PURINERGIC SIGNALING AND AUTOPHAGY REGULATE THE SECRETION OF HIGH-DENSITY LIPOPROTEIN AND HEPATIC LIPASE

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ABSTRACT

Dyslipidemia can be a comorbidity of both insulin-resistance and atherosclerosis. Hypertriglyceridemia is common in hyperglycemia and is associated with hypoalphalipoproteinemia (low HDL) and with altered nucleotide or purinergic signaling. We therefore hypothesized that extracellular nucleotides may affect hepatic lipoprotein metabolism. Our studies confirm this view and show that nucleotides regulate cellular proteolytic pathways in liver cells and thereby control lipoprotein secretion and their metabolism by hepatic lipase (HL).

Treatment of liver cells with the nucleotide, adenosine diphosphate (ADP), stimulates VLDL-apoB100 and apoE secretion, but blocks HDL-apoA-I and HL secretion. ADP functions like a proteasomal inhibitor to block proteasomal degradation and stimulate apoB100 secretion. Blocking the proteosome is known to activate autophagic pathways. The nucleotide consequently stimulates autophagic degradation in liver cells and increases cellular levels of the autophagic proteins, LC3 and p62. Confocal studies show that ADP increases cellular LC3 levels and promotes co-localization of LC3 and apoA-I in an autophagosomal degradation compartment. ADP acts through the G-protein coupled receptor, $P2Y_{13}$, to stimulate autophagy and block both HDL and HL secretion. Overexpression of $P2Y_{13}$ increases cellular LC3 levels and blocks the induction of both HDL and HL secretion, while $P2Y_{13}$ siRNA reduce LC3 protein levels and cause up to a ten-fold stimulation in HDL and HL secretion. $P2Y_{13}$ gene expression regulates autophagy through the insulin receptor (IR-β). A reduction in $P2Y_{13}$ expression increases the phosphorylation of IR-β and protein kinase B (Akt) >3-fold, while increasing $P2Y_{13}$ expression inhibits the activation of IR-β and Akt. Experiments with epitope-labeled apoA-I and HL show that activation of purinergic pathways

has no effect on the internalization and degradation of extracellular apoA-I and HL, which confirms the view that nucleotides primarily impact intracellular protein transport and degradation. In conclusion, elevated blood glucose levels may promote dyslipidemia by stimulating purinergic signaling through $P2Y_{13}$ and IR-β and perturbing the intracellular degradation and secretion of both HDL and VLDL.

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At last, but not least, a special thanks to my brother, Sujoy, who although did not really understand what I am doing or why I was in school for so many years, was curious and supportive in my efforts. Thanks also to my aunt and uncle for all their patience and encouragement over the years. A very special thank you to my parents, who from a very young age were an inspiration, believed in me even at times when I did not believe in myself and taught me that I can accomplish anything that I put my mind to!

DEDICATION

I dedicate this thesis to my mom and dad. Although you did not get a chance to see me grow up, I know you were there to guide me in spirit along the way in the journey.

Thank you for your support and I hope I made you proud.

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LIST OF ABBREVIATIONS

3-MA: 3-methyladenine
ABCA1: ATP-binding cassette subfamily A, member 1
ADP: adenosine 5'-diphosphate
Akt: protein kinase B
ALLN: N-Acetyl-L-leucyl-L-leucyl-L-norleucinal
APOA1: apoA-I gene
Apo: apolipoprotein
ARP1: apolipoprotein regulatory protein
Atg: autophagy related protein
ATP: adenosine 5'-triphosphate
CD39: ectonucleoside triphosphate diphosphohydrolase 1 or ENTDP1
CE: cholesteryl esters
CETP: cholesteryl ester transfer protein
CHD: coronary heart disease
CM: chylomicron
CRE: cytokine response element
DLPC: dilinoleoylphosphatidylcholine
DRE: drug response element
ELISA: enzyme-linked immunosorbent assay
ENTPD1: Ectonucleoside triphosphate diphosphohydrolase 1 or CD39
ER: endoplasmic reticulum
ERK1/2: extracellular-regulated protein kinase 1 and 2
F_1-ATPase: ATP synthase
FFA: free fatty acids
GLUT: glucose transporter
GPCR: G-protein coupled receptor
HDL: high-density lipoprotein
HL: hepatic lipase
HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryl-CoA reductase
HNF4: hepatocyte nuclear factor 4
HSPG: heparan sulfate proteoglycans
IDL: intermediate density lipoprotein
IF1: mitochondrial inhibitory factor 1
IGF-IR: type I insulin-like growth factor receptor
IR: insulin receptor
IR-β: insulin receptor beta subunit
IRE: insulin response element
IRS: insulin receptor substrate
LA: linoleic acid
LAPL: linoleic acid phospholipids
LC3: microtubule-associated protein 1 light chain 3
LCAT: lecithin:cholesterol acyltransferase
LDL: low-density lipoprotein
LDL-R: low-density lipoprotein receptor

LIPC: hepatic lipase gene
LPL: lipoprotein lipase
LRP1: lipoprotein receptor-like protein-1
MAPK: mitogen activated protein kinase
MG132: carbobenzoxy-Leu-Leu-leucinal
mTOR: mammalian target of rapamycin
NIDDM: non-insulin dependent diabetes mellitus
NTPDase: ecto-nucleoside triphosphate diphosphohydrolases or ecto-nucleotidase
NF-κB: nuclear factor kappa B
P1: adenosine activated GPCR
P2Y: purine nucleotide activated GPCR
$P2Y_{13}$: P2Y receptor 13
P2X: ligand-gated ion channel activated by ATP
PDK-1: phosphoinositide-dependent kinase-1
PE: phosphatidylethanolamine
PH: Pleckstrin homology domain
PI: phosphatidylionositol
PI3K: phosphatidylinositol 3' kinase
PKB: protein kinase B or Akt
PKC: protein kinase c
PL: phospholipids
PLPC: palmitoyllinoleoylphosphatidylcholine
PLTP: phospholipid transfer protein
PPARα: peroxisome proliferator-activator receptor-α
PPRE: peroxisome proliferator response element
RARE: retinoic acid response element
RCT: reverse cholesterol transport
SH2. Src homology 2 domain
siRNA: small interfering RNA or silencing RNA
SNP: single nucleotide polymorphism
SR-A: scavenger receptor A
SR-BI: scavenger receptor class B1
SREBP-1: sterol regulatory element binding protein-1
T1DM: Type 1 Diabetes Mellitus
T2DM: Type 2 Diabetes Mellitus
TG: triglyceride
Ub: ubiquitin
UPP: ubiquitin-proteasomal pathway
UPS: ubiquitin proteasome system
VLDL: very low density lipoprotein

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CHAPTER 1 – INTRODUCTION

1.1 Coronary Heart Disease & Dyslipidemia

Coronary heart disease (CHD) is a leading cause of death of both men and women not only in North America, but worldwide. CHD is characterized by the narrowing of the coronary arteries of the heart due to a buildup of fatty plaque and inflammation, resulting in a decreased supply of blood and oxygen to the heart eventually leading to a heart attack. Major risk factors for CHD fall into two categories: unmodifiable and modifiable risk factors. The unmodifiable risk factors include male gender, increasing age, race and genetic predisposition - all of which an individual cannot change [1;2]. However, individuals can decrease their risk for developing CHD by making healthy lifestyle choices and taking measures to control their modifiable risk factors. These modifiable risk factors include smoking, high cholesterol, high blood pressure, overweight and obesity, physical inactivity, poor diet, and diabetes [1;3-5]

Dyslipidemia has been a primary focus in the treatment of CHD over the last two decades. Research into developing new therapies involved devising strategies to treat hyperlipidemia by lowering plasma low density cholesterol (LDL-C) levels [6-9] and triglyceride (TG) levels [10;11] as well as to treat dyslipidemia by increasing plasma high density lipoprotein (HDL) levels [12;13]. Although there have been numerous advances in recent years, which have helped to decrease mortality from CHD, much more research is still needed to better understand the underlying molecular mechanisms that contribute to dyslipidemia for the prevention and treatment of CHD.

1.2 Lipoprotein Metabolism & Atherosclerosis

1.2.1 Lipids

Lipids are small hydrophobic or amphiphatic molecules that are responsible for multiple vital biochemical functions in the human body and in all living organisms. Lipids serve as high-density stored energy sources, structural components of cell membranes (plasma membrane and organelles), precursors to hormones and vitamins, and act as signaling molecules. There are many different lipids, but the four major classes are: phospholipids, cholesterol, triglycerides, and fatty acids. Lipids in the body are derived by dietary intake or by *de novo* production in the liver. Although the adipose tissue is the main storage site for lipids in the body, the liver plays a critical role in lipid metabolism by regulating fatty acid synthesis and lipoprotein metabolism.

1.2.2 Lipoprotein Metabolism

Lipoproteins are macromolecular structures comprised of lipids and proteins that aid in the transport of lipids in the circulation to various tissues in the body. The inner hydrophobic core of a lipoprotein is made up of triglycerides and cholesterol esters which are surrounded by an outer hydrophilic layer comprised of phospholipids, cholesterol, and apolipoproteins [14]. There are five different classes of lipoproteins: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Each class of lipoprotein has its unique function and can be distinguished based on their lipid and protein composition, density and eletrophoretic charge.

Lipoprotein metabolism/biogenesis involves two pathways: 1) the exogenous pathway involving the processing of dietary lipids, and 2) the endogenous pathway involving the

processing of lipoproteins synthesized by the liver [15]. Upon ingestion of a meal, chylomicrons (CM) are secreted from the intestines, which carry triglycerides (TG) to the liver, skeletal muscle and adipose tissue. The liver then further processes the CM remnants that are generated, to produce and secrete VLDL into the circulation, which carry TG to peripheral tissues [15]. The TG-rich VLDL is further processed by TG lipases, lipoprotein lipase (LPL) and hepatic lipase (HL), to supply free fatty acids (FFA) as energy sources to peripheral cells. This decreases the ratio of TG to cholesterol content whereby VLDL becomes converted to IDL and then LDL [15]. LDL is the lipoprotein that contains the most cholesterol and therefore supplies tissues with cholesterol. In contrast to LDL, HDL's role is to collect excess cholesterol from the peripheral tissues and to bring it back to the liver in a process called reverse cholesterol transport.

1.2.3 Apolipoproteins

Apolipoproteins are amphipathic proteins that are key structural components of lipoproteins. They assist in the solubilization and transport of lipids to tissues through the bloodstream [16]. Aside from their lipid transport role, apolipoproteins also act as co-factors for multiple different circulatory enzymes, and act as ligands for lipoprotein receptors [17]. There are two categories of apolipoproteins: 1) exchangeable, and 2) non-exchangeable. The exchangeable apolipoproteins are reversibly associated with lipoproteins and thus, can be exchanged between different types of lipoproteins [17]. The exchangeable apolipoproteins are comprised of the apoA, C and E families, each of which has numerous subclasses. The most common include apoA-I, apoA-II, apoA-IV, apoA-V, apoC-I, apoC-II, apoC-III, and apoE. ApoA-I and apoA-II are the main apolipoproteins that constitute HDL. ApoC-II is a stimulatory co-factor while apoC-III is an inhibitory co-factor for lipoprotein lipase (LPL).

ApoE plays an important role as a ligand for the LDL receptors, and the lipoprotein receptor-like protein-1 (LRP) [17]. The non-exchangeable apolipoproteins are irreversibly associated with the lipoprotein, and therefore cannot be separated [17]. The non-exchangeable apolipoproteins belong to the apolipoprotein B family. They include apoB48 and apoB100. ApoB48 is the main constituent of CMs, whereas apoB100 is the key component of VLDL and LDL. ApoB100 functions as a ligand for the VLDL and LDL receptors.

1.2.4 Dyslipidemia & Treatment

Dyslipidemia is a condition marked by abnormal plasma lipid levels due to aberrant lipoprotein metabolism resulting in either increased or decreased lipid levels. It is as result of either: 1) genetic factors such as mutations in key receptors or apolipoproteins/co-factors responsible for the metabolism of specific lipoproteins, or 2) diet and lifestyle, which are secondary factors that contribute to other conditions such as diabetes and metabolic disorders. Hyperlipidemia is the most common form of dyslipidemia characterized solely by elevations in plasma lipid levels [18]. Three common forms of hyperlipidemia include: hypercholesterolemia, hypertriglyceridemia, and combined hyperlipidemia referring to the excess of plasma cholesterol, triglycerides or both, respectively [18-20]. Another dyslipidemia that is prevalent in the general population is the decreased levels of plasma HDL or hypoalphalipoproteinemia. Low levels of HDL may impair the body's ability to remove excess cholesterol and consequently, HDL is often referred to as the "good" cholesterol.

The Canadian guidelines for plasma lipids sets target levels of total cholesterol, LDL-cholesterol (LDL-C), HDL-C, total cholesterol to HDL-C ratio , and TG for the population depending on an individual's personal risk factors for CHD [1;21]. In general, an individual should aim for a target level of < 5.2 mmol/L of total cholesterol, <3.5 mmol/L LDL-C, >1.0-

1.3 mmol/L HDL-C, <5.0 mmol/L total cholesterol to HDL-C ratio, and < 1.7 mmol/L TG [1;21]. These targets serve as a general guideline to help physicians assess an individual's risk for CHD, and targets may be more stringent on a case-by-case basis.

It has been known for many decades that low plasma HDL-C and high LDL-C is associated with an increased risk for developing CHD in humans, however high HDL-C appears to lower the risk of CHD even in the presence of high LDL-C [22]. In addition, high plasma TG levels are also associated with increased risk for CHD, but there also exists an inverse relationship between plasma TG levels and HDL [23]. Low HDL-C levels are often associated with high TG levels in both men and women, greatly increasing the risk of developing heart disease compared to men and women with high HDL cholesterol and low TG levels.

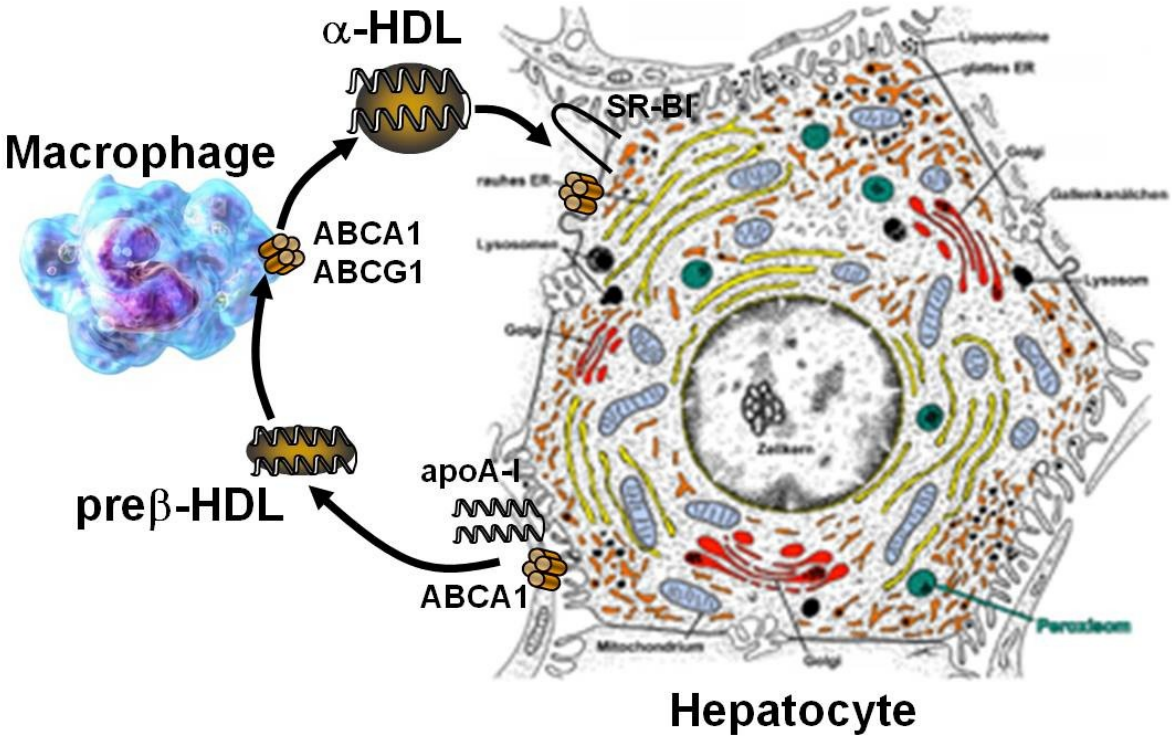
Currently, popular treatments for high LDL-C levels include: 1) statins, which inhibit HMG-CoA reductase, a rate-limiting enzyme responsible for the synthesis of cholesterol in the liver, and 2) cholesterol absorption inhibitors, such as Ezetimibe, which inhibit the absorption of cholesterol from the diet [7;24]. Unlike LDL, there are fewer and less effective treatment options for increasing HDL. The HDL-C raising therapeutics on the market are niacin and fibrates. Niacin is more commonly known as vitamin B3 and is the most effective HDL-increasing drug available, although it only modestly increases HDL by 20% [11;13]. Niacin functions by acting through the GPR109A to decrease HDL uptake and degradation by the liver, but it also has the adverse effects such causing flushing [11]. Fibrates increase plasma HDL levels by only 10% and act through peroxisome-proliferator activated receptors alpha (PPARα) to increase apoA-I transcription [13]. Other HDL-raising therapeutics that are under development include infusions of apoA-I mimetics/recombinant HDL and CETP inhibitors

[25]. Although torcetrapib increased mortality and morbidity due to off-target effects increasing aldosterone production and hyperternsion, another CETP inhibitor, dalcetrapib increased HDL-C, but did not decrease CHD risk [25-27]. In clinical development, anacetrapib and evacetrapib showed greater increases in HDL-C than dalcetrapib, while greatly reducing LDL-C and Lp(a) [25]. Studies have also showed that linoleic acid phospholipids (LAPL) such soy phosphatidylinositol (PI) and dilinoleoylphosphatidylcholine (DLPC) are also able to increase plasma HDL-C levels in humans and stimulate reverse cholesterol transport in rabbits [28-31]. DLPC was shown to be twice as effective as PI in stimulating apoA-I secretion from human hepatocytes since it contains two linoleic side chains [32]. LAPL act through phospholipase C and protein kinase C to stimulate PPAR-α, and inhibitors of cytosolic phospholipase A_2 inhibit the stimulation of apoA-I secretion by PI [32;33] There is also evidence to show that LAPL may induce HDL secretion by reducing membrane F_1-ATPase levels on hepatocytes suggesting a role for purinergic signaling [34]. In this study, we now show how DLPC may modulate purinergic signaling by regulating cellular proteolytic pathways. Interestingly, drugs that increase plasma HDL also decrease plasma TG levels in humans, which again highlights this inverse relationship between plasma HDL and TG levels. In fact, HDL raising therapies were shown to have an even more potent TG-lowering ability. Niacin, fibrate and soy PI treatment have all shown to have a significant 25-35% decrease in plasma TG levels upon administration [13;28].

1.2.5 Reverse Cholesterol Transport & Cholesterol Efflux

Reverse cholesterol transport (RCT) is a process by which excess cholesterol is retrieved from peripheral tissues by HDL and delivered back to the liver for excretion [35;36] (**Figure 1.1**). RCT is a mechanism through which the body manages cholesterol levels and

Figure 1.1. The maturation of HDL and reverse cholesterol transport. Lipid-poor pre-β-migrating apoA-I is secreted from the liver and then acquires phospholipids and cholesterol via the ATP-binding cassette, sub-family A, member 1 transporter (ABCA1) to form pre-β-HDL. Pre-β-HDL acts as an acceptor of more cholesterol from macrophages and other peripheral tissues via the ABCA1/ABCG1 transporters to form α-HDL. α-HDL functions to return the collected sterol back to the liver through selective uptake pathways involving scavenger receptor class B1 (SR-BI). The lipids are then transported for storage or excretion through a process known as reverse cholesterol transport (RCT). The resulting lipid-poor apoA-I can re-enter the maturation cycle or be degraded.


α-HDL
SR-BI
Macrophage
ABCA1
ABCG1
apoA-I
preβ-HDL
ABCA1
Hepatocyte

involves the coordination of numerous receptors and enzymes. RCT is important for the prevention of atherosclerosis since it is responsible for removing cholesterol from macrophage foam cells, which form atherosclerotic plaques [35].

ApoA-I, the main constituent of HDL is secreted by the liver and then becomes lipidated to form HDL particles [37] (**Figure 1.1**). ApoA-I collects free cholesterol through its interaction with an important membrane protein, ATP-binding cassette, sub-family A, member 1 (ABCA1). ABCA1 is responsible for the transport of cellular cholesterol and phospholipids across the plasma membrane, a process referred to as cholesterol efflux [35]. ABCA1 is expressed in the liver, macrophages, and many other tissues. ApoA-I is a major acceptor for the cholesterol and phospholipid from macrophages and other peripheral tissues through efflux to form HDL. The free cholesterol on HDL then becomes esterified in the plasma to form cholesteryl esters (CE) by lecithin:cholesterol acyltransferase (LCAT), which is another co-factor on HDL [35]. The CE on HDL can then be transferred to VLDL or LDL for receptor-mediated uptake by the liver in exchange for TG by cholesteryl ester transfer protein (CETP) [35]. Studies have also shown that HDL charge is a mediator of cholesterol mobilization [29;33;38]. It was shown that increasing the net negative charge of HDL by anionic lipids such as phosphatidylinositol (PI) results in increased excretion of cholesterol and decreased storage by blocking LCAT and CETP activity.

A second pathway involved in RCT is the scavenger receptor class B1 (SR-BI) mediated selective uptake of CE (**Figure 1.1**). As its name implies, this process involves the selective uptake of CE from HDL by SR-BI on the liver with minimal degradation of the HDL particle [39]. SR-BI mediated CE uptake is also known to be enhanced by hepatic lipase (HL) [40-44]. Hepatic lipase serves as a ligand of SR-BI and facilitates the interaction of HDL

with SR-BI, in addition to lipolysis and remodeling of HDL [40;41].

The net result of RCT is the return of CE to the liver for either storage or excretion. CE can become unesterified to form free cholesterol, which is then secreted in the bile or converted to bile acids to facilitate the absorption of dietary lipids.

1.2.6 Atherosclerosis

Atherosclerosis is a disease that is characterized by the progressive deposition of cholesterol and other lipids, calcium, and macrophages forming plaques in the inner walls of the arteries [45]. This leads to the narrowing and thickening of the arteries, which impairs blood flow to vital organs including the heart and brain resulting in a heart attack or stroke. Plaques may also rupture forming blood clots, which can travel in the circulation and deprive other tissues of blood and oxygen causing tissue damage or death. The weakening of the artery wall by the plaque formation may also result in vessel rupture or aneurysm. Interestingly, atherosclerosis has an early onset in life, where initial lesion formation has been reported as early as adolescence without the presence of any symptoms [46]. Atherosclerosis is the result of abnormal lipoprotein metabolism and RCT, as well as an impaired chronic inflammatory response. The slow, progressive accumulation of lipids and chronic inflammation over a lifetime can lead to complications from atherosclerosis and its devastating outcomes [47].

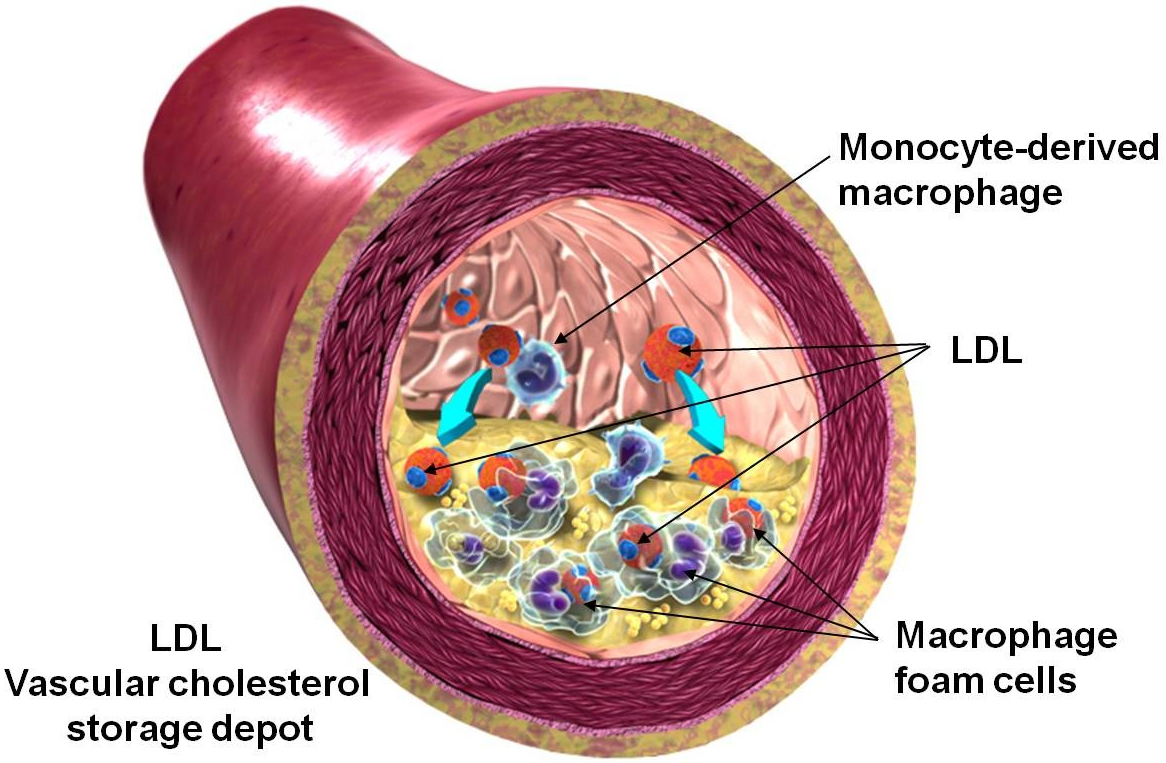
The development of atherosclerosis is a complex process that occurs over decades, which can be broken down to 5 stages: lesion initiation, inflammation, foam cell formation, fibrous plaque formation, and complex lesions and thrombus [45]. Haemodynamic forces play a critical role in determining the sites for lesion development on the endothelium of the artery wall [45]. Lesions usually form on areas of the endothelium where there is turbid blood flow such as regions where there is branching and curving of the arteries [45]. As the endothelial

cells in these regions are susceptible to injury, there is greater permeability to atherogenic lipoproteins and proinflammatory cytokines [45;48;49]. This response-to-injury hypothesis was first proposed by Ross and Glomset in 1974.

The deposition and accumulation of LDL is the hallmark of lesion development and initiates atherogenesis according to the response-to-retention hypothesis [49]. This first step triggers an immune response whereby monocyte-derived macrophages are recruited to scavenge the excess cholesterol. LDL becomes trapped in the subendothelial space undergoes oxidative modifications by neighboring smooth muscle cells, endothelial cells and macrophages[49] (**Figure 1.2**). The oxidized LDL stimulates a further inflammatory response to recruit more macrophages. Macrophages engulf the modified lipid via scavenger receptors like SR-A, to become foam cells as per the oxidative modification hypothesis [49]. This foam cell phenotype is more commonly referred to as the fatty streak in lesion development. Foam cell formation results in the recruitment of cell adhesion molecules and secretion of pro-inflammatory cytokines, which further exacerbates the inflammatory response [45]. Thus, an initial acute inflammatory response to manage excess cholesterol in the arteries becomes a chronic inflammatory response leading to atherosclerosis progression.

More advanced progression of atherosclerosis is characterized by fibrous plaques followed by complex lesion and thrombus formation. Fibrous plaques consist of foam cells as seen in fatty streaks along with extracellular accumulation of cholesterol, CE, and dead cells. This forms a necrotic core that is protected by a layer of smooth muscle cells and extracellular matrix [45]. As the fibrous plaque expands, it projects into the arterial lumen obstructing blood flow as well thickening and hardening the arteries. Fibrous plaques develop into complicated lesions upon calcification, thinning and degradation of fibrous caps [45]. The destabilization of

Figure 1.2. Macrophages scavenge LDL in the artery wall. Atherogenesis is initiated by the deposition and accumulation of LDL in the artery wall. This elicits an inflammatory response, wherein monocyte-derived macrophages are recruited to scavenge the excess cholesterol. The trapped lipoprotein lipid undergoes oxidative modifications that augment the inflammatory response and trigger the formation of macrophage foam cells and lesion development.


Monocyte-derived
macrophage
LDL
LDL
Vascular cholesterol
storage depot
Macrophage
foam cells

the structural integrity of the fibrous cap results in its rupture, exposing contents of the necrotic core, and inducing coagulation cascades and thrombus formation [45].

1.3 High-Density Lipoproteins

1.3.1 Structure & Function

HDL has been long regarded as having a protective role in the development of atherosclerosis. The composition and structure of HDL is important to its multi-faceted atheroprotective functions. HDL is the smallest (5-12nm in diameter) and most dense lipoprotein (1.063-1.21g/mL) with a ~50% protein composition and a low lipid to protein ratio [37]. ApoA-I is the primary constituent of HDL forming 70% of the apolipoprotein concentration. ApoA-II is the second most abundant apolipoprotein [47]. Structurally, HDL particles are known to have a double-belt conformation and follow the trefoil model, whereby the lipids are stabilized by 3 molecules of apoA-I that form a 3-D cage structure around it [35]. ApoC, apoE, apoA-IV and apoA-V are among other apolipoproteins that are known to associate with HDL and contribute to its highly diverse and heterogeneous nature [17]. HDL can be broken down into subspecies that can be characterized according to apolipoprotein composition, density and electrophoretic mobility [50]. The two main subclasses of HDL are HDL_2 and HDL_3. HDL_2 is more buoyant, has less apoA-II, and is associated with a decreased magnitude of postprandial lipema, compared to HDL_3 [51;52]. Recent studies have characterized more distinct subpopulations of HDL and have proposed that the quality of HDL particles may actually be more significant in mediating atheroprotection than plasma HDL concentration alone [53-55]. HDL composition and structure are therefore integral to its function.

The complex intricacies of HDL enable it to conduct its various functions. Although HDL is primarily known for its anti-atherogenic property, it also has anti-oxidant and anti-

thrombotic functions. HDL's central role in macrophage cholesterol efflux and RCT has been well characterized and received much attention contributing to its atheroprotective designation [35]. HDL particles also serve as carriers for a number of plasma remodeling enzymes such as LCAT, CETP as well as anti-inflammatory mediators such as paraoxonase and platelet-activating factor acetylhydrolase. These associated anti-inflammatory factors enable HDL to reduce the proinflammatory response that is induced by macrophage foam cells [56;57]. This is achieved by blocking LDL oxidation and endothelial inflammation, promoting nitric oxide production, and inhibiting platelet and coagulation cascade activation [58]. More recent work has shown an association of HDL with hepatic lipase (HL) and demonstrated a novel role for HDL in regulating hepatic lipase activity and TG metabolism [59-62]. Importantly, this work highlights a direct relationship between HDL and HL, which may help explain the inverse relationship between plasma HDL and TG levels in humans.

1.3.2 Gene Regulation

HDL production is primarily dependent on the regulation of apoA-I. The majority of apoA-I is synthesized in the liver with a small contribution from the small intestine. The *APOA1* gene is located on the long arm of chromosome 11q23-q24 [63]. *APOA1* was originally identified as having 4 exons and belonging to this 17kb region of DNA which also encompasses the *APOC3* and *APOA4* genes. Years later *APOA5* was identified 30kb distal to *APOA4* and from then on this ~60kb region has been referred to as the *APOA1/C3/A4/A5* gene cluster [64]. Numerous genetic studies have implicated single nucleotide polymorphisms (SNPs) in the *APOA1/C3/A4/A5* gene cluster to pertubations in lipid metabolism and CHD risk. However, results have been variable between different populations due to confounding factors such as environment [64].

Transcriptional expression of apoA-I is heavily dependent on the regulation of its promoter activity. The promoter region of *APOA1* contains a number of cis-acting elements including: apolipoprotein regulatory protein (ARP1), retinoid acid (RARE), insulin (IRE), cytokine (CRE), drug (DRE), hepatocyte nuclear factor4 (HNF4), and negative thyroid response elements [64]. The fibrate drugs are known to mediate their plasma HDL-increasing effect by activating PPARα and binding to the DRE/PPRE to stimulate both apoA-I and apoA-II transcription [65-67]. ApoA-I expression is also differentially regulated by hormones. Estrogen, thyroid hormone, glucocorticoids and insulin stimulate apoA-I expression, while androgens and retinoic acid inhibit its expression [68].

Recent studies have identified several microRNAs that play a key role in regulating HDL metabolism. Most notably, microRNA-33a and b were shown to be involved in lipid metabolism, and able to increase HDL and decrease VLDL secretion in non-human primates. Inhibition of miR-33 in mice was demonstrated to increase ABCA1 expression, increase RCT and decrease atherosclerosis [69-71].

1.3.3 Synthesis & Secretion

The biogenesis of mature spherical HDL is a complex process involving multiple lipidation and remodeling steps (**Figure 1.1**). Nascent apoA-I is originally synthesized as pro-apoA-I with a 6 amino acid prosegment that is cleaved later on and an 18 amino acid signal sequence, similar to other secreted proteins [72]. The site for the processing and proteolytic cleavage of the prosegment of apoA-I to form apoA-I has been controversial. Early studies have demonstrated that the conversion of pro-apoA-I to apoA-I occurred extracellularly, however later studies have demonstrated that human hepatic cells are also able to convert the pro-form to the processed form depending on the culture conditions and

hormonal environment of the cells [72]. The processing appears to begin in the Golgi and is completed around the time of secretion.

Hepatic apoA-I is originally secreted as a lipid-poor, pre-β-migrating apoA-I. Newly synthesized murine apoA-I from primary hepatocytes was shown to be phospholipidated early in the secretory pathway in the ER, independent of the membrane transporter, ABCA1. The majority of phospholipidation and acquisition of cholesterol by apoA-I mainly occurs in the Golgi and the plasma membrane in an ABCA1-dependent process [73;74]. Lipid-poor, pre-β-migrating apoA-I acquires phospholipids via ABCA1, which translocates the lipids from the inner leaflet of the plasma membrane to the outer leaflet, to form nascent pre-β-migrating discoidal HDL [75]. The discoidal HDL particles then obtain unesterified cholesterol with the help of ABCA1 through a mechanism that is still under investigation [37;55;76]. Studies have shown that the interaction of apoA-I with ABCA1 can result in the phosphorylation of ABCA1 via a cAMP/protein kinase A dependent pathway [77]. A high capacity binding site for apoA-I was identifited on the plasma membrane that results in ABCA1-dependent nascent HDL formation [78]. It was also found that there was a prerequisite for ABCA1 to be in a homotetrameric form for nascent HDL biogenesis [79]. However, there is some controversy in the importance of ABCA1 in mediating discoidal pre-β and mature α-HDL particles formation. A loss-of-function mutation in ABCA1 leads to Tangier's disease due to the inability to form lipidated HDL and the rapid clearance of nascent lipid-poor HDL. However, studies also suggest that ABCA1 is not required for the production of pre-β HDL, but is necessary for the formation of α-HDL [80;81]. Other research has shown that mature α-HDL can be formed even in the absence of ABCA1 [34].

The esterification of the newly acquired cholesterol on pre-β HDL particles by LCAT results in the conversion of nascent HDL to mature small spherical α-HDL. The transformation of the disc-shaped particles to spherical HDL particles is due to the hydrophobic nature of the cholesterol esters, which need to be maintained in the inner hydrophobic core away from the outer hydrophilic surface of HDL [37]. Small spherical α-HDL particles comprising two apoA-I molecules can undergo further remodeling by LCAT and PLTP to create larger spherical α-HDL particles comprising three or four apoA-I molecules either by the fusion of smaller HDL particles or the acquisition of more lipid. Given that apoA-II is also an important component of HDL, more complex HDL particles containing both apoA-I and apoA-II can be formed by the fusion of spherical apoA-I HDL with discoidal apoA-II HDL particles, since LCAT alone is unable to esterify discoidal apoA-II HDL [37]. In contrast, large spherical α-HDL particles can also be remodeled by CETP and HL, resulting in a decrease in particle size and regeneration lipid-poor pre-β-migrating apoA-I. The lipid-poor pre-β-migrating apoA-I molecules that are generated by this process can re-enter the HDL biogenesis cycle at different stages: 1) form new discoidal HDL, 2) integrated into circulating pre-existing discoidal HDL, 3) used to generate larger spherical HDL, or lastly 4) cleared from the circulation by excretion through the kidney.

1.4 MANUSCRIPT #1

Hepatic Lipase, high density lipoproteins and hypertriglyceridemia

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Key Words: triglyceride; lipolysis; proteoglycans; hepatocytes; apolipoproteins; lipoproteins; atherosclerosis; heparin

1.4.1 Abstract

Hepatic lipase (HL) is a lipolytic enzyme that contributes to the regulation of plasma triglyceride (TG) levels. Elevated TG levels may increase the risk of developing coronary heart disease (CHD) and studies suggest that mutations in the HL gene may be associated with elevated TG levels and increased CHD risk. HL facilitates the clearance of TG from the VLDL pool and this function is governed by the composition/quality of HDL particles. In humans, HL is a liver resident enzyme that is regulated by factors that release it from the liver and activate it in the bloodstream. HDL regulates the release of HL from the liver and HDL structure controls HL transport and activation in the circulation. Alterations in HDL-apolipoprotein composition can perturb HL function, by inhibiting the release and activation of the enzyme. HDL structure may therefore affect plasma TG levels and CHD risk.

1.4.2 Triglycerides and Heart Disease

Elevated plasma triglyceride (TG) levels have been viewed as a risk factor for coronary heart disease (CHD) for over a decade [1;2]. Plasma TG levels are regulated by both the synthesis and degradation of VLDL and chylomicron particles. The clearance of TG-rich lipoproteins from the circulation is controlled by the actions of lipoprotein lipase (LPL) and hepatic lipase (HL) and by the interlipoprotein exchange of TG by cholesteryl ester transfer protein. LPL is the predominant TG lipase and is responsible for hydrolyzing TG in chylomicrons and VLDL, while HL is both a phospholipase and TG lipase and plays an important role in HDL metabolism and in the conversion of VLDL to LDL [3]. Single nucleotide polymorphisms (SNPs) in the HL gene (*LIPC*) have been shown to associate with plasma lipid concentrations and increased CHD risk [4;5]. HL deficiency is a result of relatively rare *LIPC* mutations that give rise to a loss in circulating HL activity (due to impaired

secretion or inactive enzyme) and cause an increase in TG-rich HDL and VLDL remnants and increased CHD [1;6]. Common SNPs have variable functional consequences. SNPs in the *LIPC* gene can be associated with both increased or decreased plasma HDL-C levels and variable CHD risk [7;8]. Unique SNPs may consequently confer both pro- and anti-atherogenic phenotypic consequences. This may explain why bigger and more comprehensive studies have not observed an association between *LIPC* mutations and CHD risk [9]. Variable phenotypes may be partly due to secondary factors such as environment, lifestyle, and hormone levels [10], but will primarily depend on the functional consequences of SNPs on HL activity. SNPs in the *LIPC* gene may directly affect the TG-hydrolytic ability of HL and may indirectly affect HL by affecting the metabolism of HDL and its ability to control the function of HL in the circulation.

1.4.3 HL and the Liver

HL is synthesized and secreted by the liver and binds to heparan sulfate proteoglycans (HSPG) on the cell surface of hepatocytes and endothelial cells [11;12]. It has been known for over 50 years that HSPG-bound lipases can be released into the bloodstream by heparin. Hahn showed in 1943 that intravenous heparin stimulated TG hydrolytic activity in lipemic serum [13]. While lipase activity is normally undetectable in human plasma, infusion of heparin increases both HL and LPL mass and activity in the bloodstream [14]. Post-heparin HL activity measurements have been utilized to reflect the functional levels of HL in an individual and are indirectly measured by subtracting NaCl-sensitive LPL activity from total post-heparin lipase activity. Post-heparin HL activity measurements are often elevated in hyperlipidemic patients and have been linked to an increased risk for developing CHD [15;16]. This has led to the suggestion that HL may be a pro-atherogenic enzyme [17;18]. High post-heparin HL activity may

also be related to CHD risk by reflecting reduced lipolytic function. Increased post-heparin HL activity may represent an elevated storage pool of inactive HL in the liver, which results from defective release and activation of the enzyme [19-22]. Cell surface, HSPG-bound HL is catalytically inactive enzyme and studies show that HDL functions to mobilize and activate this liver-resident pool of HL [19].

1.4.4 Displacement of HSPG-bound HL

In humans, HL is primarily found associated with cell-surface HSPG on hepatocytes and endothelial cells of the liver and is therefore considered to be a liver resident enzyme [23]. Previous work has shown that specific residues in the HL protein regulate the association of HL with HSPG [24;25]. Mapping studies using peptide arrays have identified two HL-heparin binding domains, one at the N-terminus, (R310, K312, K314, R315) and another at the C-terminus (R473, K474, R476) [25]. In rodents, HL is also synthesized in the liver, but is predominantly found circulating in the bloodstream [26;27]. Murine HL appears to be more readily displaced from cell-surface HSPG due to differences in the C-terminal amino acid composition of the enzyme [27]. Human HL can be released or liberated from cell-surface HSPG by both heparin and HDL. Studies have suggested that heparin interacts directly with the TG lipases and/or competes for binding sites on cell-surface HSPG [28]. Other studies show that heparin may act through protein kinase and calcium signaling pathways to stimulate HL release [29]. HDL-dependent HL displacement is regulated by interactions between HL and HDL and affected by both the lipid and apolipoprotein composition of HDL [20-22].

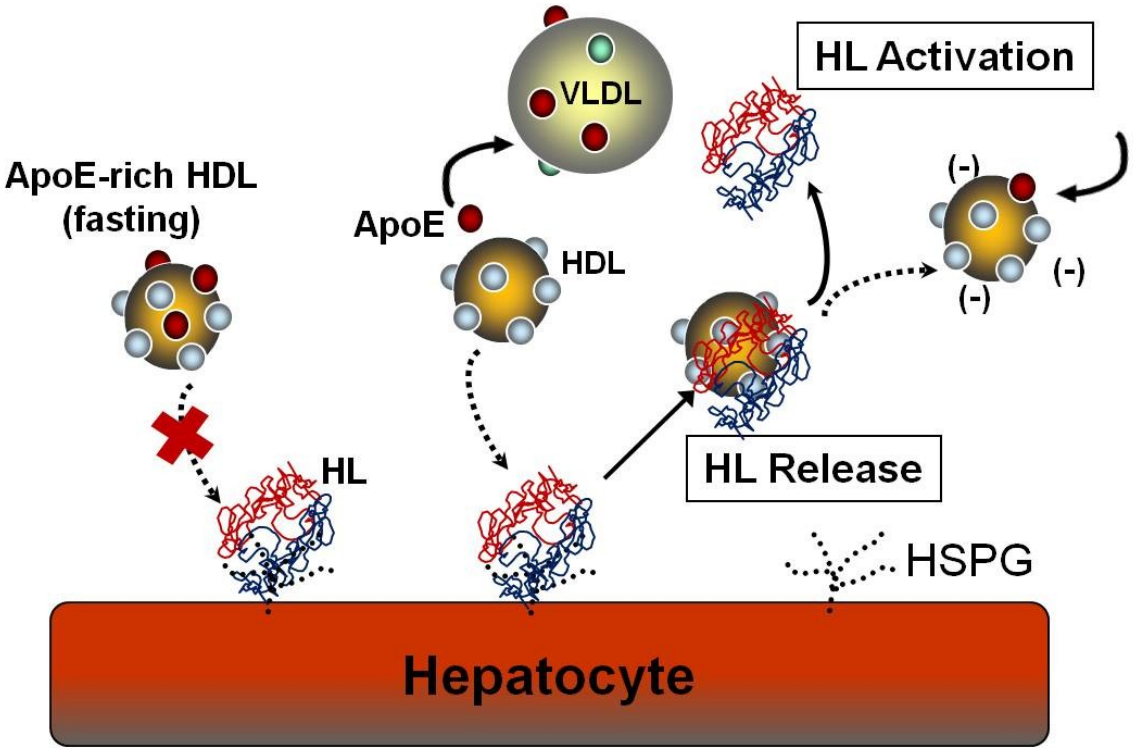
HDL composition directly affects the displacement of HL from cell-surface HSPG [20;21]. Ramsamy et al. showed that different subclasses of HDL have unique abilities to displace HL [20]. The larger, more buoyant HDL_2 fractions were more effective at displacing

HSPG-bound HL, than the smaller, dense HDL_3 fractions. Rouhani et al. showed that the various lipids in HDL had unique effects on HL displacement [21]. Increases in HDL-TG and phospholipid content directly inhibited HL displacement from the cell surface, while changes in the other lipid components of HDL had little effect on HL release. More recently, HDL and serum isolated from postprandial subjects were shown to promote increased HL displacement, relative to samples from fasted subjects [22]. The study showed that even though postprandial HDL is TG-enriched, the lipoprotein is deficient in apoE and more effective at binding to and displacing cell-surface HL [22].

HL displacement appears to be controlled by HDL apolipoproteins and is stimulated by the apoA-II content of HDL [21]. ApoA-II increases the release of HL from HSPG by enhancing the association of HL with HDL and this increased association promotes an inhibition of HL activity [30;31]. Conversely, HL displacement is inhibited by HDL-apoE [22]. Young et al. showed that HDL isolated from female subjects was significantly better at displacing HL from cell surface HSPG, relative to male HDL [22]. HDL isolated from women also contained less apoE, as compared to HDL isolated from the plasma of males. The study identified an inverse relationship between HDL-apoE content and the amount of circulating HL in the bloodstream [22]. Increased apoE content on HDL results in decreased HL release (**Figure 1.3**). Treatment of HDL with monoclonal apoE antibodies, directed against epitopes in the glutamic acid-enriched N-terminus of apoE, resulted in greater HL displacement [22], which may suggest that the binding of HL to HDL is sensitive to apoE-dependent electrostatic properties of the lipoprotein. Other work has shown that HL activity is also dependent on electrostatic events that regulate the association of HL with HDL [31;32].

Apolipoproteins are exchanged between HDL and the TG-rich lipoproteins, such as

Figure 1.3. HDL regulates the release and activation of hepatic lipase. The liver is a storage depot for catalytically inactive HL that is anchored to cell-surface HSPG. HDL binds to HL and releases the enzyme into the circulation. Fasting, apoE-rich HDL is ineffective at releasing HL from cell surface HSPG. During a postprandial response, HDL loses apoE to VLDL and the apoE-deficient HDL is more efficient at releasing HL from the cell surface. HDL compositional changes can then release HDL-bound HL and activate the catalytic activity of the circulating enzyme. HDL therefore plays an important role in the mobilization and activation of HL.


VLDL
HL Activation
ApoE-rich HDL
(fasting)
ApoE
HDL
(-)
(-)
(-)
HL
HL Release
HSPG
Hepatocyte

VLDL and IDL, during a postprandial lipemic event [33;34]. Notably, apoE and apoCs are transferred from HDL to VLDL, where they act as lipolytic co-factors and receptor ligands [33]. HDL is therefore a storage depot for apoE in the fasting state. A few hours after a meal, when plasma TG levels are high, apoE moves from HDL to the TG-rich lipoproteins [33;35]. This decrease in HDL-apoE content appears to initiate the mobilization of HL from the hepatocyte cell surface to the vascular compartment (**Figure 1.3**), where the enzyme can then act to hydrolyze circulating TG [22]. At the end of the lipemic response, apoE returns to the HDL pool and blocks the ability of HDL to release HL from the liver.

1.4.5 Regulation of HL Lipolytic Activity

In humans, there appear to be two inactive pools of HL, one that is HSPG-anchored in the liver and one that is HDL-bound and circulating in the bloodstream as an inactive enzyme. HDL therefore regulates HL activation in a two-step process, wherein HDL binds and displaces HL from cell surface HSPG, and then HDL dissociates and activates the circulating enzyme (**Figure 1.3**). Under fasting conditions, HL in the circulation appears to be catalytically inactive. HL activity can only be detected in the plasma after the enzyme is released from the liver by infusions of heparin [14]. While apoA-I and HDL are also able to liberate HL from cell surface HSPG, the association of HL with HDL directly inhibits HL activity [19;31;32]. HL is inactivated by its association with HDL particles containing both apoA-I and apoA-II [30;31;36].

HL activity is stimulated by the dissociation of HL from HDL (**Figure 1.3**) and controlled by lipoprotein electrostatic properties [31;32]. Enrichment of HDL or serum with free fatty acid or anionic phospholipids (such as PI, PA or PS) increases the net negative charge on

HDL and stimulates VLDL-TG hydrolysis by HL [32]. An increase in HDL net negative charge was shown to reduce the binding of HL to HDL and increase HL hydrolytic activity for all lipoprotein substrates. HL activity is therefore inhibited by the electrostatic-dependent association of HL with HDL [19;32]. ApoA-II has been shown to increase the association of HL with HDL and to directly inhibit TG hydrolytic activity [30;31;36]. ApoE has quite the opposite effect. ApoE blocks the association of HL with HDL [22], but stimulates the HDL lipolytic activity of HL [37]. Women have been shown to have reduced plasma apoE levels and increased circulating HL, relative to men [22]. Women also have a reduced post-heparin HL activity, which has been thought to be a consequence of an inhibitory effect of estrogen on HL transcription [38]. Reduced post-heparin activity in women may therefore be partly a consequence of reduced apoE levels and defective HL activation [22]. ApoE has been shown to directly interact with apoA-II [39] and as such, may block apoA-II dependent association of HL with HDL [31]. ApoA-II may therefore control HL displacement and activation, and its action is modulated by the amount of apoE that can reside on the HDL particle surface.

In the circulation, HDL remains associated with HL, to keep the enzyme in an inactive state, until hydrolytic activity is required. A tight regulation of HL lipolytic activity by HDL would be expected, as HL is a phospholipase and potentially lytic to cell membranes. The liberation of HL by HDL from the cell surface therefore primes HL for its hydrolytic function, by releasing the anchored-enzyme and enabling HL to gain access to circulating substrate. Increased lipase shuttling between substrate molecules has been shown to stimulate most interfacial lipolytic enzymes, such as HL [40]. Higher circulating levels of HDL-bound, inactive HL may be important to TG clearance. HDL isolated from the plasma of female normolipidemic subjects, by sequential density ultracentrifugation, was shown to contain

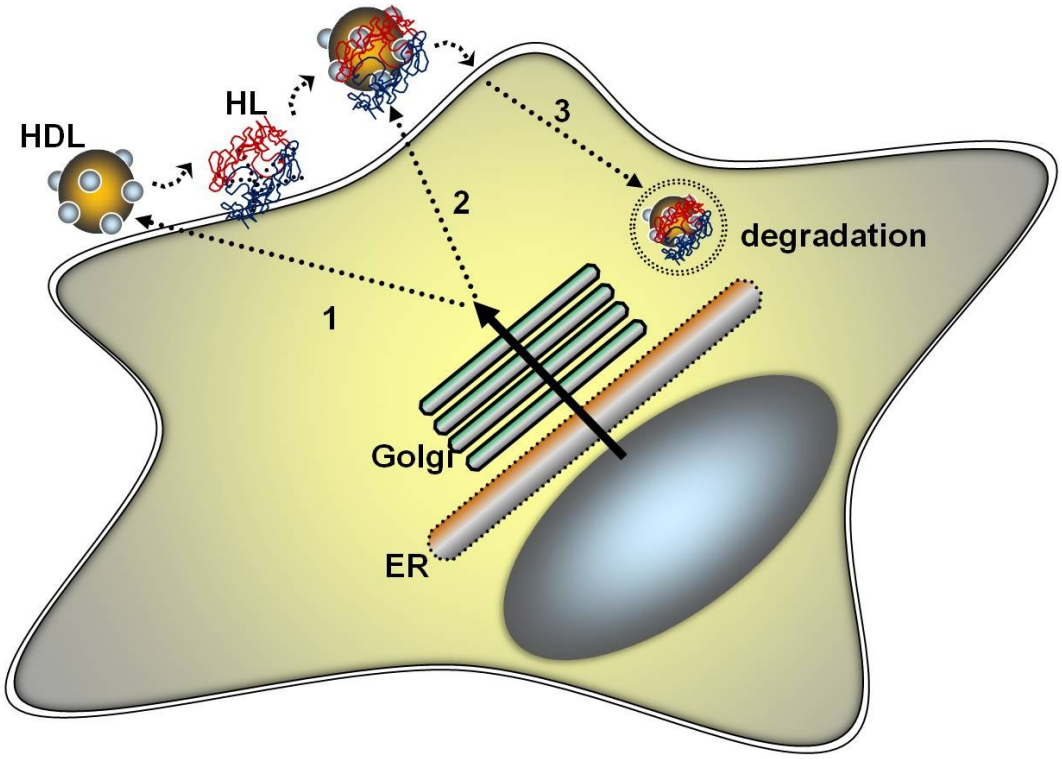
significant HL mass [22]. In contrast, HDL isolated from normolipidemic males and hyperlipidemic patients contains much less HL protein. An increased vascular pool of HL in women may therefore contribute to the reduced magnitude of postprandial lipemia, often observed in women relative to men [41]. Increased HDL-bound HL in the circulation may also affect the remodeling of HDL, since HDL_2 formation has been shown to be increased in subjects that can clear alimentary TG more rapidly [42].

1.4.6 Regulation of HL Secretion from the Liver

Since HDL is able to liberate HL from the cell-surface HSPG, it follows that hepatic HDL secretion would be expected to impact the release of HL from the liver. This view has been confirmed in studies in primary human hepatocytes and HepG2 cells, which showed that factors that increase apoA-I/HDL secretion from hepatocytes also increase HL secretion (**Figure 1.4**) [43]. Chatterjee et al. showed that overexpression of apoA-I in HepG2 cells directly stimulated HL release from the cell surface. Conversely, a knockdown of apoA-I expression with siRNA decreased HL release into the media. Therefore, newly secreted HDL may be able to bind and displace cell surface HL (**Figure 1.4**). Alternatively, HL may associate with apoA-I / HDL complexes intracellularly and be co-secreted with HDL.

HDL and HL secretion may also be affected by membrane reuptake and degradative pathways. Treatment of HepG2 cells and primary human hepatocytes with compounds that block apoA-I retroendocytosis, also affect HL release. Linoleic acid phospholipids (PL), such as dilinoleoylphosphatidylcholine, increase hepatic apoA-I secretion by 3-fold and promote a 2-fold increase in HL release [43]. PL treatment does not appear to affect HL transcription, as they have no effect on steady-state mRNA levels [43]. PL instead stimulate PPARα expression [44] and inhibit membrane nucleotide signaling events on the cell surface to block

Figure 1.4. HDL and hepatic lipase secretion are co-regulated. HDL secretion regulates HL release from the liver through three potential mechanisms: 1) newly secreted HDL binds and displaces cell surface HL, 2) HDL and HL associate intracellularly and are co-secreted, and/or 3) HDL and HL secretion are co-regulated by plasma membrane reuptake and degradation pathways.


HDL
HL
1
2
3
degradation
Golgi
ER

retroendocytic degradative pathways [45]. The data suggest that PL block membrane recycling pathways that promote the reuptake and degradation of cell surface proteins such as HL (**Figure 1.4**). HL degradation has been shown to be rate limiting to HL secretion and associated with the dimerization of the enzyme [46]. Doolittle and colleagues showed that when HL does not form an active dimeric complex, large amounts of monomeric HL accumulate in the cell and are rapidly degraded. These investigators later showed that both the maturation and homodimerization of HL and lipoprotein lipase may be governed by a chaperone protein in the endoplasmic reticulum, called lipase maturation factor 1 [47].

HL may be secreted from hepatocytes as an inactive enzyme. Stimulants of HL secretion can increase HL mass in the hepatocyte media by 2-fold, but have no affect on HL activity in the media [43]. This may be important to the regulation of HL phospholipase activity and due to an inhibitory effect of the specific species of HDL that HL is associated with in the media. As in the circulation, HL in hepatocyte media is primarily associated with larger HDL complexes containing both apoA-I and apoA-II. PL treatment increases the secretion of both apoA-I and apoA-II [43;45] and as shown by Boucher et al., the association of HL with apoA-II-enriched HDL directly inhibits HL hydrolytic activity [31].

1.4.7 Conclusion

An inverse relationship exists between blood TG and HDL levels. Low HDL levels are often associated with high TG in both men and women, and low HDL / high TG levels are related to an increased risk of developing CHD [48]. HDL is a repository for regulatory apolipoproteins and alterations in HDL apolipoprotein composition can affect TG metabolism by impacting the function of both HL and LPL. Mutations in the *LIPC* gene may have a direct effect on HL function, or may indirectly affect lipolysis by causing reduced or dysfunctional

HDL particles [7;8;10]. Stimulants of hepatic HDL production may therefore act through cofactor-pathways to stimulate lipolytic enzymes and enhance TG clearance [43]. This may partly explain why drugs that increase HDL levels, such as the fibrates and niacin, also reduce plasma TG levels [49;50].

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CHAPTER 1 – INTRODUCTION PART II

1.5 Diabetes Mellitus

1.5.1 Insulin Resistance

Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes mellitus (NIDDM), is a metabolic disorder that is primarily characterized by hyperglycemia due to insulin resistance [82]. Unlike Type 1 diabetes mellitus (T1DM), which originates from the body's inability to produce insulin, T2DM occurs due to reduced tissue response to insulin [82]. T2DM is more prevalent than T1DM, currently mostly seen in adults. Insulin resistance is a complex condition that is often associated with obesity, elevated plasma TG and apoB levels, decreased plasma HDL levels, hypertension, atherosclerosis as well as a chronic inflammatory state [83-85]. Since insulin resistance has many overlapping characteristics with CHD, T2DM is considered a risk factor for CHD [83;86].

Insulin is the key mediator in regulating blood glucose levels. It is the hormone that is responsible for increasing glucose uptake in muscle and adipose tissue and inhibiting glucose production in the liver [87]. Any disturbances to this process can lead to insulin resistance and the dysregulation of both glucose and lipid homeostasis. In addition, factors including hyperinsulinaemia, hyperglycemia, elevated circulating fatty acid levels, pro-inflammatory cytokines (IL-1β, IL-6 & TNFα), and glycation products, all contribute or even exacerbate insulin resistance by interfering with glucose uptake and insulin signaling at different stages [88;89]. Abdominal obesity in metabolic syndrome and insulin resistant states is associated with a larger, expanded adipose tissue that results in an increased turnover of free fatty acids (FFA) and increased pro-inflammatory cytokine secretion in addition to decreased anti-inflammatory cytokine and adiponectin secretion. This state promotes increased glucose, TG production

and VLDL secretion by the liver (low HDL & high TG). FFA also reduce insulin-mediated glucose uptake in muscle and increased lipid accumulation. Increased insulin secretion, hyperinsulinemia, can occur to compensate for the reduced tissue response to insulin resulting in a chronic inflammatory cycle [90].

1.5.2 Insulin Signaling

Under normal conditions, insulin is secreted after a meal from pancreatic β-cells of the islets of Langerhans, which stimulate glucose uptake in muscle and adipose tissue via glucose facilitative transporters of the GLUT family. Among the 13 members of the GLUT family, GLUTs 1-4 are the most characterized, and have tissue specific distributions and functions [91]. GLUT-1 is responsible for low-level basal glucose uptake needed for cellular respiration in all tissues, and GLUT-3 is expressed in adult neurons and during fetal development. GLUT-2 acts as a high capacity/low affinity glucose sensor stimulating glucose uptake in β-cells, the liver and the small intestine [91]. It was originally thought that GLUT-2 was regulated transcriptionally by elevations in glucose, but further research has demonstrated that GLUT-2 can co-localize with the insulin receptor (IR) forming a IR/GLUT-2 complex that is internalized upon hepatic insulin stimulation resulting in the downregulation of glucose production [92;93]. GLUT-4 is predominantly distributed in the muscle, adipose tissue, and heart, and mediates insulin-dependent glucose uptake [91]. Insulin stimulates the translocation of GLUT-4 from intracellular vesicles, where they are sequestered under a basal state, to the plasma membrane upon insulin stimulation. This results in glucose to be transported into the cell from the bloodstream [91]. GLUT-4 translocation is the rate-limiting step in glucose uptake in muscle and adipose tissue.

Both GLUT-2 and GLUT-4 translocation is regulated by insulin-dependent signaling pathways. Insulin binding to the insulin receptor (IR) initiates intracellular signal transduction. The IR is heterotetrameric and composed of two α-subunits that bind insulin and two β-subunits with intrinsic tyrosine kinase activity [91]. The binding of insulin to the α-subunits results in the autophosphorylation of the β-subunits, enabling the interaction and phosphorylation of the insulin receptor substrate (IRS) family of proteins with the IR via the phosphotyrosine binding domain [88]. Further docking sites are created on the IRS, which allow proteins containing the Src Homology 2 (SH2) domains to bind [88;91]. Two important downstream pathways that are activated this way are the phosphatidylinositol 3' kinase (PI3K) and the mitogen activated protein kinase (MAPK) pathways.

The PI3K pathway is known to be critical to glucose, protein and lipid metabolism, and also to survival. PI3Ks belonging to the Type1 group are composed of a p85 regulatory subunit and a p110 catalytic subunit. The translocation of PI3Ks from the cytosol to the docking sites on IRS molecules at the plasma membrane results in the phosphorylation of its substrate, PIP_2, generating PIP_3. PIP_3 then recruits protein serine kinases containing Pleckstrin-homology (PH) domains [88]. These include 3' phosphoinositide-dependent kinase-1 (PDK-1), Akt/ protein kinase B (PKB), and protein kinase C (PKC). PI3K activation leads to the translocation of GLUT-2 and GLUT-4 and regulates glucose uptake. The phosphorylation activity of Akt also regulates glycogen synthesis. Studies have demonstrated that although the PI3K pathway is able to stimulate GLUT-4 translocation, the CAP/Cbl/TC10, a PI3K independent pathway may also contribute to its translocation [88;91].

MAPK is involved in cellular proliferation stimulated by insulin. This occurs through two adaptor proteins, SHC and Grb2, which bind to the phosphotyrosine binding domain of

the insulin receptor along with IRS molecules. The p38 MAPK modulate glucose uptake via GLUT-4. The Erk 1/2 MAPK do not directly affect glucose uptake, but increased MAPK activity can lead to insulin resistance and cause hyperinsulinemia[87].

Insulin signaling is a complex, highly regulated mechanism involving multiple signal transduction pathways, which lead to glucose uptake and homeostasis. When cells do not respond to insulin, more insulin is secreted leading to hyperinsulinemia. The liver tries to compensate for the lack of glucose uptake in the peripheral tissues by producing more glucose via gluconeogenesis and stimulating lipogenesis via the sterol regulatory element-binding protein-1 (SREBP-1). In insulin resistance, the decreased insulin-induced activation of Akt promotes Forkhead box O1 (FoxO1) nuclear translocation and gluconeogenic gene expression [94]. The Mammalian target of rapamycin complex 1 (mTORC1) is needed for the induction of SREBP-1c expression by insulin [94]. Elevated levels of amino acids found in obesity can decrease insulin signaling by degrading IRS, stimulate FoxO1-mediated gluconeogenesis and promote lipogensis via SREBP-1c, thereby dysregulating gluconeogenesis and lipogenesis [94]. Insulin resistance is known to cause an overproduction of VLDL, which is partly due to a decrease apoB-degradation that is mediated by insulin [95]. Alterations in insulin signaling cascades at different levels such as reduced insulin receptor levels and kinase activity, decreased insulin-induced IRS-1 phosphorylation and PI3K activity due to elevated circulating free fatty acid levels, and defects in GLUT translocation also all contribute to insulin resistance. Therefore, insulin resistance and T2DM are not necessarily the result of single defect but a compounded pathophysiological disorder encompassing the dysregulation of multiple tissues including the liver, muscle, pancreas, and brain.

1.5.3 Extracellular Nucleotides & Purinergic Signaling

Insulin resistance and T2DM are primarily characterized by hyperglycemia, however studies have demonstrated that elevations in blood insulin and glucose levels also stimulate extracellular nucleotide secretion and purinergic signaling [96-98]. It was also shown that extracellular ADP acting through $P2Y_{13}$ receptors on pancreatic beta cells can inhibit insulin secretion in mice [99]. Extracellular nucleotides may exacerbate the 'metabolic syndrome' phenotype seen in CHD and insulin-resistant states since they are also known to modulate inflammatory pathways and the immune system [98;100;101].

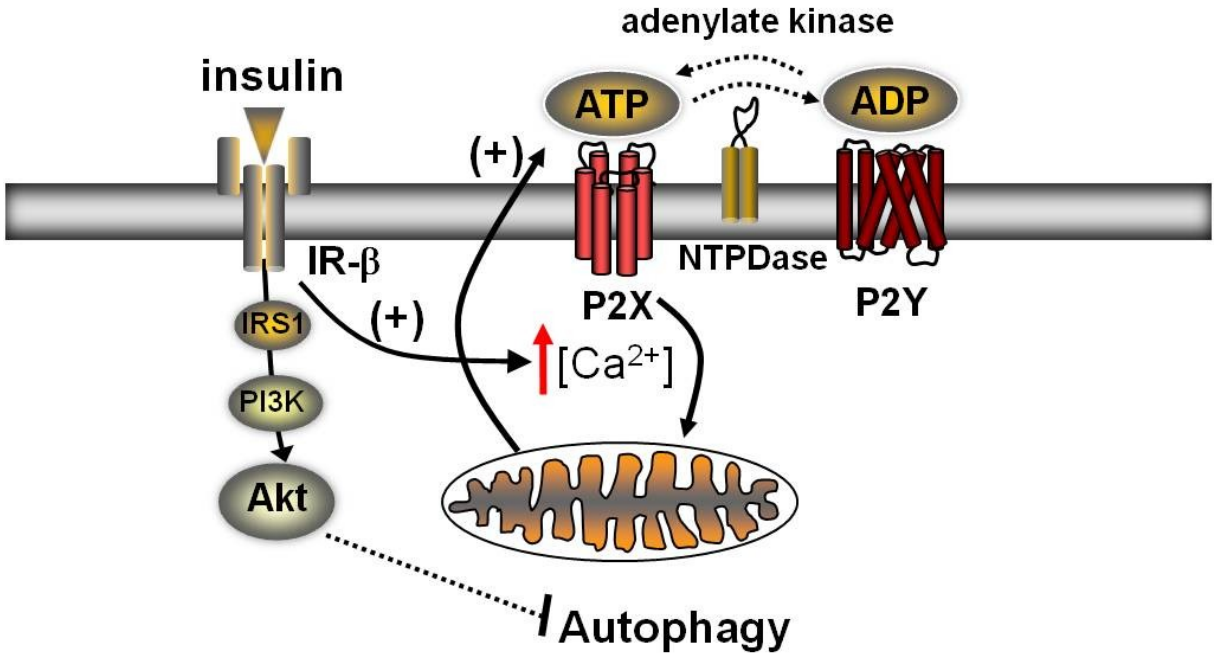
Although the role of nucleotides such as ATP and ADP as currency for energy in intracellular energy metabolism are long established, nucleotides also serve as extracellular signaling messengers that regulate inflammation, glucose, and lipid metabolism. Extracellular nucleotides, such as ATP and ADP, are secreted from red and white blood cells as well as platelets, in response to stress, injury, or hyperglycemia [102]. Their metabolism is regulated by ecto-nucleotidases (NTPDases), which convert ATP to ADP, or adenylate kinase, which converts ADP to ATP. Normally, extracellular ATP and ADP are found in nM to μM concentrations in the circulation, however upon injury and in both acute and chronic diseases, extracellular nucleotides levels in the blood are known to increase [103;104]. The secretion of nucleotides is known to stimulate nuclear factor kappa B (NF-κB) signaling as well as promote the secretion of pro-inflammatory cytokines [101;105;106]. In addition, studies have linked human single nucleotide polymorphisms in the NTPDase, CD39, to T2DM and renal disease [107], while deficiencies in murine CD39 were implicated in insulin resistance and hypertriglyceridemia [108]. Another NTPDase, F_1-ATPase, was shown to modulate HDL endocytosis by purinergic signaling through its receptor, $P2Y_{13}$ [109]. Human serum levels of its

natural inhibitor, mitochondrial inhibitory factor 1 (IF1), were shown to be positively correlated with HDL-C levels and negatively with TG levels in normolipidemic individuals [110].

Extracellular nucleotides serve as ligands for specific purinergic receptors belonging to the P1, P2Y or P2X classes. Both P1 and P2Y receptors are G-protein coupled receptors (GPCR), however P1 receptors are activated by adenosine, whereas P2Y receptors are activated by purine nucleotides (ATP, ADP, UTP, UDP). P2X receptors are ligand-gated ion channels that are activated by ATP. P2X receptors have 7 subtypes, while there are 13 subtypes of P2Y receptors that have been identified to date, all of which have distinct nucleotide specificities and tissue distributions. The two main P2Y receptors that are expressed in human liver cells are, $P2Y_1$ and $P2Y_{13}$, however $P2Y_{13}$ appears to be primarily implicated in lipoprotein metabolism [111]. Insulin secretion is also regulated by $P2Y_{13}$-mediated signaling whereby ADP inhibits insulin secretion in mouse pancreatic beta cells [99;112]. Fibroblasts from T2DM patients were also shown to have increased $P2X_7$ activity [113], and peripheral blood mononuclear cells in T2DM patients had both increased $P2X_7$ and CD39 expression, both of which were associated with glycemia and lipidemia [114]. Purinergic signaling therefore may mediate a number of important signaling pathways including insulin, MAPK, PI3K, and NF-κB signaling (**Figure 1.5**).

Extracellular ATP and ADP are responsible for mediating a vast array of processes that also impact vascular health. Some examples include proliferation and inflammation of vascular smooth muscle cells, vascular tone, and blood pressure as well as coagulation cascades involving platelets [102;115;116]. In particular, the role of extracellular ADP in platelet activation and thrombosis has been well characterized [117;118]. Clopidogrel, a widely used anti-

Figure 1.5. Extracellular nucleotides and purinergic signaling. Extracellular nucleotides are regulated by ecto-nucleotidases (NTPDase), which break down ATP and ADP, and adenylate kinase, which generates ATP from ADP. Extracellular nucleotides are ligands for purinergic receptors, which have different specificities. P2X (Ca^{2+}-gated channel) is activated by ATP, whereas P2Y (G-protein coupled receptor) is activated by ADP. Extracellular nucleotide levels and purinergic signaling is known to be modulated by cellular signaling pathways including PI3K and Ca^{2+}.


adenylate kinase
insulin
ATP
ADP
(+)
IR-β
NTPDase
P2X
P2Y
IRS1
(+)
[Ca2+]
PI3K
Akt
Autophagy

platelet therapeutic for the prevention of thromboembolic events, is known to inhibit ADP-mediated signaling through its receptor, $P2Y_{12}$ [119;120]. Antagonism of P2 receptors may have more potential benefits in the prevention and treatment of both atherosclerosis and diabetes, since common drugs used to treat diabetes such as, metformin and sulfonylureas are known to not only improve hyperglycemia, but increase plasma HDL and decrease plasma TG levels [34;121;122].

1.6 Cellular Proteolytic Pathways

1.6.1 Cellular Protein Degradation

Protein degradation is critical to numerous cellular processes and complex mechanisms exist to ensure proper regulation of degradation to prevent aberrations leading to the development of diseases such as CHD and diabetes. Protein degradation is important to cellular homeostasis and is implicated in DNA repair, growth and differentiation, regulation of gene transcription, quality control of synthesized/secreted proteins, regulation of membrane receptors, regulation of the immune system and provide a source of amino acids for energy production [123;124]. Proteins in different cellular compartments have varying turnover rates ranging from minutes for enzymes to months for structural components [124]. Due to the diverse degradative needs of the cell, cells are equipped with multiple highly selective proteolytic systems. The three major protein degradation systems in the cell are: 1) lysosomes, 2) ubiquitin-proteasome, and 3) autophagy. Lysosomal degradation of extracellular proteins was first revealed upon discovery of the lysosome organelle, but it was quickly understood that majority of intracellular proteins underwent nonlysosomal degradation with the discovery of the ubiquitin-proteasome system (UPS) [123]. Further work has demonstrated the role of

lysosomes in mediating the degradation of cytosolic proteins through a process called autophagy [124].

1.6.2 Proteasomal Degradation

Proteasomal degradation comprises the major mechanism by which intracellular proteins are degraded. The most common form of proteasomal degradation is the ubiquitin-proteasomal pathway (UPP), however there also exists ubiquitin-independent proteasomal degradation, which is not as well characterized. In the UPP, damaged or unnecessary proteins are marked for degradation by selective tagging with a small protein called ubiquitin (Ub) on Lys residues through a series of ATP-dependent reactions involving 3 ubiquitin enzymes (E1: Ub-activating enzyme, E2: Ub-conjugating enzyme, and E3: Ub-ligase). This process is repeated and the resulting polyubiquitinated protein is recognized by the 26S proteasome for proteolysis yielding small 7-9 amino acids length peptides. Interestingly, it was found that normal physiological ATP levels inhibit the proteasome, whereas 100μM ATP seems to be optimal in inducing proteasome function [125].

The 26S proteasome is found in both the nucleus and cytosol of cells. It is comprised of a 20S barrel-shaped core particle containing the proteases, and a 19S regulatory particle at each end that is responsible for recognizing Ub, unfolding the tagged-proteins by ATP hydrolysis, and translocating it to the 20S core [124]. The 20S core is composed of 4 stacked rings (2 outer α-rings and 2 inner β-rings, each with 7 subunits). The α-rings provide structural support and serve as a gate to the inner compartment, while the β-rings have proteolytic activity. Although the β1, β2 and β5 subunits all have catalytic activity, they have distinct substrate specificities, that is, chymotrypsin-like, trypsin-like, and peptidyl-glutamyl activities, respectively.

Ubiquitin-dependent proteasomal degradation plays an important role in regulating lipoprotein metabolism and diabetes. ApoB and apoE, the two main apolipoproteins on VLDL, are known to be regulated by the proteasomal pathway [126-128]. In addition, the LDL-receptor related protein and mutant forms of the LDL-R were shown to be degraded by the proteasomal pathway [129;130]. ER-associated proteasomal degradation is also critical to hepatic lipase maturation and its full activity [131;132]. Chronic insulin treatment can alter insulin signaling by promoting the degradation of IRS-1 proteins [133;134]. Proper regulation of the ubiquitin-proteasome is critical to health and the prevention of disease.

1.6.3 Lysosomal Degradation & Autophagy

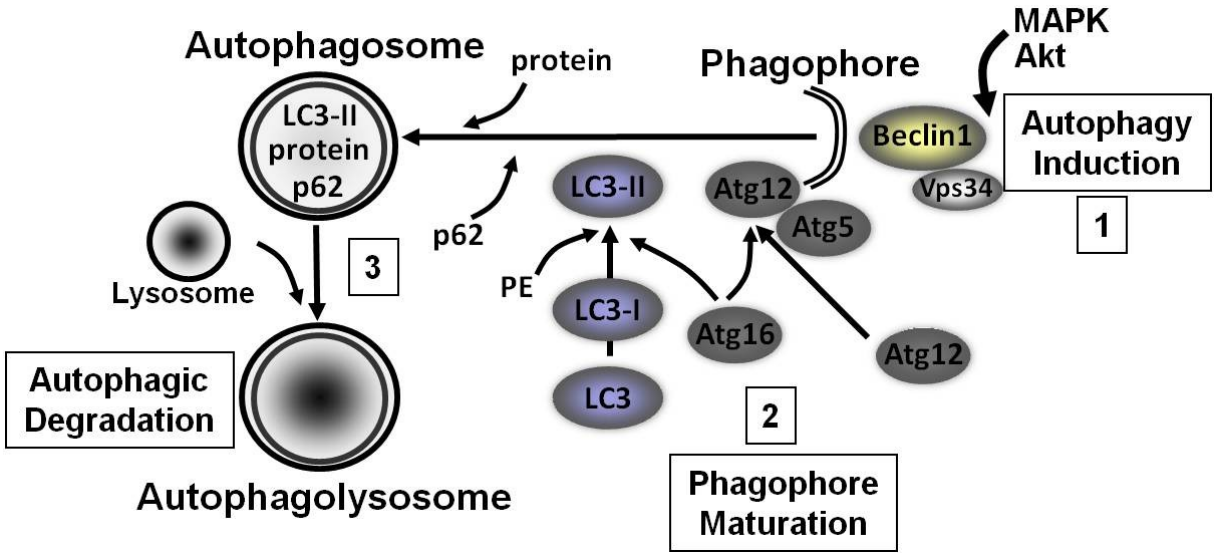
Lysosomal degradation is a cellular degradation process by which extracellular components are endocytosed or phagocytosed into the cell by vesicular transport from the plasma membrane and then fused with lysosomes. The engulfed components are then degraded by hydrolases that are active in the acidic lumen of lysosomes as well as lysosomal proteases such as cathepsins. The acidic nature of the lysosomal lumen is maintained by a vacuolar ATPase proton pump. Lysosomal degradation plays a key role in receptor-mediated endocytosis as seen in LDL uptake by the LDL-R, where clathrin-coated pits that form vesicles carry the LDL/LDL-R complex, and bud off from the plasma membrane. The clathrin coat is then removed from vesicles, the vesicle then fuses with endosomes where the LDL-R is recycled to the cell-surface and the LDL is sent to the lysosome [135-137]. Lysosomal function is necessary for proper cellular homeostasis and defects result in disorders referred to as lysosomal storage diseases.

Several years after the discovery of the lysosome, autophagic degradation was discovered in the 1960s, [138;139]. Autophagy literally means ‘self-eating’ and is described as

an adaptive cellular mechanism that is initiated by stressors such as starvation, cytokines and pathogens. Autophagy promotes the sequestering of cytosolic components (proteins, lipids and organelles) in double-membrane vesicles called autophagosomes which then fuse with lysosomes to form autolysosomes for degradation [140]. This process not only helps alleviate cells from damaged, harmful and toxic agents, but provides a source of nutrients and energy during the stress response [140]. Autophagy was first identified to be induced in the liver during starvation, then later showed to be inhibited by insulin and stimulated by glucagon [138;139;141]. Over the last 15 years, the field of autophagy has been thriving and provided new insights into the process. Eukaryotic cells were identified to have three major types of autophagy: 1) Macroautophagy, 2) Microautophagy, and 3) Chaperone-mediated autophagy (CMA) [142]. Macroautophagy is the most common form involving the formation of double-membrane autophagosome, whereas microautophagy is carried out by the direct sequestering of the cytoplasm into lysosomes [142]. Unlike macroautophagy and microautophagy, which are bulk phase degradation processes, cytosolic substrate proteins in CMA are selectively recognized and translocated across the lysosome membrane aided by receptor/translocation complexes [143;144].

The regulation of macroautophagy is widely studied and the focus of this thesis. Cell signaling pathways that are involved in nutrient deprivation or insulin signaling are known to also regulate autophagy. Insulin receptor signaling inhibit autophagy through PI3K and the phosphorylation of PKB/Akt and mTOR [140] (**Figure 1.5**). In contrast, nutrient deprivation induces autophagy by stimulating the MAPK kinase (RAF-1/MEK1/2/ERK1/2) signaling cascade [140]. Once autophagy is stimulated, an isolation membrane thought to having ER or mitochondirial origin called the phagophore is formed [140;145;146] (**Figure 1.6**). The

Figure 1.6 Overview on macroautophagy. (1) Autophagy is regulated by signaling pathways such as MAPK and Akt, which result in the formation of an isolation membrane called the phagophore. **(2)** The phagophore undergoes elongation and maturation facilitated by autophagy related proteins (Atg). It involves the ubiquitin-like conjugation of Atg12 to Atg5 and Atg16L and the subsequent association with LC3. LC3 gets cleaved by Atg4 to form LC3-I which subsequently becomes conjugated with phosphatidylethanolamine (PE) to form LC3-II. The mature phagophore sequesters some of the cytoplasm containing target proteins to form a double-membrane autophagosome. **(3)** The autophagosome fuses with a lysosome containing hydrolases to form autolysosomes that degrade the target proteins.


Autophagosome
protein
Phagophore
MAPK
Akt
LC3-II
protein
p62
Beclin1
Autophagy
Induction
Vps34
1
LC3-II
Atg12
Atg5
p62
Lysosome
3
PE
LC3-I
Atg16
Atg12
Autophagic
Degradation
LC3
2
Autophagolysosome
Phagophore
Maturation

phagophore then undergoes a number of elongation and maturation steps that requires autophagy related proteins (Atg) such as Atg12 to Atg5 and Atg16L as well as LC3-II (microtubule-associated protein light chain 3 or Atg8). LC3-II is an autophagic marker that is formed when its precursor, LC3 is cleaved by Atg4 to form LC3-I, and then C-terminally conjugated with phosphatidylethanolamine (PE) [145]. The elongated mature phagophore sequesters a part of the cytoplasm forming a double-membrane autophagosome. The autophagosome can then fuse with lysosomes or endosome forming autolysosomes, which eventually leads to the degradation of the inner membrane of the autophagosome and its contents by lysosomal hydrolases [140]. The resultant macromolecules are recycled by permeases [140;145].

It is also known that there are links and interplay between autophagy and proteasomal degradation . The complementary relationship between these two degradation pathways is best studied in neurodegenerative disease and proteinopathies, and critical to cellular protein quality control [147;148]. Proteasomal degradation is responsible for the degradation of normal proteins, and is not sufficient for the degradation of large amounts of defective or abnormal protein aggregates, which requires autophagy [148]. The 20S proteasome was shown to be involved in the processing of LC3 and furthermore p62, an autophagy marker and LC3-binding protein that can be ubiquitinated, can also mediate the autophagic degradation of polyubiquitinated proteins upon inhibition of the proteasome [149;150]. Accordingly, proteasomal inhibitors like ALLN, MG132 and Bortezomib can also stimulate autophagy [151;152].

Numerous studies have associated autophagy with CHD, atherosclerosis, and lipid metabolism in addition to its implicated roles in insulin resistance and diabetes. For example, increased autophagy can lead to cardiac hypertrophy and heart failure [153;154]. Also, other

studies have suggested that a selective stimulation in macrophage autophagic-mediated death may be beneficial to stabilizing plaques and lesions in atherosclerosis [155;156]. More recently, an inhibition in autophagy or lipophagy was shown to be associated with increased hepatic TG storage in lipid droplets [157], and autophagy was also shown to regulate macrophage cholesterol efflux [158]. Despite the recent advances of the role of autophagy and proteasomal degradation in diseases, the relationship remains poorly understood and more research is needed to characterize these pathways and explore potential therapeutic modulation.

1.7 Rationale

Both the prevalence of coronary heart disease and type 2 diabetes mellitus is on the rise in developed countries. Alterations in lipoprotein metabolism resulting in elevated plasma triglyceride and apoB levels, decreased plasma HDL levels, obesity and inflammation are common features to both of these metabolic disorders. Although great strides have been made to understand the mechanisms that contribute to each of these complex disorders, the causative linkage between these disorders has been elusive. The goal of this doctoral thesis is to gain further knowledge into common causative mechanisms that may impact both lipid and glucose metabolic disorders resulting in abnormal lipoprotein metabolism and insulin signaling, leading to CHD and T2DM. New insights into the regulation of these disorders will not only aid in their prevention, but will also help in devising new therapeutic strategies for their treatment.

1.8 Research Objectives & Introduction to Manuscripts

To determine how HDL and HL metabolism are coordinated in the liver, and to investigate the common mechanisms that cause dyslipidemia in both heart disease and insulin-resistant states, the study focused on three main hypotheses:

1. **HDL and hepatic lipase secretion are co-regulated in hepatic cells.**
2. **HDL secretion is regulated by proteolytic degradation.**
3. **HDL and hepatic lipase secretion are affected by extracellular nucleotides through degradation pathways.**

In the first part of the study (**Chapter 2-Manuscript#2**), our objective was to characterize the mechanism contributing to the inverse relationship between plasma HDL and TG levels in humans. Low HDL levels are often associated with high TG levels and increased risk for developing CHD, whereas high HDL levels are associated with low TG levels. We hypothesized that if exogenous HDL can release hepatic lipase from the liver, HDL secretion from the liver should stimulate the secretion of hepatic lipase and aid in TG clearance. This view was proven correct and demonstrated that HDL and TG metabolism may be co-regulated in the liver.

In the second part of the study (**Chapter 3-Manuscript#3**), we explored how HDL secretion may be affected by purinergic signaling and cellular degradation pathways. Elevated blood glucose levels, as seen in insulin resistance and T2DM, are associated with abnormal plasma lipoprotein metabolism and elevated circulating nucleotide levels. We hypothesized that extracellular nucleotides and purinergic signaling may inhibit hepatic HDL secretion by affecting protein degradation. This view was confirmed and we showed that purinergic signaling affects lipoprotein secretion by coordinating both proteasomal degradation and autophagy, through an inhibition of insulin receptor signaling.

In the third part of the study (**Chapter 4-Manuscript#4**), we hypothesized that if HDL secretion is regulated by purinergic signaling and autophagy, and HL secretion parallels HDL secretion, then HL secretion may also be regulated by extracellular nucleotides and autophagy. Hyperglycemia is known to exacerbate postprandial lipemia and insulin resistance stimulates hypertriglyceridemia due to an overproduction and impaired degradation of TG-rich lipoproteins. HL secretion, may therefore be impaired by extracellular nucleotides and purinergic signaling, similar to HDL. This hypothesis appears valid and my studies have shown that a modulation of HL secretion is not associated with extracellular HL recycling, but a consequence of a selective control of autophagy and secretion pathways.

In the last section (**Chapter 5-Manuscript#5**), we put into context the implications of our purinergic signaling and HDL findings to what is known in the field in a review article that we recently published. We discuss the contributions of purinergic signaling to dyslipidemia and inflammatory diseases such as cardiovascular disease and diabetes.

Altogether, these findings suggest that both HDL and TG metabolism are co-regulated in the liver by purinergic signaling-mediated autophagy of HDL and HL, and insulin receptor signaling.

1.9 References

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CHAPTER 2-MANUSCRIPT#2

Hepatic high-density lipoprotein secretion regulates the mobilization of cell-surface hepatic lipase

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2.1 Abstract

HDL acts much like heparin to liberate hepatic lipase (HL) from cell surface proteoglycans and stimulate triglyceride clearance. Experiments were undertaken to evaluate the effects of factors that stimulate the secretion of HDL from the liver on the release of HL. Treatment of HepG2 cells with linoleic acid phospholipids (LAPL) (12 μM) promotes a similar increase in the accumulation of both HDL and HL in the cell media. LAPL also induce both apoA-I and HL release from primary human hepatocytes. Dilinoleoylphosphatidylcholine has a greater effect on both apoA-I secretion and HL release than palmitoyllinoleoylphosphatidylcholine. HL released from HepG2 cells is inactive and associated with a large HDL complex containing both apoA-I and apoA-II. Inclusion of the PPARα inhibitor, MK-886, or MAPK inhibitor, U0126, completely blocks the LAPL-induced apoA-I and HL accumulation in the media. LAPL treated cell lysates, however, showed no change in HL protein expression nor HL mRNA. LAPL-induced HL release appears to be a consequence of the displacement ability of newly secreted HDL. Overexpression of pre-pro-apoA-I in HepG2 cells increased HL release, while siRNA inhibition of the apoA-I gene reduced HL in the media. The data shows that factors that stimulate HDL secretion in hepatocytes act to also increase the release of HL. This may partly explain why HDL therapeutics often impact plasma triglyceride levels.

2.2 Introduction

Human hepatic lipase (HL) is a 66kDa protein that is synthesized and secreted by the liver and hydrolyzes triglycerides (TG) and phospholipids in plasma lipoproteins [1]. HL is primarily found associated with heparan sulfate proteoglycans (HSPG) on the hepatocyte cell surface and studies have shown that HL is catalytically inactive when associated with HSPG

[2;3]. Heparin is able to release HL from cell surface HSPG and stimulate the hydrolysis and clearance of TG from the blood [4;5]. Heparin is thought to displace HL by competing for binding sites on the HSPG [5] or through a more complex route involving protein kinase signaling [6]. HDL can act similar to heparin and can also displace HL from the cell surface HSPG [2;3]. A high post-heparin HL activity therefore appears a measure of increased inactive liver-bound HL. Elevated post-heparin HL activity is common in patients with low HDL levels [7;8] and may be related to an inefficient displacement and activation of HL [3].

Linoleic acid phospholipids (LAPL), such as dilinoleoylphosphatidylcholine (DLPC), stimulate apoA-I and HDL secretion from hepatocytes [9]. Linoleic acid itself has no hepatic HDL secretory activity. LAPL induced HDL secretion was shown to involve a phospholipase C / protein kinase C activation of mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor-α (PPARα) signaling pathways through upregulation of PPARα protein expression [9]. HDL secretion and the accumulation of apoA-I in the hepatocyte media is the net result of both apoA-I synthesis and the reuptake and degradation of apoA-I. However, apoA-I synthetic pathways do not appear to be upregulated by LAPL and instead, increased HDL secretion appears to be the consequence of a decrease in the reuptake and degradation of apoA-I [10;11].

Addition of purified HDL or apoA-I to the media of HepG2 cells stimulates the release of HL into the media [2;3]. We therefore hypothesized that an increased secretion of HDL by liver cells would stimulate the release of HL from the cell-surface and increase HL accumulation in the cell media. As expected, we show that factors that stimulate hepatic apoA-I secretion, act to promote HL release into the media, similar to exogenously added apoA-I [2;3]. LAPL act through MAPK and PPARα pathways to stimulate the secretion of

apoA-I and apoA-II and the release of HL. Evidence suggests that HL accumulation in the media may be a product of cell surface displacement, resulting from HDL and HL interactions at the cell surface. The data shows that HDL and triglyceride metabolism may be co-regulated.

2.3 Materials and Methods

Chemicals – The phospholipids, palmitoyllinoleoylphosphatidylcholine (PLPC) and dilinoleoylphosphatidylcholine (DLPC), were purchased from Avanti Polar Lipids Inc., Alabaster, AL. Linoleic acid sodium salt was acquired from Sigma, Oakville, ON. MK-886, a non-competitive PPARα-inhibitor, and Clofibrate (PPARα-agonist), were from Cayman Chemicals, Ann Arbor, MI. The selective MEK 1/2 inhibitor, U0126 was acquired from Cell Signaling Technology, Beverly, MA along with the mouse monoclonal anti-human β-actin antibody. Antibodies for apoA-I used for ELISA was obtained from Biodesign, Saco, ME and the mouse monoclonal anti-human apoA-I antibodies (4H1 and 5F6) were obtained from Dr. Marcel in the Lipoprotein and Atherosclerosis Research Group at the University of Ottawa Heart Institute. The goat polyclonal anti-human apolipoprotein A-II antibody was from Chemicon International, Billerica, MA. The mouse monoclonal anti-human HL (XHL3-6a) antibody was from Dr. Bensadoun, Cornell University. Unless otherwise stated, drugs and inhibitors were of analytical grade and solubilized in DMSO.

Cell Culture and Treatment - HepG2 cells were cultured in normal glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Almost confluent cells were treated to stimulation with drugs for 24h under serum-starved conditions, as indicated. Briefly cells were treated as indicated, with or without various inhibitors/agonist, namely 10μM MK-886 (PPARα inhibitor), U0126

(MEK1/2 inhibitor) or Clofibrate (PPARα agonist) in serum-free DMEM for 30min prior to the addition of 12μM DLPC and then incubated for 24h. For time-course experiments, cells were incubated with or without 12μM DLPC for the indicated timepoints. Concentrations up to 24μM DLPC had no cytotoxic effects. All inhibitors were used at the recommended IC_{50} concentration for particular targets and have been previously reported from our laboratory [9;11].

Primary Human Hepatocytes - Collagen-coated, and HIVI, hepatitis B and C, mycoplasma, bacteria, yeast, and fungi test-negative primary human hepatocytes (PHH) were obtained from freshly donated livers supplied by Lonza Walkersville (Walkersville, MD). PHH were incubated overnight in hepatocyte medium supplemented with transferrin, insulin, and recombinant human epidermal growth factor (rhEGF). The hepatocytes were incubated for 24 h with or without drugs as for HepG2 cells, and then conditioned medium and cell protein were collected for analysis.

Preparation of phospholipids – One milligram of palmitoyllinoleoylphosphatidylcholine (PLPC) or dilinoleoylphosphatidylcholine (DLPC) (in chloroform) was dried under N_2 gas and 1mL of DMSO added and then vortexed for 1 min. The mixture was then sonicated in a Branson 5200 waterbath sonicator for 3 × 10 min pulses, with brief vortexing between pulses.

ApoA-I ELISA – ApoA-I in conditioned medium, from each treatment, was analyzed by ELISA on a 96-well plate as previously described [9]. Briefly, the Nunc Immuno-maxisorp 96-well plates were coated overnight with a mouse anti-human apoA-I monoclonal antibody. Samples and standards were incubated in the wells for 2 h, followed by a 1 h incubation with a horseradish peroxidase-linked goat anti-human apoA-I antibody. Both antibodies were purchased from Biodesign. K-blue Max TMB substrate (Neogen Corporation, Lexington, KY) was added to each well and the reaction was stopped with a 1 M HCl solution; and the

absorbance was recorded at 470nm on a microplate reader. The ELISA assay was shown to not be sensitive to apoA-I conformation interference.

HL Immunoanalysis – After incubation with the inhibitors/agonists and/or phospholipids for the indicated times and doses, the conditioned media was removed and briefly centrifuged to remove any cells. The cells were washed twice with ice-cold PBS on ice and then lysed with NP40 lysis buffer (Biosource, Camarillo, CA) [50mM Tris, pH 7.4, 250mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na_3VO_4, 1% NP40 and 0.02% NaN_3] supplemented with 1mM PMSF and 1X protease inhibitor cocktail (Sigma, Saint Louis, MO) [AEBSF, aprotinin, bestatin hydrochloride, E-64, EDTA and leupeptin hemisulfate salt]. Total protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL) and 30µg of cell lysate or 30µL of 1:1 dilution of conditioned media in Laemmli sample buffer containing β-mercaptoethanol was separated by 8% SDS-PAGE. The proteins were transferred onto a PVDF membrane, and probed using a 1:5000 dilution of the mouse monoclonal anti-human HL antibody and a 1:20,000 dilution of the goat anti-mouse IgG linked HRP secondary antibody (KPL, Gaithersburg, MD) in 1%BSA/TBST. Blots were developed using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) on the Fluorochem AlphaImager. Band intensities were analyzed using the Spot-densitometer application of the AlphaEaseFC software. Cell lysate blots were then stripped and probed for β-actin for normalization, while the band intensities of conditioned media blots were normalized to total cell protein.

Immunoanalysis of Non-denaturing Gradient Gel Electrophoresis – Conditioned media from HepG2 cells stimulated with DLPC were electrophoresed in triplicate on a 4-20% Tris-Glycine Novex gel (Invitrogen, Carlsbad, CA) under non-denaturing conditions for 19 hours

at 100V alongside high molecular weight native markers (Amersham, Piscataway, NJ). The gel was then soaked in 0.1% SDS for 15min to give the proteins a slight negative charge in order for unidirectional transfer onto a PVDF membrane for 4 hours at 125V in Tris-Glycine transfer buffer containing 20% methanol. The membrane was allowed to dry at which point the molecular weight markers were outlined and the membrane was cut in three to probe for apoA-I, apoA-II and HL. The apoA-I membrane was blocked with 5% milk/TBST and then probed for apoA-I using a 1:2500 dilution of the mouse monoclonal anti-human apoA-I antibodies (4H1 and 5F6) and a 1:20,000 dilution of the goat anti-mouse IgG linked HRP secondary antibody (KPL, Gaithersburg, MD) in 1% milk/TBST. ApoA-II was probed by blocking in 1% BSA/TBST followed by incubation with 1:1000 dilution of the goat polyclonal anti-human apolipoprotein A-II antibody in 1% BSA/TBST and 1:10,000 dilution of the donkey anti-goat IgG-HRP secondary antibody in 1% BSA/TBST (Santa Cruz Biotechnology, Santa Cruz, CA). HL was probed as described in Western blot analysis for HL. Blots were developed using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) on the Fluorochem AlphaImager. Densitometry profiles were obtained using the 1D-Multi application of the AlphaEaseFC software.

Quantitative RT-PCR - Total RNA was isolated using TRI-reagent (Sigma, Oakville, ON) and treated with DNase using the DNA-free DNase treatment kit (Ambion, Inc., Austin, TX) according to manufacturer's specifications. RNA purity and integrity were assessed and first strand cDNA synthesis was performed with 2μg of RNA using the SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and then purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). cDNA was quantified using the NanoDrop ND-1000 spectrophotometer and 2ng of

purified cDNA was then subjected to 30 rounds of amplification using the Eppendorf Mastercycler thermal cycler using human HL RT-PCR primer sequences and conditions obtained from Gonzalez-Navarro *et al.* [12] and normalized to GAPDH. PCR products were run on 1.5% high-resolution agarose gel and visualized under UV using the Bio-Rad Gel-Doc System using Quantity One software. Real-time quantitative RT-PCR was performed using human HL forward, 5'-GGAGGAATCTGTTCAACTCTCTCG-3'; HL reverse, 5'-AGAAAGACGATTGCTGGGGG-3'GAPDH forward, 5'-GACATCAAGAAGGTGGTGAA-3'; and GAPDH reverse, 5'-CCACATACCAGGAAATGAGC-3' primer sequences, reagents and conditions according to Sirvent *et al.* [13] with the minor change of conducting 45 cycles of amplification on a Roche Lightcycler 480.

ApoA-I Overexpression in HepG2 cells - The pCMV5 vector containing the full length pre-pro-apoA-I cDNA subcloned into the *Pst*I and *Bam*HI was a kind gift from Dr. Mary Sorci-Thomas (Wake Forest University). The pre-pro-apoA-I-pCMV5 and the control pCMV5 plasmids were both grown and purified using the Plasmid Maxi kit (Qiagen Inc., Mississauga, ON), according to manufacturer's specifications. HepG2 cells were transiently transfected with pCMV5 plasmid alone or the pre-pro-apoA-I-pCMV5 plasmid by reverse (fast forward) transfection (where the cells were seeded and transfected on the same day at the same time) using FuGENE HD (Roche Applied Science, Laval, QC). In brief, complexes were prepared per manufacturer's instructions with a FuGENE HD-to-DNA volume-to-mass ratio of 6:2 (µl to µg) in 100µL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). HepG2 cells were trypsinized and seeded in 12-well plates at a density of 500,000 cells/well in a volume of 1mL in normal growth media containing 10% FBS in the absence of penicillin/streptomycin and then 100µL of the transfection complexes were immediately

added to the suspended cells. The cells were incubated with the complexes for 24h and then switched to serum-free DMEM for another 24h. The cells were then harvested and the media collected for analysis for apoA-I by ELISA and HL by immunoblotting as described previously. Protein in the conditioned media was normalized to total cell protein. Transfection of the pCMV5 plasmid alone showed no adverse cytotoxic effects compared to non-transfected cells.

siRNA Knockdown of apoA-I in HepG2 cells – HepG2 cells were transiently transfected with All Stars Negative siRNA or four different apoA-I siRNA sequences (separately) from the Flexitube Gene Solution siRNA kit (Qiagen Inc., Mississauga, ON) by reverse transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In brief, complexes were prepared per manufacturer's specifications with a Lipofectamine 2000- to- siRNA volume-to-mole ratio of 2:40 (μL to ρmol) in 200μL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). HepG2 cells were seeded, transfected and harvested as described in the apoA-I overexpression studies. Protein in the conditioned media was normalized to total cell protein. Transfection of the All Stars Negative siRNA alone showed no adverse cytotoxic effects compared to non-transfected cells.

Statistical Analysis – Values are shown as mean ± SD and $P<0.05$ was considered significant. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) on ranks by a pairwise multiple comparison using the Student-Newman-Keuls post-hoc test (SigmaStat; Systat Software, Inc., San Jose, CA).

2.4 Results

Effect of LAPL on apoA-I secretion and HL release from human hepatocytes - Treatment of HepG2 cells with different linoleic acid phospholipids (LAPL) has shown that a

stimulation of apoA-I secretion is associated with increased HL accumulation in the media **(Figure 2.1, panels A and B)**. Linoleic acid alone (24 μM) had no effect on apoA-I secretion or HL release, relative to control DMSO incubations. PLPC (12 μM), which contains one linoleoyl acyl chain, stimulated a 2-fold increase in apoA-I **(panel A)** and a 1.5-fold increase in HL release/accumulation **(panel B)** at 24 h. DLPC (12 μM), which contains two linoleoyl groups, stimulated a 3-fold increase in apoA-I secretion and 2.2-fold increase in HL mass in the media. Thus, HL release into the media parallels apoA-I secretion, and the extent of both apoA-I secretion and HL accumulation in the media is dependent upon the number of linoleic acid groups in the phospholipid.

Experiments with primary human hepatocytes were undertaken to confirm observations in HepG2 cells. **Figure 2.1**, panel A inset, shows that DLPC was able to induce a 2-fold increase in apoA-I secretion from primary hepatocytes and a 1.75-fold increase in HL release **(Figure 2.1, panel B inset)** at 24 h. LAPL therefore appear able to stimulate apoA-I secretion and HL accumulation to a similar extent in both HepG2 cells and primary human hepatocytes.

Effect of LAPL on cellular HL in HepG2 cells - To determine whether the increase in HL accumulation in the media, after treatment with LAPL, is associated with increased cellular levels of HL, HepG2 cells were treated with DLPC for 24 h and then HL mass was quantified. To compare media to lysate HL levels, equivalent volumes of cell lysate and media ($1/30^{th}$ total volume) were probed on the same gel. **Figure 2.2** shows that treatment of HepG2 cells with DLPC increased media HL levels but had no effect on cellular HL. When cellular HL protein levels were normalized to β-actin, cellular HL mass was shown to be similar between control and treated cells (data not shown). When media and lysate HL mass levels were

Figure 2.1. Linoleic acid phospholipids (LAPL) stimulate apoA-I secretion and HL release from human hepatocytes. HepG2 cells were treated with LA (24μM), PLPC or DLPC (12 μM) for 24h, conditioned media was collected and apoA-I secretion **(panel A)** and HL **(panel B)** release into the media was quantified by immunoblotting. Primary human hepatocytes (PHH) were treated with DLPC (12 μM) for 24h and apoA-I **(inset A)** and HL **(inset B)** levels in the media were quantified. Integrated density values (IDV) from immunoanalysis are expressed as mean ± SD. At least 3 independent experiments were performed with HepG2 cells and 2 independent triplicate experiments were performed with primary human hepatocytes. **P*<0.05 and **P<0.001 vs DMSO.

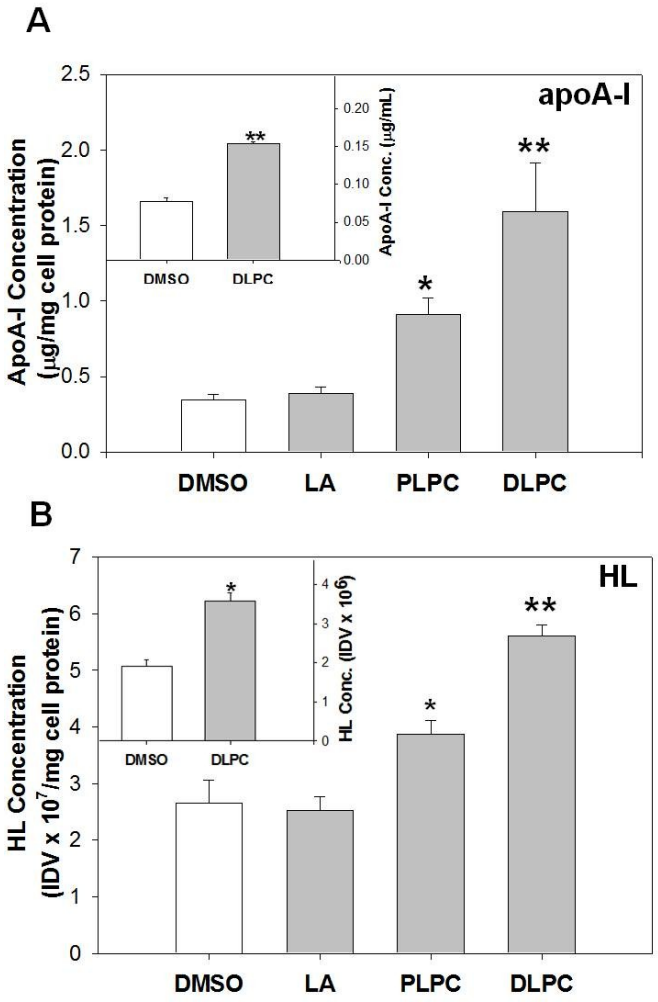
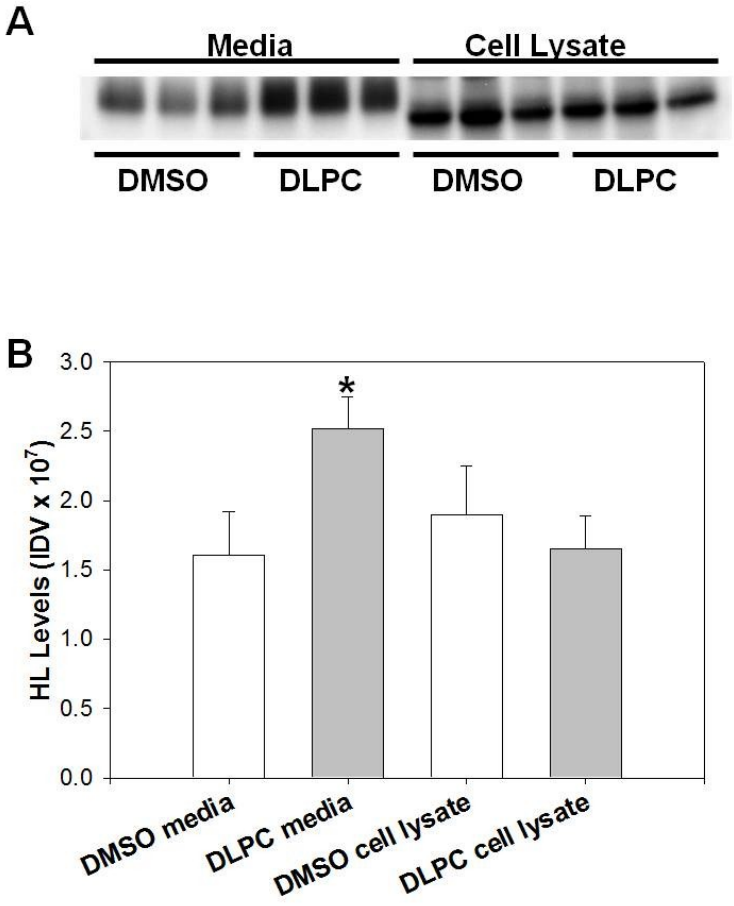

A
apoA-I
ApoA-I Concentration (μg/mg cell protein)
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
LA
PLPC
DLPC
**
*
**
ApoA-I Conc. (μg/mL)
0.20
0.15
0.10
0.05
0.00
DMSO
DLPC
B
HL
HL Concentration (IDV x 10^7/mg cell protein)
7
6
5
4
3
2
1
0
DMSO
LA
PLPC
DLPC
*
*
**
HL Conc. (IDV x 10^6)
4
3
2
1
0
DMSO
DLPC

Figure 2.2. HL cellular protein levels are unaffected by LAPL. HepG2 cells were treated with 12 μM DLPC for 24h. HL mass in equivalent volumes of cell lysate and media ($1/30^{th}$ total volume) were measured by immunoblotting for HL **(panel A)**. Cellular and media HL levels were then quantified and graphically illustrated **(panel B)**. Values are expressed as mean ± SD and are representative of 3 independent experiments. *$P<0.05$ vs DMSO media.


A
Media
Cell Lysate
DMSO
DLPC
DMSO
DLPC
B
HL Levels (IDV x 10^7)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
DMSO media
DLPC media
DMSO cell lysate
DLPC cell lysate

compared, DLPC significantly increased media HL levels, from 60% that of control cellular levels, to ~120% **(Figure 2.2B)**. Experiments were also undertaken to quantify HL levels on the plasma membrane. Cell-surface biotinylation and reisolation experiments were conducted to determine cell-surface HL levels after DLPC treatment. There was no significant difference in plasma membrane HL levels 24h after DLPC treatment compared to control cells **(Supplementary Figure 2.1, panel A)**. Displacement experiments were also performed with heparinase I and heparin to determine the amount of HL that could be released into the media from the HepG2 cell-surface after DLPC treatment. Similar to the cell-surface biotinylation experiments, there was no significant difference in the amount of HL displaced from the DLPC-treated HepG2 cells compared to control cells **(Supplementary Figure 2.1, panel B)**.

Effect of Time and Dose on DLPC-induced apoA-I secretion and HL release - Figure 2.3 shows that apoA-I secretion parallels HL release into the media over the 24 h incubation. A low level of secretion of apoA-I and release of HL was observed prior to the 12 h time point. A similar secretion profile was observed for control incubations. The net accumulation of apoA-I and HL increased between the 12 h and 24 h time points with DLPC treatment. The effect of DLPC concentration on apoA-I and HL was also evaluated. HepG2 cells were treated with 0, 6, 12 and 24 μM DLPC for 24 h and media was probed for apoA-I and HL. Both apoA-I and HL accumulation increased with DLPC dose and reached maximum levels in the media at 12 μM DLPC (data not shown). Treatment of HepG2 cells with DLPC was also shown to increase apoA-II secretion (data not shown).

HL association and activity – Figure 2.4 illustrates densitometry profiles of immunoblots of conditioned DLPC media samples that were electrophoresed on non-denaturing gradient gels

Figure 2.3. Effects of time on DLPC-induced apoA-I and HL release. HepG2 cells were treated with 12 μM DLPC for the indicated time points (4, 8, 12 and 24h). The conditioned media was collected and an ELISA was performed to determine the amount of apoA-I that was secreted **(panel A)**. Immunoblotting was conducted to quantify HL in the media **(panel B)**. Integrated density values (IDV) from immunoanalysis are expressed as mean ± SD. At least 3 independent experiments were performed. **P<0.001 vs DMSO at 24h.

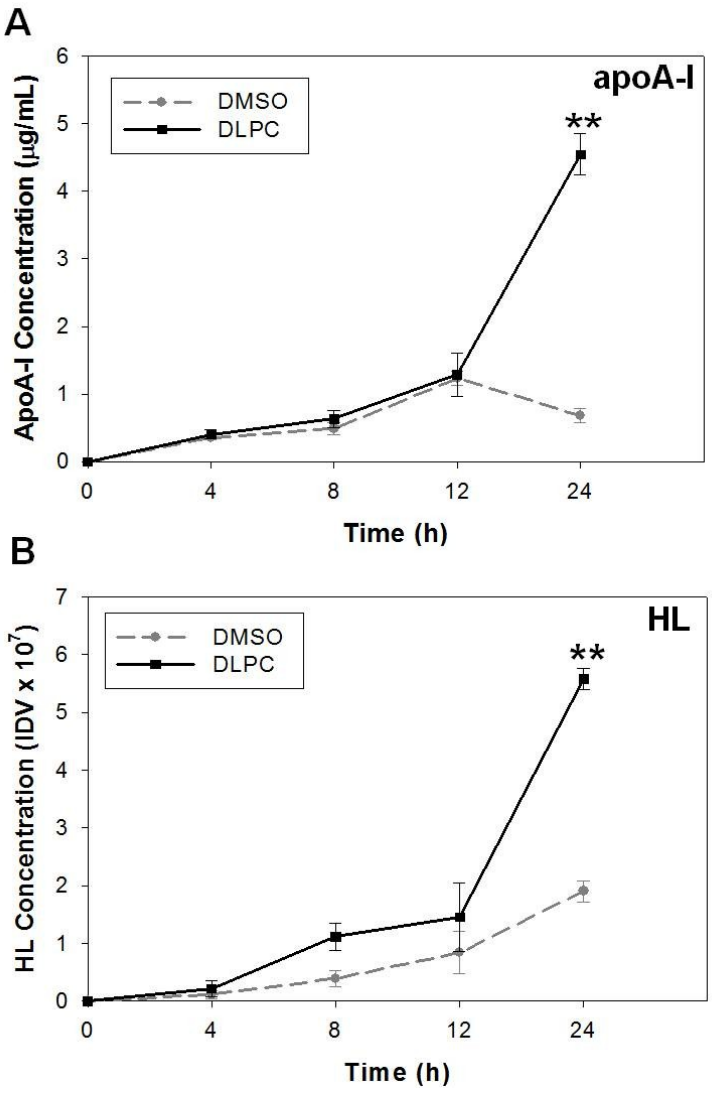
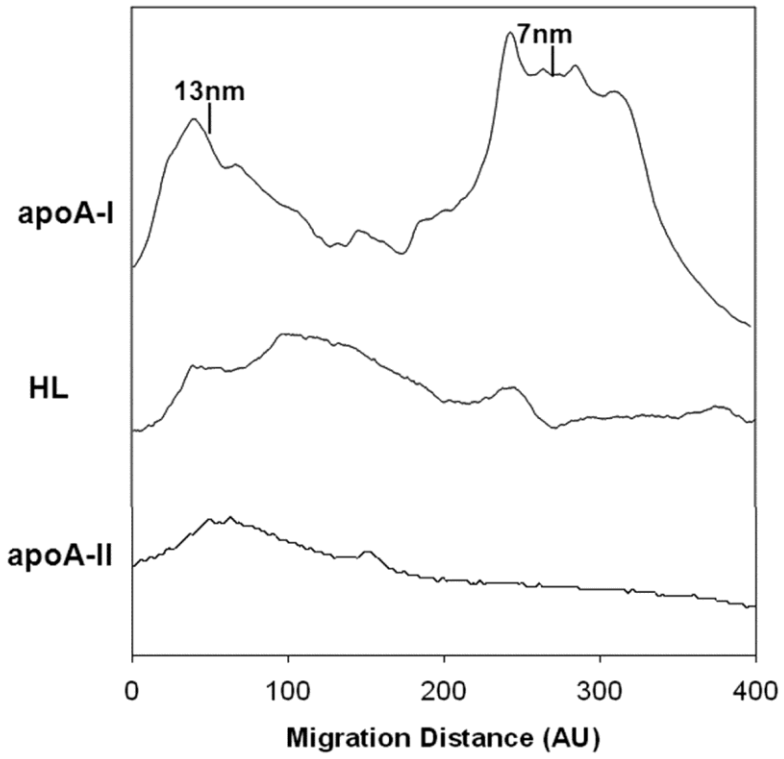

A
apoA-I
DMSO
DLPC
**
ApoA-I Concentration (μg/mL)
0
1
2
3
4
5
6
0
4
8
12
24
Time (h)
B
HL
DMSO
DLPC
**
HL Concentration (IDV x 10^7)
0
1
2
3
4
5
6
7
0
4
8
12
24
Time (h)

Figure 2.4. Association of HL with apoA-I and apoA-II on HDL. Non-denaturing gradient gel electrophoresis was performed on conditioned media from DLPC treated HepG2 cells and immunoblotted for apoA-I, apoA-II and HL. Densitometry profiles show an association of HL with 9-14nm HDL complexes containing both apoA-I and apoA-II. Results are representative of at least 3 independent experiments.


7nm
13nm
apoA-I
HL
apoA-II
0
100
200
300
400
Migration Distance (AU)

and probed for HL, apoA-I and apoA-II. HL is associated with HDL particles containing both apoA-I and apoA-II and ranging in size from 9-14 nm. Previous studies have also shown that HL is primarily associated with an 11 nm HDL complex and that HL and apoA-I non-denaturing gradient profiles are very similar after a 45 min displacement experiment [14]. HL activity measurements in the treated media samples showed that the secreted HDL-associated HL was inactive (data not shown). This is consistent with our earlier work, which showed that while apoA-II stimulates HL displacement [14], it directly inhibits HL activity [15].

Effect of signaling inhibitors on DLPC-induced apoA-I secretion and HL release - LAPL act via the Ras, MAPK, and PPARα signaling pathways to stimulate apoA-I secretion [9;11]. Experiments were conducted to examine the effects of the MEK 1/2 inhibitor, U0126, and the PPARα inhibitor, MK-886, on HL release. **Figure 2.5** shows that similar to apoA-I secretion **(panel A)**, the stimulation of HL release by DLPC **(panel B)** was completely blocked when HepG2 cells were pre-treated with 10μM U0126 for 30 min. Pre-treatment of the HepG2 cells with 10μM of the PPARα inhibitor, MK-886 also inhibited the DLPC-induced release of HL **(Figure 2.5 panel B)**.

Effect of clofibrate and DLPC on HL release - Experiments were undertaken to examine the effects of clofibrate on HL release. **Figure 2.6** shows that clofibrate modestly stimulates the accumulation of apoA-I and HL in HepG2 cell media **(panel A and panel B, respectively)**. Clofibrate however, augments the apoA-I secretion effects of DLPC and significantly increases the effects of DLPC on HL release.

Effect of LA phospholipids on HL RNA levels - Experiments were performed to determine whether the increase in HL release seen in hepatocytes treated with LAPL was related to a

Figure 2.5. MEK1/2 and PPARα inhibition block DLPC-induced apoA-I secretion and HL release. HepG2 cells were pre-treated with 10μM of the MEK 1/2 inhibitor, U0126, or the PPARα inhibitor, MK-886, for 30min prior to addition of 12 μM DLPC for 24h. The conditioned media was collected and an ELISA was performed to determine the amount of apoA-I that was secreted **(panel A)**. Immunoblotting was conducted to determine HL accumulation **(panel B)**. Both apoA-I and HL accumulation results are normalized to total cell protein amounts and shown as fold change relative to DMSO controls. Values are expressed as mean ± SD. At least 3 independent experiments were performed. ***P*<0.001 vs DMSO, #*P*< 0.05, and †*P*<0.001 vs DLPC.

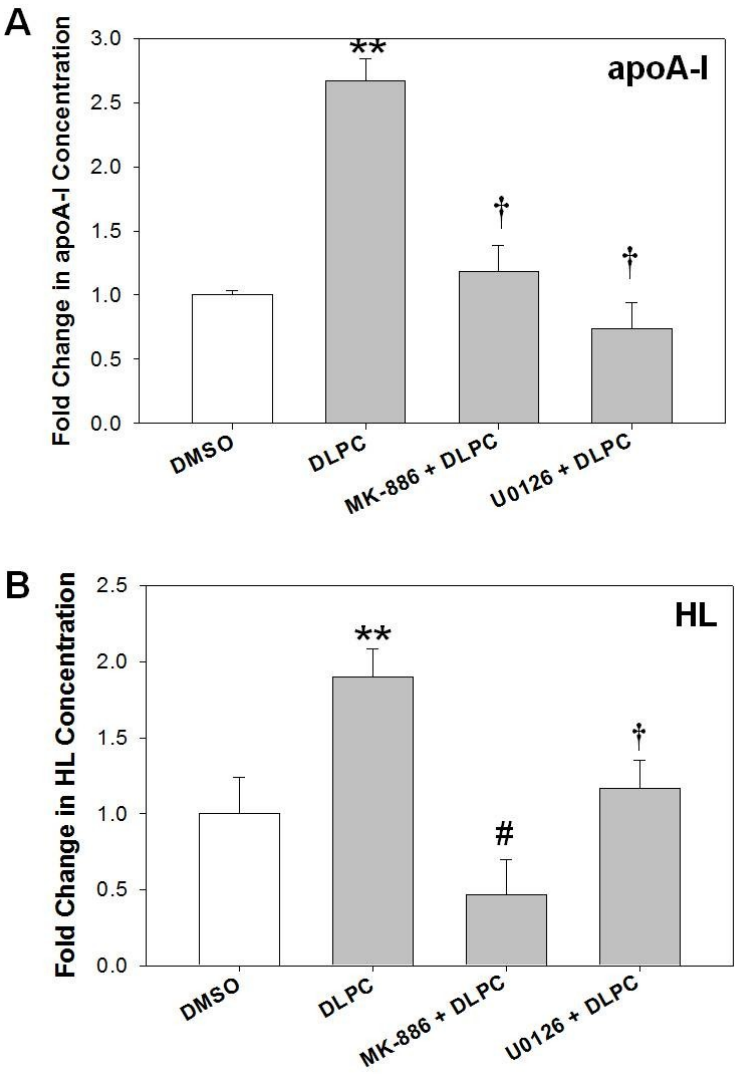
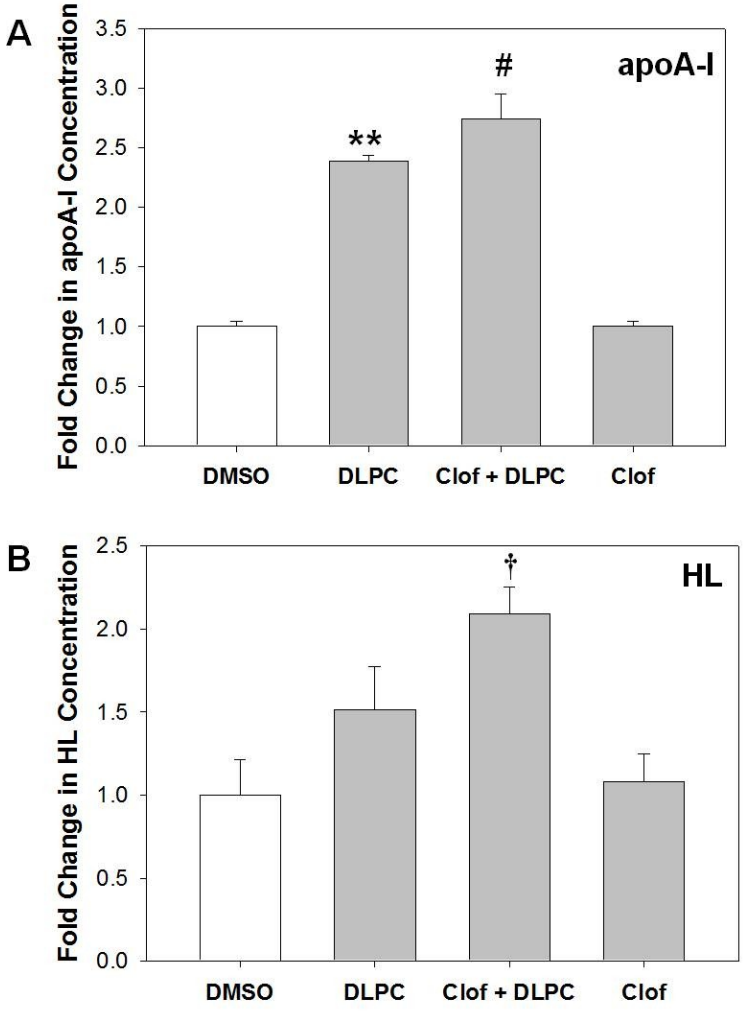

A
apoA-I
Fold Change in apoA-I Concentration
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
†
†
DMSO
DLPC
MK-886 + DLPC
U0126 + DLPC
B
HL
Fold Change in HL Concentration
2.5
2.0
1.5
1.0
0.5
0.0
**
#
†
DMSO
DLPC
MK-886 + DLPC
U0126 + DLPC

Figure 2.6. Clofibrate augments DLPC-induced apoA-I and HL release. HepG2 cells were pre-treated with 10μM of clofibrate for 30min prior to addition of 12 μM DLPC for 24h. The conditioned media was collected and an ELISA was performed to determine the amount of apoA-I that was secreted **(panel A)**. Immunoblotting was conducted to quantify HL in the media **(panel B)**. Both apoA-I and HL accumulation results are normalized to total cell protein amounts and shown as fold change relative to DMSO controls. Values are expressed as mean ± SD. At least 3 independent experiments were performed. ***P*<0.001 vs DMSO, #*P*< 0.05, and †*P*< 0.001 vs DLPC.


A
apoA-I
Fold Change in apoA-I Concentration
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
#
DMSO
DLPC
Clof + DLPC
Clof
B
HL
Fold Change in HL Concentration
2.5
2.0
1.5
1.0
0.5
0.0
†
DMSO
DLPC
Clof + DLPC
Clof

transcriptional stimulation resulting in increased cellular HL mRNA levels. HepG2 cells were treated with DLPC for 24 h and cell lysates were collected for isolation of total RNA. RT-PCR experiments were initially conducted to determine if LAPL treatment affected steady-state HL mRNA levels. **Figure 2.7, panel A**, shows that DLPC treatment did not result in any change in HL mRNA levels by RT-PCR. This result was then confirmed by real-time quantitative PCR, which also showed that HL RNA levels were unaffected by DLPC treatment (normalized to GAPDH), as compared to DMSO controls **(Figure 2.7, panel B)**.

Effect of apoA-I overexpression on HL release– HepG2 cells were transiently transfected with pre-pro-apoA-I to determine whether increased apoA-I secretion was directly associated with increased HL accumulation in the media. **Figure 2.8, panel A**, shows that the transient transfection with apoA-I-pCMV5 construct resulted in a 2.2-fold increase in apoA-I secretion, as compared to pCMV5 alone or non-transfected cells. This increase in apoA-I secretion was associated with a 1.3-fold increase in HL in the cell media **(Figure 2.8, panel B)**. ApoA-I siRNA knockdown experiments showed that a 40% knockdown of apoA-I secretion from HepG2 cells was associated with a 30% decrease in HL accumulation in the media **(Figure 2.9)**. These experiments confirm that apoA-I secretion is linked to the release and accumulation of HL in the HepG2 cell media.

2.5 Discussion

Ramsamy et al. previously showed that apoA-I and HDL can act similar to heparin and liberate HL from cell surface HSPG [2]. Previous reports showed that HSPG-bound HL has reduced lipolytic activity and that its liberation from HSPG by heparin or HDL increases TG hydrolysis [2;5]. Elevated HDL levels would therefore be expected to decrease liver-bound HL, and result in enhanced TG clearance [16]. This heparin-like nature of HDL was shown to be

Figure 2.7. Steady-state HL mRNA levels are unaffected by LAPL. HepG2 cells were treated with 12 μM DLPC for 24h. Total RNA was isolated and RT-PCR was carried out with primers specific for human HL and GAPDH, respectively. The RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide **(panel A)**. The results were then confirmed by real-time quantitative RT-PCR **(panel B)**. Values are expressed as mean ± SD. At least 3 independent experiments were performed.

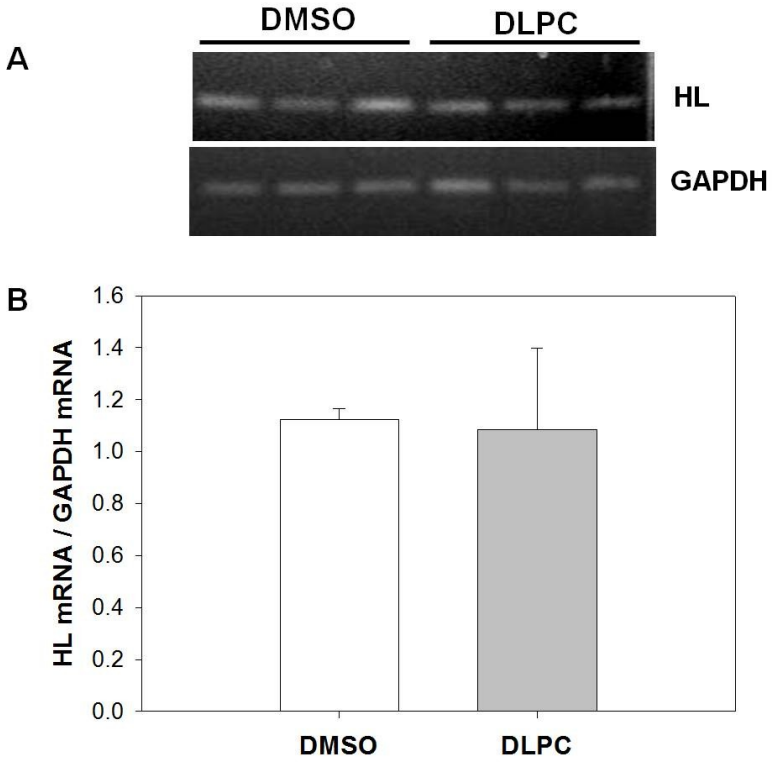

A
DMSO
DLPC
HL
GAPDH
B
HL mRNA / GAPDH mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
DLPC

Figure 2.8. Effect of pre-pro-apoA-I overexpression on HL release. HepG2 cells were transiently transfected with pre-pro-apoA-I-pCMV5 or the control pCMV5 plasmid by reverse transfection. Conditioned media was collected 48h post-transfection and an ELISA was performed to determine the amount of apoA-I that was secreted **(panel A)**. Non-transfected and pCMV5 transfected HepG2 cells secreted ~6-7μg apoA-I /mg cell protein, whereas pre-pro-apoA-I transfected HepG2 cells secreted ~15-17μg apoA-I/mg cell protein. Immunoblotting was conducted to quantify HL in the media **(panel B)**. Both apoA-I and HL quantification results are normalized to total cell protein amounts. Values are expressed as mean ± SD. At least 3 independent experiments were performed. **P*<0.05 and **P<0.001 vs pCMV5.

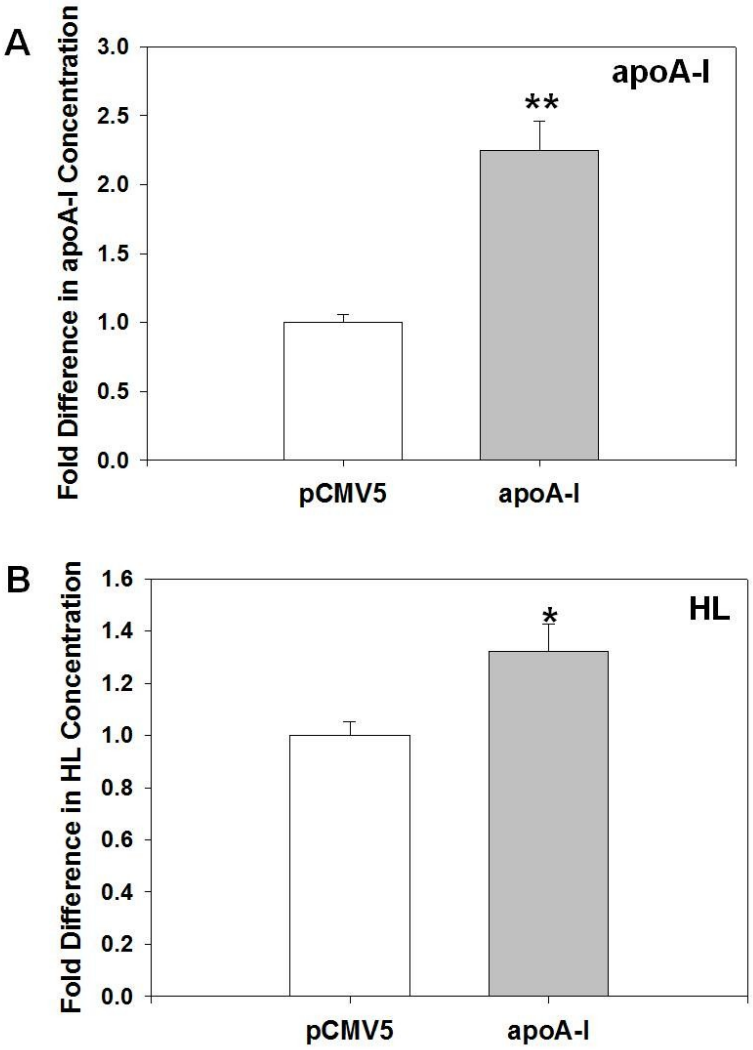
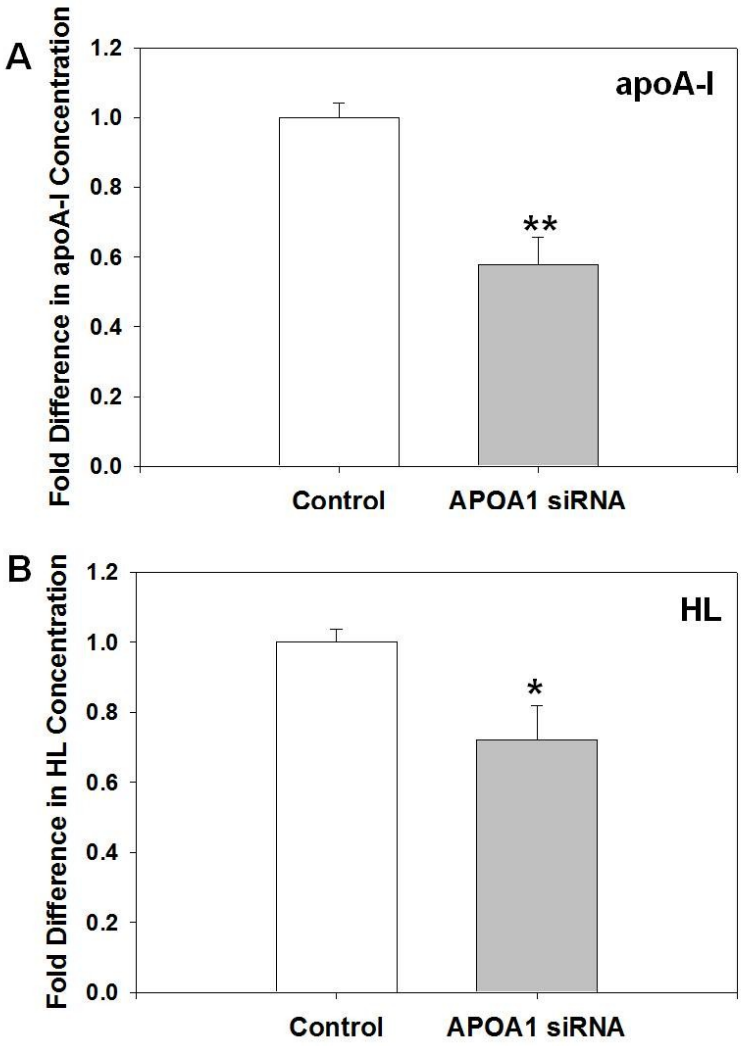

A
Fold Difference in apoA-I Concentration
3.0
2.5
2.0
1.5
1.0
0.5
0.0
apoA-I
**
pCMV5
apoA-I
B
Fold Difference in HL Concentration
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HL
*
pCMV5
apoA-I

Figure 2.9. Effect of siRNA knockdown of apoA-I on HL release. HepG2 cells were transiently transfected with four different siRNA sequences for apoA-I or the control negative siRNA by reverse transfection. Conditioned media was collected 48h post-transfection and an ELISA was performed to determine the amount of apoA-I that was secreted **(panel A)**. Immunoblotting was conducted to quantify HL in the media **(panel B)**. Both apoA-I and HL quantification results are normalized to total cell protein amounts and shown as fold change relative to control negative siRNA and non-transfected cell media. Values are expressed as mean ± SD of the four different siRNA sequences. At least 2 independent experiments were performed averaging the results of the 4 siRNA sequences. **P*<0.05 and **P<0.001 vs control.


A
apoA-I
Fold Difference in apoA-I Concentration
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
APOA1 siRNA
B
HL
Fold Difference in HL Concentration
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
APOA1 siRNA

dependent upon both the lipid and apolipoprotein composition of HDL and has given rise to a novel mechanism by which HDL may impact lipolysis and TG clearance [3;14]. This work has led to a clearer understanding of why low HDL levels, high post-heparin HL activity and elevated plasma TG levels are related [16;17]. It appears that the heparin-like displacement ability of HDL may play a central role in regulating TG clearance. Cell culture studies showed that HDL, when added to the cell media, will displace cell surface HL and liberate the enzyme into the media [3]. As such, it would be expected that an accumulation of newly synthesized HDL in the media would have a similar effect on HSPG-bound lipases. This view has now been confirmed. Experiments show that LAPL are able to stimulate both HDL secretion and HL release from hepatocytes. Factors that stimulate HDL accumulation in the cell media, also stimulate HL accumulation. HL accumulation therefore appears to be a product of HDL-dependent release from the cell membrane. The displacement process appears due to protein-protein interactions and related to the apolipoprotein composition of HDL [14;15]. ApoA-I can directly displace HL by binding to the lipase [14] and apoA-II can enhance HL displacement by increasing the association of HL with HDL [14;15].

HL release and HDL secretion are sensitive to LAPL and incubation time **(Figure 2.3)**. DLPC treatment over a 24 h period showed two different phases of protein secretion. During the first 12 h period, a low level of steady accumulation was observed for both apoA-I and HL in the media. After the first 12 h, however, there was a marked increase in both apoA-I and HL accumulation in the media. This high-output accumulation of HL could be attributed to an increased protein synthesis or decreased degradation of cellular HL. Since analyses showed no change in cellular HL mRNA levels and total HL protein expression after DLPC treatment **(Figures 2.7 and Figure 2.2B)**, HL synthetic pathways did not appear to be have

been upregulated. Increased media HL mass may instead be related to an increased displacement of cell surface HL and a decrease in the cellular recycling and degradation of the enzyme. Both cell-surface biotinylation and displacement experiments conducted 24h after DLPC treatment showed that cell-surface HL levels are unaffected by DLPC treatment **(Supplementary Figure 2.1)**. Cell surface HL levels therefore also remain somewhat constant. This suggests that hepatocytes are able to maintain membrane HL levels, by quickly replenishing any cell-surface HL lost to the media.

HL release appears the result of the accumulation of extracellular HDL, which in turn acts similar to heparin to displace HL from the HSPG of the hepatocyte cell surface [2]. The direct effect of apoA-I on HL release into the media was confirmed by both overexpression of pre-pro-apoA-I and siRNA knockdown of apoA-I in HepG2 cells. As with LAPL studies, a stimulation in apoA-I secretion was shown to be directly related to the accumulation of HL in the cell media **(Figure 2.8)**. Accumulation of the overexpressed apoA-I appears to have the same effect at liberating cell surface HL, as adding exogenous apoA-I or HDL to the media [2;5]. ApoA-I siRNA knockdown studies showed the opposite effect. A knockdown of apoA-I secretion resulted in a decrease in HL release into the media **(Figure 2.9)**.

DLPC also increased secretion of another apolipoprotein, apoA-II (data not shown) and our previous work has shown that apoA-II directly stimulates HL displacement [14]. ApoA-II has also been shown to inhibit HL activity [15], which may explain why the HL in HepG2 cell media was shown to be inactive (data not shown). HL is associated with large HDL complex containing both apoA-I and apoA-II **(Figure 2.4)**. The average size of the complex, 11 nm, may indicate an HL-HDL stoichiometric composition of a dimer of HL and a 7-8 nm HDL particle. This is similar to that reported by Rouhani et al., where HL and apoA-I were also

shown to be associated with a 11 nm complex [14]. Since HL is associated with an apoA-II–enriched HDL, this interaction would be expected to result in a catalytically inactive enzyme. HL in pre-heparin plasma has also been shown to be catalytically inactive [2;3] and associated with HDL (Young et al., unpublished observation). HDL inactivation of circulating HL may be important to preventing adverse membrane lytic events from the enzyme phospholipase activity. HL may then be activated in the vascular compartment by factors that reduce the association of HL with HDL. We have shown that HL activity is regulated by HDL charge and activated by anionic lipids, such as free fatty acids, which promote the release of HL from HDL [18].

Numerous studies have shown linkage between the cellular metabolic pathways thought to regulate HDL and TG metabolism [16;17;19;20]. Drugs that have been shown to affect plasma HDL levels are also known to affect TG levels [21;22]. Both niacin and the fibric acid derivatives (fibrates) are examples of drugs that affect plasma HDL and TG. The fibrate drugs act through PPARα to impact HDL through a transcriptional regulation of the apoA-I gene and TG metabolism by inhibiting TG synthesis and stimulating TG clearance [22;23]. PPARα agonists have been shown to enhance TG clearance by increasing plasma TG hydrolytic activity [24;25]. They stimulate lipoprotein lipase (LPL) expression [26] and inhibit apoC-III secretion [27-29]. LAPL appear to act similar to the fibrate PPARα agonists, and stimulate HDL secretion and HL release, in a PPARα dependent fashion. Clofibrate has a small effect on HL release but appears to act synergistically with DLPC to enhance HL release from HepG2 cells **(Figure 2.6B)**. Niacin treatment is believed to raise HDL levels without impacting apoA-I gene transcription [30]. LAPL may act similarly, as DLPC does not appear to affect cellular apoA-I and HL mRNA levels. It therefore seems that therapeutic compounds that increase

hepatic HDL secretion and plasma HDL levels would be expected to promote the simultaneous release of HL.

A stimulation of HDL secretion therefore appears to impact plasma TG metabolism by promoting the release of cell surface bound lipases. LAPL are more effective than fibrates at stimulating hepatic HDL secretion [9] and therefore would be expected to have a greater impact on plasma TG levels. Soy phosphatidylinositol is enriched in linoleic acid, much like PLPC, and oral administration of the lipid to healthy normal subjects for 2 weeks caused an almost 40% reduction in plasma TG levels [31]. LAPL therefore appear to have value as both HDL and TG regulatory therapeutics.

2.6 Acknowledgements

We appreciate the gift of the apoA-I plasmid construct from Dr. Mary Sorci-Thomas (Wake Forest University). These studies were supported by a grant from the Heart and Stroke Foundation of Ontario, T5593 (D.L.S.).

2.7 Supplementary Information

Supporting Information Available

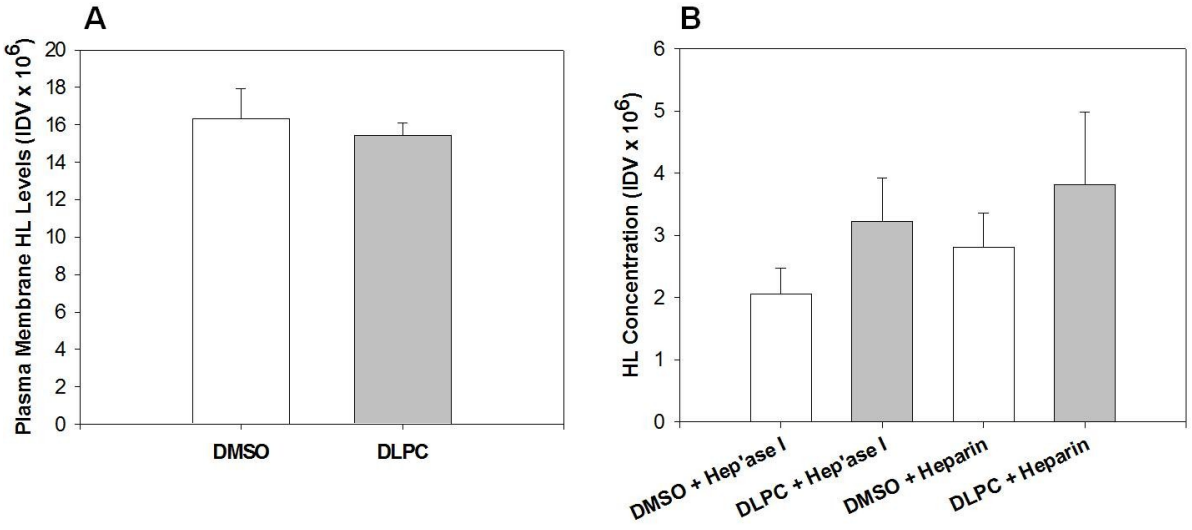
Plasma membrane HL levels after DLPC treatment is shown in Supplementary Figure 1, panel A and B. This material is available free of charge via the Internet at http://pubs.acs.org.

Biotinylation and Isolation of Membrane HL - Plasma membrane proteins were biotinylated and isolated using a Cell Surface Labeling Accessory Pack (Pierce Chemical, Rockford, IL, USA) according to manufacturer's protocol. Briefly, cell monolayers were biotinylated with EZlink-sulfo-NHS-LC-biotin at 4°C for 30 min with gentle agitation. The cells were harvested after addition of Quenching solution and then washed 3 times with TBS using centrifugation at 500 x g for 3 minutes each. Cells were then lysed using lysis buffer

supplemented with protease inhibitors, followed by low power sonication and centrifugation to disrupt the cells and were incubated on ice for 30 min. Cleared cell lysates were obtained for each sample as supernatant by centrifugation at 10,000 x g for 2 min. at 4°C. Columns packed with Immobilized NeutrAvidin Gel were used to isolate labeled proteins. Finally SDS-PAGE sample buffer supplemented with 50 mM DTT was used to elute labeled proteins by centrifugation at 1,000 x g for 2 min. Protein assay was performed by BCA method with minor modifications. Samples were first treated with sodium deoxycholate and followed TCA precipitation to nullify the effect of DTT. An equal amount of proteins were then electrophoresed and immunoblotted for HL as described previously.

Heparinase and Heparin Displacement Studies – Treatment media was removed from HepG2 cells 24h after treatment with DLPC and cells were washed twice with PBS. Either 3U/mL heparinase I or 150IU/mL of heparin in serum-free DMEM were then added to the cells and incubated for 2 h. The media was collected and the cells harvested as described in HL immunoanalysis.

Figure S2.1. HepG2 cell surface HL levels remain unchanged after DLPC treatment. HepG2 cells were treated with 12 μM DLPC for 24h at which point cell-surface biotinylation was conducted or displacement experiments were performed using heparinase I or heparin. Immunoblotting of the isolated membrane proteins from cell-surface biotinylation experiments was conducted to determine cell surface HL levels **(panel A)**. The conditioned media from the displacement studies was collected and immunoblotted to determine the amount of HL released **(panel B)**. Integrated density values (IDV) from immunoanalysis are expressed as mean ± SD. At least 3 independent experiments were performed.


A
Plasma Membrane HL Levels (IDV x 10^6)
20
18
16
14
12
10
8
6
4
2
0
DMSO
DLPC
B
HL Concentration (IDV x 10^6)
6
5
4
3
2
1
0
DMSO + Hep'ase I
DLPC + Hep'ase I
DMSO + Heparin
DLPC + Heparin

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CHAPTER 3-MANUSCRIPT#3

Extracellular nucleotides inhibit insulin receptor signaling, stimulate autophagy and control lipoprotein secretion

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3.1 Abstract

Hyperglycemia is associated with abnormal plasma lipoprotein metabolism and with an elevation in circulating nucleotide levels. We evaluated how extracellular nucleotides may act to perturb hepatic lipoprotein secretion. Adenosine diphosphate (ADP) (>10 μM) acts like a proteasomal inhibitor to stimulate apoB100 secretion and inhibit apoA-I secretion from human liver cells at 4h and 24h. ADP blocks apoA-I secretion by stimulating autophagy. The nucleotide increases cellular levels of the autophagosome marker, LC3-II, and increases co-localization of LC3 with apoA-I in punctate autophagosomes. ADP affects autophagy and apoA-I secretion through $P2Y_{13}$. Overexpression of $P2Y_{13}$ increases cellular LC3-II levels by ~50% and blocks induction of apoA-I secretion. Conversely, a siRNA-induced reduction in $P2Y_{13}$ protein expression of 50% causes a similar reduction in cellular LC3-II levels and a 3-fold stimulation in apoA-I secretion. $P2Y_{13}$ gene silencing blocks the effects of ADP on autophagy and apoA-I secretion. A reduction in $P2Y_{13}$ expression suppresses ERK1/2 phosphorylation, increases the phosphorylation of IR-β and protein kinase B (Akt) >3-fold, and blocks the inhibition of Akt phosphorylation by TNFα and ADP. Conversely, increasing $P2Y_{13}$ expression significantly inhibits insulin-induced phosphorylation of insulin receptor (IR-β) and Akt, similar to that observed after treatment with ADP. Nucleotides therefore act through $P2Y_{13}$, ERK1/2 and insulin receptor signaling to stimulate autophagy and affect hepatic lipoprotein secretion.

3.2 Introduction

Chronic hyperglycemia in insulin resistance is known to increase the risk of cardiovascular disease and to be associated with elevated plasma apoB100 and low HDL levels [1;2]. Elevated blood glucose is also known to stimulate nucleotide secretion and

purinergic signaling [3;4]. Under stress or injury, blood and vascular cells release nucleotides, such as ATP and ADP [5;6]. Extracellular nucleotide concentration in the bloodstream is normally in the nM-μM range [7;8], but can increase significantly in disease states [5;9;10]. Purinergic signaling events stimulate mitogen-activated protein kinase (MAPK) pathways and trigger the release of pro-inflammatory cytokines [6;11;12]. Extracellular nucleotides thereby directly impact the development of cardiovascular disease by promoting an "injury response" in circulating blood cells and vascular tissues [11-13].

Extracellular nucleotides affect hepatic lipoprotein metabolism through membrane G-protein coupled receptors (GPCR) [14;15]. Compounds that stimulate HDL secretion from the liver appear to act through an inhibition of nucleotide signaling. Niacin has been shown to act through GPCR pathways to stimulate the secretion of HDL [16;17] and niacin is thought to inhibit the cellular degradation of apoA-I through an inhibition of nucleotide signaling [18]. We have shown that linoleic acid phospholipids (i.e. DLPC) also act through nucleotide signaling pathways to stimulate HDL secretion [19]. These phospholipids uniquely affect MAPK and protein kinase B (Akt) signaling [20] to block apoA-I degradation in liver cells [21].

Factors that stimulate or inhibit HDL secretion from the liver appear to have the opposite effect on the secretion of the LDL protein, apoB100. ApoB100 secretion from liver cells is regulated by protein folding and proteasomal degradation [22;23] and proteasomal inhibitors are known to stimulate the secretion of apoB100 [23]. Proteasomal inhibitors also stimulate cellular autophagic pathways [24;25]. Autophagy is an adaptive cellular "stress response" that promotes the lysosomal degradation of cytosolic components when a cell is stimulated by stressors, i.e. nutrient deprivation, extracellular signals, hormones, cytokines and pathogens [26;27]. Autophagy is designed to protect the cell by eliminating harmful cellular

components through catabolism and recycling. Nucleotides act much like proteasomal inhibitors to stimulate apoB100 secretion and autophagy. The nucleotide, adenosine diphosphate (ADP), significantly increases apoB100 secretion from liver cells and increases the levels of the autophagy marker, microtubule-associated protein 1 light chain 3 (LC3-II). Autophagy has been shown to be associated with cardiovascular disease and studies suggest that excessive autophagy can lead to cardiac hypertrophy and heart failure [28;29]. Pharmacological intervention to regulate cellular autophagy may therefore have therapeutic value in the treatment of cardiovascular disease.

This study shows that ADP acts through the specific GPCR, $P2Y_{13}$, to stimulate autophagy and block HDL secretion. While stimulation in purinergic signaling would be expected to affect cellular autophagy through MAPK pathways [26;30], we now show that ADP also acts through $P2Y_{13}$ to block insulin receptor (IR-β) signaling and prevent the activation of Akt. The inhibition of insulin signaling pathways and Akt phosphorylation are known to stimulate autophagy [26;27]. ADP therefore stimulates autophagy and inhibits HDL secretion by both a stimulation of MAPK and inhibition of Akt. The study suggests that elevations in circulating nucleotide levels in hyperglycemic states may affect hepatic lipoprotein secretion through a stimulation in purinergic signaling and a coordinated regulation of both proteasomal and autophagic protein degradation.

3.3 Materials and Methods

Reagents: Dilinoleoylphosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Adenosine 5'-diphosphate sodium salt (ADP), adenosine triphosphate (ATP), chloroquine diphosphate salt as well as the PI3 kinase inhibitors, 3-methyladenine (3-MA) and Wortmannin were purchased from Sigma-Aldrich (Oakville, ON). The antibody to

$P2Y_{13}$ was obtained from Abcam (Cambridge, MA). The LC3 polyclonal antibody was purchased from MBL International (Woburn, MA). Antibodies to phosphorylated ERK1/2 (p44/p42), phosphorylated Akt (Ser473), phosphorylated mTOR (Ser2448), phosphorylated IR-β (Tyr1345) and β-actin, as well as the mTOR inhibitor, rapamycin, were all obtained from Cell Signaling Technology (Danvers, MA). Human TNFα was purchased from Calbiochem (San Diego, CA). The monoclonal antibody to human apoA-I was purchased from Meridian Life Sciences, Inc (Saco, ME). The antibody to apoB (1D1) was obtained from Dr. Milne and Dr. Marcel (University of Ottawa Heart Institute). Affinity purified peroxidase linked goat anti-mouse and anti-rabbit antibodies were purchased from GE Healthcare (UK). All Stars Negative control small interference RNA (siRNA) were purchased from Qiagen (Mississauga, ON) and human $P2Y_{13}$ siRNA were purchased from Thermo Scientific Dharmacon (Lafayette, CO). Human $P2Y_{13}$ plasmid was purchased from Origene (Rockville, MD). Inhibitors were of analytical grade and were solubilized in dimethyl sulfoxide (DMSO).

Cells and Cell Culture: Human hepatocarcinoma, HepG2, cells were regularly maintained in Dulbecco's modified Eagle medium (DMEM) (5g/L glucose) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Passages 4-10 were used and cells that were 80% confluent were treated with DLPC, nucleotides and/or inhibitors for the indicated times and concentrations under serum-free conditions. Cell viability was evaluated after all treatment conditions.

Preparation of DLPC Micelles: DLPC micelles were prepared in DMSO by sonication as previously described [20]. Purity of all phospholipids was >99%.

Knockdown of Human $P2Y_{13}$ by Small Interference RNA: HepG2 cells were transiently transfected with All Stars Negative control siRNA from Qiagen (Mississauga, ON) or two different $P2Y_{13}$ siRNA sequences (ACCUUCAUCAUCUACCUCAAAUU or GACACUCAUGCUUCCUUUCAAUU) from Thermo Scientific Dharmacon (Lafayette, CO), by reverse transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 12-well plates. In brief, complexes were prepared per manufacturer's specifications with a Lipofectamine 2000-to-siRNA volume-to-mole ratio of 2:40 (μL:ρmol) in 200μL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). Lipofectamine-siRNA complexes were added to the cells immediately after the cells were seeded at a density of 500,000 cells/well in a volume of 1mL of normal growth media containing 10% FBS in the absence of penicillin/streptomycin . The cells were treated with ADP, TNFα or DLPC in serum-free DMEM 48h after transfection. Cell media and lysate samples were harvested at the indicated timepoints for both immunoblot and ELISA analysis. Transfection of the control and test siRNA caused no cytotoxic effects.

Overexpression of Human $P2Y_{13}$ by Plasmid: The pCMV6 vector containing the full-length human $P2Y_{13}$ cDNA was purchased from Origene (Rockville, MD). HepG2 cells were transiently transfected with control plasmid or the pCMV6-$P2Y_{13}$ plasmid by reverse transfection using FuGENE HD (Roche Applied Science, Laval, QC). Complexes were prepared per manufacturer's instructions with a FuGENE HD-to-DNA volume-to-mass ratio of 6:2 (μl to μg) in 100μL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). HepG2 cells were trypsinized and seeded in 12-well plates at a density of 500,000 cells/well in a volume of 1mL in normal growth media containing 10% FBS in the absence of penicillin/streptomycin and then 50μL of the transfection complexes were immediately added

to the suspended cells. The cells were treated with ADP, TNFα or DLPC in serum-free DMEM 48h after transfection. Cell media and lysate samples were harvested at the indicated timepoints for both immunoblot and ELISA analysis. Transfection of the control and test plasmid caused no cytotoxic effects.

ApoA-I ELISA: ApoA-I concentration in conditioned media and cell lysate samples were analyzed by ELISA according to manufacturer's instructions as previously described [20]. 96-well plates were coated overnight with a mouse anti-human apoA-I monoclonal antibody (Meridian Life Sciences, Inc, Saco, ME). Wells were blocked with BSA and then samples/standards were incubated in the wells for 2h, followed by a 1h incubation with a horseradish peroxidase-linked goat anti-human apoA-I antibody. K-blue Max TMB substrate (Neogen, Inc) was added to each well, the reaction was stopped with 0.2N HCl, and the absorbance was recorded at 450 nm. ApoA-I concentration in the conditioned media and cell lysate samples were normalized to total cell protein.

Immunoblot Analysis: After treatment for the indicated timepoints, cells were washed twice with ice-cold PBS. Cells were lysed in NP-40 lysis buffer (Biosource, Camarillo, CA) supplemented with 1mM PMSF and 1X protease inhibitor cocktail (Sigma, Saint Louis, MO). Cell protein concentrations were determined by the BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA). Cell lysate samples containing equal total protein (30μg) were separated by SDS-PAGE and analyzed by Western blot using specific antibodies to apoA-I, apoB100, $P2Y_{13}$, LC3, p62, p-ERK1/2, p-Akt, p-mTOR, p-IR-β, and β-actin. Blots were exposed using the Alpha Innotech FluorChem™ HD Imager and band intensities were quantified with the Alpha Ease FC™ software.

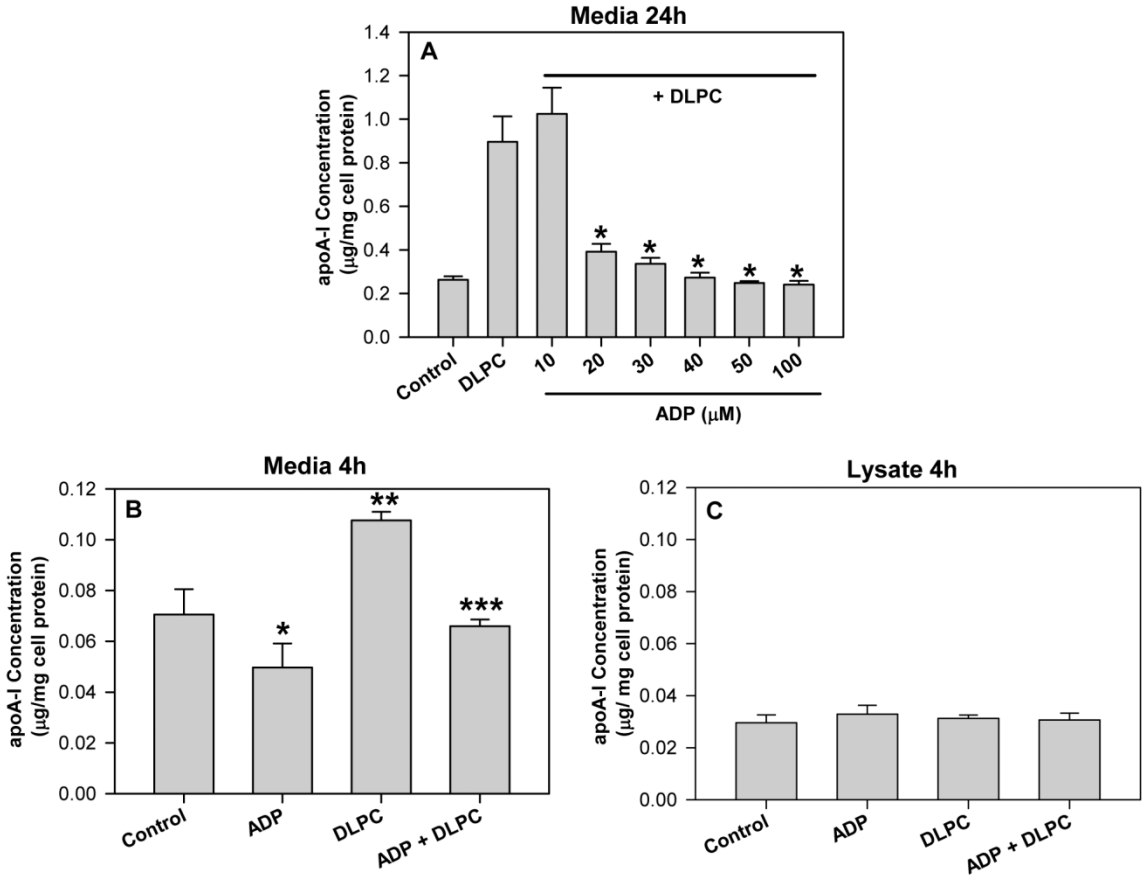
Immunofluorescence and Colocalization: After treatment, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then blocked in 10% FBS for 30 min. The cells were then immunostained with 1:200 rabbit polyclonal anti-LC3 (MBL International, Woburn, MA) and 1:200 mouse anti-apoA-I antibodies (Meridian Life Sciences, Saco, ME) for 1h and then with 1:1000 Alexa Fluor 488 goat anti-rabbit IgG (H+L) and 1:1000 Alexa Fluor 647 donkey anti-mouse IgG (H+L) (Invitrogen, Burlington, ON) for an additional 1h. Images were acquired on an Olympus 1X80FV1000 confocal laser microscope using Olympus Fluoview FV1000 software and colocalization was quantified using FV10-ASW V2.1.

Statistical Analysis: Values are shown as Mean ± SD for at least 3 independent experiments and $P<0.05$ was considered significant. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) on ranks by a pairwise multiple comparison using the Student-Newman-Keuls post-hoc test (SigmaStat; Systat Software, Inc., San Jose, CA).

3.4 Results

Extracellular nucleotides inhibit hepatic apoA-I secretion: Previous work has shown that membrane ATPases control the nucleotide-dependent endocytosis of HDL [14;31] and that inhibition of ADP production by membrane ATPases may promote HDL secretion [18;19]. To determine if ADP directly affects hepatic HDL secretion, experiments were undertaken to evaluate the effect of ADP on apoA-I secretion from liver cells. As we have previously shown [19], dilinoleoylphosphatidylcholine (DLPC) (12 μM) stimulates a ~4-fold increase in apoA-I secretion (accumulation in the media) from HepG2 cells over 24h (**Figure 3.1A**). Pre-treatment of the cells with 20-100 μM ADP completely blocked the induction of apoA-I secretion by DLPC (**Figure 3.1A**). Treatment with ATP (100 μM) had a lesser effect than

Figure 3.1. Extracellular nucleotides block the induction of apoA-I secretion. HepG2 cells were pre-treated with adenosine diphosphate (ADP) (10 to 100 μM)) for 30 min. and then incubated with 12 μM DLPC in serum-free DMEM media. **(A)** Conditioned media was collected after 24h and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs DLPC. **(B)** Conditioned media was collected after 4h treatment with 100μM ADP +/- DLPC and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.01 vs ADP, **P<0.001 vs Control, ***P<0.001 vs DLPC. **(C)** Cell lysates were collected after 4h of treatment and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the cell lysate is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments.


Media 24h
A
+ DLPC
apoA-I Concentration (μg/mg cell protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
DLPC
10
20
30
40
50
100
ADP (μM)
Media 4h
B
apoA-I Concentration (μg/mg cell protein)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control
ADP
DLPC
ADP + DLPC
Lysate 4h
C
apoA-I Concentration (μg/ mg cell protein)

ADP and only blocked the induction of apoA-I secretion by 60% (**Figure S3.1**). The effects of DLPC and ADP on apoA-I secretion were also evident after short time periods. ADP (100 μM) was able to significantly reduce basal apoA-I secretion at 4h (**Figures 3.1B and 3.2C**). DLPC stimulated the secretion of apoA-I after 4h and ADP completely blocked this DLPC-induction of apoA-I secretion. Lower doses of ADP (25 and 50 μM) also inhibited apoA-I secretion at 4h (not shown). ADP treatment had no effect on cellular apoA-I levels after 4h (**Figure 3.1C)** or 24h (not shown). ADP therefore significantly reduced apoA-I mass (media + lysate) at 4h by 20% (ADP alone) to 30% (ADP+DLPC).

Adenosine diphosphate stimulates hepatic apoB100 and apoE secretion: Experiments were undertaken to determine if ADP might affect the secretion of other apolipoproteins. In contrast to that observed with apoA-I, ADP significantly increased apoB100 secretion (**Figure 3.2A**) and apoE secretion (**Figure 3.2B**) from HepG2 cells after 24h. ApoB100 secretion is regulated by proteasomal degradative pathways [22;23;32] and therefore the effect of ADP on apolipoprotein secretion was compared to that of proteasomal inhibitors. ADP and ALLN (25 μM) decreased apoA-I levels in the media to a similar extent at 4h (**Figure 3.2C**), but increased the concentration of apoB100 in the media (**Figure 3.2D**). The work suggests that ADP may regulate apolipoprotein secretion in a similar fashion to that observed with proteasomal inhibitors.

Adenosine diphosphate stimulates autophagy: Studies have shown that proteasomal inhibitors also act to stimulate autophagy [24;25] and therefore experiments were undertaken to determine if ADP affects autophagy and impacts the level of the autophagy marker, LC3. HepG2 cells were treated with 100 μM ADP for 4h and then cell lysates were probed for LC3.

Figure 3.2. ADP stimulates apoB100 and apoE secretion. (A&B) HepG2 cells were incubated with 100 μM adenosine diphosphate (ADP) for 24h in serum-free DMEM media. Conditioned media was collected and immunoblotted for apoB100 **(A)** and apoE **(B)**. Histograms represent band densitometry analysis of apoB100 or apoE, normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.01 vs Control. **(C&D)** HepG2 cells were treated with 100 μM adenosine diphosphate (ADP) or 25 μM ALLN (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal) for 4h in serum-free DMEM media. **(C)** Conditioned media was collected and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs Control. **(D)** ApoB100 concentration in the media was determined by Western blot and histograms represent band densitometry analysis of apoB100, normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs Control.

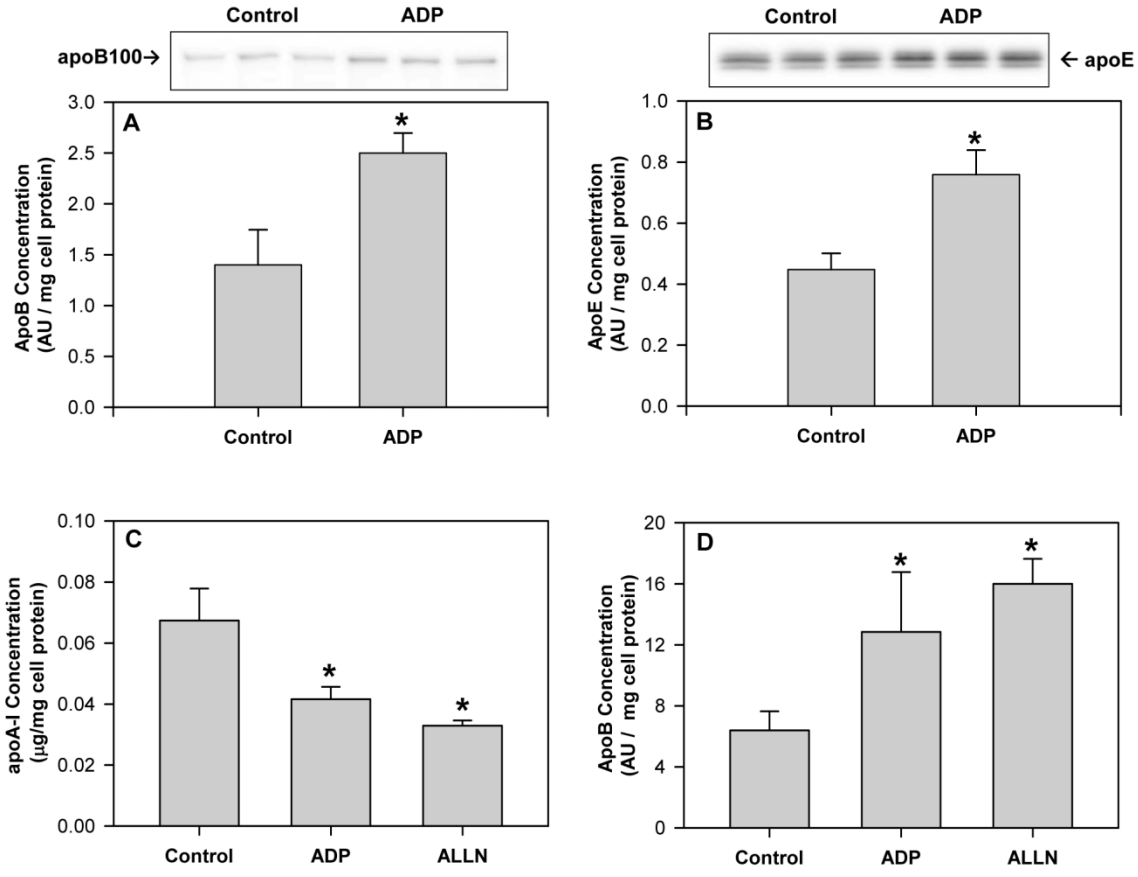
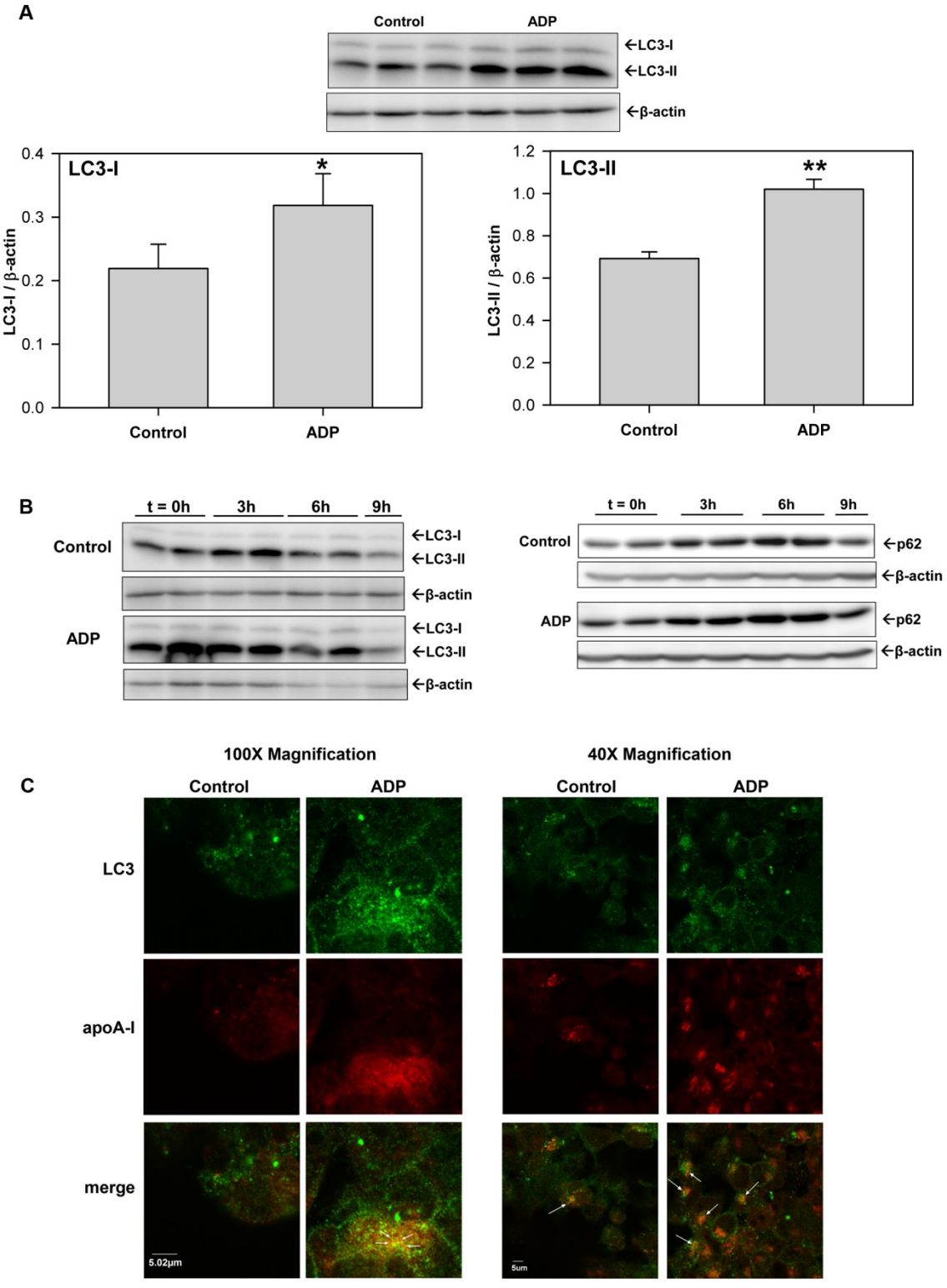

Control
ADP
apoB100→
A
ApoB Concentration (AU / mg cell protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
ADP
Control
ADP
← apoE
B
ApoE Concentration (AU / mg cell protein)
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
ADP
C
apoA-I Concentration (µg/mg cell protein)
0.10
0.08
0.06
0.04
0.02
0.00
*
*
Control
ADP
ALLN
D
ApoB Concentration (AU / mg cell protein)
20
16
12
8
4
0
*
*
Control
ADP
ALLN

Figure 3.3A shows that ADP significantly increased both LC3-I and LC3-II levels at 4h. The data suggests that ADP stimulates autophagy and this view is confirmed by the data in **Figure 3.3B**. To determine if ADP uniquely affects the activation versus flux of LC3 in HepG2 cells, 9h time course studies were undertaken. **Figure 3.3B** shows that serum starvation (**control**) increases LC3-II levels in HepG2 cell lysates at 3h, which return to below basal levels by 9h (**left panel blots**). ADP increases LC3-II levels more quickly than the control starvation (within the 30 min pretreatment) and maintains increased cellular LC3-II levels for 6h (**Figure 3.3B, left panel blots**). Similar results were seen with another autophagy marker protein, p62 (**Figure 3.3B, right panel blots**).

Immunofluorescent experiments using laser confocal microscopy confirm the Western blot data and further showed that apoA-I and LC3 are colocalized within autophagosomes. **Figure 3.3C** (**and Figure S3.2**) illustrates 4h confocal micrographic images of indirect immunofluorescent stained apoA-I and LC3 in fixed and permeabilized HepG2 cells. Control starvation conditions show the staining pattern for both LC3 (green) and apoA-I (red) and illustrate low levels of colocalization of the two proteins in the merged images (yellow-orange). Similar to that shown in **Figures 3.3A&B**, ADP increases cellular LC3 levels relative to control, and confocal images show LC3 to be localized to punctate autophagosomal structures within the cell (**Figure 3.3C**). When LC3 and apoA-I immunofluorescent images for ADP treated cells are merged, the images show significantly ($P<0.001$) more yellow-orange structures in ADP-treated vs control cells (23.8% $\pm$ 5.2% vs 7.5% $\pm$ 3.0%), which indicates that ADP significantly increases the colocalization of apoA-I with LC3. ADP-dependent increases in LC3-II levels were completely inhibited by treatment with 3-methyladenine (3-MA) (5 mM) or U0126 (10 μM), while LC3-II levels were further increased

Figure 3.3 ADP stimulates autophagy and increases cellular LC3-II. (A) HepG2 cells were treated with 100 μM adenosine diphosphate (ADP) for 4h in serum-free DMEM media. Cell lysates were immunoblotted for LC3. Histograms represent band densitometry analysis of LC3-I and LC3-II, normalized to β-actin and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs Control, **P<0.001 vs Control. **(B)** HepG2 cells were serum-starved (Control) or pretreated for 30 min. with 100 μM adenosine diphosphate (ADP) in serum-free DMEM media and then lysates were harvested at the indicated timepoints (0, 3, 6 & 9h). **(Left panels)** Cell lysates were immunoblotted for LC3 and β-actin. **(Right panels)** Cell lysates were immunoblotted for p62 and β-actin. Blots are representative of 2 independent experiments. **(C)** HepG2 cells were serum-starved (Control) or treated with 100 μM ADP in serum-free DMEM media for 4h. Cells were fixed and permeabilized and then LC3 and apoA-I were detected by indirect immunofluorescence using primary antibodies against human LC3 and apoA-I, and Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit Ab (green for LC3) and Alexa Fluor 647 anti-mouse Ab (red for apoA-I)) by confocal microscopy. Micrograph 100x and 40x images of representative cells from 2 independent experiments done in quadruplicate are shown.


A
Control
ADP
←LC3-I
←LC3-II
←β-actin
LC3-I
LC3-I / β-actin
0.4
0.3
0.2
0.1
0.0
*
Control
ADP
LC3-II
LC3-II / β-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
ADP
B
t = 0h
3h
6h
9h
Control
←LC3-I
←LC3-II
←β-actin
ADP
←LC3-I
←LC3-II
←β-actin
t = 0h
3h
6h
9h
Control
←p62
←β-actin
ADP
←p62
←β-actin
C
100X Magnification
40X Magnification
Control
ADP
Control
ADP
LC3
apoA-I
merge
5.02μm
5um

by treatment with chloroquine (50 μM) or Wortmannin (10 μM) (**Figure S3.3**).

$P2Y_{13}$ expression affects autophagy and apoA-I secretion: Extracellular ADP affects cellular apoA-I metabolism through the GPCR, $P2Y_{13}$ [15] in hepatocytes and therefore the effect of $P2Y_{13}$ expression on autophagy and apoA-I secretion were evaluated. Transfecting HepG2 cells with a pCMV6-$P2Y_{13}$ plasmid promoted a ~50% increase in $P2Y_{13}$ expression (**Figure 3.4, Western blot and inset**) and a parallel increase in LC3-II levels (**Figure 3.4A**). Treatment of liver cells with a pCMV6-$P2Y_{13}$ plasmid blocked the basal secretion of apoA-I at 4h, similar to that observed after treatment with ADP, while a combination of pCMV6-$P2Y_{13}$ and ADP had no additional effect on apoA-I secretion (**Figure 3.4B**). Increasing $P2Y_{13}$ expression in the liver cells significantly reduced the DLPC-induction of apoA-I secretion after 24h (**Figure 3.4C**).

Reducing $P2Y_{13}$ expression had the opposite effect. Transfecting HepG2 cells with $P2Y_{13}$ siRNA promoted a >50% reduction in $P2Y_{13}$ protein expression (**Figure 3.5, Western blot and inset**) and caused a similar reduction in LC3-II levels (**Figure 3.5A**). $P2Y_{13}$ siRNA significantly stimulated apoA-I secretion at both 4h and 24h (**Figures 3.5B and 3.5C**). After a 4h incubation, ADP was unable to block apoA-I secretion in cells treated with $P2Y_{13}$ siRNA, but conversely, increased apoA-I secretion relative to $P2Y_{13}$ siRNA treatment alone (**Figure 3.5B**). Treatment with DLPC (12 μM) or $P2Y_{13}$ siRNA for 24h stimulated apoA-I secretion by ~2.5-fold, while treatment with both DLPC and $P2Y_{13}$ siRNA promoted a ~10-fold stimulation in apoA-I secretion from HepG2 cells (**Figure 3.5C**). $P2Y_{13}$ siRNA completely blocked the effect of ADP on LC3-II (**Figure S3.4**).

Nucleotides and $P2Y_{13}$ regulate MAPK signaling. Cellular autophagy is regulated by the activation of MAPK (ERK1/2) and therefore the importance of ERK1/2 in ADP-dependent

Figure 3.4. Increasing $P2Y_{13}$ expression stimulates autophagy and blocks apoA-I secretion. HepG2 cells were transfected with either a control pCMV plasmid (pCMV) or a pCMV plasmid expressing human $P2Y_{13}$ (pCMV-P2Y13). Cell lysates were collected 48h after transfection and immunoblotted for $P2Y_{13}$ to measure protein overexpression **(Upper left panel)**. Histograms represent band densitometry analysis of $P2Y_{13}$, normalized to β-actin **(inset A)** and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs pCMV. **(A)** Cell lysates were immunoblotted for LC3 and histograms represent band densitometry analysis of LC3-II, normalized to β-actin and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs pCMV. **(B)** Transfected cells were treated with 100 μM ADP in serum-free DMEM media for 4h, conditioned media was collected and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs pCMV Control. **(C)** Transfected cells were treated with 12 μM DLPC in serum-free DMEM media for 24h, conditioned media was collected and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.01 vs pCMV Control, **P<0.01 vs pCMV+DLPC.

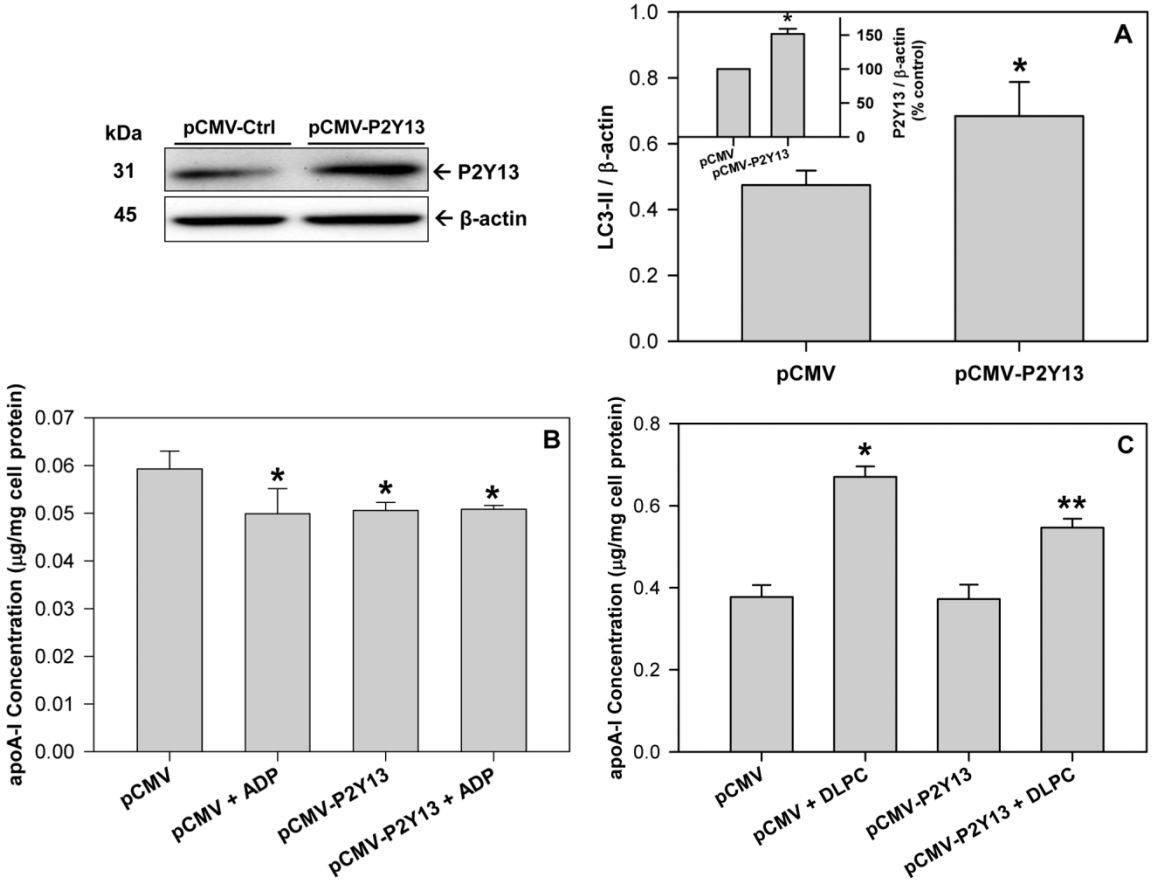
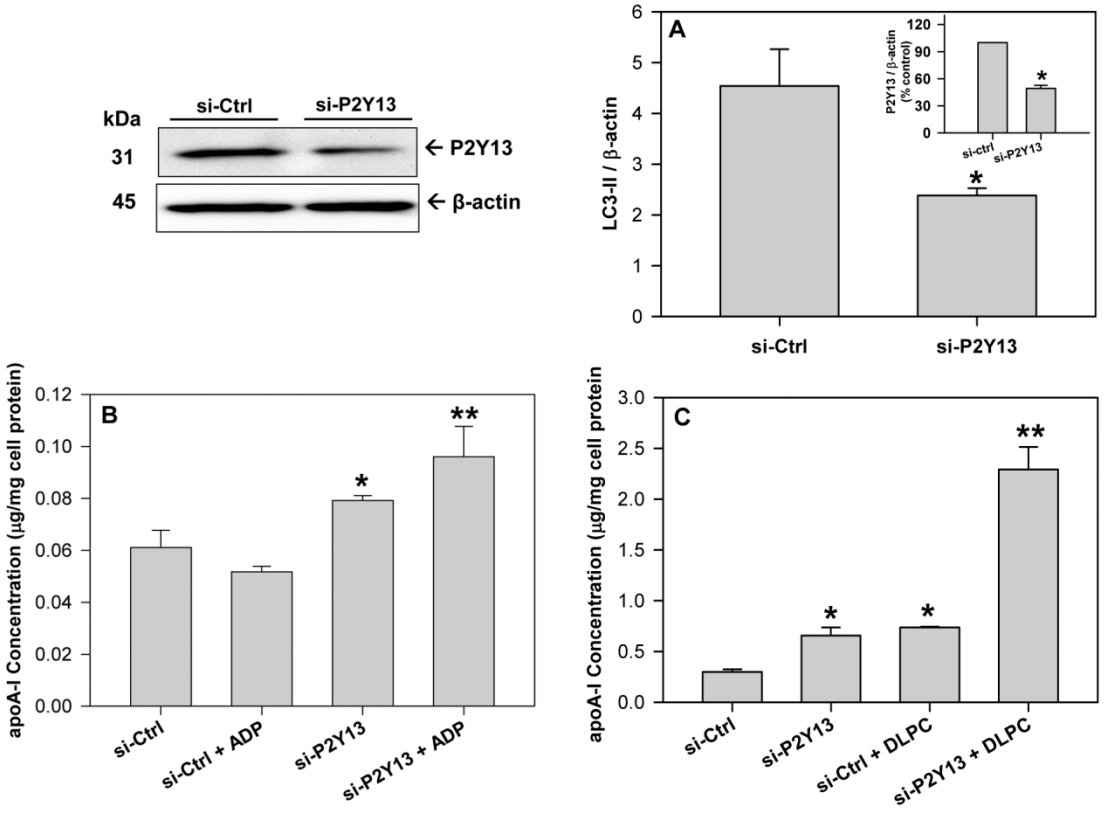

kDa
pCMV-Ctrl
pCMV-P2Y13
31
45
← P2Y13
← β-actin
A
LC3-II / β-actin
P2Y13 / β-actin (% control)
pCMV
pCMV-P2Y13
B
apoA-I Concentration (μg/mg cell protein)
pCMV
pCMV + ADP
pCMV-P2Y13
pCMV-P2Y13 + ADP
C
apoA-I Concentration (μg/mg cell protein)
pCMV
pCMV + DLPC
pCMV-P2Y13
pCMV-P2Y13 + DLPC

Figure 3.5. Reducing $P2Y_{13}$ expression blocks autophagy and stimulates apoA-I secretion. HepG2 cells were transfected with either a negative control (si-ctrl) or a siRNA against human $P2Y_{13}$. Cell lysates were collected 48h after transfection and immunoblotted for $P2Y_{13}$ to confirm protein knockdown **(Upper left panel)**. **(A)** Cell lysates were immunoblotted for LC3 and histograms represent band densitometry analysis of LC3-II, normalized to β-actin, and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs si-Ctrl. **(Inset A)** Histograms represent band densitometry analysis of $P2Y_{13}$ normalized to β-actin and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs si-Ctrl. **(B)** Transfected cells were treated with 100 μM ADP in serum-free DMEM media for 4h, conditioned media was collected and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs si-Ctrl, **P<0.05 vs si-P2Y13 **(C)** Transfected cells were treated with 12 μM DLPC in serum-free DMEM media for 24h, conditioned media was collected and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.01 vs si-Ctrl, **P<0.001 vs si-Ctrl + DLPC.


kDa
si-Ctrl
si-P2Y13
31
45
← P2Y13
← β-actin
A
LC3-II / β-actin
si-Ctrl
si-P2Y13
P2Y13 / β-actin (% control)
B
apoA-I Concentration (μg/mg cell protein)
si-Ctrl
si-Ctrl + ADP
si-P2Y13
si-P2Y13 + ADP
C
apoA-I Concentration (μg/mg cell protein)
si-Ctrl
si-P2Y13
si-Ctrl + DLPC
si-P2Y13 + DLPC

purinergic signaling was investigated. The nucleotide, ADP, stimulates ERK1/2 phosphorylation over a 30 min period and DLPC blocked the activation of ERK1/2 by ADP (**Figure 3.6A**). $P2Y_{13}$ expression also affects ERK1/2 phosphorylation and a 50% reduction in cellular $P2Y_{13}$ expression caused a significant reduction in ERK1/2 phosphorylation after 24h **(Figure 3.6B).** Cell viability was unaffected by the $P2Y_{13}$ siRNA.

Nucleotides and $P2Y_{13}$ regulate insulin receptor signaling. Cellular autophagy is activated by the inhibition of protein kinase B (Akt) [27]. DLPC stimulates the phosphorylation of Akt at 5 min. and ADP completely blocks the activation of Akt by DLPC (**Figure 3.7A**). ADP also inhibits the activation of Akt by insulin. **Figure 3.7B** shows that insulin stimulates Akt phosphorylation in HepG2 cells and that both tumor necrosis factor α (TNFα) and ADP inhibit insulin-induced Akt phosphorylation by ~50%. Reducing $P2Y_{13}$ expression by treatment with $P2Y_{13}$ siRNA appeared to inhibit cellular autophagic pathways by stimulating the phosphorylation of Akt. A reduction in cellular $P2Y_{13}$ expression significantly increases the phosphorylation of IR-β and Akt **(Figure 3.7C&D)** by >3-fold. $P2Y_{13}$ gene silencing significantly augmented the insulin-induced phosphorylation of Akt (**Figure S3.5**) and blocked the inhibitory effect of TNFα and ADP on IR-β and Akt phosphorylation (**Figure 3.7C&D and S3.5)**. Similar results were observed for the insulin-like growth factor receptor (IGF-1R) (not shown). $P2Y_{13}$ overexpression had the opposite effect. Transfection of HepG2 cells with pCMV6-$P2Y_{13}$ plasmid significantly reduced the phosphorylation of IR-β (**Figure S3.6**). Increasing $P2Y_{13}$ expression significantly inhibited the insulin-induced phosphorylation of IR-β and Akt (**Figure S3.6**), similar to that observed after treatment of HepG2 cells with ADP (**Figure 3.6C**). $P2Y_{13}$ expression significantly affected Akt phosphorylation, but had no effect on mTOR (**Figure S3.7**). ADP actually increased mTOR phosphorylation, while the

Figure 3.6. Extracellular nucleotides and $P2Y_{13}$ expression regulate ERK1/2 signaling. **(A)** HepG2 cells were pre-treated with 12 μM DLPC for 30 min. and then incubated with and without ADP (100 μM) for 0, 5, 15 and 30min in DMEM serum-free media. Cell lysates were immunoblotted for phosphorylated ERK1/2. Histograms represent densitometry analysis of p-ERK1/2 normalized to β-actin and expressed as mean percent change ± SD of 3 independent experiments. *P<0.01 vs Ctrl, **P<0.05 vs ADP 5min. **(B)** HepG2 cells were transfected with either negative control (si-Ctrl) or $P2Y_{13}$ siRNA (si-$P2Y_{13}$) and incubated for 24h. Cell lysates were immunoblotted for phosphorylated and total ERK1/2. Histograms represent band densitometry analysis of the ratio of phospho-ERK1/2 (p-ERK1/2) to total ERK1/2 (t-ERK1/2) and are expressed as mean ± SD for 3 independent experiments. *P<0.05 vs. control siRNA.

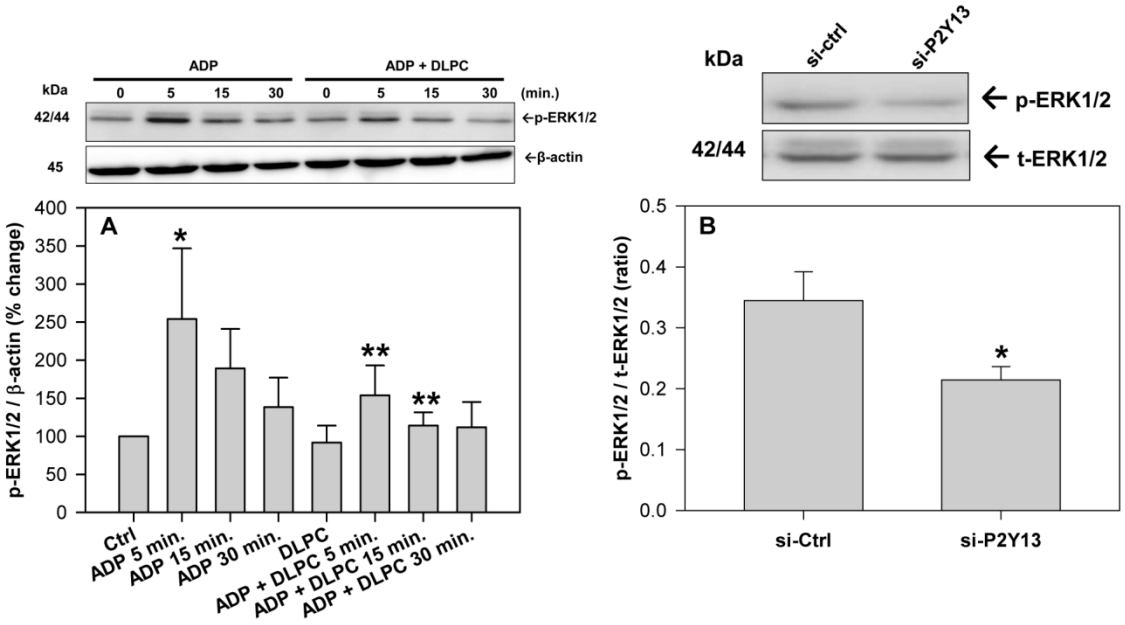
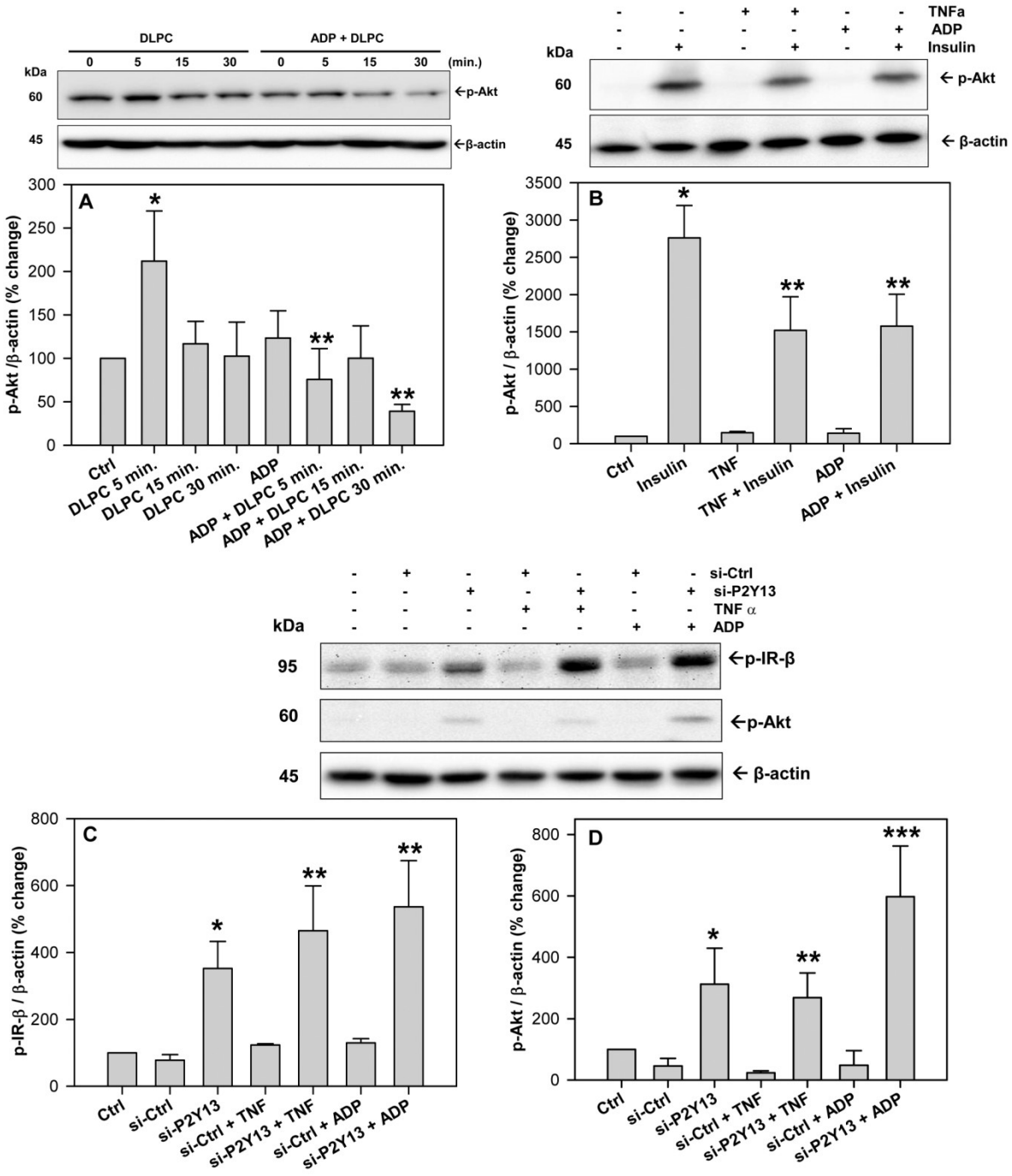

ADP
ADP + DLPC
kDa
0
5
15
30
0
5
15
30
(min.)
42/44
←p-ERK1/2
45
←β-actin
A
p-ERK1/2 / β-actin (% change)
400
350
300
250
200
150
100
50
0
*
**
**
Ctrl
ADP 5 min.
ADP 15 min.
ADP 30 min.
DLPC
ADP + DLPC 5 min.
ADP + DLPC 15 min.
ADP + DLPC 30 min.
kDa
si-ctrl
si-P2Y13
42/44
← p-ERK1/2
← t-ERK1/2
B
p-ERK1/2 / t-ERK1/2 (ratio)
0.5
0.4
0.3
0.2
0.1
0.0
*
si-Ctrl
si-P2Y13

Figure 3.7. Extracellular nucleotides and $P2Y_{13}$ expression regulate insulin receptor signaling. (A) HepG2 cells were pre-treated with 12 μM DLPC for 30 min. and then incubated with and without ADP (100 μM) for 0, 5, 15 and 30min in DMEM serum-free media. Cell lysates were immunoblotted for phosphorylated Akt (Ser473). Histograms represent densitometry analysis of p-Akt normalized to β-actin and expressed as mean percent change ± SD of 3 independent experiments. *P<0.01 vs Control, **P<0.001 vs DLPC 5min. **(B)** HepG2 cells were pre-treated with adenosine diphosphate (ADP) (100 μM) or TNFα (10 ng/ml) for 5 min. and then with human insulin (100 nM) for 5 min in DMEM serum-free media. Cell lysates were immunoblotted for phosphorylated Akt (Ser473). Histograms represent densitometry analysis of p-Akt normalized to β-actin and expressed as mean ± SD for 3 independent experiments. *P<0.001 vs Ctrl, **P<0.001 vs. insulin alone. **(C&D)** HepG2 cells were transfected with either negative control (si-Ctrl) or $P2Y_{13}$ siRNA (si-$P2Y_{13}$) and incubated for 48h. Cells were then treated with adenosine diphosphate (ADP) (100 μM) or TNFα (10 ng/ml) for 5 min. in DMEM serum-free media. **(C)** Cell lysates were immunoblotted for phosphorylated insulin receptor (IR-β) (Tyr1345). Histograms represent densitometry analysis of p-IR-β normalized to β-actin and expressed as mean ± SD for 3 independent experiments. *P<0.01 vs si-Ctrl, ** P<0.001 vs si-Ctrl. **(D)** Cell lysates were also immunoblotted for phosphorylated Akt (Ser473). Histograms represent densitometry analysis of p-Akt normalized to β-actin and expressed as mean ± SD for 3 independent experiments.*P<0.01 vs si-Ctrl, **P<0.05 vs. si-Ctrl, ***P<0.001 vs si-Ctrl.


DLPC
ADP + DLPC
0 5 15 30 0 5 15 30 (min.)
kDa
60
45
←p-Akt
←β-actin
A
p-Akt /β-actin (% change)
Ctrl
DLPC 5 min.
DLPC 15 min.
DLPC 30 min.
ADP
ADP + DLPC 5 min.
ADP + DLPC 15 min.
ADP + DLPC 30 min.
TNFa
ADP
Insulin
B
p-Akt / β-actin (% change)
Ctrl
Insulin
TNF
TNF + Insulin
ADP
ADP + Insulin
si-Ctrl
si-P2Y13
TNF α
ADP
95
←p-IR-β
C
p-IR-β / β-actin (% change)
si-Ctrl + TNF
si-P2Y13 + TNF
si-Ctrl + ADP
si-P2Y13 + ADP
D

mTOR inhibitor, rapamycin, significantly reduced p-mTOR levels (**Figure S3.8**).

3.5 Discussion

Insulin resistance and hyperglycemia have been shown to perturb plasma lipoprotein metabolism and increase apoB100 levels, but decrease HDL [1;2]. Insulin resistance is consequently a well accepted risk factor for the development of cardiovascular disease [1]. High blood glucose levels stimulate ATP production and promote the release of nucleotides from circulating blood cells, endothelial cells and smooth muscle cells [3;4]. ATP is unstable in the circulation and is quickly converted to ADP [7;8]. Elevations in blood nucleotide levels can impact cardiovascular disease [4;5;13] and inhibition of ADP-dependent thrombosis with $P2Y_{12}$ receptor inhibitors has already shown significant cardiovascular therapeutic value [33;34]. Niacin has also been shown to have cardiovascular therapeutic value and this molecule appears to act through another G-protein coupled receptor [16] to block purinergic signaling [18] and atherogenesis [35].

Our studies show that HDL secretion is regulated similarly in primary human hepatocytes and HepG2 liver cells and that the linoleic acid phospholipid, dilinoleoylphosphatidylcholine (DLPC), can stimulate HDL/apoA-I secretion [19;20;36]. DLPC appears to act much like niacin to prevent purinergic signaling by inhibiting F1-ATPase and blocking the production of ADP [19]. This view has been confirmed by the present work, which shows that ADP is a potent antagonist to the induction of apoA-I secretion by DLPC. **Figure 3.1** shows that an [ADP] >10 μM completely blocked the induction of apoA-I secretion by DLPC at 24h and also at 4h. Conversely, ADP can directly stimulate apoB100 and apoE secretion from human liver cells at 24h (**Figure 3.2A&B**). The normal physiological concentration of ADP in the bloodstream has been reported to be ~15μM [8], but elevated blood

glucose levels can increase nucleotide secretion and accumulation in the circulation [3;4]. High circulatory nucleotide levels would therefore be expected to block hepatic apoA-I secretion and stimulate apoB100 output. This is indeed similar to what is thought to occur in hyperglycemic, insulin resistant patients [1;37;38]. This may suggest that elevations in blood nucleotide levels may be partly causative to abnormal plasma lipoprotein levels.

Since apoB100 secretion is known to be regulated by proteasomal degradation, we evaluated the effect of proteasomal inhibitors on both apoB100 and apoA-I secretion. ADP and the proteasomal inhibitor, ALLN, appear very similar and both inhibit apoA-I secretion, but stimulate apoB100 secretion at 4h (**Figure 3.2C&D**). ADP may therefore act similar to proteasomal inhibitors to stimulate autophagy [24;25]. ADP stimulates autophagy in HepG2 cells and increases the level of autophagic markers, LC3-I and LC3-II, and p62 over a 6h period (**Figure 3.3**). The ability of ADP to stimulate apoB100 secretion (**Figure 3.2**) and increase p62 levels may indicate that ADP blocks proteasomal degradation, since p62 levels are known to rise when the proteasome is inhibited [26;27]. Confocal studies confirmed the higher levels of LC3 after treatment with ADP. Micrographs showed that LC3-II was located in punctate autophagosomes within the liver cells and clearly showed that higher levels of LC3 and apoA-I were colocalized within autophagosomes in ADP treated cells (**Figure 3.3C**). The view that a stimulation in autophagy may inhibit apoA-I secretion is consistent with earlier work, which has shown that serum deprivation, a treatment well-known to directly stimulate autophagy (**Figure 3.3B** and [26;27]), also inhibits apoA-I secretion of HepG2 cells by ~50% over the first hour [39].

Human liver cells contain two ADP-receptors, $P2Y_1$ and $P2Y_{13}$, but HDL metabolism is primarily affected by $P2Y_{13}$ [15;40;41]. ADP is a potent agonist to $P2Y_{13}$ and stimulates a rapid

(10 min) endocytic recycling pathway for extracellular apoA-I [14;15;31]. This recycling pathway has been shown contribute to apoA-I lipidation, cholesterol efflux and apoE resecretion, but does not promote significant apoA-I degradation [42-44]. Conversely, it is known that apoA-I secretion is affected by cellular degradation [18;21] and activation of $P2Y_{13}$ appears to stimulate degradation pathways. The direct effect of $P2Y_{13}$ on both autophagy and apoA-I secretion from human liver cells is clearly illustrated by increasing or silencing $P2Y_{13}$ gene expression. $P2Y_{13}$ overexpression significantly increased cellular LC3-II levels and decreased apoA-I secretion (**Figure 3.4**), similar to that seen with exogenous ADP. Conversely, $P2Y_{13}$ gene silencing with siRNA significantly decreased LC3-II levels, increased both basal apoA-I secretion and the DLPC induction in apoA-I secretion, and blocked the effects of ADP on autophagy and apoA-I secretion (**Figure S3.4 and Figure 3.5**). $P2Y_{13}$ expression in HepG2 cells therefore appears to regulate apoA-I secretion through cellular autophagic pathways.

In contrast to this work, studies in $P2Y_{13}$-deficient mice have shown that reducing $P2Y_{13}$ expression caused a small reduction in plasma HDL levels *in vivo* [45;46]. This may be partly due to the fact that mice are not a human equivalent model for the study of HDL metabolism, since numerous liver-specific signaling pathways differ in mice [47]. Nucleotide signaling also differs significantly in rodents [40;48]. In studies with rat hepatocytes, ADP acts through $P2Y_2$ receptors to increase [IP3] and $[Ca^{2+}]$ [48]. This does not occur in human cells. Treatment of both primary human liver cells and HepG2 cells with ADP or UDP has no effect on cellular [IP3] and $[Ca^{2+}]$ [40]. In human liver cells, ADP stimulates MAPK and reduces [cAMP] [15;41], both of which are known to reflect activation of $P2Y_{13}$ [49;50]. We show that ADP stimulates ERK1/2 in human liver cells (**Figure 3.6A**), but ADP has the opposite effect in murine pancreatic cells and inhibits ERK1/2 phosphorylation [51]. ADP and $P2Y_{13}$-dependent

signaling may therefore be very different in humans and rodents and this may explain why $P2Y_{13}$-deficient mice show no major lipoprotein phenotype [45;46].

Nucleotide signaling through P2Y receptors is well known to activate MAPK pathways [49;50] and MAPK is a well-known activator of cellular autophagy [26]. DLPC may therefore stimulate apoA-I secretion by blocking MAPK activation and preventing the autophagic degradation of apoA-I. DLPC can block an ADP-dependent activation of ERK1/2 in HepG2 cells (**Figure 3.6A**), similar to that shown in neuronal cells, where DLPC blocked ERK1/2 activation by TNFα and hydrogen peroxide [52]. Treatment of liver cells with the MEK1/2 inhibitor, U0126, completely blocked the ADP-dependent increase in LC3-II levels (**Figure S3.3)** and $P2Y_{13}$-gene silencing also muted ERK1/2 activation (**Figure 3.6B**). Our data suggests that MAPK is not alone in regulating autophagy and apoA-I secretion. ADP can block the activation of Akt by DLPC and insulin (**Figure 3.7A&B**) and Akt is an established inhibitor of autophagy [26;30]. Therefore, while nucleotides may affect cellular autophagic pathways through MAPK pathways, the insulin signaling-dependent Akt pathways may also play an important role in the purinergic regulation of autophagy and HDL secretion. Akt can inhibit autophagy directly, and indirectly through mTOR [26]. Our work, however suggests that ADP stimulates autophagy through mTOR-independent pathways, since $P2Y_{13}$ expression had no effect on mTOR phosphorylation, while ADP increased p-mTOR levels (**Figures S3.7& S3.8**).

If ADP-dependent signaling through $P2Y_{13}$ impacts Akt-dependent pathways, it follows that $P2Y_{13}$ expression would be expected to affect insulin receptor signaling. Consistent with this view, a reduction in $P2Y_{13}$ expression directly stimulates the phosphorylation of IR-β and Akt by >3-fold (**Figure 3.7**), increases insulin-dependent

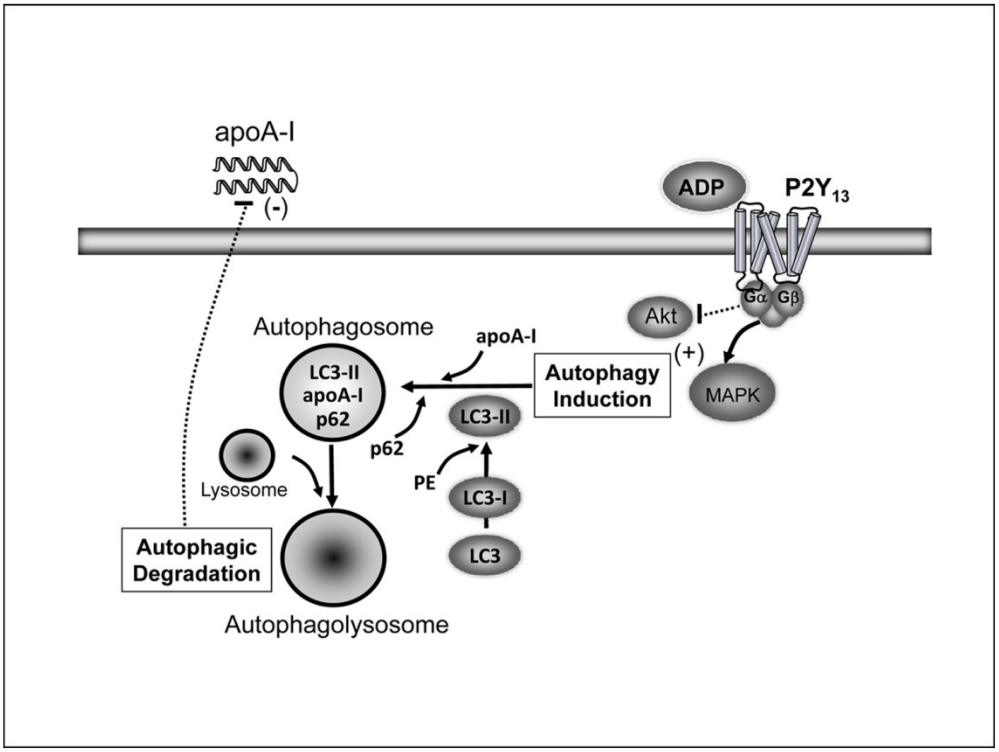
signaling (**Figure S3.5**) and completely blocks the inhibition of insulin receptor signaling by TNFα and ADP (**Figure 3.7**). Increasing $P2Y_{13}$ expression has the opposite effect and significantly inhibits insulin-induced phosphorylation of IR-β and Akt **(Figure S3.6)**. Nucleotide signaling through $P2Y_{13}$ may therefore affect insulin receptor signaling. This work appears consistent with other studies showing that ADP acts through $P2Y_{13}$ to inhibit insulin secretion from pancreatic beta cells [51;53].

This work shows that lipoprotein secretion and insulin signaling pathways are affected by hepatic membrane purinergic receptor signaling. Elevations in blood glucose promote the synthesis and secretion of nucleotides from circulating blood cells and vascular tissues. Nucleotides that accumulate in the circulation can then act through purinergic receptors, i.e. $P2Y_{13}$, to stimulate mitogenic pathways and inhibit insulin receptor signaling. Enhanced purinergic signaling in insulin resistance may give rise to an inhibition of proteasomal degradation and a chronic induction of cellular autophagy (**Figure 3.8**). The net result is a stimulation of apoB100 secretion from the liver and a reduction in apoA-I secretion. This may partly explain the well-described lipoprotein phenotype associated with insulin resistance [1].

3.6 Acknowledgements

We thank Dr. Nihar Pandey for his advice and technical assistance in the insulin signaling experiments. The work was supported by a grant from the Heart and Stroke Foundation of Ontario (D.L.S). C.C is the recipient of a Government of Ontario Graduate Scholarship.

Figure 3.8. Extracellular nucleotides act through $P2Y_{13}$ to stimulate autophagy. Elevations in blood glucose promote the secretion and accumulation of nucleotides in the circulation. Nucleotides act through $P2Y_{13}$ to activate mitogenic pathways, inhibit insulin receptor signaling and stimulate autophagic protein degradation. Enhanced purinergic signaling in insulin resistance may give rise to a chronic induction of cellular autophagy and a reduction in apoA-I secretion from the liver.


apoA-I
(-)
ADP
P2Y13
Gα
Gβ
Akt
(+)
MAPK
Autophagosome
apoA-I
LC3-II
apoA-I
p62
Autophagy
Induction
LC3-II
p62
PE
LC3-I
LC3
Lysosome
Autophagic
Degradation
Autophagolysosome

3.7 Supplemental Figures

Figure S3.1. ATP decreases apoA-I levels in the media at 24h. HepG2 cells were pre-treated with adenosine diphosphate (ADP) or adenosine triphosphate (ATP) (100 μM) for 30 min. and then incubated with 12 μM DLPC in serum-free DMEM media. Conditioned media was collected after 24h treatment and apoA-I concentration was quantified by ELISA. ApoA-I concentration in the media is normalized to total cell protein and expressed as mean ± SD of 3 independent experiments *P<0.01 vs DLPC, **P<0.001 vs DLPC.

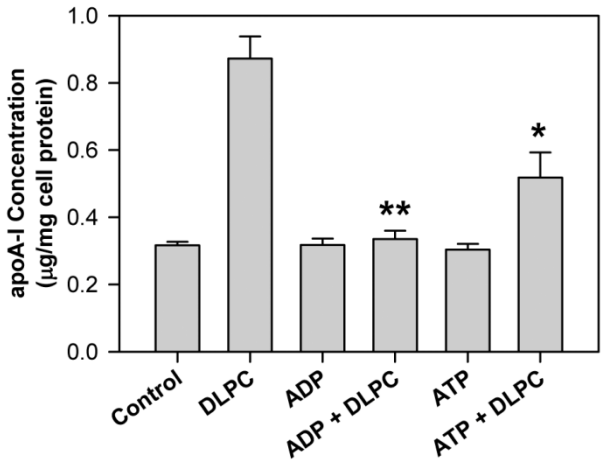

apoA-I Concentration
(μg/mg cell protein)
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Control
DLPC
ADP
ADP + DLPC
ATP
ATP + DLPC

Figure S3.2. ADP stimulates autophagy. HepG2 cells were serum-starved (Control) **(A)** or treated with 100 μM ADP **(B)** in serum-free DMEM media for 4h. Cells were fixed and permeabilized and then apoA-I and LC3 were detected by indirect immunofluorescence using confocal microscopy. Original images of representative micrographs at 100X magnification from 2 independent experiments performed in quadruplicate are shown.

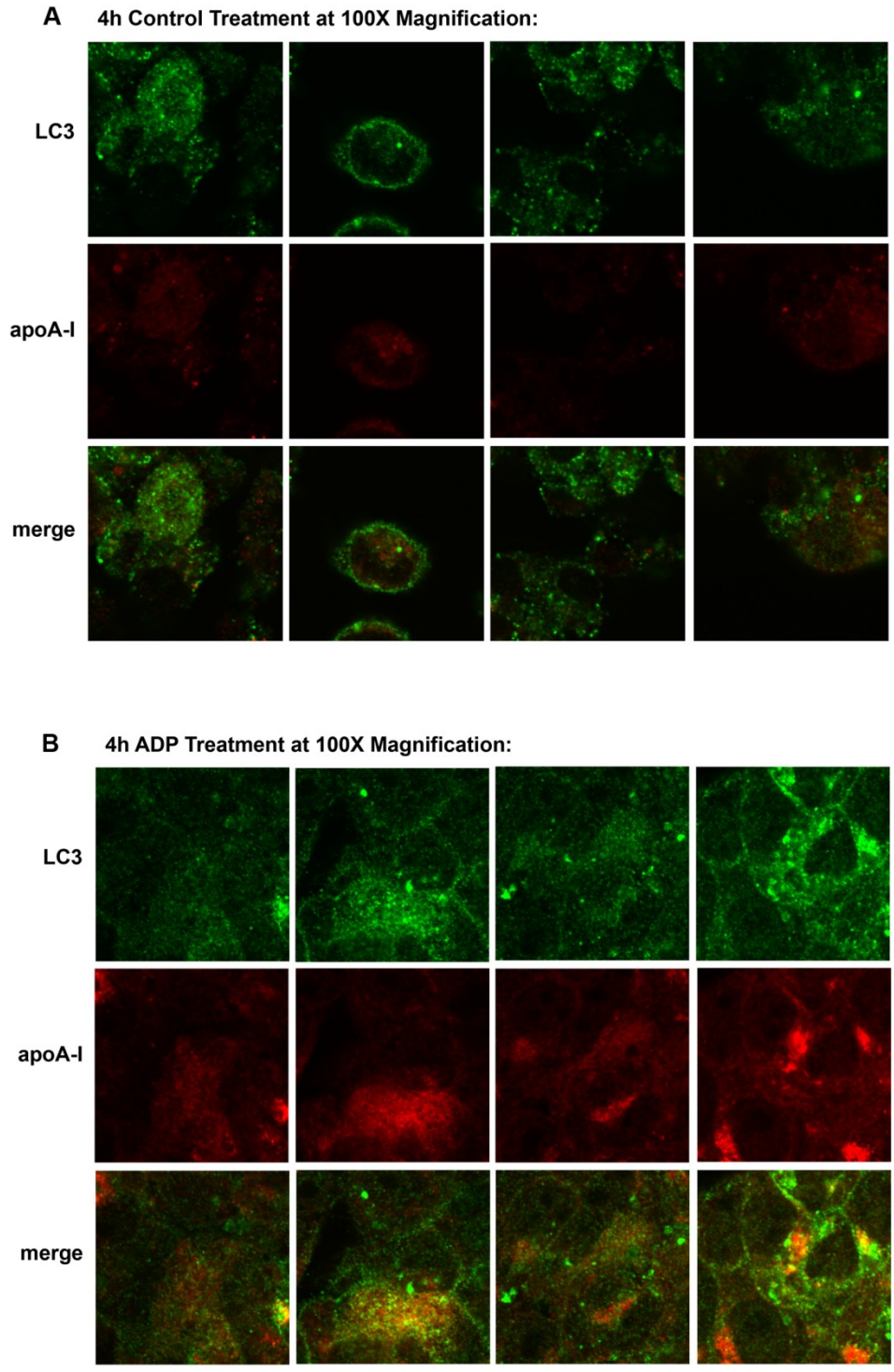

A
4h Control Treatment at 100X Magnification:
LC3
apoA-I
merge
B
4h ADP Treatment at 100X Magnification:
LC3
apoA-I
merge

Figure S3.3. Effect of autophagy inhibitors on cellular LC3-II. HepG2 cells were pre-treated with 50 μM chloroquine, 5mM 3-methyladenine (3-MA), 10 μM wortmannin or 10 μM U1026 for 30min ± 100μM ADP for 4h in serum-free DMEM media. Cell lysates were immunoblotted for LC3. Histograms represent band densitometry analysis of LC3-II normalized to β-actin and expressed as percent change ± SD of 3 independent experiments. *P<0.05 vs Control and **P<0.01 vs Control.

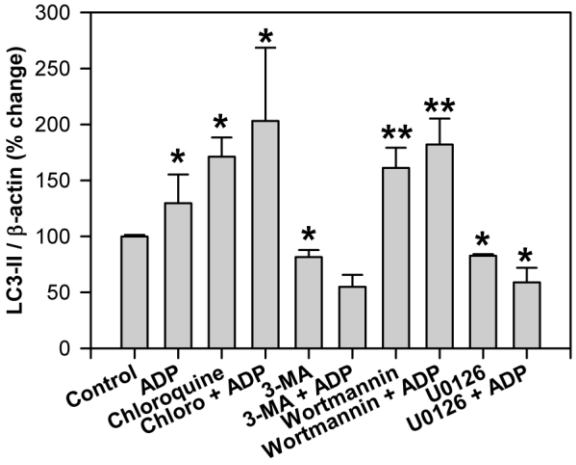

LC3-II / β-actin (% change)
300
250
200
150
100
50
0
*
*
*
*
**
**
*
*
Control
ADP
Chloroquine
Chloro + ADP
3-MA
3-MA + ADP
Wortmannin
Wortmannin + ADP
U0126
U0126 + ADP

Figure S3.4. $P2Y_{13}$ knockdown inhibits the ADP-dependent stimulation in autophagy.
HepG2 cells were transfected with either negative control (si-Ctrl) or $P2Y_{13}$ siRNA (si-P2Y13) and incubated for 48h. Cells were then incubated with ADP (100 μM) for 4h in DMEM serum-free media. Cell lysates were immunoblotted for LC3 and blots are representative of 3 independent experiments.

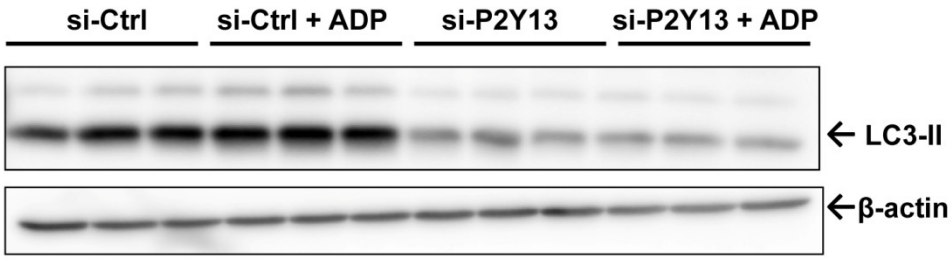

si-Ctrl
si-Ctrl + ADP
si-P2Y13
si-P2Y13 + ADP
← LC3-II
←β-actin

Figure S3.5. Reducing $P2Y_{13}$ expression augments insulin receptor signaling. HepG2 cells were transfected with either negative control (si-Ctrl) or $P2Y_{13}$ siRNA (si-P2Y13) and incubated for 48h. Cells were then pre-incubated with ADP (100 μM) for 5 min. and then with human insulin (100 nM) for 5 min in DMEM serum-free media. Cell lysates were immunoblotted for phosphorylated Akt (Ser473). Histograms represent densitometry analysis of p-Akt normalized to β-actin and expressed as mean percent change ± SD for 2 independent experiments performed in triplicate.*P<0.05 vs si-Ctrl, **P<0.01 vs si-P2Y13.

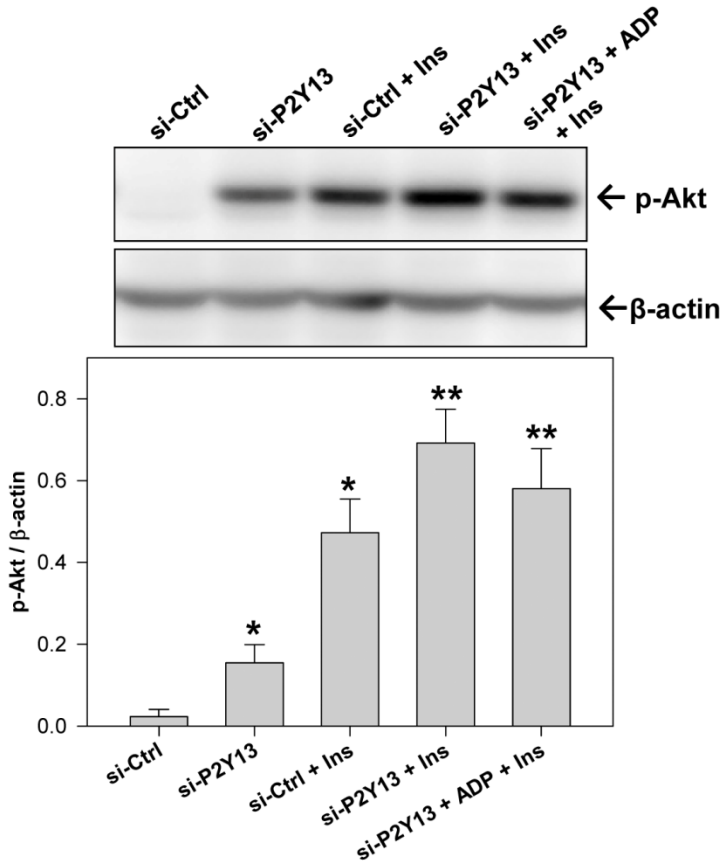

si-Ctrl
si-P2Y13
si-Ctrl + Ins
si-P2Y13 + Ins
si-P2Y13 + ADP + Ins
← p-Akt
←β-actin
p-Akt / β-actin
0.0
0.2
0.4
0.6
0.8
*
*
**
**
si-Ctrl
si-P2Y13
si-Ctrl + Ins
si-P2Y13 + Ins
si-P2Y13 + ADP + Ins

Figure S3.6. $P2Y_{13}$ overexpression blocks insulin receptor signaling. HepG2 cells were transfected with either a control pCMV plasmid (pCMV) or a pCMV plasmid expressing human $P2Y_{13}$ (pCMV-P2Y13). Cell lysates were collected 48h after transfection and immunoblotted for $P2Y_{13}$ to measure protein overexpression **(inset, panel A)**. Cells were then treated with human insulin (100 nM) for 5 min in DMEM serum-free media. Cell lysates were immunoblotted for insulin receptor (p-IR-β) **(A)** and phosphorylated Akt (Ser473) **(B)**. Histograms represent densitometry analysis normalized to β-actin and are expressed as mean percent change ± SD for 3 independent experiments. **(A)***P<0.001 vs pCMV and **P<0.01 vs pCMV + Insulin. **(B)** *P<0.001 vs pCMV and ** P<0.05 vs pCMV + Insulin.

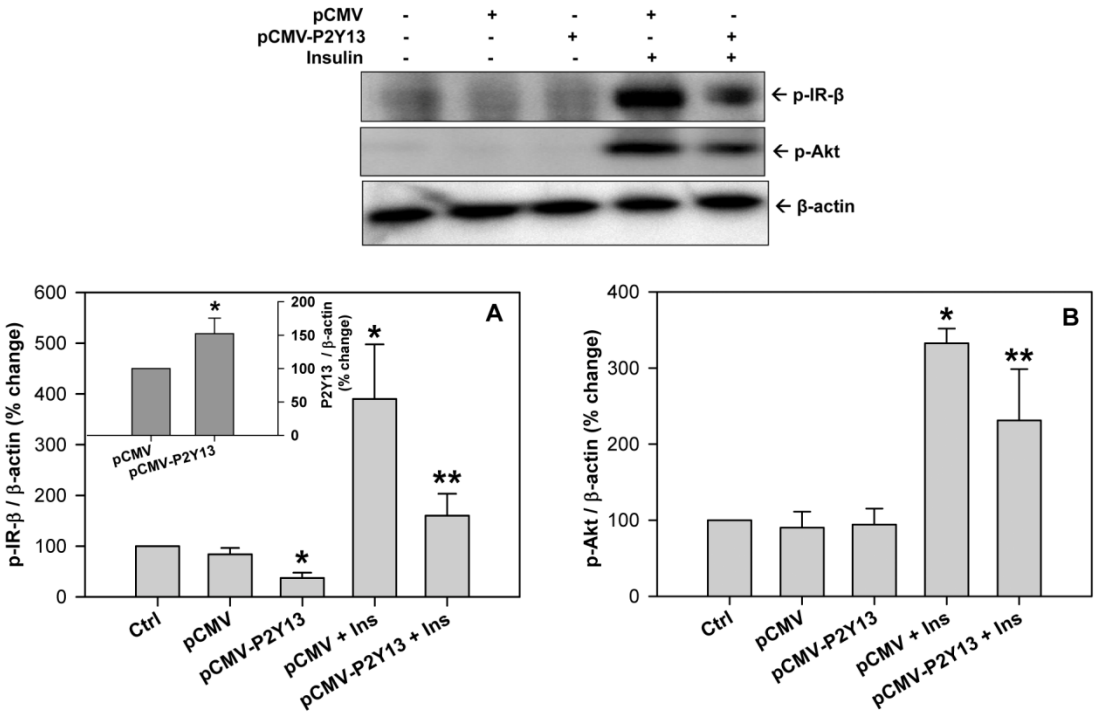

pCMV - + - + -
pCMV-P2Y13 - - + - +
Insulin - - - + +
← p-IR-β
← p-Akt
← β-actin
A
p-IR-β / β-actin (% change)
600
500
400
300
200
100
0
P2Y13 / β-actin (% change)
200
150
100
50
0
pCMV
pCMV-P2Y13
Ctrl
pCMV
pCMV-P2Y13
pCMV + Ins
pCMV-P2Y13 + Ins
B
p-Akt / β-actin (% change)
400
300
200
100
0
Ctrl
pCMV
pCMV-P2Y13
pCMV + Ins
pCMV-P2Y13 + Ins

Figure S3.7. Reducing $P2Y_{13}$ expression had no effect on the phosphorylation of mTOR. HepG2 cells were transfected with either a negative control (si-ctrl) or a siRNA against human $P2Y_{13}$ (si-P2Y13). Cell lysates were collected 48h after transfection and immunoblotted for phosphorylated Akt (Ser473) and phosphorylated mTOR (Ser2448). Histograms represent densitometry analysis of p-Akt and p-mTOR normalized to β-actin and expressed as mean ± SD of 3 independent experiments. *P<0.001 vs si-Ctrl.

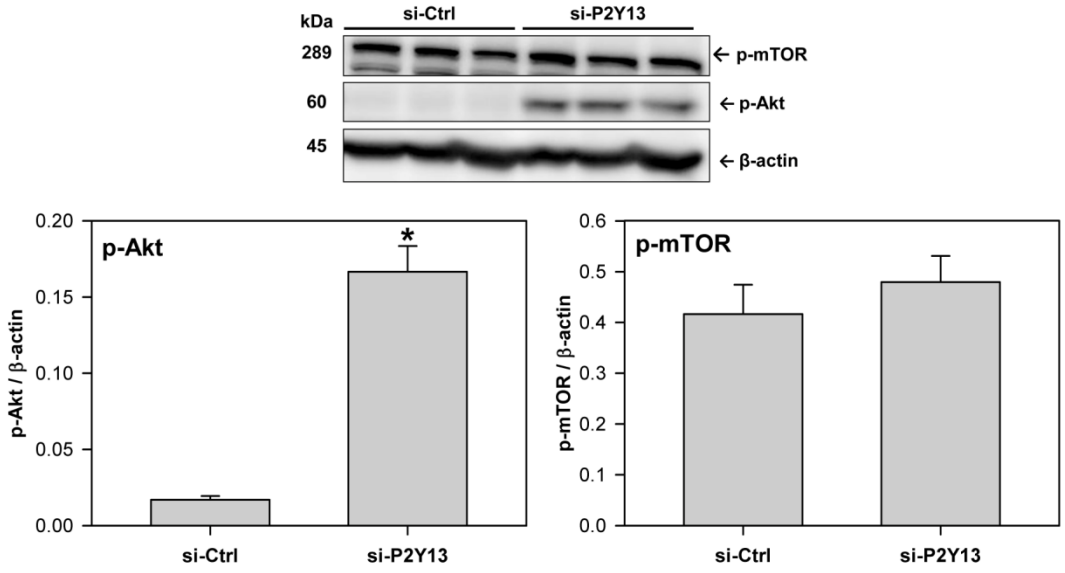
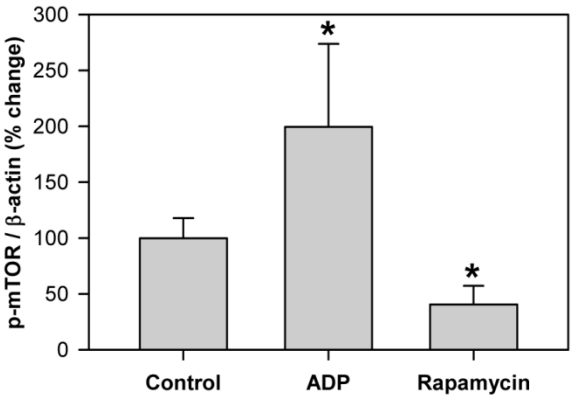

kDa
si-Ctrl
si-P2Y13
289
← p-mTOR
60
← p-Akt
45
← β-actin
p-Akt
*
p-Akt / β-actin
0.20
0.15
0.10
0.05
0.00
si-Ctrl
si-P2Y13
p-mTOR
p-mTOR / β-actin
0.6
0.5
0.4
0.3
0.2
0.1
0.0
si-Ctrl
si-P2Y13

Figure S3.8. ADP increases mTOR phosphorylation. HepG2 cells were treated with 100 μM ADP or 250 nM rapamycin for 4h. Cell lysates were immunoblotted for phosphorylated mTOR (Ser2448). Histograms represent densitometry analysis of p-mTOR normalized to β-actin and expressed as mean percent change ± SD of 3 independent experiments. *P<0.05 vs control.


p-mTOR / β-actin (% change)
300
250
200
150
100
50
0
*
*
Control
ADP
Rapamycin

3.8 References

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CHAPTER 4 - MANUSCRIPT #4

Hepatic Lipase Secretion is Regulated by Purinergic Signalling and Autophagy

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Author contributions: C.C and D.L.S. conceived and designed the experiments. C.C. performed the experiments while both C.C. and D.L.S. analyzed and interpreted the data. C.C. performed the statistical analyses. C.C. and D.L.S. wrote the manuscript. C.C. and D.L.S. reviewed and edited the manuscript.

Key Words: Hepatic lipase, HDL, nucleotide, $P2Y_{13}$, autophagy

4.1 Abstract

Elevated blood glucose levels are associated with increased circulating nucleotides and abnormal triglyceride metabolism. Triglyceride clearance may therefore be perturbed by nucleotide signaling. Extracellular nucleotides control lipoprotein metabolism in hepatic cells, therefore we investigated the effect of purinergic signaling on the lipoprotein remodeling enzyme, hepatic lipase (HL). The nucleotide, adenosine diphosphate (ADP) (100μM), blocks hepatic lipase (HL) secretion and inhibits the stimulation of HL secretion from liver cells after 24h. Previous work has shown that ADP and the proteasomal inhibitor, ALLN, stimulate cellular autophagy. Treatment of cells with ALLN (25 μM) significantly reduces cellular HL levels and HL secretion at 4h. Conversely, treatment with the autophagy inhibitor, 3-methyladenine (5 mM), significantly increases cellular HL levels and stimulates HL secretion. HL secretion therefore appears to be regulated by autophagy. ADP acts through the G-protein coupled receptor, $P2Y_{13}$, to stimulate autophagy and therefore $P2Y_{13}$ expression would affect HL secretion. Overexpression of $P2Y_{13}$ blocks HL secretion, much like ADP, while siRNA-targeted reduction in $P2Y_{13}$ protein expression has the opposite effect and stimulates the secretion of HL by 5 to 8-fold. $P2Y_{13}$ stimulates endocytic recycling pathways, and therefore experiments were performed to determine the effect of extracellular ADP on the uptake and degradation of V5-epitope tag-labeled HL (HL-V5). Two isoforms of HL-V5, at 62 and 68 kDa, are secreted from HepG2 cells, but only the 62 kDa protein undergoes reuptake / internalization. The smaller HL isoform progressively accumulates in the cell over 24h, with no detectible modification or degradation.Treatment of HepG2 cells with ADP reduces the total cell association of HL-V5 at 30 min and 4h, but has no effect on HL-V5 internalization or degradation. Receptor-associated protein (RAP) is known to block LRP1-

dependent endocytosis and 24h treatment with RAP increases HL secretion from HepG2 cells. Treatment with RAP (100 nM) reduces the total cell association of HL-V5, but has no effect on the internalization of HL-V5. This work shows that extracellular HL is not transported through LRP1 to degradation pathways. HL secretion appears to be controlled by autophagic proteolytic pathways and extracellular nucleotides block HL secretion by stimulating autophagy.

4.2 Introduction

Hypertriglyceridemia is a consequence of insulin resistance [1] and is due to an over-production and impaired degradation of triglyceride (TG)-rich postprandial lipoproteins [2;3]. Hepatic lipase (HL) plays a central role clearing TG-rich very-low lipoproteins (VLDL) from the circulation [4] and HL lipolytic function may be impaired in insulin resistance [5;6]. Hyperglycemia is known to perturb postprandial lipemia. The clearance of VLDL from the circulation is impaired in insulin resistance and VLDL levels are elevated [5;7].

Elevated blood glucose levels are associated with an increase in nucleotide secretion from vascular cells and increased purinergic signaling [8-10]. Circulating nucleotide levels are regulated by ecto-enzymes that degrade nucleotides. A deficiency in the endonucleotidase, CD39/ENTPD1, has been shown to cause insulin resistance and hypertriglyceridemia in mice [11] and polymorphisms in the CD39 gene are associated with type 2 diabetes [12]. Studies show that extracellular nucleotides affect insulin signaling and glucose metabolism [13;14] and also significantly perturb hepatic lipoprotein secretion [15].

Extracellular nucleotides appear to control lipoprotein secretion from liver cells [15]. Lipoprotein secretion is regulated by cellular proteolytic pathways and nucleotides act like proteasomal inhibitors to block proteasomal degradation and stimulate cellular autophagy.

Consequently, the nucleotide, adenosine diphosphate (ADP), stimulates hepatic apoB100 secretion from liver cells and blocks HDL secretion [15]. ADP activates purinergic signaling through the G-protein coupled receptor, $P2Y_{13}$, and stimulates the autophagic degradation of apoA-I. Previous work has shown that the metabolism of apoA-I in liver cells affects the secretion of HL [16]. Since apoA-I secretion is suppressed by a purinergic stimulation in autophagy, a similar consequence to HL secretion would be expected.

The present study shows that HL secretion from liver cells is also inhibited by ADP and purinergic signaling through $P2Y_{13}$. Conversely, HL secretion is stimulated by autophagic inhibitors and suppression of $P2Y_{13}$ expression. The work shows that a modulation of HL secretion is not associated with extracellular HL recycling, but a consequence of a selective control of cellular proteolytic degradation and secretion pathways.

4.3 Materials and Methods

Reagents: Dilinoleoylphosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Adenosine 5'-diphosphate sodium salt (ADP), the proteasomal inhibitor, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), and the PI3 kinase inhibitor, 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (Oakville, ON). Receptor-associated protein (RAP) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY) The antibody to $P2Y_{13}$ and scavenger receptor SR-BI were obtained from Abcam (Cambridge, MA). The LRP1 antibody was purchased from Epitomics, Inc. (Burlingame, CA) and the LDL-R antibody was obtained from Dr. Thomas Lagace (University of Ottawa Heart Institute). The human hepatic lipase antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), the V5 antibody was from Invitrogen Corporation (Burlington, ON), and the β-actin antibody was obtained from Cell Signaling Technology (Danvers, MA).

Affinity purified peroxidase linked goat anti-mouse and anti-rabbit antibodies were purchased from GE Healthcare (UK). All Stars Negative control small interference RNA (siRNA) and human LRP1 siRNA were purchased from Qiagen (Mississauga, ON) and human $P2Y_{13}$ siRNA were purchased from Thermo Scientific Dharmacon (Lafayette, CO). Human $P2Y_{13}$ plasmid was purchased from Origene (Rockville, MD). The pcDNA6-human HL-V5-6XHis plasmid construct was a kind gift from Dr. Miklos Peterfy (UCLA, Los Angeles, CA). Inhibitors were of analytical grade and were solubilized in dimethyl sulfoxide (DMSO).

Cells and Cell Culture: Human hepatocarcinoma, HepG2, cells were regularly maintained in Dulbecco's modified Eagle medium (DMEM) (5g/L glucose) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Passages 4-10 were used and cells that were 80% confluent were treated with DLPC, nucleotides and/or inhibitors for the indicated times and concentrations under serum-free conditions. Cell viability was evaluated after all treatment conditions.

Preparation of DLPC Micelles: DLPC micelles were prepared in DMSO by sonication as previously described [17]. Purity of all phospholipids was >99%.

Knockdown of Human $P2Y_{13}$ by Small Interference RNA: HepG2 cells were transiently transfected with All Stars Negative control siRNA from Qiagen (Mississauga, ON) or two different $P2Y_{13}$ siRNA sequences (ACCUUCAUCAUCUACCUCAAAUU or GACACUCAUGCUUCCUUUCAAUU) from Thermo Scientific Dharmacon (Lafayette, CO), by reverse transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 12-well plates. In brief, complexes were prepared per manufacturer's specifications with a Lipofectamine 2000-to-siRNA volume-to-mole ratio of 2:40 (μL:pmol) in 200μL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). Lipofectamine-siRNA complexes

were added to the cells immediately after the cells were seeded at a density of 500,000 cells/well in a volume of 1mL of normal growth media containing 10% FBS in the absence of penicillin/streptomycin . The cells were treated with ADP or DLPC in serum-free DMEM 48h after transfection. Cell media and lysate samples were harvested at the indicated timepoints for immunoblot analysis. Transfection of the control and test siRNA caused no cytotoxic effects.

Overexpression of Human $P2Y_{13}$ by Plasmid: The pCMV6 vector containing the full-length human $P2Y_{13}$ cDNA was purchased from Origene (Rockville, MD). HepG2 cells were transiently transfected with control plasmid or the pCMV6-$P2Y_{13}$ plasmid by reverse transfection using FuGENE HD (Roche Applied Science, Laval, QC). Complexes were prepared per manufacturer's instructions with a FuGENE HD-to-DNA volume-to-mass ratio of 6:2 (µl to µg) in 100µL of Opti-MEM I Reduced Serum Media (Invitrogen, Carlsbad, CA). HepG2 cells were trypsinized and seeded in 12-well plates at a density of 500,000 cells/well in a volume of 1mL in normal growth media containing 10% FBS in the absence of penicillin/streptomycin and then 50µL of the transfection complexes were immediately added to the suspended cells. The cells were treated with ADP or DLPC in serum-free DMEM 48h after transfection. Cell media and lysate samples were harvested at the indicated timepoints for immunoblot analysis. Transfection of the control and test plasmid caused no cytotoxic effects.

Immunoblot Analysis: After treatment for the indicated timepoints, cells were washed twice with ice-cold PBS. Cells were lysed in NP-40 lysis buffer (Biosource, Camarillo, CA) supplemented with 1mM PMSF and 1X protease inhibitor cocktail (Sigma, Saint Louis, MO). Cell protein concentrations were determined by the BCA Protein Assay (Thermo Fisher

Scientific, Waltham, MA). Cell lysate samples containing equal total protein (30μg) were separated by SDS-PAGE and analyzed by Western blot using specific antibodies to HL, V5, LRP1, LDL-R, SR-BI, and β-actin. Blots were exposed using the Alpha Innotech FluorChem™ HD Imager and band intensities were quantified with the Alpha Ease FC™ software.

HL-V5 Endocytosis Assay: HepG2 cells were transiently transfected with the pcDNA6-human HL-V5-6XHis plasmid construct obtained from Dr. Peterfy (UCLA, Los Angeles, CA) by reverse transfection using FuGENE HD (Roche Applied Science, Laval, QC) as described for $P2Y_{13}$ overexpression. The conditioned media containing the secreted HL-V5 was collected 48h after transfection and pooled. Wildtype (untransfected) HepG2 cells were either pre-treated with 100μM ADP, 100μM MDC, 100nM RAP or left untreated for 30min in serum-free DMEM. HL-V5 conditioned media was then added to the pre-treated cells for either 30min or 4h for endocytosis. Cells were then harvested to determine the total cell association or internalization of HL-V5. Cells for total cell association were washed gently in PBS and then lysed, whereas cells for internalization were acid washed twice with 150mM NaCl and 10mM acetic acid at pH 3.5 as previously described in [18]. Cell media and lysate samples were then subjected to immunoblot analysis and probed for V5.

Statistical Analysis: Values are shown as Mean ± SD for at least 3 independent experiments and $P<0.05$ was considered significant. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) on ranks by a pairwise multiple comparison using the Student-Newman-Keuls post-hoc test (SigmaStat; Systat Software, Inc., San Jose, CA).

4.4. Results

Effect of extracellular nucleotides on HL secretion: We have previously shown that factors that stimulate HDL secretion also stimulate HL secretion [16], and that extracellular ADP blocks HDL secretion by stimulating autophagy. HL secretion may therefore also be inhibited by ADP-induced autophagy. The linoleic acid phospholipid, DLPC, acts to block purinergic signaling [15;19] and stimulate both HDL and HL secretion [16]. **Figure 4.1A** shows that DLPC (12 μM) stimulates the secretion of HL from HepG2 cells at 24h, while the nucleotide, adenosine diphosphate (ADP) (100 μM) blocks HL accumulation in the media and inhibits the induction of HL secretion by DLPC. We have previously shown that ADP stimulates autophagy similar to the proteasomal inhibitor / autophagy stimulant, ALLN. We now show that ALLN also has a similar inhibitory effect on HL secretion as that observed with HDL. ALLN significantly reduced HL secretion from liver cells at 4h, (**Figure 4.1B**) and reduced cellular HL levels by ~35% (**Figure 4.1C**). ADP and ALLN therefore stimulate autophagic degradation and decrease the total HL mass (media + cell lysate), in much the same manner to that previously reported for apoA-I [15]. Conversely, inhibition of cellular autophagy significantly increased the total HL mass. To determine how inhibition of autophagy may affect HL secretion we evaluated the effect of the autophagy inhibitor, 3-methyladenine (3-MA). Treatment of liver cells with 3-MA (5 mM) significantly increased HL secretion after 4h, while treatment with both ADP and 3-MA blocked the HL secretion stimulatory effect of 3-MA (**Figure 4.2A**). Treatment with 3-MA also significantly increased cellular levels of HL (**Figure 4.2B**). This work confirms the view that both HDL and HL secretion are co-regulated [16] and inhibited by cellular autophagy [15].

Figure 4.1. Extracellular nucleotides block the induction of HL secretion. (A) HepG2 cells were pre-treated with 100 μM adenosine diphosphate (ADP) for 30 min. and then incubated with 12 μM DLPC in serum-free DMEM media for 24h. Conditioned media was collected after 24h and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P=0.05 vs. Control; ***P<0.05 vs. DLPC. **(B&C)** HepG2 cells were treated with 25μM ALLN (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal) or 100μM adenosine diphosphate (ADP) for 4h in serum-free DMEM media. **(B)** Conditioned media was collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 2 independent experiments performed in triplicate. *P<0.05 vs. Control. **(C)** Cell lysates were collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL expressed as mean ± SD of 2 independent experiments performed in triplicate. *P<0.01 vs. Control.

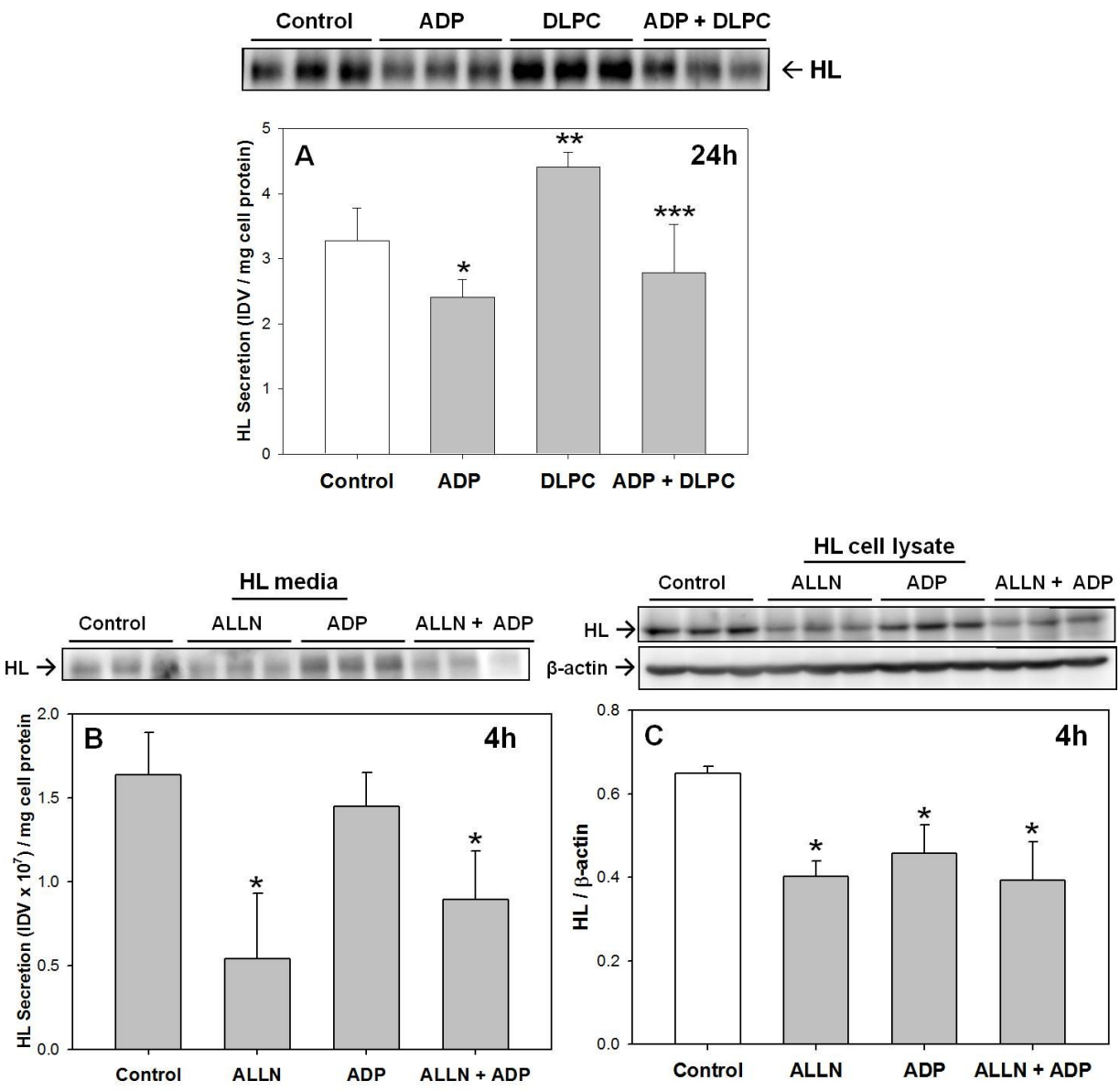
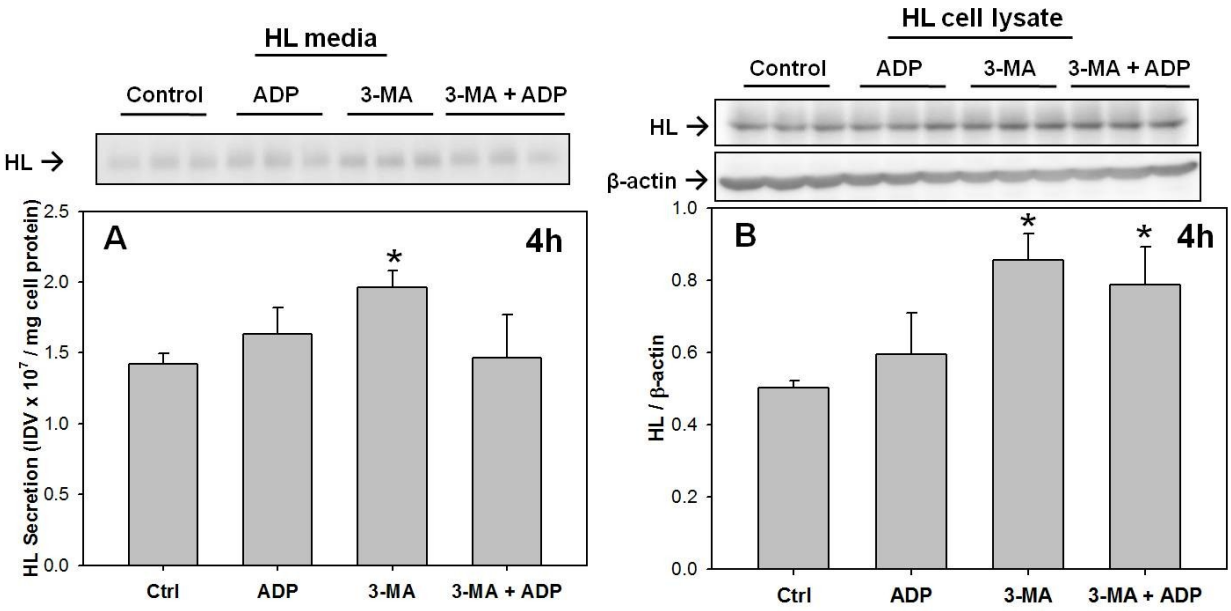

Control
ADP
DLPC
ADP + DLPC
← HL
A
24h
HL Secretion (IDV / mg cell protein)
Control
ADP
DLPC
ADP + DLPC
HL media
HL cell lysate
ALLN
ALLN + ADP
HL →
β-actin →
B
4h
HL Secretion (IDV x 10^7) / mg cell protein
C
HL / β-actin

Figure 4.2. The autophagy inhibitor, 3-methyladenine, increases HL. HepG2 cells were treated with 5mM methyladenine (3-MA) or 100μM adenosine diphosphate (ADP) for 4h in serum-free DMEM media. **(A)** Conditioned media was collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 2 independent experiments performed in triplicate. *P<0.05 vs. Control. **(B)** Cell lysates were collected and immunoblotted for HL normalized to β-actin expressed as mean ± SD of 2 independent experiments performed in triplicate. *P<0.01 vs. Control.


HL media
Control
ADP
3-MA
3-MA + ADP
HL →
A
4h
*
HL Secretion (IDV x 10^7 / mg cell protein)
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
ADP
3-MA
3-MA + ADP
HL cell lysate
Control
ADP
3-MA
3-MA + ADP
HL →
β-actin →
B
4h
*
*
HL / β-actin
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
ADP
3-MA
3-MA + ADP

Effect of $P2Y_{13}$ expression on HL secretion: The G-protein coupled receptor, $P2Y_{13}$, has been shown to regulate cell metabolism and autophagy in liver cells, and extracellular ADP stimulates autophagy through activation of $P2Y_{13}$ [15;20]. Experiments were therefore undertaken to determine the effect of $P2Y_{13}$ expression on HL secretion from liver cells. Transfecting HepG2 cells with the $P2Y_{13}$-pCMV6 plasmid promotes a 50% increase in $P2Y_{13}$ protein expression and decreased HL secretion similar to treatment with exogenous ADP (**Figure 4.3A**). Conversely, transfecting HepG2 cells with $P2Y_{13}$ siRNA promotes a 50% reduction in $P2Y_{13}$ protein expression and stimulates the secretion of HL by 5 to 8-fold (**Figure 4.3B**).

Extracellular HL uptake and metabolism: $P2Y_{13}$ has been shown to stimulate the endocytosis of HDL [20]. Therefore to determine the effect of ADP on HL endocytic pathways, the binding, uptake and metabolism of V5-epitope-tagged HL (HL-V5) was characterized. HL-V5 was isolated in cell media after transfection of HepG2 cells with a pcDNA6-HL-V5 expression vector [21]. Wild-type (untransfected) cells were then incubated with exogenous HL-V5 and the binding and internalization of HL-V5 was measured immunochemically. HepG2 cells secrete two different species of HL-V5, at 62 and 68 kDa and the V5 mAb detects both species equally (**Figure 4.4, t=0**). We have previously shown that HepG2 cells secrete two isoforms of HL [22] and earlier studies suggest the proteins differ in their extent of glycosylation [23]. **Figure 4.4** shows that only the 62kDa HL-V5 can become re-associated and taken up by the cell. The 62kDa HL-V5 is progressively internalized over time and after 24h the media becomes almost completely depleted of the protein (**Figure 4.4, upper panel**). Concomitantly, the 62kDa HL is selectively internalized into HepG2 cells and accumulates unmodified in the cell over 24h. Internalized 62kDa HL-V5 was not degraded over time, but

Figure 4.3. $P2Y_{13}$ expression regulates HL secretion. (A) HepG2 cells were transfected with either a control pCMV plasmid (pCMV) or a pCMV plasmid expressing human $P2Y_{13}$ (pCMV-P2Y13). Cell lysates were collected 48h after transfection and immunoblotted for $P2Y_{13}$ to measure protein overexpression **(upper panel)**. Conditioned media was collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs pCMV+DLPC. **(B)** HepG2 cells were transfected with either a negative control siRNA (si-ctrl) or a siRNA against human $P2Y_{13}$ (si-P2Y13). Cell lysates were collected 48h after transfection and immunoblotted for $P2Y_{13}$ to confirm protein knockdown **(upper panel)**. Conditioned media was collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.001 vs si-Ctrl, **P<0.001 vs si-Ctrl + DLPC.

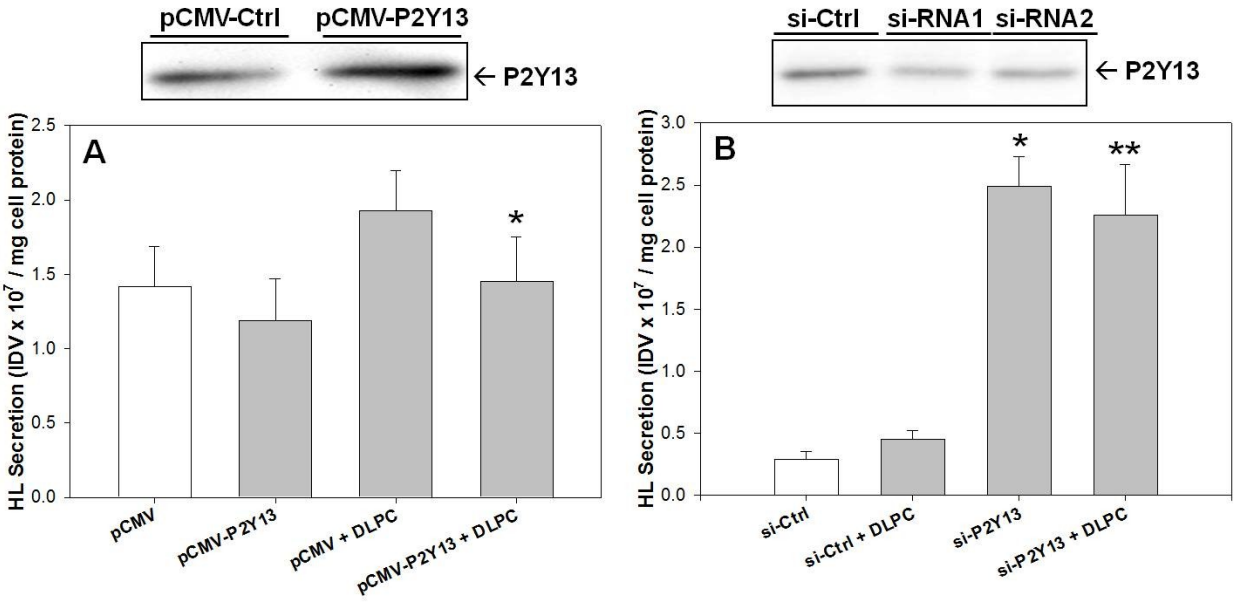
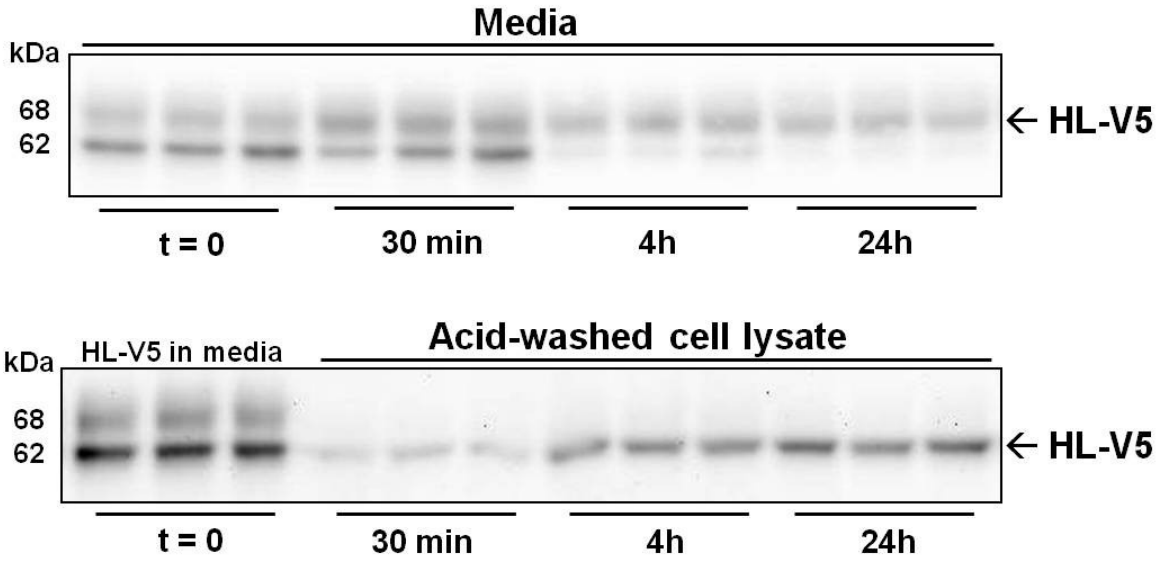

pCMV-Ctrl
pCMV-P2Y13
← P2Y13
A
HL Secretion (IDV x 10^7 / mg cell protein)
2.5
2.0
1.5
1.0
0.5
0.0
*
pCMV
pCMV-P2Y13
pCMV + DLPC
pCMV-P2Y13 + DLPC
si-Ctrl
si-RNA1
si-RNA2
← P2Y13
B
HL Secretion (IDV x 10^7 / mg cell protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
si-Ctrl
si-Ctrl + DLPC
si-P2Y13
si-P2Y13 + DLPC

Figure 4.4. The endocytic uptake of epitope-labeled HL in liver cells. HepG2 cells were transfected with either a control pCMV plasmid (pCMV) or a pCMV plasmid expressing N-terminus V5-epitope tagged human hepatic lipase (pCMV-HL-V5) for 24h. Conditioned media containing HL-V5 was collected and then added to wildtype, untransfected HepG2 cells over a 24h period to monitor the binding, internalization and re-secretion of HL. Both conditioned media **(upper panel)** and acid-washed cell lysates to measure internalization **(lower panel)** from the recipient HepG2 cells were collected at different timepoints (0.5, 4 & 24h) and immunoblotted for HL-V5. The immunoblots are representative of 2 independent experiments that were performed in triplicate.


Media
kDa
68
62
← HL-V5
t = 0
30 min
4h
24h
HL-V5 in media
Acid-washed cell lysate
kDa
68
62
← HL-V5
t = 0
30 min
4h
24h

was retained intact in the cell for 24h (**Figure 4.4, lower panel**). The effect of ADP on the metabolism of exogenous HL-V5 was then investigated. Treatment of HepG2 cells with 100μM ADP slightly reduced the total cell association of HL-V5 at 0.5h (**Figure 4.5A**), but had no effect on HL-V5 endocytosis. Treatment with ADP had no effect on the internalization of HL-V5 at 0.5h or 4h (**Figures 4.5A&B**). No HL-V5 degradation was detectable after short or long-term incubations for control or ADP treated cells.

Effect of LRP1 on HL secretion: The LRP1 receptor is thought to play a role in HL secretion [24]. We therefore evaluated the effect of ADP on the expression of LRP1 and other membrane receptors. Treatment of HepG2 cells with ADP for 24h increased both LRP1 and LDL receptor levels, but only had modest effects on SR-BI (**Figure 4.6A**). The receptor-associated protein (RAP) is thought to block LRP1 endocytosis and treatment of HepG2 cells with RAP for 24h significantly increased HL secretion (**Figure 4.6B**). This may suggest that LRP1 may affect HL secretion by controlling HL endocytosis. In contrast, **Figure 4.6C** shows that treatment with 100nM of the RAP significantly reduced the total cell association of HL-V5 with HepG2 cells, but had no effect on the internalization of HL-V5 at 30 min. To determine whether LRP1 expression affects HL secretion, HepG2 cells were treated with four different LRP1-specific siRNA. Reducing LRP1 expression by 50-90% significantly reduced HL secretion from liver cells (**Figure 4.6D**).

4.5 Discussion

Hypertriglyceridemia is a comorbidity of insulin resistance [1] and one that is thought to exacerbate perturbations in insulin signaling pathways that cause elevated blood glucose [25]. Elevations in blood triglyceride (TG) are a consequence of impaired TG-rich lipoprotein

Figure 4.5. The effect of ADP on HL endocytosis. **(A&B)** HepG2 cells were pre-treated with 100μM ADP for 30min prior to the addition of HL-V5 conditioned media for 0.5h **(A)** or 4h **(B)**. Cell lysates were collected without acid-wash for total cell association (TCA) or with acid-wash for internalization, and immunoblotted for HL-V5. Histograms represent band densitometry analysis of HL-V5 total cell association (TCA) and internalization (Int) expressed as mean ± SD of 2 independent experiments performed in triplicate. (NS = not significant).

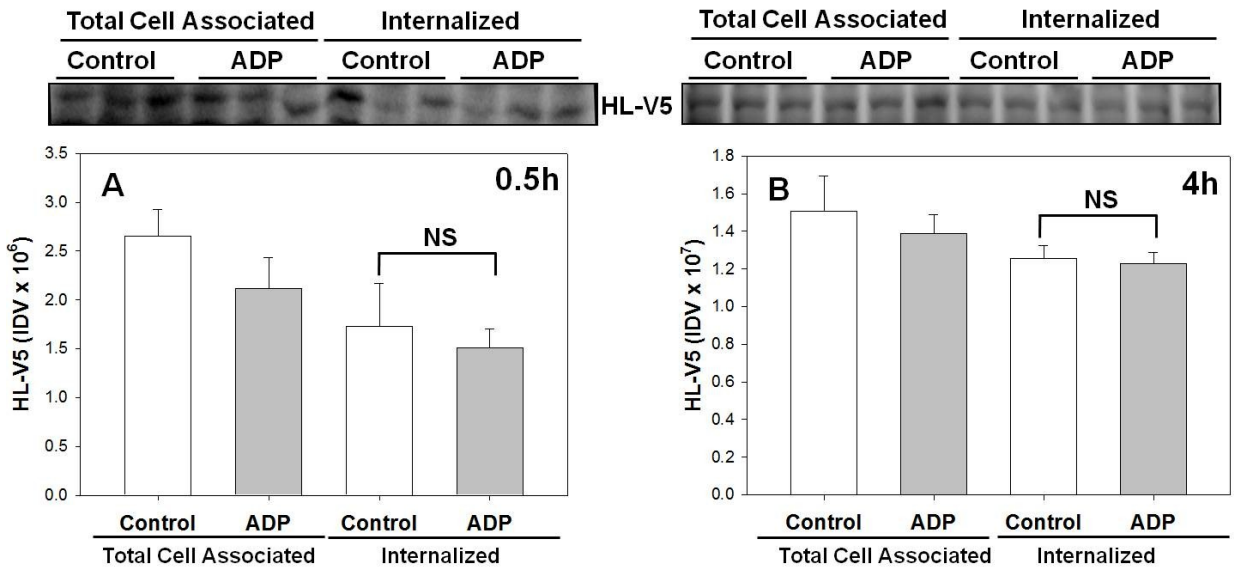
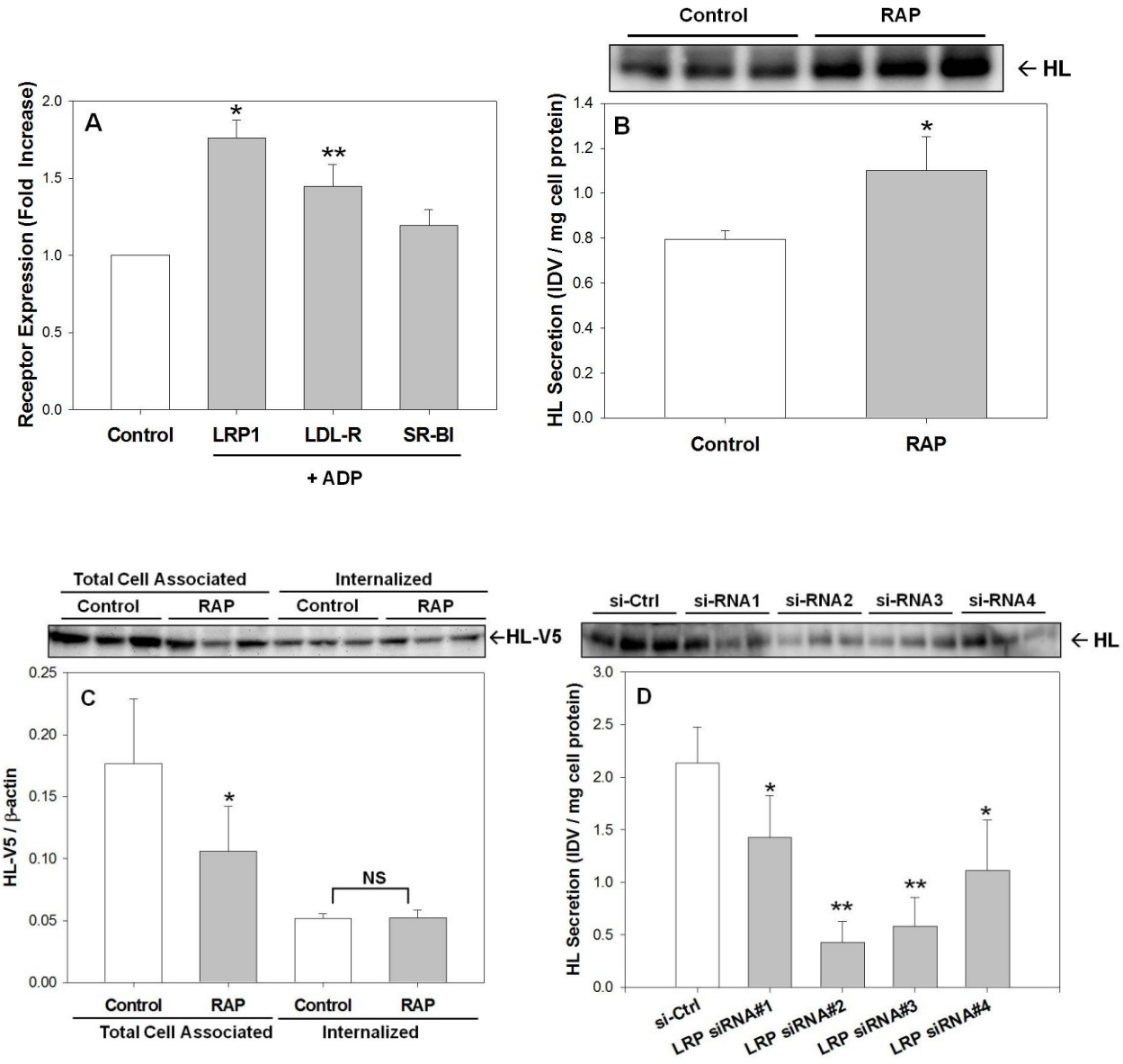

Total Cell Associated
Internalized
Control
ADP
Control
ADP
HL-V5
Total Cell Associated
Internalized
Control
ADP
Control
ADP
A
0.5h
NS
HL-V5 (IDV x 10^6)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
ADP
Control
ADP
Total Cell Associated
Internalized
B
4h
NS
HL-V5 (IDV x 10^7)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
ADP
Control
ADP
Total Cell Associated
Internalized

Figure 4.6. The effect of LRP1 on HL secretion from liver cells. (A) HepG2 cells were treated with 100μM adenosine diphosphate (ADP) for 24h in serum-free DMEM media. Cell lysates were collected and immunoblotted for LRP1, LDL-R and SR-BI. Histograms represent band densitometry analysis of LRP1, LDL-R and SR-BI normalized to β-actin and expressed as mean ± SD of 2 independent experiments performed in triplicate. *P<0.01 vs Control and **P<0.05 vs Control. **(B)** HepG2 cells were treated with 500nM receptor-associated protein (RAP) for 24h in serum-free DMEM media. Conditioned media was collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs. Control. **(C)** HepG2 cells were pre-treated with 100nM receptor-associated protein (RAP) for 30min prior to the addition of HL-V5 condition media for 30min. Cell lysates were collected without acid-wash for total cell association (TCA) or with acid-wash for internalization, and immunoblotted for HL-V5. Histograms represent band densitometry analysis of HL-V5 total cell association (TCA) and internalization (Int) normalized to β-actin and expressed as mean ± SD of 2 independent experiments performed in triplicate. (NS = not significant) **(D)** HepG2 cells were transfected with either a negative control siRNA (si-ctrl) or four different siRNAs against human LRP1 (LRP1 siRNA). Cell lysates were collected 48h after transfection and immunoblotted for LRP1 **(upper panel)**. Conditioned media was collected and immunoblotted for HL. Histograms represent band densitometry analysis of HL concentration in the media normalized to total cell protein and expressed as mean ± SD of 3 independent experiments. *P<0.05 vs si-Ctrl and **P<0.01 vs si-Ctrl.


A
Receptor Expression (Fold Increase)
2.0
1.5
1.0
0.5
0.0
*
**
Control
LRP1
LDL-R
SR-BI
+ ADP
Control
RAP
← HL
B
HL Secretion (IDV / mg cell protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
RAP
Total Cell Associated
Internalized
Control
RAP
Control
RAP
←HL-V5
C
HL-V5 / β-actin
0.25
0.20
0.15
0.10
0.05
0.00
*
NS
Control
RAP
Control
RAP
Total Cell Associated
Internalized
si-Ctrl
si-RNA1
si-RNA2
si-RNA3
si-RNA4
← HL
D
HL Secretion (IDV / mg cell protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
**
si-Ctrl
LRP siRNA#1
LRP siRNA#2
LRP siRNA#3
LRP siRNA#4

clearance [2] and over-production of VLDL [3]. Insulin resistance may also be associated with an increase in nucleotide secretion [8-10]. Increased circulating nucleotide levels may contribute to hypertriglyceridemia by increasing VLDL levels in the bloodstream [11;15]. Gene knock out studies in mice have shown that a deficiency in the NTPDase1 can cause elevated blood nucleotide levels, insulin resistance and hypertriglyceridemia [11].

VLDL-TG and apoB100 levels are elevated in insulin resistance [5;7] and this may be partly due to a stimulatory effect of extracellular nucleotides on hepatic VLDL production. Lipoprotein secretion is controlled by cellular proteolytic pathways and nucleotides act much like proteasomal inhibitors to block proteasomal degradation and stimulate autophagy [15]. Extracellular nucleotides, i.e. adenosine diphosphate (ADP), stimulate both apoB100 and apoE secretion from liver cells and thereby promote the accumulation of VLDL in the cell media [15].

Nucleotides would also be expected to promote VLDL accumulation by blocking VLDL degradation. Hepatic lipase (HL) promotes the hydrolysis and clearance of VLDL from the circulation and thereby regulates the production of LDL [4]. Circulatory VLDL levels are elevated in insulin resistance, as a consequence of reduced HL lipolytic function and impaired clearance of VLDL [5;6] We have previously shown that HL lipolytic function is regulated by HDL secretion from liver cells and by apolipoprotein exchange between HDL and VLDL [26]. HL secretion from liver cells is controlled by lysosomal degradation pathways [27] and therefore a nucleotide stimulation of autophagic-lysosomal degradation would be expected to reduce HL secretion.

As expected, treatment of human liver cells with ADP for 24h blocked both basal and DLPC-induced HL secretion (**Figure 4.1A**), much the same as that observed with ADP on

HDL secretion [15]. ADP significantly reduced cellular HL levels at 4h, much like the autophagic stimulator, ALLN (**Figure 4.1C**), but only ALLN significantly reduced HL secretion at 4h. Both ADP and ALLN had a more pronounced inhibitory effect on HDL secretion at 4h [15], which may suggest that cellular transport pathways for HDL and HL may differ. Conversely, the autophagic inhibitor, 3-MA stimulates HL secretion from liver cells (**Figure 4.2A**) and significantly increases HL levels in the cell (**Figure 4.2B**). This is strong evidence to suggest that HL secretion is sensitive to nucleotide-induced cellular autophagy and this may explain why chaperone proteins, i.e. lipase maturation factor 1, are needed to protect HL from lysosomal degradation during cellular transport [28].

We showed that ADP acts through the membrane G-protein coupled receptor, $P2Y_{13}$, to stimulate the autophagic degradation of apoA-I [15]. Since the metabolism of apoA-I in liver cells affects the secretion of HL [16], it would be expected that ADP may also act through $P2Y_{13}$ to regulate HL secretion. In agreement with this view, $P2Y_{13}$ expression affects HL secretion in much the same manner as that shown for HDL (**Figure 4.3**). A reduction in $P2Y_{13}$ expression increases HL secretion by 5 to 8-fold, while $P2Y_{13}$ over-expression only modestly suppresses HL secretion (**Figures 4.3A&B**). It is notable that gene silencing had a much greater effect on HL secretion than over-expression of $P2Y_{13}$. Similar observations were made between $P2Y_{13}$ expression and HDL secretion [15]. This appears consistent with the view that intracellular degradation pathways are normally hyperactive and most intracellular HL is targeted to degradation [27]. HL secretion may therefore be less sensitive to stimulation in autophagic degradation, but very sensitive to inhibition of degradation. Our work has clearly shown that $P2Y_{13}$ expression controls cellular autophagy by directly affecting LC3 levels [15]. This new work provides further evidence that $P2Y_{13}$ and autophagy regulate HL secretion.

To determine if purinergic stimulation of autophagy affects extracellular HL uptake and degradation, we utilized an epitope-labeled HL construct to characterize HL endocytic pathways. HL-V5 was produced in HepG2 cells [27] and then used to track the metabolism of extracellular HL in untransfected cells. HepG2 cells produce two HL-V5 isoforms at 62kDa and 68 kDa, but only the smaller 62 kDa species is taken up by the cell (**Figure 4.4**). HL-V5 is readily internalized into HepG2 cells, but neither ADP nor RAP affects the endocytosis of HL-V5 (**Figures 4.5&4.6**). Nor did these reagents affect the degradation of the endocytosed HL-V5. Proteins that were endocytosed in control and ADP treatment conditions remained intact in the HepG2 cells for up to 24h. In contrast, previous work has shown that treatment of HepG2 cells with RAP blocked endocytosis and degradation of ^{125}I-HL [29]. This may suggest that iodinated HL is degraded more rapidly and this is consistent with our previous work, which showed that iodination stimulates HDL uptake and degradation in the liver [30].

While RAP had no effect on the endocytic uptake of HL-V5, RAP treatment over 24h significantly increased HL secretion (**Figure 4.6B**), similar to that reported by others [24]. This confirms that LRP1 plays a role in HL secretion. However, if RAP acts to downregulate LRP1 endocytic degradation pathways, a reduction in LRP1 expression should stimulate HL secretion. In contrast, treating liver cells with LRP1 siRNA significantly inhibited HL secretion (**Figure 4.6C**). This may indicate that LRP1 and RAP assist in the intracellular transport of HL in liver cells and this is consistent with the view that both RAP and LRP1 have been touted to be ER chaperones [31]. The work suggests that HL secretion is not associated with extracellular HL recycling, but a consequence of autophagic degradation in lysosomes.

Elevated circulating nucleotide levels and purinergic activation in insulin resistance may therefore perturb triglyceride [15;32]. Circulating nucleotide levels are affected by nucleotide degradative enzymes and therefore, altered function of cellular nucleotidases may be implicated in insulin resistance and hyperlipidemia. Human polymorphisms in the endonucleotidase, CD39, gene were shown to be associated with type 2 diabetes and diabetic nephropathy [12]. A suppression in CD39 expression was also shown to cause insulin resistance and hypertriglyceridemia in mice [11]. These studies support the view that abnormal purinergic signaling may contribute to insulin resistance and hypertriglyceridemia [32]. Purinergic inhibitors may therefore have therapeutic value to treat metabolic and cardiovascular disease [33;34]. Inhibition of $P2Y_{12}$-dependent purinergic signaling pathways has shown significant cardiovascular therapeutic value [35;36].

4.6 Acknowledgements

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CHAPTER 5 – MANUSCRIPT#5

Purinergic Signaling, Dyslipidemia and Inflammatory Disease

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Abbreviations: ADP, adenosine diphosphate; Akt, protein kinase B; apoA-I, apolipoprotein A-I; ATP, adenosine triphosphate; CD73, ecto-5'-nucleotidase; F_1-ATP synthase, F_1-ATPase; HDL, high density lipoprotein; IF1, mitochondrial inhibitory factor 1; IR-β, insulin receptor β; LC3, microtubule-associated protein 1 light chain 3; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; NTPDase, nucleoside triphosphate diphosphohydrolase; P2X, ion channel purinergic receptor; P2Y, G-protein-coupled purinergic receptor; T2D, type 2 diabetes

5.1 Abstract

Metabolic syndrome is a compound obesity disorder, wherein the abnormal metabolism of glucose and lipid is associated with the development of chronic inflammatory diseases. The prevalence of this disease is increasing in the developed world, but the causative linkage between these metabolic disorders has remained obscure. Metabolic disease may be associated with chronic nucleotide secretion, purinergic signaling and activation of inflammatory pathways. Purinergic signaling has been implicated in impaired glucose metabolism and inflammatory disease and may contribute to dyslipidemia. Our research shows that purinergic signaling also disrupts hepatic lipoprotein metabolism by blocking insulin receptor signaling and by activating cellular autophagic pathways. Chronic stimulation of purinergic signaling may therefore be causative to glucose and lipid metabolic disorders and associated with the development of cardiovascular disease.

5.2 Introduction

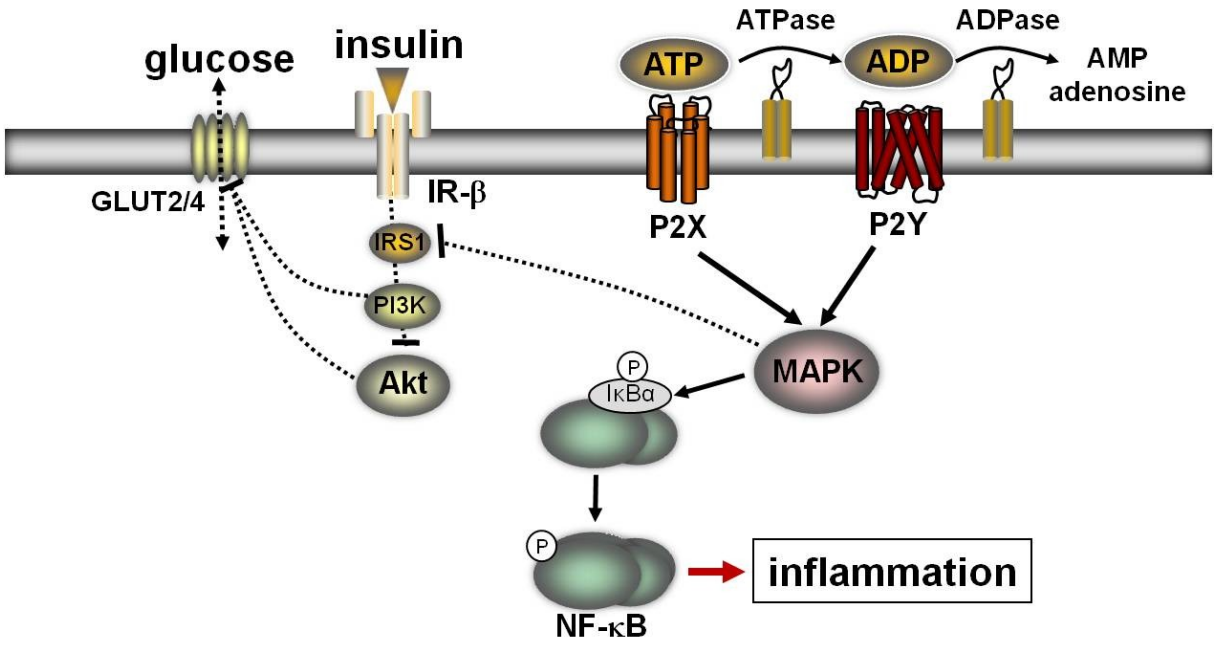
While nucleotides are well known for their important role in intracellular energy metabolism, it is now established that they also play a role as extracellular messengers to modulate the immune and inflammatory response [1-3]. In healthy tissues, extracellular nucleotide concentration is maintained at low concentrations to minimize purinergic signaling [4]. In fresh blood samples, nucleotide levels are normally in low μM concentrations [5;6], but can increase both acutely and chronically in various disease states [2;7;8]. Extracellular nucleotide levels are controlled by cellular secretion and extracellular degradation. Nucleotide secretion is affected by intracellular [Ca^{2+}] and P2X7 [9]. The gated ion channel, P2X7 receptor, has been shown to stimulate nucleotide secretion and activate both the immune and inflammatory response [3;10]. High blood glucose levels directly stimulate the release of adenosine

triphosphate (ATP) from endothelial tissues and circulating blood cells [11-13], but ATP is unstable in the circulation and is rapidly degraded to ADP, AMP and adenosine, by unique ecto-enzymes. Nucleotides are degraded by membrane ATP metabolizing proteins and specific ectonucleotidases, including NTPDase1, 2, 3, 8 (ATPase and ADPase) and CD73 (AMPase) [14;15].The extracellular nucleotide milieu acts through specific P2X and P2Y receptors to promote a purinergic signaling response (**Figure 5.1**). Short-lived nucleotide signaling may positively affect glucose metabolism by stimulating insulin secretion from pancreatic beta cells [16;17]. Sustained purinergic signaling appears to have the opposite effect and inhibits insulin receptor (IR-β) signaling [18;19] and insulin secretion [20;21]. The acute release of nucleotides, by stress or injury, also activates nuclear factor kappa B [1;22] and triggers the release of pro-inflammatory cytokines [8;23]. Chronic nucleotide signaling may therefore be involved in the pathophysiology of both metabolic and cardiovascular diseases [2;24].

5.3 Nucleotides and disease

Extracellular nucleotides are implicated in the development of coronary artery disease through a number of putative mechanisms [2;7;25] and therapeutic inhibition of purinergic signaling has a well-established utility in the treatment of cardiovascular disease [24;26]. Nucleotides activate thrombosis pathways in the bloodstream and inhibition of nucleotide-dependent platelet activation has shown significant cardiovascular therapeutic value [26;27]. Abnormal nucleotide metabolism may also contribute to the development of type 2 diabetes (T2D), metabolic syndrome, and dyslipidemia. Fibroblasts from T2D patients show 2-3 fold increase in ATP secretion [28] and enhanced inflammatory and cytotoxic responses through the P2X7 receptor [29]. P2X7 expression appears to be elevated in peripheral blood mononuclear cells from T2D patients and significantly correlated with LDL-cholesterol [30]. Adipocyte P2X7

Figure 5.1. Regulation of extracellular nucleotides and purinergic signaling pathways. Extracellular nucleotide levels are modulated by ecto-nucleotidases (ATPase and ADPase). Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) act through P2X and P2Y receptors to promote inflammation by activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB). Nucleotides can also affect glucose metabolic pathways by inhibiting insulin receptor-β (IR-β) and protein kinase B (Akt) signaling.


glucose
insulin
ATPase
ADPase
ATP
ADP
AMP
adenosine
GLUT2/4
IR-β
P2X
P2Y
IRS1
PI3K
Akt
MAPK
P
IκBα
P
NF-κB
inflammation

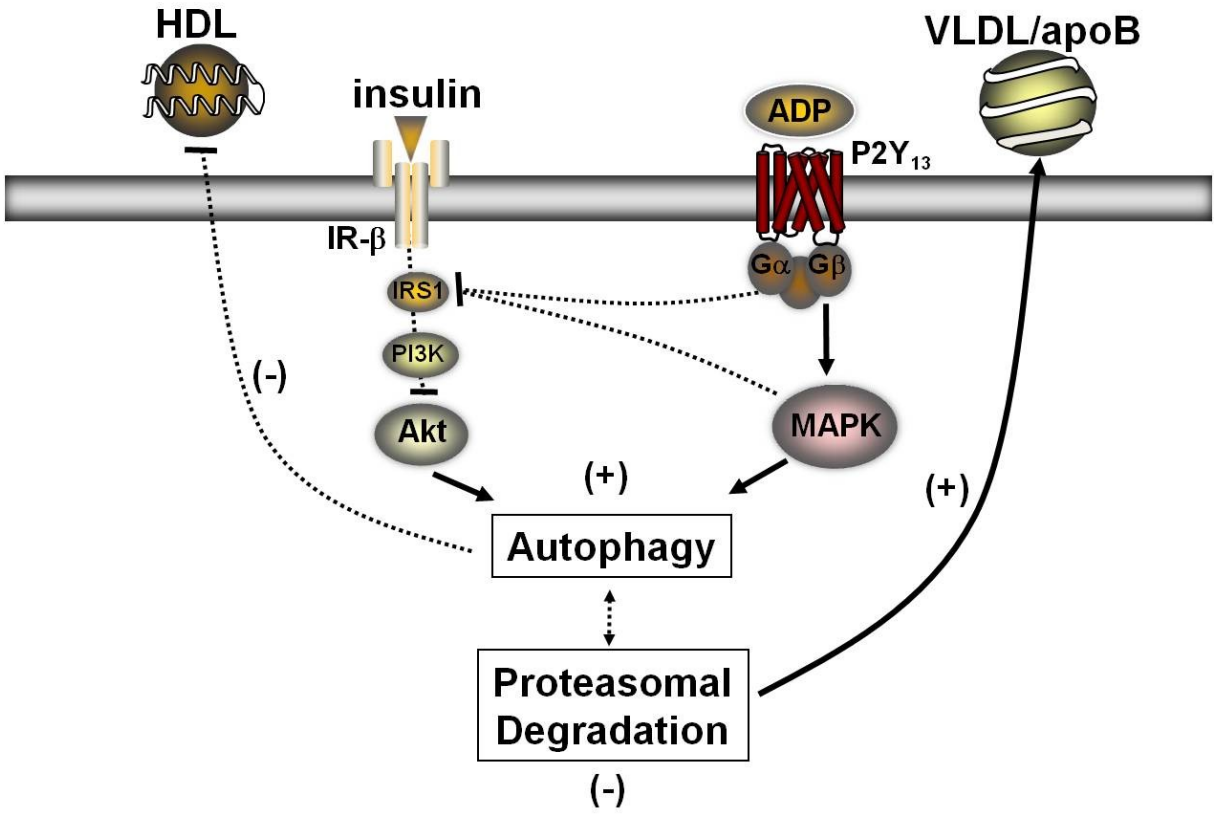
expression is elevated in patients with metabolic syndrome and associated with an enhanced inflammatory response [31]. Nucleotide degradation may also be impaired in diabetes, as polymorphisms in NTPDase1/CD39 are associated with T2D and diabetic nephropathy [32]. *In vivo* murine studies corroborate this view and show that a deficiency in CD39 can cause insulin resistance and hypertriglyceridemia [33]. Alterations in nucleotide secretion and/or degradation therefore appear to be associated with dyslipidemia, which suggests that purinergic signaling influences lipoprotein metabolism.

A role for extracellular nucleotides in plasma lipoprotein metabolism was proposed with the discovery of a plasma membrane form of F_1-ATP synthase, now called ecto-F_1-ATP synthase or F_1-ATPase. F_1-ATPase was identified to be an HDL receptor that functions to bind with apoA-I and to regulate HDL endocytosis [34]. F_1-ATPase was also shown to stimulate extracellular ADP production [35;36] and promote purinergic signaling through the G-protein coupled receptor, $P2Y_{13}$ [37;38]. F_1-ATPase is inhibited by mitochondrial inhibitory factor 1 (IF1) [34] and serum IF1 levels have been shown to be positively correlated with HDL-cholesterol levels and negatively correlated with serum triglyceride levels in normolipidemic subjects [39]. This is consistent with other work, which suggested that therapeutic modulation of circulating HDL levels may be associated with the expression of F_1-ATPase, ADP production and purinergic signaling [19;40;41].

5.4 Nucleotides, lipoproteins, and cellular protein degradation

Lipoprotein secretion is affected by proteolytic degradation pathways, which appear to be controlled by purinergic signaling [19]. Nucleotides therefore affect lipoprotein secretion by regulating intracellular protein degradation (**Figure 5.2**). Pertubations in the lipidation or folding of the LDL protein, apoB100, result in the ubiquitination and transport of the protein

Figure 5.2. Regulation of lipoprotein secretion by purinergic signaling. Extracellular nucleotides act through $P2Y_{13}$ receptors to activate MAPK, inhibit Akt and regulate cellular proteolytic pathways. A purinergic stimulation in autophagy blocks HDL secretion from liver cells and stimulates VLDL-apoB secretion.


HDL
insulin
ADP
VLDL/apoB
P2Y13
IR-β
Gα
Gβ
IRS1
PI3K
Akt
MAPK
(-)
(+)
(+)
Autophagy
Proteasomal Degradation
(-)

for proteasomal degradation [42-44]. Inhibitors of proteasomal degradation are known to stimulate apoB100 secretion from liver cells and extracellular ADP acts much like a proteasomal inhibitor to affect lipoprotein secretion [19]. Both ADP and the proteasomal inhibitor, ALLN, stimulate apoB100 secretion and inhibit HDL secretion at 4h. This appears due to a co-regulation of proteasomal and autophagic protein degradation, since proteasomal inhibitors are known to stimulate autophagy [45-48]. Proteasomal inhibitors activate autophagy and increase expression of the autophagic proteins, Atg5, Beclin-1 and Atg7 [47;48], which then gives rise to the transport and accumulation of Atg8/LC3 in autophagic vacuoles [45]. Much like proteasomal inhibitors, ADP also stimulates autophagy and significantly increases cellular LC3 levels [19].

Autophagy is a cellular stress response that promotes the lysosomal degradation of cytosolic components when stimulated by stressors, i.e. nutrient deprivation, extracellular signals, cytokines and pathogens [49;50]. ADP stimulates autophagy, increases autophagic protein levels and decreases HDL secretion from liver cells (**Figure 5.2**) in similar fashion to that observed by serum deprivation [19]. Serum deprivation is known to stimulate autophagy [49;50] and to inhibit HDL/apoA-I secretion from liver cells [51]. Both ADP and serum deprivation stimulate an autophagic response in hepatic cells and increase LC3-II and p62 levels over a three to six hour period. Confocal micrographs of ADP-treated liver cells show increased LC3 levels in punctate autophagosomes and significant colocalization of apoA-I with LC3 [19]. The work shows that HDL secretion from liver cells is inhibited by cellular autophagic pathways, which may suggest that HDL secretion and lipidation are oppositely regulated by autophagy. Autophagy is also known to stimulate ABCA1-mediated cholesterol efflux from macrophages and promote the transport and clearance of cholesterol [52].

5.5 Purinergic signaling and autophagy

Extracellular nucleotides act through membrane P2 receptors to stimulate purinergic signaling. ADP activates the P2Y receptor class and affects cellular metabolism through a stimulation of mitogen-activated protein kinase (MAPK) and inhibition of adenylate cyclase [53]. Lipoprotein secretion is directly affected by MAPK, but is less sensitive to cAMP-dependent pathways, since chemical inhibition of adenylate cyclase had no effect on HDL secretion, while blocking MAPK pathways affects both autophagy and HDL secretion [19]. MAPK signaling is known to affect cellular autophagy [50] and ADP directly stimulates MAPK pathways [19;37;54]. Insulin receptor (IR-β) signaling is also known to regulate cellular autophagic pathways [49] and ADP inhibits IR-β signaling [19]. IR-β signaling is negatively associated with autophagy, since the phosphorylation of protein kinase B (Akt) has been shown to inhibit autophagy [49;55]. ADP blocks insulin signaling and reduces both IR-β and Akt phosphorylation by ~50%, similar to that observed with tumor necrosis factor alpha. ADP therefore regulates autophagy and lipoprotein secretion through both MAPK and Akt signaling pathways (**Figure 5.2**).

Human liver cells contain two ADP-receptors, $P2Y_1$ and $P2Y_{13}$, but lipoprotein metabolism appears to be primarily affected by $P2Y_{13}$ [37;54;56]. Chemical inhibitors of $P2Y_1$ have no effect on HDL secretion, while modulation of $P2Y_{13}$ expression directly affects HDL secretion from liver cells. $P2Y_{13}$ overexpression increases cellular LC3-II levels and decreases HDL secretion, while $P2Y_{13}$ gene silencing decreases LC3-II levels and increases HDL secretion [19]. Consistent with the view that $P2Y_{13}$ expression regulates HDL secretion through cellular autophagic signaling pathways, $P2Y_{13}$ expression also regulates both MAPK and Akt signaling. A reduction in $P2Y_{13}$ expression causes a parallel reduction in ERK1/2

phosphorylation, but increases the phosphorylation of IR-β, IGF-1R and Akt. Increasing $P2Y_{13}$ expression has the opposite effect. Nucleotide signaling through $P2Y_{13}$ therefore blocks insulin receptor signaling [19] (**Figure 5.1**).

5.6 Conclusion

Lipoprotein metabolism is directly affected by nucleotides and cellular autophagy and therefore inhibition of hepatic purinergic signaling should directly affect circulating lipoprotein levels. Niacin may impact cardiovascular disease and lipoprotein metabolism through purinergic and G-protein coupled signaling pathways [40;57;58]. Niacin has been shown to increase circulating HDL levels by blocking hepatic apoA-I degradation [59]. Niacin reduces cell surface levels of F_1-ATPase in hepatocytes and thereby blocks the production of extracellular ADP [40]. Linoleic acid phospholipids also block ADP production by inhibiting the cell surface expression of F_1-ATPase and thereby stimulate HDL secretion from liver cells [41]. These phospholipids appear to mute purinergic signaling and cellular autophagy by stimulating Akt phosphorylation and blocking MAPK activation [19]. An activation of Akt and inhibition of autophagy may therefore be important in increasing plasma HDL levels. Metformin and sulfonylurea drugs are popular anti-diabetic drugs that have been well described to reduce plasma glucose levels and improve circulating HDL and triglycerides [60]. Both classes of drugs are known to stimulate Akt phosphorylation and may therefore also block cellular autophagy [61;62]. This may suggest that augmentation of insulin receptor signaling and inhibition of cellular autophagy may positively affect both glucose and lipoprotein metabolism. This dual metabolic property may represent a common mechanistic feature of both the insulin-sensitizing drugs and some therapeutics that are utilized to treat patients with disorders in lipid metabolism.

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CHAPTER 6 – DISCUSSION

6.1 Introduction: Dyslipidemia, Purinergic Signaling and Inflammatory Disease

Dyslipidemia is a common metabolic disorder and often associated with atherosclerosis and insulin-resistant states, such as T2DM. Low plasma HDL levels and elevated TG levels increase the risk of developing CHD and are also prevalent in hyperglycemic and insulin-resistant states. Hyperglycemia can also be associated with elevations in circulating nucleotides and chronic purinergic signaling can stimulate inflammatory pathways [1]. Inflammation is known to impact HDL levels [2], and membrane ATPases and purinergic receptors have also been implicated in HDL metabolism [3;4]. However, the common mechanism by which dyslipidemia may be linked to both atherosclerosis and hyperglycemia is not well understood. We therefore investigated this relationship in this thesis. Taken together, this dissertation provides novel mechanisms by which HDL and TG metabolism may be coordinated by purinergic signaling and autophagy, and with insulin receptor signaling. The significance and implications of these findings to the field will be discussed in detail.

6.2 Purinergic Signaling, Autophagy & Lipoprotein Metabolism

Studies have demonstrated that F_1-ATPase can stimulate the production of extracellular ADP and regulate the hepatic endocytosis of HDL through the $P2Y_{13}$ receptor [3-5]. The direct effect of extracellular ADP and ATP on hepatic apoA-I/HDL secretion and its relation to HL secretion was therefore examined in this study. It was demonstrated that extracellular ADP acts similar to proteasomal inhibitors and stimulates autophagy. Treatment of hepatic cells with extracellular ADP resulted in alterations in apolipoprotein and HL secretion. ApoB and apoE secretion (**Figure 3.2 B&D**) were stimulated, while apoA-I and

HL secretion (**Figure 3.1 A&B, Figure 3.2C & Figure 4.1 A&B**) were inhibited by treatment with ADP. This study is the first to demonstrate that apoA-I/HDL secretion is regulated by autophagy. We showed that the decrease in apoA-I/HDL and HL secretion with ADP treatment not only paralleled increased levels of the autophagy markers, LC3-II and p62 (**Figure 3.3 A&B**), but was also associated with increased co-localization of apoA-I with LC3 (**Figure 3.3C**). The importance of purinergic signaling in regulating apoA-I and HL secretion were further investigated by examining the effect of $P2Y_{13}$ receptor expression. $P2Y_{13}$ was shown to be the main ADP receptor that may regulate HDL endocytosis in human hepatocytes [3;4]. Our findings showed that $P2Y_{13}$ overexpression was associated with an increase in LC3-II levels (**Figure 3.4A**) and a corresponding decrease in both apoA-I (**Figure 3.4B&C**) and HL (**Figure 4.3A**) secretion. In contrast, $P2Y_{13}$ knockdown by siRNA was associated with a decrease in LC3-II levels (**Figure 3.5A**) and a corresponding increase in both apoA-I (**Figure 3.5B&C**) and HL (**Figure 4.3B**) secretion. Thus, our study demonstrated a novel linkage between a purinergic receptor and autophagy in the regulation of HDL and HL secretion. It showed that extracellular nucleotides might inhibit HDL secretion and promote the secretion of TG-rich lipoproteins like VLDL (**Figure 5.2**). Further experimentation examining the effect of extracellular nucleotides and purinergic signaling on intracellular transport and degradation of apoA-I and HL would further strengthen the link between autophagy and lipoprotein metabolism. ^{35}S-Met/Cys Pulse-Chase studies in the presence or absence of ADP or purinergic would help to further confirm that purinergic signaling induces degradation of apoA-I and HL. In addition, complementary confocal studies tracking the colocalization of apoA-I and HL with Rab proteins involved in the trafficking and recycling of proteins would demonstrate how nucleotide-induced stimulation of autophagy can divert apoA-I and HL from

secretory pathways to autophagic degradation in hepatic lysosomes. Isolation of autophagosomes from hepatocytes and co-immunoprecipitaion studies with LC3 and apoA-I or HL may also show more direct association of apoA-I and HL in autophagosomes.

The observed effects of purinergic signaling on lipoprotein and TG metabolism in the liver is in accordance with recent observations in normolipidemic human subjects, which showed that serum levels of mitochondrial inhibitory factor 1 (IF1), an inhibitor of F_1-ATPase that decreases extracellular ADP, was positively correlated with HDL-C levels and negatively with TG levels [6]. In addition, chronic stimulation of $P2Y_{13}$ with cangrelor, a partial $P2Y_{13}$ chemical agonist, decreased HDL-C levels in mice [7]. This provides evidence that purinergic signaling is important in lipoprotein metabolism and supports the idea that HDL and TG metabolism may be co-regulated.

6.3 Co-Regulation of High-Density Lipoprotein & Hepatic Lipase Secretion

It has been known for many decades that there exists an inverse relationship between plasma HDL-C and TG levels such that high HDL-C is often associated with low TG levels in humans and reduced risk of developing CHD [8]. This association suggested a possible role for HDL in affecting TG metabolism and studies have implicated a role for HDL composition in regulating the TG lipase, HL. Early work from our laboratory demonstrated the importance of HDL lipid and apolipoprotein composition in regulating HL release from HSPG and HL lipolytic activity [9-14]. This thesis therefore set forth to examine the hepatic regulation and coordination of this process and evaluate how endogenous HDL secretion may in turn impact HL secretion.

To determine how apoA-I metabolism may affect HL secretion, apoA-I overexpression and siRNA knockdown studies were conducted in hepatic cells. ApoA-I

siRNA knockdown resulted in a parallel decrease of both apoA-I and HL secretion (**Figure 2.9**). Chemical inhibition of PPARα and MAPK pathways that are known to affect HDL also showed corresponding decreases in both apoA-I and HL secretion (**Figure 2.5**). It would also be interesting to determine whether exogenous apoA-I or conditioned media from DLPC-treated HepG2 cells would be able to rescue the inhibition of HL secretion by the PPARα and MAPK chemical inhibitors, and would provide more direct evidence that apoA-I is the cause of HL release. The secreted HL was associated with large HDL complexes containing both apoA-I and apoA-II (**Figure 2.4**). The association of secreted HL with apoA-II may explain why HL showed no lipolytic activity, since apoA-II increases the liberation of HL from HSPG [13], but it also inhibits HL catalytic activity [12]. This appears to be a regulatory mechanism by which lipolysis can be controlled, wherein dissociation of HL from HDL is necessary for HL-mediated lipolysis [15]. Altogether these results indicate that HDL and HL metabolism are co-regulated and that HDL is a key mediator in TG metabolism. We now know that HDL and HL may be co-regulated by both cell surface displacement events and cellular degradation. It is important to note that HL also has an established role in regulating HDL levels by hydrolyzing postprandial TG-rich HDL [16;17]. Under normal metabolic conditions, it is likely that this co-regulation of HDL and HL is needed to the manage lipid storage and degradative pathways. We now provide additional evidence to suggest that both processes are also regulated by autophagy, a proteolytic degradative pathway. Abnormalities in both HDL and TG metabolism in dyslipidemia may therefore be associated with purinergic perturbations in the systemic regulation of protein degradation. HDL plays an important role in the liberation and activation of HL, but we now have evidence to suggest that a purinergic response redirects both apoA-I and HL from secretory pathways to autophagic degradation.

We also showed that the linoleic acid phospholipid, dilinoleoylphosphatidylcholine (DLPC), stimulates apoA-I/HDL secretion from human hepatocytes, by affecting intracellular signaling and degradation pathways [18;19]. My investigations show that DLPC had a similar effect on HL. Treatment of both HepG2 cells and primary human hepatocytes with DLPC resulted in a time-dependent increase in both apoA-I and HL secretion (**Figure 2.3**). The stimulation in both HDL and HL secretion was associated with the linoleic acid content of the phospholipid. Linoleic acid alone was unable to stimulate secretion of apoA-I or HL, while DLPC (two linoleic acid chains) was more potent than PLPC (one linoleic acid chain) at stimulating apoA-I secretion (**Figure 2.1**). The mechanism through which linoleoyl phospholipids stimulate apoA-I secretion is not well understood but may involve the actions of phospholipase C (PLC) and cytosolic phospholipase A2 (cPLA2) [19;20]. Linoleic acid is anti-inflammatory and can inhibit NF-κB activation, unlike saturated fatty acids like palmitate [21]. Conjugated linoleic acid may also improve glucose tolerance and insulin sensitivity in obese mice [22]. Therefore, linoleoyl specific phospholipids may have a therapeutic role in lipoprotein metabolism and metabolic disease.

The increase in HL secretion by DLPC was associated with no change in HL mRNA or cellular protein levels, similar to that seen previously with apoA-I (**Figure 2.7 and Figure 2.2**) [18;19]. Pulse-Chase experiments with ^{35}S-Met/Cys examining the effect of DLPC on apoA-I and HL synthesis and degradation would also provide more insight into the mechanism by which DLPC stimulates apoA-I and HL secretion. The stimulation of both apoA-I and HL secretion by DLPC instead appeared related to its ability to decrease membrane F_1-ATPase levels [20]. The data suggested that DLPC may act to reduce extracellular ADP levels and decrease the intracellular degradation of apoA-I and HL. The

corollary was indeed seen with ADP pretreatment of hepatic cells, which resulted in the inhibition of the DLPC-induced stimulation of both apoA-I (**Figure 3.1A&B**) and HL secretion (**Figure 4.1A**) and a stimulation of autophagic degradation pathways (**Figure 3.3 & 3.7A**). The co-regulation of HDL and HL by purinergic signaling was further confirmed with $P2Y_{13}$ overexpression and knockdown studies, which showed $P2Y_{13}$ expression regulates both autophagy and apoA-I and HL secretion (**Figure 3.4, 3.5 and 4.3**). It is noteworthy that siRNA knockdown of $P2Y_{13}$ had a greater effect on both apoA-I and HL secretion than overexpression of $P2Y_{13}$. This may a consequence of intracellular degradation pathways being normally hyperactive, whereby apoA-I and HL secretion may be less sensitive to a stimulation of degradation, but very sensitive to an inhibition of degradation [23].

6.4 Lipoprotein Metabolism, Protein Transport & Degradation

The net secretion of proteins from the liver is dependent on both the synthesis and degradation of the protein. Since our studies with DLPC showed no increase in apoA-I or HL at the mRNA and cellular level, we evaluated the role of transport and degradation (**Figure 2.7 and Figure 2.2**) [18;19]. The potential proteolytic degradation processes involved in lipoprotein metabolism include proteasomal degradation, lysosomal degradation and autophagy and there is known interplay between these degradation pathways [24-27]. It might be expected that there would be some coordination between the different degradation pathways in the cell, wherein if one degradation pathway is inhibited, proteins would be shuttled to another degradation pathway. This would prevent the accumulation of unnecessary proteins that might have detrimental consequences [28-30]. As such, it is known that inhibition of proteasomal degradation stimulates autophagy [26;27]. Our results confirm this view and highlight the crosstalk between proteasomal and autophagic degradation.

The role of degradation in lipoprotein metabolism has been best characterized for VLDL secretion, which is regulated by both proteasomal degradation and autophagy. The main apolipoproteins on VLDL, apoB100 and apoE are shown to undergo both ubiquitin-dependent and –independent proteasomal degradation [31-33]. More recent studies have also showed the role of autophagic degradation in regulating apoB100 [34;35]. Autophagy is thought to play a role in tracking the lipidation of apoB. Studies have suggested that proteasomal degradation plays a major role in the quality control of apoB (pre-VLDL) in the ER, whereas autophagy may be more important post-translationally after lipidation in the Golgi [36]. However, while this work suggests that autophagy may inhibit apoB100 secretion [34;35;37;38], our study shows quite the opposite. An induction of autophagy was associated with an increase in apoB secretion (**Figure 3.2A&D)**. While there are notable differences between the cell type/species and experimental protocols utilized, our data is completely consistent with the proteasomal inhibitor data [31]. This is not surprising given that studies have demonstrated that proteasomal inhibition can stimulate autophagy [26;27]. ADP and ALLN both appear to stimulate autophagy, inhibit the proteasome and stimulate apoB secretion. This may partly explain why insulin resistance is associated with elevated apoB levels. Insulin-resistance is known to cause an overproduction of VLDL, which is partly a consequence of a decrease in insulin-mediated apoB degradation [38]. Insulin resistance may also stimulate circulating nucleotide levels, increase purinergic signaling, and inhibit Akt activation [39-42]. This may result in a stimulation in autophagy, inhibition of proteasome and a stimulation in apoB/VLDL secretion [43]. Hepatic cells therefore have a complex mechanism to coordinate the different degradation pathways and regulate apoB levels.

Our study clearly illustrated the crosstalk between autophagy and proteasomal degradation, but also implicated a new role for autophagy in mediating apoA-I/HDL secretion. HDL biogenesis involves hepatic secretory pathways and maturation pathways that coordinate the lipidation of apoA-I. The mechanism through which HDL maturation is controlled has remained elusive, but is thought to involve the internalization and lipidation of apoA-I [44;45]. HDL internalization is associated with the localization of apoA-I in endosomes and recycling endosomes containing apoE [44;46]. The biogenesis of HDL and its lipidation involving ABCA1 is modulated by calpain-mediated intracellular degradation [47-49]. HL maturation and secretion is also dependent on proteasomal degradation mediated by the ER [50-52], but the role of autophagy in HL and HDL secretion has not been investigated until now.

Given that purinergic signaling has been shown to affect the internalization and endocytosis of HDL in hepatocytes [3-5], we investigated the role of purinergic signaling on the endocytosis of HDL and HL using a novel epitope tagged endocytosis assay. The endocytic uptake of exogenous HL-V5 in wildtype hepatic cells showed the progressive internalization and accumulation of HL-V5 in the cell over the 24h with a corresponding decrease of HL-V5 in the media (**Figure 4.4**). This indicated that there was no degradation of HL-V5 since there were no smaller molecular weight degradation products detected by the V5 antibody. In the presence of ADP, there was no significant change in HL-V5 total cell association or internalization at 30min or 4h (**Figure 4.5**) suggesting that ADP does not affect HL endocytosis. We also made similar observations with a DDK-epitope tagged apoA-I construct, which showed no change in apoA-I-DDK total cell association, internalization or degradation with ADP (data not shown). This is in agreement with studies that have demonstrated exogenous apoA-I is recycled and not degraded [44;45]. Together this suggests that nucleotides

do not impact secretion through endocytic pathways, but may regulate the secretion of hepatic apoA-I and HL through a nucleotide-induced autophagic response that diverts these proteins from secretory pathways to lysosomal degradation.

ADP significantly increased the expression of both the LDL-R and LRP1 with 24 h treatment (**Figure 4.6A**). The endocytosis of both HL and LPL in hepatocytes is mediated by LRP and HSPG [53-55], thus we examined the effect of LRP1 and its inhibitor, RAP, on the modulation of HL secretion. We showed that as previously reported [53], treatment of hepatic cells with RAP for 24h stimulated HL secretion (**Figure 4.6B**). While endocytosis studies using ^{125}I-HL showed that RAP inhibited ^{125}I-HL endocytosis and degradation [55], RAP did not have a significant effect on HL-V5 internalization, but decreased the total cell association of HL-V5 **(Figure 4.6C)**. This difference may be attributed to altered cellular metabolism of ^{125}I versus V5-tagged HL. Our laboratory has previous shown that iodinated proteins are not metabolized normally in liver cells [56]. LRP siRNA knockdown studies were then conducted to determine if reducing LRP expression would stimulate HL secretion. We saw quite the reverse. LRP knockdown almost completely blocked HL secretion (**Figure 4.6D**). This suggests that LRP and RAP may regulate HL secretion by facilitating the intracellular transport of HL to the secretory pathway. This is in agreement with work that has demonstrated that LRP and RAP play roles as ER chaperones in addition to their role in endocytosis [57]. In summary, this thesis emphasizes the importance of transport and autophagic degradative pathways over recycling pathways in regulating HDL and HL secretion.

6.5 Effect of Purinergic Signaling on Insulin Receptor Signaling

The effects of starvation and insulin signaling on autophagy were first identified in the 1960s. Insulin was shown to inhibit autophagy whereas starvation and glucagon had the

opposite effect and stimulated autophagy [58]. During starvation conditions, when blood glucose levels decrease, glucagon is secreted by the pancreas to elevate blood glucose and fatty acid levels. Glucagon promotes catabolic reactions and therefore cells induce proteolytic pathways such as autophagy to regulate energy homeostasis. Work by Ranganathan and Kottke demonstrated that serum starvation inhibits apoA-I secretion from HepG2 cells as early as 1h [59]. Serum deprivation is known to induce cellular stress and autophagic responses [60], which may both be associated with increased exogenous ADP levels [61]. Our studies showed that ADP-mediated autophagy inhibited apoA-I and HL secretion from liver cells, in a very similar fashion to serum starvation. Serum is comprised of a plethora of components including albumin, growth factors and hormones, i.e. insulin [62;63]. Insulin receptor signaling and autophagy both share the PI3K/Akt and MAPK signaling pathways and therefore insufficient insulin during serum starvation may inhibit Akt phosphorylation and stimulate autophagy similar to that shown with ADP treatment.

Both ADP treatment and $P2Y_{13}$ expression inhibited insulin receptor signaling by blocking IR-β and Akt phosphorylation (**Figure 3.7 & Figure S3.6**) compared to serum-starved control cells. $P2Y_{13}$ knockdown had the opposite effect and stimulated IR-β and Akt phosphorylation even in the absence of insulin stimulation suggesting that modulation of $P2Y_{13}$ alone may be novel regulator of the insulin receptor and insulin signaling (**Figure 3.7**). Moreover, ADP treatment stimulated MAPK signaling by increasing ERK1/2 phosphorylation, whereas $P2Y_{13}$ knockdown inhibited ERK1/2 phosphorylation (**Figure 3.6**). Purinergic-mediated PI3K and/or MAPK signaling has been demonstrated in vascular smooth muscle cells and HUVECs, but not in hepatocytes [64;65]. We showed in human hepatic cells that ADP-mediated purinergic signaling inhibits PI3K-mediated Akt phosphorylation and

increases MAPK signaling. Studies have also shown that ADP induced $P2Y_{13}$ activation can inhibit insulin secretion in mouse pancreatic beta cells [66;67]. Our findings are consistent with these studies, and show how chronic purinergic signaling negatively impacts insulin receptor signaling and may lead to insulin resistance and diabetes.

Purinergic signaling is dependent on the availability of circulating nucleotides, which is a consequence of the net cellular secretion and extracellular degradation by specific ecto-nucleotidases (NTPDases). Single nucleotide polymorphisms in human NTPDase1 or CD39 were associated with T2DM and diabetic nephropathy [68]. Similarly, a deficiency in CD39 in mice was shown to be associated with the development of insulin resistance and hypertriglyceridemia [40]. Peripheral blood mononuclear cells from T2DM patients showed increased CD39 and P2X7 expression which was associated with glycemia and correlated with LDL-C [69]. These studies corroborate our results and show an association between purinergic signaling, insulin resistance/diabetes and dyslipidemia. However, our results provide a mechanism by which this may occur. Our studies show purinergic signaling impacts insulin receptor signaling and autophagy, which in turn regulates lipoprotein metabolism. We showed that enhanced purinergic signaling promotes an inhibition of proteasomal degradation and a chronic induction of cellular autophagy. The net result is a stimulation of apoB100 secretion from the liver and a reduction in apoA-I secretion (**Figure 5.2**). This may partly explain the well-described high VLDL and low HDL phenotype associated with insulin resistance.

Studies have demonstrated that stress, injury and disease can promote increased secretion of extracellular nucleotides from cells. Circulating nucleotides are known to impact the immune system and increase inflammation by stimulating NF-κB signaling and the

secretion of pro-inflammatory cytokines [61;70-72]. Low HDL, hypertriglyceridemia, and inflammation are common phenotypes observed in metabolic and cardiovascular diseases. We showed that ADP is similar to the cytokine, TNFα, both of which reduce the insulin-induced phosphorylation of Akt (**Figure 3.7B**). $P2Y_{13}$ knockdown reversed the inhibitory effect of TNFα and ADP on IR-β and Akt phosphorylation (**Figure 3.7C&D).** TNFα is a well described pro-inflammatory cytokine that activates NF-κB [73;74] and we have also showed that like TNFα, ADP is able to also promote NF-κB phosphorylation (data not shown). Altogether, our results indicate that inhibition of purinergic signaling in the liver can reverse the inhibitory effects of both nucleotides and inflammatory cytokines on insulin receptor signaling. Consequently, purinergic inhibitors may be able to increase HDL and HL secretion and prevent activation of inflammatory pathways, which may have value in the treatment of metabolic and cardiovascular disease [75;76].

6.6 Conclusion

In conclusion, this dissertation demonstrates a novel mechanism by which HDL and TG metabolism may be coordinated by purinergic and insulin receptor signaling. Nucleotides appear to regulate cellular protein transport and autophagy and control the secretion of apoA-I and HL. The work shows that HDL and HL secretion may be co-regulated by autophagy and may partly explain the inverse relationship between HDL and TG levels in human plasma [8]. It also may help explain why drugs used in the treatment of diabetes such as metformin and sulfonylureas compounds are able to reduce plasma glucose levels and improve circulating HDL and TG levels [77]. These drugs stimulate the phosphorylation of Akt and may thus inhibit autophagy [78;79]. Stimulation of both insulin receptor signaling and inhibition of autophagy may improve both glucose and lipoprotein metabolism. This work provides the first

mechanistic hypothesis to explain how purinergic signaling may 1) perturb lipoprotein metabolism, 2) block insulin receptor signaling & glucose uptake, and 3) stimulate inflammatory pathways. This research suggests that enhanced purinergic signaling may be partly causative to insulin resistance, dyslipidemia and coronary heart disease. These findings may aid in the prevention and development of new therapeutic strategies for both glucose and lipid metabolic disorders.

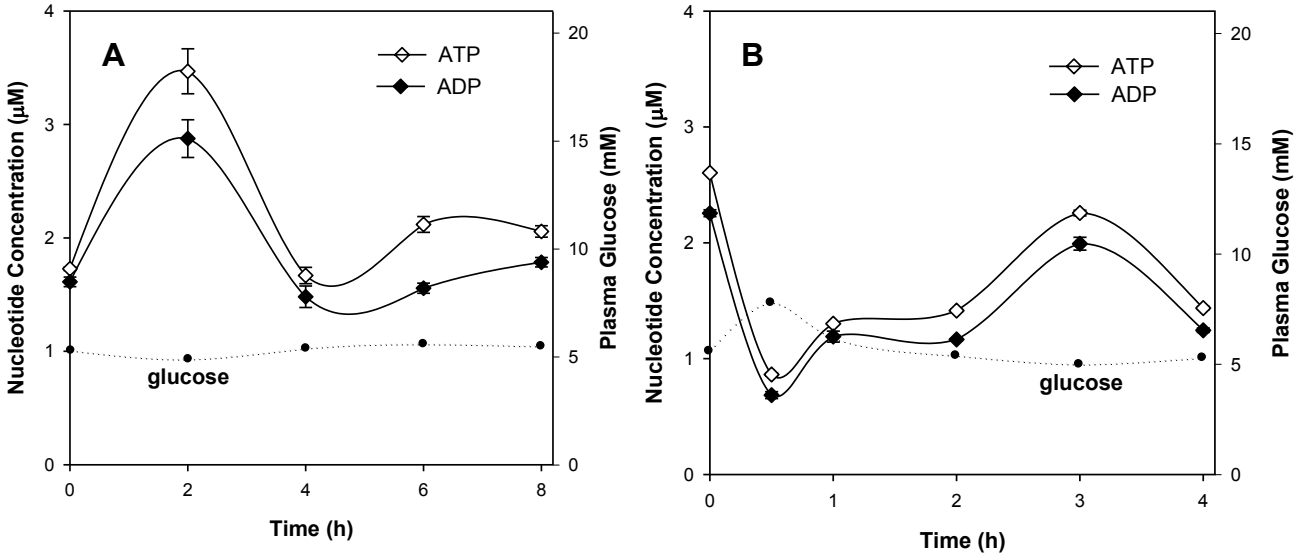
6.7 Future Directions

This thesis had provided many new insights into the role of purinergic signaling in the modulation of insulin receptor signaling and lipoprotein metabolism. However, many questions still remain to be addressed in future studies. Further experimentation should investigate how purinergic stimulation of autophagy can divert apoA-I and HL from secretory pathways to autophagic degradation in lysosomes of hepatocytes. Experiments are underway to characterize the kinetics of protein transport and degradation conducting classical radioactive pulse-chase experiments with purinergic stimulation or inhibition. We are also tracking the colocalization of apoA-I and HL with different Rab proteins that are involved in the trafficking and recycling of proteins by confocal microscopy. Future studies will also examine the role of other hepatic P2 receptors, namely P2X4 and P2X7, which are activated by ATP, in insulin receptor signaling and autophagy and their coordination with $P2Y_{13}$ in lipoprotein metabolism. It will also be important to identify the major hepatic ecto-nucleotidases that regulate extracellular nucleotide levels.

Since insulin resistance and diabetes are complex metabolic diseases that affect multiple tissues including the liver, pancreas, adipose tissue, and muscle, it would be informative to determine the impact of purinergic signaling in each of these cell types and

their contribution to metabolic disease. Most importantly, it is necessary to corroborate the significance of these findings *in vivo* in human subjects, to clarify their importance to human disease. It is well described that ADP-mediated purinergic signaling plays a critical role in thrombosis, and thus drugs targeting $P2Y_{12}$ receptor inhibition have been effective in preventing CHD [80;81]. However, little is known about the factors that regulate nucleotide levels in the bloodstream. It would therefore be important to determine the effect of diet on the postprandial circulating nucleotide response in normal subjects, diabetics, and hyperlipidemics. A preliminary study performed in a normal glycemic/lipidemic subject showed that while plasma ATP and ADP levels increased 2-fold and peaked 2 hours after a 600 calorie meal, plasma nucleotide levels fell to <50% basal levels for ~ 2 hours after a partial oral glucose tolerance test (**Figure 6.1**). This may suggest that fat and/or protein in the diet may affect plasma nucleotides levels differently than glucose. Further experimental work is needed to evaluate the effect of glucose, carbohydrates, fat, and protein on nucleotide levels in the bloodstream and the contribution of ecto-nucleotidase expression and activity in human plasma. These human studies should provide a greater understanding of the role of diet to the development of diabetes and coronary heart disease, and aid in developing novel treatment strategies.

Figure 6.1. Plasma nucleotide levels increase during a postprandial response. (A) In a normal human subject, ATP & ADP levels in the plasma increase 2-fold, peak 2 hours after a 600-calorie meal, and then return to basal levels by 4 hours. **(B)** In contrast, a partial (37.5g glucose) oral glucose tolerance test in a normal subject causes a significant reduction in plasma nucleotide levels, which lasted for up to 3 hours.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Plasma Glucose (mM)
Time (h)
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ATP
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Plasma Glucose (mM)
Time (h)

6.8 References

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Publications:

1. Sparks, D.L. and **C. Chatterjee** (2012) Purinergic Signaling, Dyslipidemia and Inflammatory Disease. Cell Physiology and Biochemistry 30(6):1333-39.

2. **Chatterjee, C.** and D.L. Sparks (2012) Extracellular nucleotides inhibit insulin receptor signaling, stimulate autophagy and control lipoprotein secretion. PLoS One 7(5):e36916.

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Published Abstracts:

1. **Chatterjee, C.,** and D.L. (2010) Sparks. Hepatic Lipase Secretion is Affected by Hepatocyte ApoE Metabolism. Arterioscer. Thromb Vasc. Biol. 30(11): e281, P520.

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Conference Presentations:

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