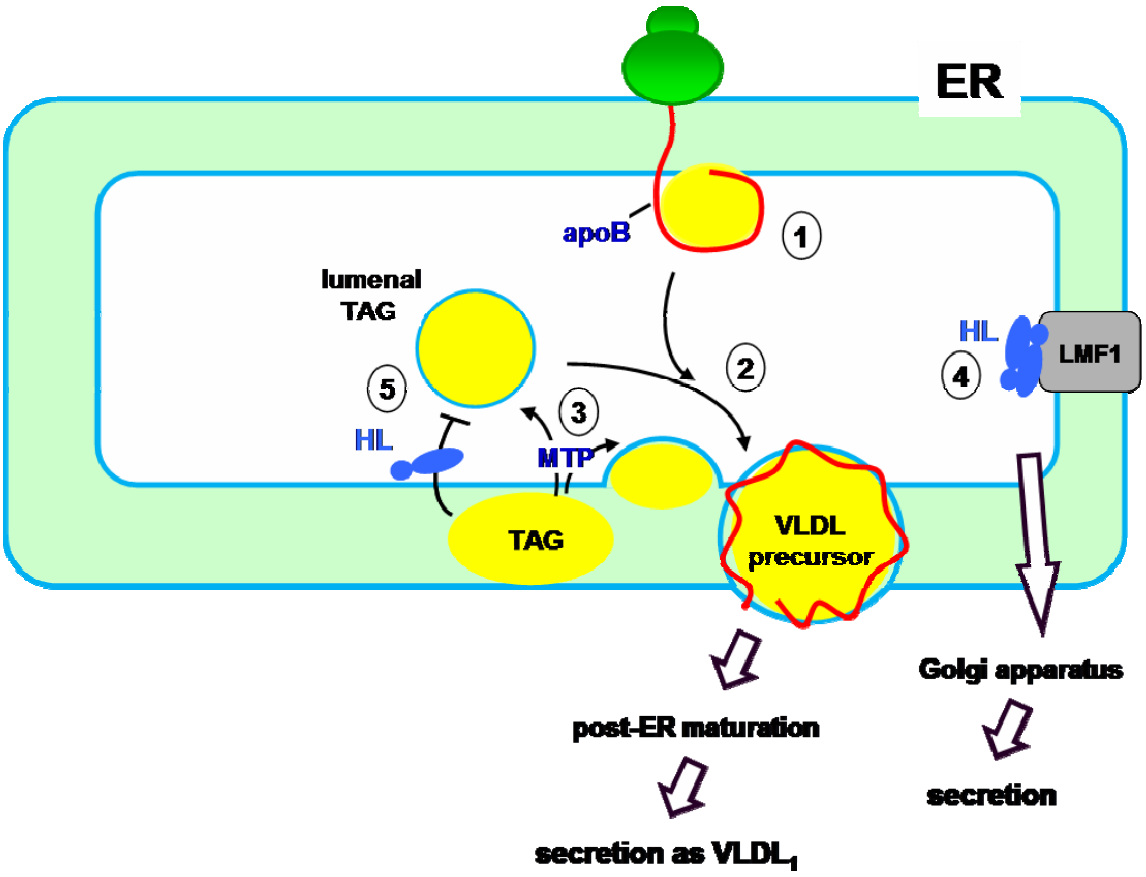
Consideration has to be given to the fact that hHL was overexpressed in a cell line that expresses and secretes endogenous rat HL (rHL). Since HL is active as a dimer, it is possible that the McA-RH7777 cells expressing hHL are also producing and secreting heterodimers of hHL and rHL and could play an important role in the various phenotypic effects observed of hHL expression in McA-RH7777 cells. Our study is not the first of its kind where a secretory protein has been described to possess intracellular roles. Transfection studies showed that recombinant apoA-V expressed in McA-RH7777 cells was unexpectedly associated with cytosolic lipid droplets, despite the fact that apoA-V possesses the signal peptide and is a secretory protein [249]. Studies from the Yao lab have shown that stable expression of apoC-III, a hepatic secretory protein which regulates LPL activity and VLDL lipolysis and clearance, in McA-RH7777 cells enhanced the hepatic assembly and secretion of TAG-rich VLDL [158;159]. There have been reports showing that LPL expression increased the lipid content of adipocytes [250], and suppression of either LPL or EL reduced the lipid content of macrophages [251], but the molecular mechanisms were not further elucidated. Thus, there have been reports of secretory proteins having intracellular functions, and in particular, secretory lipases affecting intracellular lipid levels.

RT-PCR analysis revealed that hHL protein (irrespective of catalytic function) may play a regulatory role in the modulation of cellular lipid homeostasis. This is not the first report suggesting that HL may be involved in hepatic lipid homeostasis. USF-1 (a key regulator of lipid homeostasis) stimulates HL expression [92]. As such, it may be possible that through the regulation of HL expression, USF-1 controls cellular lipid homeostasis including genes involved in TAG synthesis and β-oxidation. The mechanism by which hHL modulates cellular lipid homeostasis is unclear. It is at least known that the regulation does not require the catalytic function of hHL. Since HL has not been reported in the nucleus, maybe its regulatory role occurs indirectly through other protein mediators. It must be noted that an upregulation of relative gene expression does not always correlate with an increase in protein levels as there exist translational and post-translational regulatory mechanisms. Attempts were made to measure the steady-state levels of DGAT2 by western blot analysis from cell lysates as well as from microsomal lysates, but DGAT2 levels could not be detected. The increase in relative gene expression of *MTTP* may also suggest that HL impaired MTP activity thereby creating a feedback loop which stimulated the production of MTP. That MTP activity was impaired by HL expression has to be confirmed, but could potentially lead to the reasoning behind the reduction in the TAG associated with the LLD in HL expressing cells. Alternatively, *MTTP* expression may be increased to compensate for the HL-mediated reduction in LLD-associated TAG. The increase in the relative expression of *Cpt1α* may also suggest that there was an influx of FA going to the mitochondria for oxidation in hHL- and hHLSG-expressing cells.

Fig. 4.1. hHL expression impairs partitioning of TAG into LLD leading to the secretion of lipid-poor VLDL particles.

ApoB acquires lipid during translation and translocation (1) and the nascent apoB-lipid particle acquires additional TAG in a step-wise fashion from the LLD (2). The activity of MTP is required for partitioning of TAG into the ER membrane or lumen (LLD) for VLDL assembly (3). Before they homo-dimerize and acquire catalytic activity by the action of the LMF1 (4), newly synthesized monomers of hHL reside in the ER lumen. hHL can reduce the partitioning of TAG into the microsomal lumen (5) leading to the assembly and secretion of lipid-poor VLDL particles. apoB, apolipoprotein B; ER, endoplasmic reticulum; hHL, human hepatic lipase; LMF1, lipase maturation factor 1; MTP, microsomal triglyceride transfer protein; TAG, triacylglycerol; and VLDL, very low density lipoprotein. Modified from Sundaram *et al.* [92].

Fig. 4.1


ER
apoB
1
lumenal
TAG
2
HL
4
LMF1
5
3
HL
MTP
TAG
VLDL
precursor
Golgi apparatus
post-ER maturation
secretion
secretion as VLDL1

The classic view of cell-surface association of HL, LPL and EL was that these enzymes are anchored at the endothelium by interactions with HSPGs. However, it is now clear that GPIHBP1 is important for binding of LPL to the endothelium [19]. HL activity is normal in patients with nonfunctional GPIHBP1 showing that HL and LPL have different cell-surface binding sites [252]. Therefore, at present, HL is considered bound to the cell surface mainly by interactions with HSPG. Heparinase treatment of HL-expressing McA-RH7777 cells showed that HSPG mediated about 90% of HL-mediated cell-surface lipoprotein association [253]. Thus assuming the process was complete, the use of heparin in our labeling procedures effectively removed HL from the cell surface.

Studies describing HL-mediated cell surface lipoprotein sequestration used exogenously supplied labeled lipoproteins to HL-expressing cells [177;178]. In our study, however, comparing the respective levels and lipoprotein profiles of secreted [^{3}H]TAG- and [^{35}S]apoB-100-associated particles from HL-expressing cells in the presence or absence of heparin revealed newly synthesized lipoprotein particles associated with the cell surface. Since the levels of TAG-associated VLDL particles from the control cells were not increased with the addition of heparin, the HL +/- heparin data indicated that VLDL particles were associated with the cell surface mainly through an HL-mediated process. This HL-mediated cell-surface sequestration of secreted TAG- or apoB-100-containing lipoproteins was independent of its catalytic function. This is the first report of endogenously secreted VLDL particles being sequestered at the cell surface through an HL-mediated process.

Besides removing HSPG-bound proteins [254], heparin has been shown to possess other functions. Two-hour exposure to heparin in smooth muscle cells resulted in receptor-mediated endocytosis of this compound [255]. Long term (> 20-h) exposure to heparin (5.4 μg/mL) in a human parenchymal hepatoma cell line HepG2 stimulated transcription and

secretion of hHL [93]. Since only the coding sequence of hHL was cloned into the mammalian vector for subsequent transfection in McA-RH7777 cells (see Materials and Methods), the regulatory sequences controlling heparin activation are not present upstream of the integrated hHL. The levels of endogenous rHL, although minor relative to the levels of overexpressed hHL, could have been increased by heparin [the actual levels of rHL and hHL were not directly measured]. As such, the increase of catalytically active rHL into the media would theoretically increase the hydrolysis of media TAG thereby decreasing the total level of ^{3}H-TAG in the conditioned media. However, the level of ^{3}H-TAG in the conditioned media was actually increased in the presence than in the absence of heparin from HL-expressing cells. This suggested that the potential effect of heparin on rHL was minor. In another study, long term exposure (2-day) to heparin (3 μM) in human alveolar cells increased Forkhead family member FoxA1 protein expression [256]. Fox transcription factors regulate differentiation, development, as well as metabolism [257], and Foxa1 has been reported to suppress lipid accumulation in adipocytes [257]. It is not reported whether heparin incubation increases FoxA1 in hepatocytes, but it is possible that the presence of heparin in 2-h labeling media could have affected the transcription of this and other genes involved in cell lipid homeostasis. However, the levels of cell-associated ^{3}H-TAG did not change in the presence of or absence of heparin suggesting that the short-term (2-h) heparin incubation did not grossly affect cellular lipid metabolism. Besides affecting clearance of lipoproteins, there are no reports of heparin affecting apolipoprotein levels in hepatocytes, nor are there studies of heparin affecting the buoyant density of lipoprotein particles or the proteasomal degradation of proteins.

That HL has an intracellular and non-catalytic role in reducing the levels of newly synthesized TAG that associate with the microsomal lumenal lipid droplet (thereby impairing

the assembly and secretion of lipid-rich VLDL) is novel and warrants further investigation. Initial follow up studies to better understand the global cellular effect of HL expression could involve confirming the +heparin data with heparinase treatment, measuring the protein levels and activities of MTP, DGAT2 and CPT1α, and determining whether FAs are preferentially shuttled for oxidation, PC or TAG synthesis in HL-expressing cells. As the phenotype was detected for newly synthesized lipids and proteins, it would be of interest to determine whether the mass of TAG and apoB-100 is altered within the cell or media of HL-expressing cells. Future directions of the research could include determining whether HL impairs the formation of the LLD or reduces pre-existing LLD, whether HL is associated with the LLD or microsomal membrane, and which proteins HL associates with in the microsome. Further, structure-function analysis could be performed on HL to determine which domains (lipid binding, HSPG binding, etc) are important for the phenotype. Interestingly, point mutations of HL can be made to mimic naturally occurring mutations to determine if the phenotype is observed or abolished. In an effort to confirm the phenotype, short interfering RNA (siRNA) against HL can be employed, and the phenotype can be mimicked in another cell type.

The major weakness of the current study is that the differences between hHL- or hHLSG-expressing and neo control cells were not always statistically significant, and as such some experimental conclusions were drawn from the observable trend in the data. As it is nearly impossible to reduce the variation in these multi-step experiments, one way to reduce standard deviation and show statistical significance is to increase the number of independent experiments. An alternate approach to show differences between hHL- or hHLSG-expressing and neo control cells is to perform different types of experiments. For instance, supporting immunocytochemistry analysis confirming biochemical data, or mass analysis confirming metabolic labeling results could have been performed. However, the major strength of the

current study is that a novel intracellular role for hHL was determined in the assembly and secretion of VLDL which fits with the accepted inverse correlation between HL expression and VLDL-TAG levels observed in mouse models and humans.

Our cell culture studies revealed a novel intracellular role of HL that adds to the complexity of HL in lipoprotein metabolism and the controversy surrounding a pro- or anti-atherogenic role for HL. As explained in Chapter I and eloquently summarized by Zambon et al. [62], HL deficiency is associated with diminished conversion of remnant VLDL-to-IDL and IDL-to-LDL, and to reduced hepatic uptake of remnants and IDL particles. Remnant lipoproteins and IDL are major determinants of atherosclerosis, suggesting that HL deficiency creates an atherogenic profile. On the other hand, high HL activity may result in an increased atherosclerotic risk by promoting the formation of small, dense LDL particles. Additionally, HL is also synthesized by macrophages suggesting that HL may also contribute to the progression of atherosclerosis by enhancing the retention of VLDL, IDL and small LDL at the site of the atheroma. Our study showed that at the level of the liver, HL impairs the assembly and secretion of the VLDL (the precursor particle for IDL and LDL). As HL has been shown to be expressed in macrophages, it is of interest to determine the intracellular role of HL within this cell type since it is one of the important contributors to the progression of atherosclerosis.

4.2 Human hepatic lipase impacts hepatic apoA-I production and secretion

Raising HDL levels is of utmost importance since it has cardio-protective properties; thus understanding how this lipoprotein class is regulated is critical. The use of the human HL chimera hHLmt has shed light on another protein factor that is involved in apoA-I secretion and HDL biogenesis, HL. The findings of this study show that hHL or hHLmt expression suppresses apoA-I secretion and suggest that HL has a role in the synthesis and secretion of apoA-I/nascent HDL formation from the hepatocyte. The discovery that hHL expression impaired apoA-I synthesis and secretion is novel. Importantly, since hHL and hHLmt shared the same phenotype but had differential properties, hHLmt could be used as a tool to better understand the mechanism whereby hHL impaired the synthesis and secretion of apoA-I.

We have shown that the hHLmt protein exhibited a reduced rate of apoA-I secretion relative to hHL, and hHLmt was mainly distributed in the ER fraction. It should be noted that Nycodenz fractions considered distal Golgi (fractions 1-3) could be contaminated by endosomes [106]. Regardless if the bands in fraction 3 of the Nycodenz fraction show hHL destined for secretion or hHL within endosomes, the data suggest that hHLmt is mainly distributed in the ER. The reduced rate of secretion might have been due to an inherent protein folding problem of the hHLmt-expressing cells due to the overexpression of a chimeric protein. However, since some hHLmt was secreted at the expected size and since secretion of apoE from both cell types was comparable, the data suggest that the reduced rate of secretion of hHLmt, relative to hHL, may be due to differences in sequence elements between hHL and hHLmt. In a study by Masuno et al., short term use of chlorate was used to inhibit the sulphation of GAG and LPL synthesis and secretion was studied [258]. It was shown

that the chlorate treated cells were unable to transport LPL to the Golgi and thus LPL accumulated in the ER [258]. These data suggest that the binding of LPL to HSPG is necessary for intracellular transport of LPL. Similarly in our study, hHLmt, which is unable to bind HSPG, seems to accumulate in the ER and is poorly secreted.

We have also shown that the expression of either hHL or hHLmt reduces apoA-I production and secretion and that hHLmt is mainly distributed in the ER. Thus, it is attractive to speculate that the HL residing in the ER is the site at which apoA-I impairment occurs. Co-immunoprecipitation experiments with anti-HL and -apoA-I antibodies under non-denaturing conditions revealed that HDL from normolipidimic individuals displaced hHL from the cell surface of Chinese hamster ovary cells (stably expressing hHL) through a direct interaction between HDL and hHL [242]. If apoA-I and HL have the potential to associate *in vitro*, it is possible that apoA-I and HL can associate within the cell as well, considering both of these proteins are within the secretory pathway.

In McA-RH7777 cells, stable expression of hHL resulted in a dose dependent increase in the levels of cell-associated and secreted ^{35}S-apoA-I. The reason for the discrepancy in apoA-I level between transient and stable expression of hHL in hepatocytes is uncertain. The phenotype in McA-hHL cells could be due to chronic exposure of an overexpressed protein. Alternatively, the proteome and signaling pathways between primary hepatocytes and hepatoma cells may be different at handling hHL expression. Furthermore, there may also be different signaling pathways and/or transcriptional regulation of apoA-I between mouse and rat hepatic cells.

It has been suggested that *de novo* synthesized apoA-I associates with phospholipids and cholesterol prior to its secretion as a nascent HDL particle [209]. It was also reported that phospholipid association was required for apoA-I stability [259]. It is attractive to speculate that

the intracellular hydrolytic activity of HL could negatively impact the stability of newly synthesized nascent-HDL (apoA-I) particles. However, the levels of cell-associated ^{35}S-apoA-I in HL-null or wildtype hepatocytes was comparable. Nevertheless, the levels of secreted ^{35}S-apoA-I from HL-null hepatocytes cells were increased relative to wildtype. *In vivo* studies have also shown that HL-null mice exhibited increased steady-state levels of plasma apoA-I relative to control mice [65;260]. The idea that HL may play a role in the secretion of apoA-I was initially suggested by Lambert et al. [260] where the fractional catabolic rate of apoA-I in the HL-null was comparable to that from wildtype mice.

In view of either hHL- or hHLmt-expression resulting in a similar phenotype in terms of apoA-I levels, it suggests that the N-terminal ~400 amino acids of the hHL protein is the effector sequence that either directly associates with apoA-I to mediate the observed changes, or hHL associates with lipid(s) or protein effector(s) at this region to bring about these changes. Besides the described catalytic region, lipid binding domains and N-glycosylation sites, putative protein domains within the N-terminal region of hHL (based on the Expasy Prosite http://expasy.org/ algorithms) include protein kinase C phosphorylation sites, an amidation site, casein kinase II phosphorylation sites, and N-myristoylation sites. Accordingly, Park et al. [261] have reported that activation of LPL might require some modification such as phosphorylation in the Golgi. It has also been shown that protein kinase A and protein kinase C phosphorylation signaling pathways are involved with ABCA1 in the lipidation of apoA-I [196]. This ABCA1-mediated phosphorylation could be altered in HL-expressing cells considering HL can associate with the membrane in ER [90], thereby limiting apoA-I lipidation and potentially impairing its stability and subsequent secretion. However, it has been reported that apoA-I associates with lipid in the ER mainly through ABCA1-independent mechanisms [209]. As such, the mechanisms by which HL negatively impacts

apoA-I in the ER may or may not involve the impairment of lipidation of apoA-I, and may or may not involve the ABCA1. ApoA-I secretion was reduced from PLTP-null relative to wildtype primary hepatocytes [259]. The authors attributed this decrease in apoA-I secretion to the observed change in the PC/sphingomyelin ratio which lead to the destabilization of the apoA-I protein [259]. They thus speculated that PLTP might play a role in the formation of nascent HDL within the secretory pathway, such as in the association of phospholipids with newly synthesized apoA-I [259].

Notably, the observed rates of synthesis and secretion show that the synthesis of hHL or hHLmt reached a plateau after 2-h labeling while the secretion of hHL and hHLmt continued to increase up to 4-h. This may suggest what has previously been described, that the rate limiting step of the production of HL is the formation of functional dimers [84]. That is, a large amount of inactive HL monomers is synthesized, processed into dimers, and then secreted [84]. It is also worth mentioning that the dose of hHL or hHLmt required to decrease the steady-state cell-associated or secreted levels of apoA-I was higher (10 – 20 pfu/cell) than the dose required to decrease the levels of newly synthesized or secreted apoA-I (5 pfu/cell). This is most probably because the 'steady-state' levels of apoA-I encompass many different pools of apoA-I protein, including newly synthesized, cell-surface associated, and apoA-I re-uptaken for the purposes of lipidation or degradation [212]. As such, hHL or hHLmt expression impaired the rate of synthesis of apoA-I, as demonstrated by the [^{35}S]met/cys labeling experiments, but most probably did not affect the other pools of apoA-I that were detected by immunoblot analysis. Additionally, the steady-state levels of cell-associated apoA-I were significantly decreased in high dose hHL- or hHLmt-expressing cells relative to control. However, in the presence of heparin, the steady-state levels of cell-associated apoA-I were only slightly reduced in high dose hHL- or hHLmt-expressing cells. The reason for this

is unclear. Furthermore, it was not clear why the cell-associated and secreted steady-state levels of apoA-I were increased in hepatocytes infected with control adenovirus as compared to 'no infection' control cells. It has been described that viral infection *in vivo* elicits host responses including lowering plasma apoA-I levels [262], however such responses to infections have not been described *ex vivo*/*in vitro*. Moreover, apoE was used as a control in our study to show that hHL- (or hHLmt-) expression specifically decreased the levels of apoA-I. The rates of synthesis or secretion of apoE did not change in hHL- or hHLmt-expressing cells relative to control. However, although the effect of hHL- or hHLmt-expression was more potent at reducing the levels of steady-state apoA-I, the steady-state levels of secreted apoE were also significantly decreased in high dose hHL- or hHLmt-expressing cells in the absence of heparin. In the presence of heparin however, the steady-state levels of secreted apoE were unchanged relative to control. These ± heparin immunoblot studies suggest that a pool of apoE protein is associated with the cell surface (via HSPG) in the absence of heparin.

Our study has revealed that the effect of hHLmt expression on the levels of secreted apoA-I was comparable to that of hHL. This is in stark contrast to what was previously reported [46]. The discrepancy in the results could be due to the differences in the way the levels of hHL and hHLmt were normalized. In the 2004 study, it was shown that infection with 1.8×10^{10} VP of either the hHL- or hHLmt-encoding adenovirus in mice produced similar levels of plasma post-heparin HL activity towards triolein [46]. It was thus assumed that the infection of the primary cells with 20 VP/cell of either adenovirus would also result in similar levels of hHL and hHLmt protein [46]. However, previous studies showed that the apparent activity of hHLmt towards triolein was lower than that of hHL *in vitro* [45]. In the current study, the hepatic cells were infected with equal amounts of adenovirus, measured by particle forming unit (pfu)/cell, and comparable steady-state levels of cell-associated HL

protein were detected by immunoblot. The discrepancy in the data between the two studies could also be due to the differences in the genetic backgrounds of the primary cells used. In the 2004 study, primary hepatocytes were isolated from wildtype C57BL/6 mice, whereas in the present study, primary cells from HL-null mice were used. A potential problem that could arise from the differential genetic background stem from the fact that HL is functional as a homodimer. The expression of hHL or hHLmt in hepatocytes that synthesize and secrete endogenous mHL could potentially lead to the formation of hetero-dimers between hHL or hHLmt and mHL, and these enzymes could have contributed to the reported phenotype. Experimental differences aside, this study may suggest that the profound decrease in plasma apoA-I levels observed in hHLmt-expressing C57BL/6 mice relative to hHL or control mice [46] occurred through mechanisms independent of differential secretion of apoA-I between hHL- or hHLmt-expressing hepatic cells.

The purpose of this study was to determine whether hHL possessed an intracellular role in the production and/or secretion of hepatic derived apoA-I (nascent HDL). Transfection studies using primary hepatocytes and/or hepatoma cell lines revealed that hHL expression does play a role in impacting the synthesis (and secretion) of apoA-I. The study also partially unraveled mechanisms underlying this phenomenon. In the case of HL expression in primary hepatocytes, the N-terminus of hHL directly or indirectly impaired apoA-I (nascent-HDL) at the ER.

That HL expression impaired apoA-I production in HL-null primary hepatocytes but promoted apoA-I production in McA-RH7777 cells was unexpected, novel and warranted further investigation. It would be of interest to determine whether HL expression affects the relative expression of apoA-I in primary cells or McA-RH7777 cells. Future direction of the research could include determining whether HL expression affects apoA-I protein stability

and/or secretion efficiency, and whether the catalytic activity or lipid-binding of HL are required for the phenotype. To elucidate more directly whether HL affects the HDL class, it would be of interest to determine to which lipoprotein class(es) apoA-I is mostly associated within and secreted from HL-expressing cells. To gain some perspective on HL expression and hepatic HDL biogenesis, the protein levels, distribution and activities of ABCA1 and LCAT can be measured in HL-expressing cells. Along these lines, it would be important to identity the proteins with which HL interacts. Additionally, in an effort to confirm the phenotype by an alternative approach, siRNA against HL could be administered to the cells and the levels and lipidation of apoA-I measured.

As mention in section 4.1, the major weakness of the current study is that the differences between HL-expressing and control cells were not always statistically significant, and some experimental conclusions were drawn from the observable trend in the data. The number of replicates within each experiment or the number of independent experiments could have been increased to show statistical significance in some cases. The major strength of the current study is that a novel intracellular role for hHL was determined in the production and secretion of apoA-I, and in the case of HL expression in HL-null primary hepatocytes, the phenotype fits with the observed inverse correlation between HL expression and apoA-I/HDL-cholesterol levels observed in mouse models and humans. As explained in Chapter 1, HL remodels lipid-rich HDL (via both its lipolytic and selective-lipid uptake functions) into lipid-poor apoA-I/HDL particles which are hypothesized to promote RCT and regress atherogenesis. On the other hand, lipid-poor apoA-I can be efficiently removed from plasma presumably by the liver and kidneys thereby reducing apoA-I/HDL and its anti-atherogenic abilities. Our data show that HL also possesses an intracellular role in reducing apoA-I production (in the case of HL-null hepatocytes) thereby contributing to the reduction

in apoA-I/HDL in plasma observed in HL-expressing organisms. The observation that HL expression reduces apoA-I production and secretion from HL-null hepatocytes also corroborates plasma levels of HDL-cholesterol in type I diabetes mellitus (T1DM) patients. The normal stimulatory action of insulin on HL expression and activity is not observed in T1DM and thus low levels of HL are associated with increased levels of HDL-cholesterol in the patients.

Our cell culture studies revealed a novel intracellular role of HL that adds to the complexity of HL in lipoprotein metabolism and the controversy surrounding a pro- or anti-atherogenic role for HL. As explained in Chapter I and eloquently summarized by Zambon et al. [62], HL deficiency is associated with diminished conversion of remnant VLDL-to-IDL and IDL-to-LDL, and to reduced hepatic uptake of remnants and IDL particles. Remnant lipoproteins and IDL are major determinants of atherosclerosis, suggesting that HL deficiency creates an atherogenic profile. On the other hand, high HL activity may result in an increased atherosclerotic risk by promoting the formation of small, dense LDL particles. Additionally, HL is also synthesized by macrophages suggesting that HL may also contribute to the progression of atherosclerosis by enhancing the retention of VLDL, IDL and small LDL at the site of the atheroma. Our study showed that at the level of the liver, HL impairs the assembly and secretion of the VLDL (the precursor particle for IDL and LDL). As HL has been shown to be expressed in macrophages, it is of interest to determine the intracellular role of HL within this cell type since it is one of the important contributors to the progression of atherosclerosis.

4.3 Concluding remarks

The purpose of this thesis investigation was to determine whether hHL possessed an intracellular role in the production and/or secretion of hepatic derived lipoproteins. Transfection studies using primary hepatocytes and/or hepatoma cell lines revealed that hHL expression does play a role in TAG lipidation of VLDL particles and in modulating the synthesis (or secretion) of apoA-I. The investigation also partially unraveled mechanisms underlying these phenomena. Specifically, the hHL protein (irrespective of catalytic activity) reduced LLD-associated TAG thus forming lipid-poor VLDL particles, and also seemed to modulate cellular lipid homeostasis in McA-RH7777 cells. In the case of HL expression in primary hepatocytes, the N-terminus of hHL directly or indirectly impaired apoA-I (nascent-HDL) at the ER. Thus, not only does HL aid in the clearance of lipoproteins in plasma, it has now been discovered that HL can mediate the production and secretion of these particles in the hepatocyte, adding to the complexity of HL in lipoprotein metabolism.

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CONTRIBUTION OF COLLABORATORS

Section 3.1 Human hepatic lipase impacts hepatic VLDL assembly and secretion

Dr. Robin Parks (generation of empty-vector pAdEasy adenovirus)

Shumei Zhong (generation of McA-RH7777 cells stably expressing hHLSG)

Erik Yao (performance of RT-PCR analysis)

Section 3.2 Human hepatic lipase impacts hepatic apoA-I production and secretion

Dr. Ann White (generation of the anti-hHL polyclonal antibody)

CIRRICULUM VITAE

MICHELLE BAMJI-MIRZA

EDUCATION

Ph. D. in Biochemistry (in progress) — **University of Ottawa**
Thesis: Defining an Intracellular Role of Hepatic Lipase in the Formation of High Density Lipoproteins and Very Low Density Lipoproteins — *Ottawa, ON*, Thesis submission **March 2011**

M. Sc. Human Genetics — **McGill University**
Thesis: Functional Characterization of the Cytochrome c Oxidase Assembly Factor COX11 using RNA Interference — *Montreal, QC* **August 2004**

B. Sc. (High Honours) Biochemistry and Biotechnology — **Carleton University**
Thesis: Expression of Synapsin-II in BL21-RIL-CodonPlus *Escherichia coli* Cells — *Ottawa, ON* **June 2002**

WORK EXPERIENCE

National Research Council of Canada (NRC) — **May – Aug 2002**
Institute for Biological Sciences, *Ottawa, ON* — Summer Student

- Alzheimer's disease research – assisted with the assembly of expression constructs.

University of Ottawa — **May – Aug 2001**
Department of Cellular and Molecular Medicine, *Ottawa, ON* — Summer Student

- Assisted laboratory research by expressing and purifying recombinant proteins that have roles in biomineralization.

Apotex Inc. — **July – Aug 2000**
Ottawa, ON — Summer Student

- Aided product research by cryosectioning animal tissue, and performing immunohistochemistry and microscopy analysis.

Children's Hospital of Eastern Ontario (CHEO) **1998 – 2000**
Molecular Genetics Laboratory, Research Institute, *Ottawa, ON* Summer Student

- Contributed to two research projects (Spinal Muscular Atrophy and Simpson-Golabi-Behmel syndrome) by performing cell culture, cloning, and immunoblot analysis.

PUBLICATIONS

Bamji-Mirza M, and Yao Z. Phospholipases. *American Oil Chemists Society Lipid Library.* 2011. Jan 12 (submitted).

Bamji-Mirza M, Sundaram M, Zhong S, Yao EF, Parks RJ, and Yao Z. Secretion of triacylglycerol-poor VLDL particles from McA-RH7777 cells expressing human hepatic lipase. *J. Lipid Res.* 2011. 52(3):540-8.

Zhong S, Magnolo AL, Sundaram M, Zhou H, Yao EF, Di Leo E, Loria P, Wang S, **Bamji-Mirza M**, Wang L, McKnight CJ, Figeys D, Wang Y, Tarugi P, and Yao Z. Nonsynonymous Mutations within APOB in Human Familial Hypobetalipoproteinemia: Evidence for Feedback Inhibition of Lipogenesis and Postendoplasmic Reticulum Degradation of Apolipoprotein B. *J Biol Chem.* 2010. 285(9):6453-64.

Lo JC, Wang Y, Tumanov AV, **Bamji M**, Yao Z, Reardon CA, Getz GS, and Fu YX. Lymphotoxin Beta Receptor-Dependent Control of Lipid Homeostasis. *Science.* 2007. 316(5822): 285-8.

ORAL AND POSTER PRESENTATIONS

Bamji-Mirza M, Sundaram M, Zhong S, and Yao Z - Expression of Catalytically Active Human Hepatic Lipase in McA-RH7777 Cells Results in Impaired VLDL Assembly and Secretion
Canadian Lipoprotein Conference, *Niagara on the Lake, ON* **2010**

Bamji-Mirza M, Wang Y, Sundaram M, Bou Khalil M, and Yao Z - Retention of Hepatic Lipase in the Endoplasmic Reticulum Results in Impaired Secretion of ApoA-I from Primary Mouse Hepatocytes
Arteriosclerosis, Thrombosis and Vascular Biology Conference, *Atlanta, GA* **2008**

Bamji M, Bou Khalil M, Loh M, Brown RJ, and Yao Z - Sequence Elements within Human and Mouse Hepatic Lipase that Determine Substrate Specificity
Canadian Lipoprotein Conference, *Montebello, QC* **2005**

Bamji M - Your Genes Unzipped (Invited talk)
Environment Canada Information Technology Symposium, *St-Sauveur, QC* **2005**

Bamji M and Shoubridge E - Functional Identification of Human Cytochrome C Oxidase Assembly Factors using RNA Interference
BioNorth: 10th Annual International Life Sciences Conference, *Ottawa, ON* **2003**

ACHIEVEMENTS AND AWARDS

Graduate Research Assistantship	University of Ottawa	**2004 – present**
Doctoral Admission Scholarship	University of Ottawa	**2004 – 2008**
Graduate Research Assistantship	McGill University	**2002 – 2004**
Deans' Honour List	Carleton University	**2002**
President's Scholarship	Carleton University	**1998**
Faculty of Science Entrance Scholarship	Carleton University	**1998**

LABORATORY SKILLS

Maintenance of mouse colonies; Mouse procedures (including liver perfusion, primary hepatocyte isolation, and intravenous injection); Mammalian cell culture and the generation of stable cell lines; Metabolic labeling of lipids and proteins; Recombinant protein expression; Protein purification by column chromatography; Protein characterization using protein assays, chromatography and electrophoresis; Production and use of adenoviruses *in vitro* and *in vivo;* Enzyme kinetic determinations; Bacterial culture and aseptic techniques; Recombinant DNA techniques; PAGE and immunoblotting; Cryosectioning and immunohistochemistry; Subcellular fractionation

PERSONAL, COMPUTER AND LANGUAGE SKILLS

PERSONAL: Superior communication skills - both written and oral; Advanced problem-solving and multi-tasking skills; Strong interpersonal skills; Proven team leadership ability; Excellent organizational skills; Effective in a team or independently; Highly motivated

COMPUTER: Proficient in Microsoft Excel, Outlook, PowerPoint, and Word; Adobe Reader and Photoshop; Stat Plus

LANGUAGE: Fluent English and Intermediate French language skills. Graduated with French Immersion Certificate

ACADEMIC AND EXTRA CURRICULAR ACTIVITIES

- Member, Biochemistry Microbiology & Immunology Graduate Student Association, University of Ottawa **(2009 - 2011)**
- Vice President - Outreach Program, Women in Science & Engineering Society, University of Ottawa Chapter **(2009/10)**
- Member, American Heart Association **(2008)**

- Judge, Canada-Wide Science Fair, University of Ottawa **(2008)**
- Attendee, National Course on Lipids, Lipoproteins & Atherosclerosis. The Stroke, Cardiovascular, Obesity, Lipid, Atherosclerosis Research Training Program. University of Alberta **(2005)**
- Representative - Montreal Neurological Institute, Human Genetics Students Society, McGill University **(2004/5)**
- Internal Operations Coordinator, Women in Science & Engineering Society, Carleton University Chapter **(1998/9)**