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Effects of Phosphatidylinositol on ApoA-I Metabolism: Implications in HDL Metabolism

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Effects of Phosphatidylinositol on ApoA-I Metabolism: Implications in HDL Metabolism

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Abstract

Coronary heart disease (CHD) is the leading cause of morbidity and mortality in the developed world. Attempts to prevent CHD using LDL lowering medications have been only partly successful and new approaches are under investigation. Significant efforts are being made to develop therapeutics that raise plasma HDL levels to aid in the prevention of CHD. HDL levels are believed to be inversely associated with the risk of developing CHD. Naturally occurring phospholipids such as phosphatidylinositol (PI), have been shown to increase plasma apoA-I levels and HDL levels in animal models and human subjects; but the mechanism remains to be elucidated. Since in humans, HDL is primarily synthesized in the liver, the objective of the present study was to evaluate the underlying molecular mechanism of PI-induced apoA-I and HDL secretion from liver cells. We show that PI doubles apoA-I/HDL secretion at 24h in a model hepatocyte, HepG2, cell culture system. PI-induced apoA-I secretion is unaffected by PI-3-kinase inhibitors but is sensitive to various MAP kinase inhibitors. While the p38MAPK inhibitor SB203580 has no effect on PI-induced apoA-I secretion, the MEK1/2 inhibitor U0126 blocks PI-induced apoA-I secretion. Inhibition of the JNK MAPK pathway by SP600125 also blocks PI mediated apoA-I secretion. Real-time PCR shows no changes in cellular apoA-I mRNA and suggests that PI is not impacting the transcription of the apoA-I gene. However, the degradation of apoA-I is decreased in PI treated HepG2 cells. Collectively, the data from these investigations suggest that PI acts through mitogen and stress-activated protein kinase pathways to increase plasma apoA-I levels by decreasing the degradation of apoA-I.

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List of Abbreviations

ABCAI: ATP-Binding Cassette transporter type AI
ABCGI: ATP-Binding Cassette transporter type GI
ACAT-1: Acyl CoA:cholesterol Acyltransferase-1
ANOVA: Analysis of Variance
Apo: Apolipoprotein
ARP-1: ApoA-I Repressor Protein-1
CE: Cholesterol Ester
CETP: Cholesterol Ester Transfer Protein
CHD: Coronary Heart Disease
CM: Chylomicron
DLPC: 1,2-dilinoleoyl-sn-glycero-3-phosphocholine
DMPC: 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DOPA: 1,2-dioleoyl-sn-glycero-3-phosphate
ERK1/2: Extracellular-regulated protein kinase 1 and 2
FC: Free Cholesterol
HDL: High Density Lipoprotein
HepG2: Human Hepatoblastoma Cell Line
HNF-4: Hepatocyte Nuclear Factor-4
IDL: Intermediate Density Lipoprotein
IRCE: Insulin Response Core Element
JNK: c-Jun-N-terminal kinase
LCAT: Lecithin:Cholesterol-acyltransferase
LDL: Low Density Lipoprotein
LDLr: LDL receptor
MAPK: Mitogen-activated Protein Kinase
MEK: MAPK kinase
MTP: Microsomal Triglyceride Transfer Protein
PA: Phosphatidic Acid
PC: Phosphatidylcholine
PCR: Polymerase Chain Reaction
PE: Phosphatidylethanolamine
PI: Phosphatidylinositol
PI3K: Phosphatidylinositol-3-kinase
PL: Phospholipid
PLC: Phospholipase C
POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PPAR: Peroxisome-Proliferator Activator Receptor
PS: Phosphatidylserine
RTK: Receptor Tyrosine Kinase
SP1: Specific Protein-1
SR-A: Scavenger Receptor-A
SR-BI: Scavenger Receptor-BI
TG: Triglyceride

VCAM-1: Vascular Cell Adhesion Molecule 1
VLDL: Very Low Density Lipoprotein

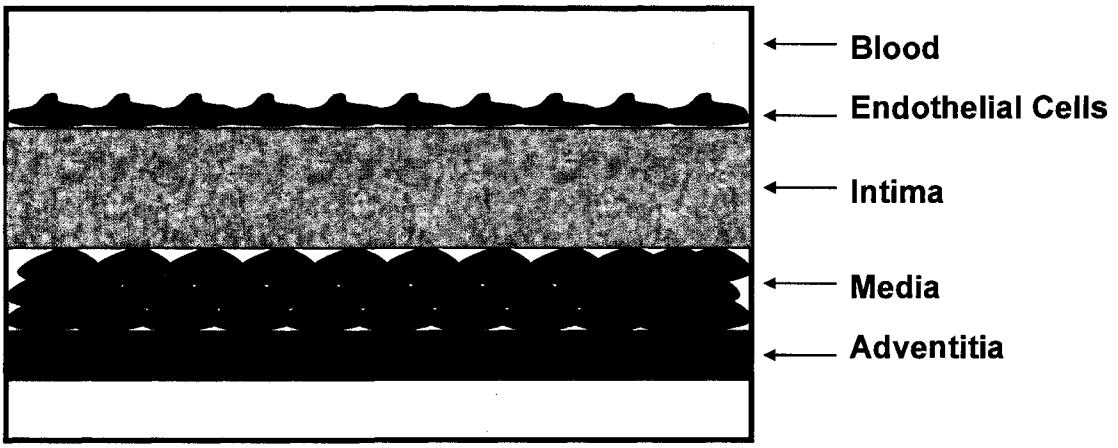
Chapter 1 – Introduction

1.1 Atherosclerosis: Inflammation and lipid accumulation

Coronary heart disease (CHD) is the leading cause of morbidity and mortality in the developed world. The primary cause of heart disease is atherosclerosis. This is a progressive disease of the large arteries which is characterized by accumulation of lipids and inflammation in the arteries. Much of the evidence regarding atherosclerotic plaque development has been found using a variety of animal models. Specifically, mice models deficient in apolipoprotein E (apoE) or the low-density lipoprotein receptor (LDLr) have been the most popular models for studying atherosclerosis (1). Unfortunately, atherosclerosis is a progressive disease which can be seen in development from birth. This constant progression can have lethal consequences for many people who are susceptible to heart disease. It is now known that atherosclerosis is not only a consequence of ageing, but an inflammatory condition which can create artery rupture and thrombosis. Figure 1.1.1 illustrates the structure of an artery and the major sites where the process of atherosclerosis occurs.

The primary stage of atherosclerosis is called lesion formation. The lesions begin as fatty streaks under the endothelial cells of the large arteries. The endothelium is composed of a complex of tight junctions which enable the selective uptake between blood and tissue. The initial stages are propagated by macrophages and their ability to take up cholesterol derived from oxidized-LDL (2). Areas of the artery which are in the area of branching or curvature, where flow is disturbed, have an increased permeability of oxidized-LDL and therefore more incidence of fatty streak formation

Figure 1.1.1 : Structure of a normal large artery
A large artery consists of three distinct layers. The intima, the innermost layer, is bound by a monolayer of endothelial cells on the luminal side, and internal elastic lamina on the peripheral side. It is composed of mostly proteoglycans and collagen. The middle layer, the media, consists of smooth muscle cells. The adventitia, the outer layer, consists of connective tissue with fibroblasts and smooth muscle cells (114).


Blood
Endothelial Cells
Intima
Media
Adventitia

(3). LDL can passively diffuse through the endothelium and get trapped in the artery wall where it can be susceptible to modifications including oxidation (4, 5).

The next stage of atherosclerosis is inflammatory. This stage is marked by the recruitment of monocytes and leukocytes to the artery wall. This process is triggered by the aforementioned oxidized-LDL accumulation in the artery wall. The oxidized-LDL stimulates the production of pro-inflammatory molecules from endothelial cells such as adhesion molecules and growth factors. One such adhesion molecule has been identified as vascular cell adhesion molecule 1 (VCAM-1) (6, 7). This adhesion molecule as well as chemotactic factors allow for the endothelium to recruit and adhere to leukocytes and T cells forming the inflammation.

The development of both early and late atherosclerotic lesions is marked by macrophage foam cells. These cells contain huge amounts of cholesterol ester obtained from oxidized-LDL recruited by scavenger receptors (8). The receptors allow for the oxidized-LDL to be taken up fast enough to make a foam cell. These scavenger receptors include scavenger receptor A (SR-A) and CD36. Mice lacking these receptors have a reduction in atherosclerotic lesions (9, 10). Free cholesterol that is obtained from the oxidized-LDL is esterified by acyl CoA:cholesterol acyltransferase-1 (ACAT-1) and stored in lipid droplets.

The lesion progression continues from fatty streaks to fibrous plaque formation. This stage entails the migration of smooth muscle cells from the medial layer of the artery wall past the internal elastic lamina and into the intimal space. These smooth muscle cells will proliferate and take up modified lipoproteins and produce extracellular matrix proteins that lead to the development of the fibrous plaque (11).

Various cytokines and growth factors secreted by macrophages are important for smooth muscle cell migration and proliferation (12).

The final and most problematic phase of atherosclerosis is plaque rupture and thrombosis. This stage is thought to be responsible for the narrowing of arteries and consequently responsible for many cardiovascular events. The stability of the fibrous cap as well as its thickness are factors which can impact this final stage. Vulnerable caps usually are thin with increased number of inflammatory cells and ruptures generally occur on the perimeter of the plaque. Plaque rupture exposes the plaque lipids and tissue factor to blood which will start the coagulation cascade and thrombosis (13, 14). The coagulation response leads to the narrowing of arteries and potential for cardiac infarction.

Atherosclerosis is a chronic disease which needs a lifetime treatment. One of the major new therapies that are being investigated is increasing high density lipoprotein (HDL). The anti-atherogenic effects of HDL and a potential HDL therapy will be introduced and discussed in the coming chapters.

1.2 Lipoprotein Metabolism

1.2.1 *General properties of lipoproteins*

Lipoprotein metabolism and atherosclerosis are very intimately linked. As mentioned before, LDL is very closely linked with the progression of atherosclerosis. Conversely, HDL has been shown to be anti-atherogenic as it has the ability to take cholesterol from macrophages thereby preventing foam cell formation and plaque

build-up in the arteries. Human studies have shown an inverse correlation between HDL levels and atherosclerotic cardiovascular disease (15). It is crucial that the plasma levels of both HDL and LDL be monitored as it is obvious that they play a huge role in this disease.

Lipoprotein and lipid metabolism are very closely associated with one another. Lipoproteins are the mode by which lipids are transported through the bloodstream. Lipoproteins have a distinct structure and composition which allows each class of lipoprotein to have a unique role in lipid metabolism. In large part, the characterization of a lipoprotein is determined by its components. In general, all lipoproteins are composed of free cholesterol (FC), cholesterol ester (CE), phospholipids (PL), triglycerides (TG), and an apolipoprotein component. The most common classification of lipoproteins is based on their density or mobility on agarose gel-electrophoresis (16).

Cholesteryl esters and triglycerides are the major neutral lipids which are transported through the blood stream. They are insoluble in aqueous solutions and must therefore be protected from the plasma by a coating of amphipathic molecules. Lipoproteins are spherical macromolecular complexes that are comprised of a neutral core of hydrophobic molecules (CE and TG) enveloped by a surface monolayer of amphipathic molecules, primarily phospholipids, free cholesterol, and specific proteins called apolipoproteins. The phospholipids are comprised of two hydrophobic acyl chains and a hydrophilic phosphate group linked to a glycerol backbone and a head group which distinguishes the various phospholipids. The combination of the hydrophobic chains and a hydrophilic head group creates an amphiphilic molecule. The most common phospholipids are phosphatidylserine (PS), phosphatidylcholine

(PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), and phosphatidylinositol (PI) (17, 18). The specific composition of human lipoproteins can be found in table 1.2.1 (19). As seen in table 1.2.1, the various lipoproteins have distinct differences between their surface and core lipids. In addition, each lipoprotein has a unique complement of amphipathic proteins known as apolipoproteins (see table 1.2.2).

Lipoproteins have been classified into six main classes. As mentioned earlier, these classes have been determined by their density and electrophoretic mobility. The main classes of lipoproteins are chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate density lipoprotein (IDL), low-density lipoproteins (LDL), and high-density lipoprotein (HDL). HDL has been subdivided using ultracentrifugal and gel-filtration separations into HDL_2 and HDL_3. HDL_1 subclass is enriched in apoE and is not very abundant and commonly disregarded (20). As seen in table 1.2.1, the composition of lipid and protein determines the density of the lipoprotein. CM have been shown to have only 2% protein while HDL have about 55% protein by weight. The lipids are inversely correlated to the density showing CM to have 86% TG in the core while HDL only has 3% TG in the core. Lipoproteins have also been classified by their electrophoretic mobility on agarose gels into α, preβ, and β lipoproteins, corresponding to HDL, VLDL, and LDL density classes. CM do not migrate on agarose gels (see table 1.2.2) (19).

Table 1.2.1: Chemical Composition of Human Lipoproteins

	Surface Components (% of dry Mass)			Core Lipids (% of dry mass)	
Lipoprotein Class	**FC**	**PL**	**Apolipoproteins**	**TG**	**CE**
Chylomicrons	2	7	2	86	3
VLDL	7	18	8	55	12
IDL	9	19	19	23	29
LDL	8	22	22	6	42
HDL_2	5	33	40	5	17
HDL_3	4	35	55	3	13

Adapted from Havel and Kane 2001.

Table 1.2.2: Physical Properties of Human Lipoproteins

Class	Density (g/mL)	Electrophoretic Mobility	Diameter (nm)	Molecular weight (Da)	Protein Associated
Chylomicron	0.93	Remains at origin	75 - 1200	$50\text{-}1000\text{x}10^6$	A-I, A-II, B-48, C-I, C-II, CIII, E
VLDL	0.93-1.006	pre-β migration	30 - 80	$10\text{-}80\text{x}10^6$	A-I, B-100, C-I, C-II, CIII, E
IDL	1.006-1.019	Slow pre-β migration	25 - 35	$5\text{-}10\text{x}10^6$	B-100, C-I, C-II, CIII, E
LDL	1.019-1.063	β-lipoprotein	18 - 25	$2.3\text{x}10^6$	B-100
HDL_2	1.063-1.125	α-lipoprotein	9 - 12	$3.6\text{x}10^6$	A-I, A-II, AIV, C-I, C-II, CIII, E
HDL_3	1.125-1.210	α-lipoprotein	5 - 9	$1.75\text{x}10^6$	A-I, A-II, AIV, C-I, C-II, CIII, E

Adapted from Havel and Kane 2001.

1.2.2 *Apolipoproteins*

Apolipoproteins have a very important role in lipid metabolism as they have a huge impact on the function of each lipoprotein class. Apolipoproteins have been subdivided into to two categories: the non-exchangeable and the exchangeable apolipoproteins. The two non-exchangeable apolipoproteins are apoB-48 and apoB-100. The apoB containing lipoproteins are responsible for the bulk transport of lipids in the blood stream. ApoB-48 and apoB-100 are synthesis in the intestine and liver, respectfully. The secretion of these two apolipoproteins gives rise to the CM and VLDL lipoprotein species (21).

ApoB-48 and apoB-100 have been found to be products of the same gene (22, 23). It was later determined that apoB-48 is produced in the intestine due to an in-frame stop codon where Gln-2153 would be in apoB-100 (24, 25). The production of apoB-48 in the intestine is essential for the production of CM and transport of exogenous lipids. While apoB-48 is responsible for exogenous transport, apoB-100 is responsible for the bulk transport of endogenous lipids. ApoB-100 is the largest of the apolipoproteins stretching over 4536 amino acids (26). Like apoB-48, it can not be produced without lipids as it is highly susceptible to degradation (27). In fact, it is the only protein that requires lipidation for secretion. It is proposed that apoB-100 and apoB-48 are secreted in a two-step lipidation process. The first step is co-translational lipidation. This lipidation prevents pre-mature degradation of apoB and produces a molecule referred to as a precursor VLDL (28). The assembly of this precursor is dependent upon microsomal triglyceride transfer protein (MTP). Although its specific

role remains unclear, it is proposed that it also is required for lipid accumulation of TG and CE in the second step. The second step is post-translational and occurs as the protein is passed through the golgi. It is here that the VLDL matures by incorporating TG into the lipoprotein (29). ApoB has recently been shown to be a better indicator of risk for CHD than LDL-cholesterol or other commonly used cholesterol indices (30). ApoB containing lipoproteins continue to be the focus of lipid lowering therapeutics as efforts continue to minimize the incidence of CHD.

The exchangeable lipoproteins are much smaller than the apoB apolipoproteins. They also have the ability to transfer between lipoproteins, and acquire lipids in circulation (31). Unlike the apoB proteins, the exchangeable lipoproteins are derived from separate genes of common descent. Furthermore, each exchangeable lipoprotein contains 4 exons and 3 introns. The only exception to this is apoA-IV (32). These apolipoproteins are for the most apart clustered on chromosome 11 and 19. ApoA-I, C-III, and A-IV were found to occur in cluster of about 22kb on chromosome 11 (33, 34, 35). ApoE, apoC-I, and apoC-II were clustered within 4kb on chromosome 19 (36). Finally, the structures of most of the exchangeable apolipoproteins are similar and consist primarily of alpha-helices produced by their unique multiple repeats of 22 amino acids (22-mer). Each of these 22-mers is a tandem array of two 11-mers. This repeating structure was first observed in apoA-I by several scientists (37, 38, 39) and has since been extended to the other exchangeable apolipoproteins.

ApoE has been studied very thoroughly over the years. Its structure and function have been very well defined. Structurally, apoE is 299 residues in length and contains two distinct α-helical domains (40). The N-terminal domain is a 4-helix

bundle which is responsible for binding to the LDLr (41, 42). In order to bind to the LDLr, apoE must be bound to phospholipids or lipoproteins. The lipid binding domain is found in the α-helical C-terminus (43). ApoE binds to mainly CM, VLDL, and LDL and aid in the interaction of these lipoproteins with the LDLr (44). It has been shown that apoE is present in three isoforms and each variation can impact the function of apoE and lead to lipoprotein abnormalities and diseases. The three isoforms are apoE2, apoE3 and apoE4. They differ at position 112 and 158. ApoE3 is the most common isoform and contains a cysteine and an arginine, respectively, whereas apoE2 has two cysteines and apoE4 has two arginines at these positions (45). ApoE4 is perhaps the most serious of the conditions and could lead to early onset of both Alzheimer's disease and CHD (46, 47).

As mentioned earlier, there are three apoC apolipoproteins in circulation. The apoC group of apolipoproteins are often portrayed as one family of proteins because of their similarity in size, distribution among lipoproteins, and coincident purification. ApoCs are found primarily on CM, VLDL, and HDL and each have a unique function in lipid metabolism (48). ApoC-I, apoC-II, and apoC-III are mostly produced in the liver and to a lesser extent in the intestine. They are relatively small proteins ranging in size from 57-79 amino residues in length (49, 50, 51). Each apoC apolipoprotein exerts a similar role as an inhibitor of lipoprotein binding to receptors. It has been shown that they all inhibit apoE mediated binding to the LDLr by displacing apoE from VLDL and CM. ApoC-I has been shown to inhibit binding to the LDLr by masking or altering the conformation of apoE rather than displacing it (52). ApoC-III has been implicated in abolishing apoB binding to the LDLr by masking its binding

domain (53). Although apoCs can prevent binding of apoB and apoE to the LDLr, the apoCs main role in lipid metabolism has been related to enzyme activity. ApoCs appear to affect lecithin:cholesterol acyltransferase (LCAT). ApoC-I was shown to activate LCAT, whereas apoC-II and apoC-III inhibit LCAT activity (54, 55). ApoCs also play a major role in lipoprotein lipase (LPL) activity. ApoC-II has been shown to be an essential activator of LPL. ApoC-II can bind to CM/LDL or to LPL directly and activate lipolysis of TG. Conversely, apoC-I and apoC-III have been shown to inhibit LPL activity (56). Finally, apoC-III has been shown to inhibit hepatic lipase (HL) (57). The importance of apoC-I, apoC-II, and apoC-III is clear from its impact on lipoproteins and the enzymes responsible for the transport of cholesterol and triglyceride to specific tissues. These apolipoproteins affect major metabolic pathways and could play a major role in the prevention of many hyperlipidemia diseases.

1.2.3 *Exogenous and Endogenous Lipid Transport*

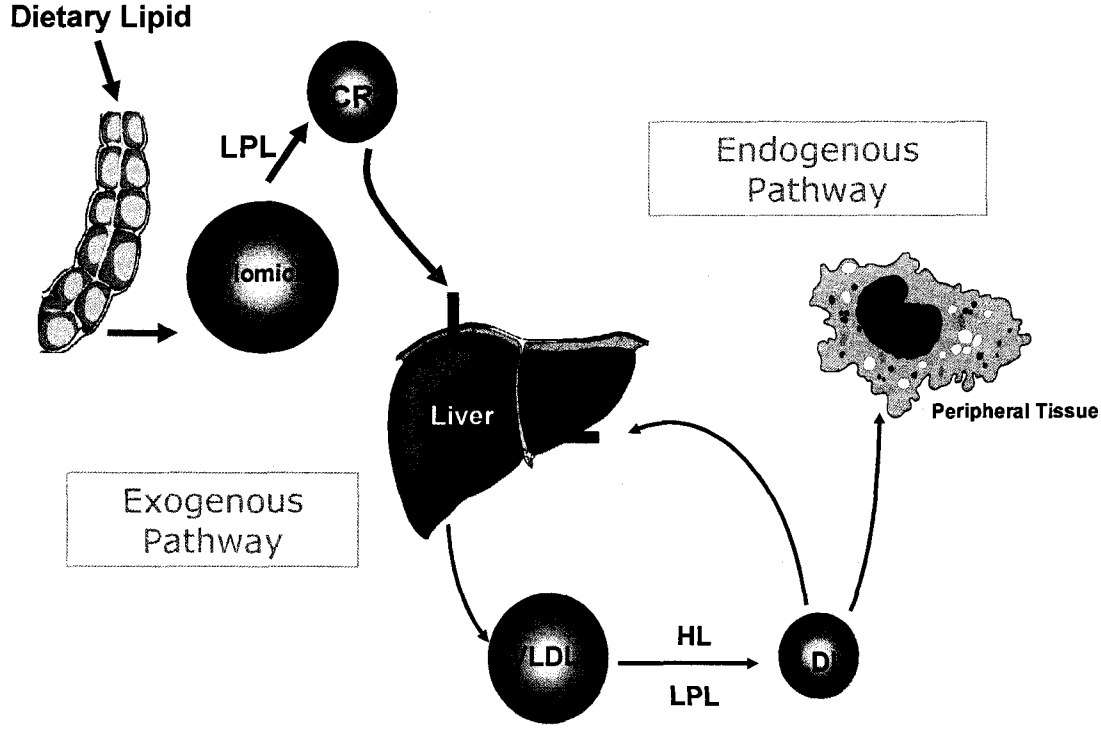
The aforementioned apolipoproteins, enzymes and lipoproteins all play a part in the bulk transfer of lipid and cholesterol in circulation. They all work in magnificent unison to regulate circulating lipids. There are two main pathways of lipid transport, which are exogenous and endogenous. Functionally, there are three main classes of lipoproteins which partake in these pathways. They are CM, VLDL, and LDL. In the exogenous pathway, cholesterol and triglycerides are digested in the intestine. They are passed through the intestine and become part of a CM particle. This particle contains apoB-48 as well as some apoC and apoA-I. The apoA-I is shed to HDL as

soon as the CM particle reaches circulation. Through the action of LPL, the CM particle loses most of its TG load becoming a CM remnant and they are taken up by the liver. The liver is the hub of both pathways. It is here that the exogenous pathway ends and the endogenous pathway begins. ApoB-100 containing VLDL particles are produced in the liver and through the action of both LPL and HL lose there TG load in circulation. Some of the VLDL in circulation will return to the liver, however, those that do not return become LDL particles. These particles are rich in cholesterol and can stay in circulation for upwards of two days. These LDL are eventually taken up by the liver via the LDLr (see figure 1.2.1) (20). This prolonged exposure to circulation can of course lead to atherosclerosis as the LDL has more time to deposit cholesterol in peripheral tissues. This effect of lipid deposition can only be countered by the actions of HDL and its main apolipoprotein apoA-I.

1.2.4 *ApoA-I*

ApoA-I is the major apolipoprotein associated with HDL. ApoA-I constitutes approximately 70% of the apolipoprotein content of HDL. As such, it has been

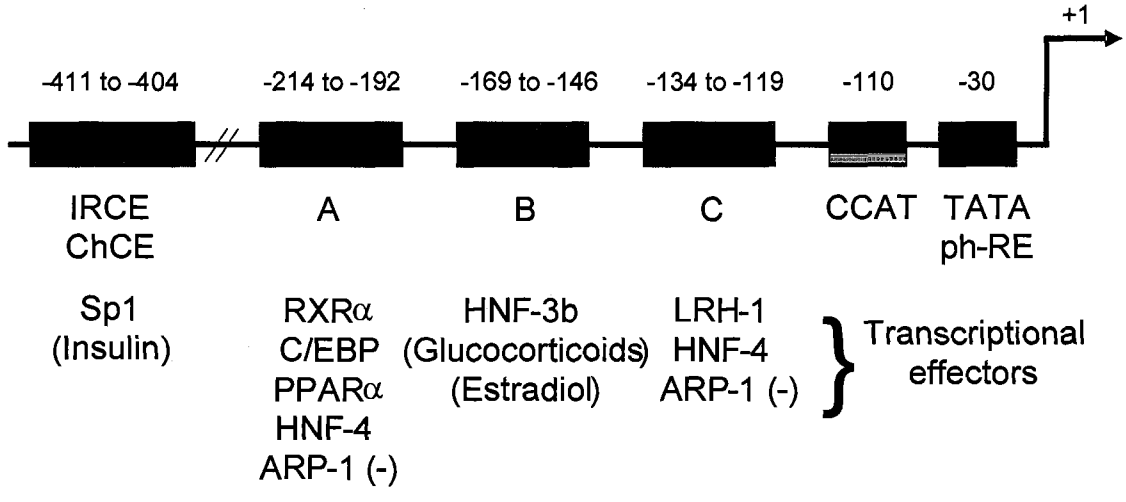
Figure 1.2.1: Exogenous and Endogenous Lipoprotein Metabolism
Lipids which are digested in the intestine are secreted into circulation as CM. LPL hydrolyzes the lipids from CM resulting in CM remnants. The remnants are taken up by the liver. VLDL is produced in the liver and secreted into circulation as the major carrier of cholesterol. Via the actions of HL and LPL, VLDL is converted to LDL. LDL can then be taken up by peripheral cells or taken back up by the liver via the LDLr.


Dietary Lipid
LPL
Endogenous Pathway
Liver
Peripheral Tissue
Exogenous Pathway
HL
LPL

inversely correlated with the risk of developing CHD and appears to be protective against atherosclerosis (58).

As described earlier, apoA-I is part of the exchangeable lipoprotein family and is primarily produced in the liver and intestine. At these sites, the synthesis and secretion of apoA-I has been studied in great detail. The apoA-I gene has been shown to contain 4 exons and is located at chromosome 11 (59). ApoA-I gene regulation is a complex mix of promoters and enhancers which drive the apoA-I transcription to its maximal output. In the intestine, full gene expression is achieved when each of the proximal apoA-I promoter, apoA-I enhancer, and apoC-III enhancer are activated. However, in the liver, much of the apoA-I production can be obtained from just the activity of the proximal apoA-I promoter (60). The apoA-I promoter has been mapped in great detail (see figure 1.2.2). Four distinct regulatory elements have been mapped to the apoA-I promoter ranging from -199 to -411. These regulatory elements have been termed A, B, C, and IRCE (insulin response core element). Various factors have been shown to bind to the promoter and up regulate or down regulate apoA-I transcription. Thyroid hormones, retinoids, and estrogens have been shown to increase apoA-I promoter activity in site B (-169 to -146) (61). While HNF-4 (hepatocyte nuclear factor-4) and ARP-1 (apoA-I repressor protein-1) have been shown to decrease promoter activity in sites A (-214 to 192) and C (-134 to -119). Finally, the ubiquitous transcription factor SP1 (specific protein-1) binds to the IRCE (-411 to -404) and is responsible for the induction of apoA-I transcription via insulin (62). Effectors such as insulin, can also influence mitogenic-activated protein kinase (MAPK) pathways.

Figure 1.2.2: ApoA-I Promoter
Organization of regulatory elements within the apo A-I gene promoter. Several regulatory elements mediating numerous transcriptional responses (hormonal, metabolic, and tissue-specific), as well as the factors that mediate both positive and negative effects on apo A-I gene expression, are shown. In some cases (estradiol, glucocorticoids, and pH), the promoter region modulating the effect has been identified but the factors mediating the process have not been reported. A cytokine response element mediating the suppressive effects of TNF- and IL-1ß is located within site A. TR, thyroid hormone receptor; IRCE/ChRE, insulin response core element/carbohydrate response element; pHRE, pH response element (93).


+1
-411 to -404
-214 to -192
-169 to -146
-134 to -119
-110
-30
IRCE
ChCE
A
B
C
CCAT
TATA
ph-RE
Sp1
(Insulin)
RXRα
C/EBP
PPARα
HNF-4
ARP-1 (-)
HNF-3b
(Glucocorticoids)
(Estradiol)
LRH-1
HNF-4
ARP-1 (-)
Transcriptional
effectors

Activation of MAPK pathways can lead to activation of SP1. Currently, both Ras-raf-MAP kinase and phosphatidylinositol-3-kinase (PI3K) are effective in activating SP1 suggesting a role in apoA-I synthesis (63).

In general, the MAPK pathway is a highly regulated signal transduction system. The signaling process commences when a receptor, such as the receptor tyrosine kinase (RTK), phosphorylates a guanine nucleotide exchange factor (GEF) called Ras. Ras can then be inactivated by a GTPase-activating protein (GAP) to terminate the signal. Activated Ras triggers a series of downstream serine/threonine phosphorylations which constitute the MAP kinase cascade. There are three major kinases in the pathway including MAP kinase-kinase-kinase, MAP kinase-kinase, and MAP kinase. One of the major cascades in humans is termed the MEK/ERK cascade where the kinases are Raf, MEK1/2, and ERK1/2. The activation of a MAP kinase requires both a threonine and a tyrosine phosphorylation which are separated by one amino acid. Once activated, the MAP kinase relays signals downstream by phosphorylating various proteins in the cell, including gene regulatory proteins and other protein kinases (64).

After the apoA-I transcriptional process is complete, the final product is a 267 amino acid protein. It is synthesized as a pre-pro-protein. The pre-peptide is 18 amino acids long and the pro-protein is 6 amino acids long. As the apoA-I pre-pro-protein is being translocated to the cell surface, the pre-protein is cleaved off by a signal peptidase (65, 66). The resulting pro-apoA-I undergoes an early phospholipidation in the endoplasmic reticulum (ER) that is followed by another phosphorylation in the golgi. It has been shown previously that ABCAI plays an important role in apoA-I lipidation and secretion. Phospholipidation of newly synthesized apoA-I is largely

dependent on ABCAI (115). More specifically, the phospholipidation at the ER appears to be ABCAI independent, while phospholipidation at the golgi is ABCAI dependent. This mildly lipidated apoA-I also acquires some cholesterol in the ER and golgi, but the major accumulation remains at the cell surface (67). Finally, the pro-apoA-I is secreted and the pro-segment is cleaved via an unidentified metallo-enzyme. The secreted protein of apoA-I can then associate with an ATP binding cassette transporter A1 (ABCA1) where cholesterol accumulation and HDL formation can commence (68).

1.2.5 *HDL Metabolism*

A fully mature apoA-I can associate with ABCA1 and obtain small amounts of free cholesterol, phospholipids, and sphingomyelin. This particle is referred to as lipid-poor apoA-I or nascent HDL (69). ABCAI mutations in humans (Tangier disease) have demonstrated that HDL metabolism is dependent on ABCAI. ABCAI mutation results in having very little HDL due to rapid catabolism of lipid-poor apoA-I (116). The nascent HDL can then become part of an existing HDL (70) or easily accumulate its own cholesterol and phospholipids to generate a HDL particle. The nascent HDL accumulates cholesterol and lipid from peripheral tissues and through the action of LCAT become a spherical HDL_3 particle (see figure 1.2.4). ApoA-I is an essential co-factor for LCAT and therefore essential for the esterification of cholesterol and production of spherical HDL (71). In circulation, HDL_3 particles can exchange CE for TG with apoB containing lipoproteins like VLDL or LDL. This process is facilitated

by an enzyme called cholesterol ester transfer protein (CETP). The HDL_3 particle accumulates the majority of its cholesterol via ATP-binding cassette transporter G1 (ABCG1) found on the surface of macrophages (72). As the cholesterol accumulates in the HDL_3 particles they get larger. This ensuing particle is defined as HDL_2. The HDL_2 particle can be recognized by scavenger receptor BI (SR-BI) which is located in the cell membrane of hepatocytes (73). This process of 'reverse cholesterol transport' (see figure 1.2.5) is the major reason why HDL has an anti-atherogenic effect. HDL's ability to take cholesterol from macrophages and deliver it to the liver for excretion makes it the focus for current therapies for preventing atherosclerosis and CHD.

Figure 1.2.3: Generic structural model of HDL
HDL consists of a core of neutral lipids, primarily TG, and CE surrounded by a PL monolayer, and its major lipoprotein, ApoA-I.

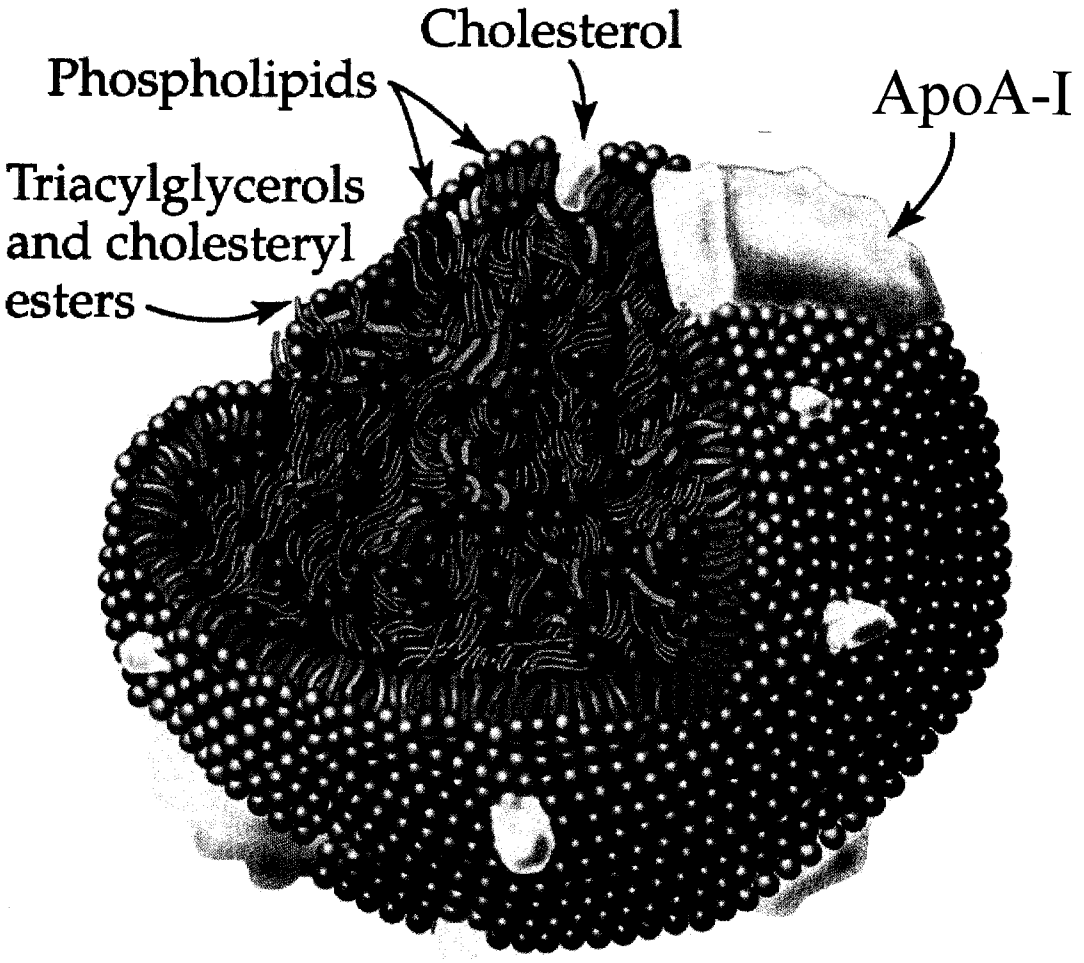
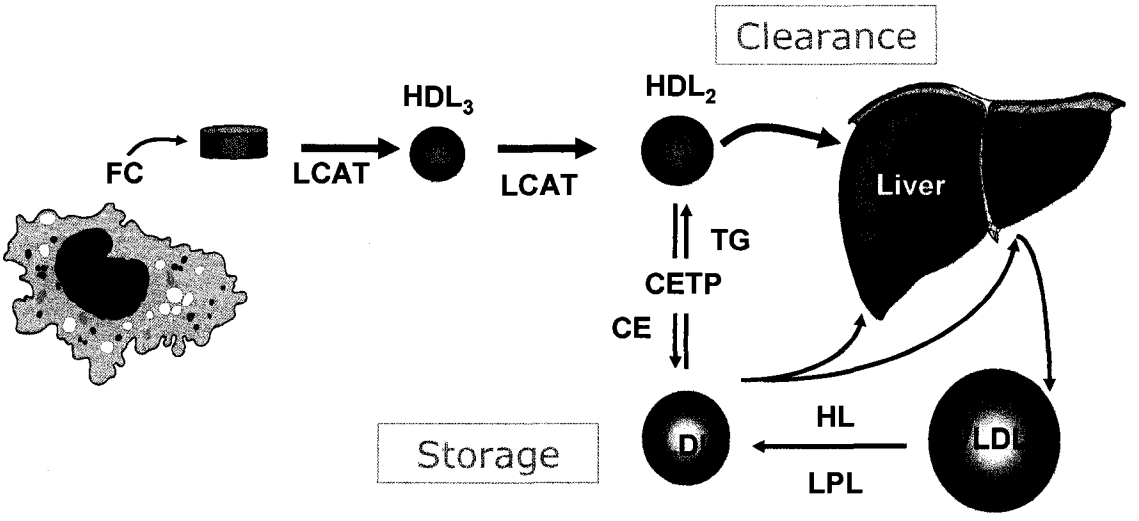

Cholesterol
Phospholipids
ApoA-I
Triacylglycerols
and cholesteryl
esters

Figure 1.2.4: Reverse Cholesterol Transport
Free lipid-poor apoA-I can bind to transporters (ABCAI) on peripheral cells and takes up both free cholesterol and phospholipids. Through the action of LCAT, the discoidal HDL becomes a HDL_2. This molecule can than be taken up by the liver, via SR-BI, in order to return cholesterol to the liver. HDL_2 can also exchange TG for CE with LDL or VLDL due to CETP.


Clearance
HDL3
HDL2
FC
LCAT
LCAT
Liver
TG
CETP
CE
Storage
D
HL
LPL
LD

1.2.6 HDL Therapeutics

In past years, much of the research going into preventing CHD and atherosclerosis has been aimed at lower LDL cholesterol. Statins are the most frequently used lipid-lowering and anti-atherogenic agents. However, recent strategies to elevate HDL cholesterol have been in the forefront. Unlike LDL cholesterol, elevating HDL cholesterol concentrations may protect against CHD (74). Epidemiological studies have shown that each 1% increase in HDL cholesterol is associated with a 2% reduction in the risk of developing CHD (75). This association can be even stronger in the presence of additional risk factors.

There are currently five major initiatives in place to elevate HDL cholesterol levels. The current therapies include nicotinic acid, CETP inhibitors, peroxisome-proliferator activator receptor (PPAR) agonists, ApoA-I mimetics, and phospholipid therapies. Nicotinic acid or niacin has been the longest standing elevator of HDL (76). It primarily raises HDL cholesterol by inhibiting the uptake and degradation of apoA-I, providing an abundant source of apoA-I for the production of HDL (77). CETP inhibitors have had success in human trials. The latest CETP inhibitor, Torcetrapib, increased HDL levels by more than 90% in phase I clinical trials (78). However, due to a recent set back, CETP inhibitors have lost favor but are still a promising therapeutic for raising HDL (79, 80). Fibrates (PPAR agonists) have been shown to increase HDL cholesterol levels, while being associated with a reduction in CHD events (81). The primary fibrate used thus far has been Gemfibrozil. Although it has been beneficial, it's modest elevation in HDL and potential dangers with statin

combination have placed it in the background. Currently, apoA-I based strategies include infusion of recombinant phospholipids/apoA-I complexes (rHDL), recombinant proapoliportein A-I, and apoA-I mimetic peptides. The simple basis of this technique is to increase circulating levels of lipid-poor apoA-I thereby increasing efflux of cholesterol. This strategy has been promising with studies showing regression of plaque development due to perhaps increased efflux from macrophages via ABCAI transporters (82, 83). Finally, phospholipid-directed therapies have found there way to the forefront. This is a novel and side-effect free therapy and as such has given phospholipid therapeutics the spot light for the time being. At this time two phospholipid species have been studied in detail. They are synthetic phospholipids, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and a soy lecithin derivative, phosphotidylinositol (PI). DMPC has been shown to increase HDL and reduce aortic lesion size in apoE knockout mice (84). PI was recently tested in phase I clinical trials and showed an increase in HDL upwards of 25% in just two weeks (85). It is the focus of my research to elucidate the mechanism of action of PI and to define the role of another potential phospholipid therapeutic, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC).

1.3 Rationale and Objectives

Lipid therapeutic intervention has focused on reducing the amount of low density lipoprotein (LDL) in the circulation as high LDL is a significant risk factor in the development of CHD. However, recent studies have shown that increasing HDL can be as or more effective than lowering LDL in treating and preventing CHD. HDL is considered to be inversely related to the development of CHD. Currently there are 54 million Americans that have low HDL.

Phosphatidylinositol (PI) is a well known intracellular signaling precursor molecule and has been shown to have a profound impact on lipoprotein and lipid metabolism. In recent human trials, oral administration of PI has resulted in significant increases in HDL and apoA-I and reductions in TG levels. The exact mechanism by which PI acts to increase the levels of HDL and apoA-I in the blood is unknown.

The objective of this research is to elucidate the cellular regulatory pathways that PI acts through to increase the synthesis and secretion of apoA-I. The specific aims of the research are 4 fold: 1) Develop an in vitro model to study apoA-I synthesis and secretion, 2) Investigate the signaling cascades through which PI acts in cultured cells, 3) Investigate the effects of PI on apoA-I synthesis and secretion, and 4) Investigate the effects of PI on apoA-I binding, uptake and degradation.

The experiments detailed within this thesis reveal insight into the mechanism by which PI elevates the levels of apoA-I. The model proposed suggests that PI increases apoA-I in HepG2 cells mediated by the MEK/ERK and JNK MAPK cascades. The stimulation of these pathways does not involve the elevation of apoA-I

gene expression. However, PI appears to reduce the amount of apoA-I degradation resulting in an increase in apoA-I in the cell media.

Chapter 2 – Experimental Procedures

2.1 Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), L-α-phosphatidylinositol (PI) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The human hepatoblastoma cell line (HepG2) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) high glucose (25mmol/L) and low glucose (5.5mmol/L), and penicillin/streptomycin were purchased from invitrogen (Burlington, ON, CAN). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO, USA). 6-well, 12-well, and 10cm^2 plates were purchased from Corning (New York, NY, USA). MEK1/2 and p38MAPK inhibitors and their inactive isoforms U0126, U0124, SB203580, and SB202474 respectively, were purchased from Calbiochem (La Jolla, CA, USA). U73122 (PI-PLC inhibitor), D-609 (PC-PLC inhibitor), SP600125 (JNK inhibitor), Sulindac Sulfide (RAS inhibitor), Wortmannin (PI3K inhibitor) and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Mouse anti-human apoA-I monoclonal antibodies 4H1 and 5F6 were obtained from the lab of Dr. Yves Marcel. Mouse anti-human apoA-I monoclonal antibody, horseradish peroxidase-linked goat anti-human apoA-I antibody and K-Blue MAX substrate (TMB) were purchased from Cedarlane (Hornby, ON, CAN). Immuno Maxisorp 96-well plates were purchased from Nunc (Rochester, NY, USA). Rabbit anti-human phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody, rabbit anti-human p44/42 MAP Kinase antibody, and horseradish peroxidase-linked anti-rabbit

IgG were purchased from Cell Signaling (Danvers, MA, USA). Novex 12% polyacrylamide gels were purchased from Invitrogen (Burlington, ON, CAN). Laemmli sample buffer was purchased from Bio-Rad (Hercules, CA, USA). Tri-reagent was purchased from Sigma (St. Louis, MO, USA). Superscript II RNAse H-RT Kit was purchased from Invitrogen (Burlington, ON, CAN). Primers for the ApoA-I gene were designed on the Lightcycler LC Probe Design software (Roche) and purchased from Alpha DNA (Montreal, PQ, CAN). LightCycler Fast Start DNA Master SYBR Green I was purchased from Roche (Indianapolis, IN, USA). pDNR-LIB vector which contains the full length cDNA (containing pre- and pro-peptide sequence) of human apoA-I (BC005380) was supplied by OpenBiosystems (Huntsville, AL, USA). Radionuclide [α-^{32}P]-dCTP was purchased from PerkinElmer (Shelton, CT, USA). Iodine^{125} and PD-10 Sephadex Columns were purchased from Amersham Biosciences (Piscataway, NJ, USA). Iodo-gen Pre-coated Iodination tubes were purchased from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

2.2 Methods

2.2.1 *Cell Culture of HepG2 Cells*

HepG2 cells were cultured in high or low glucose Dulbecco's Modified Eagle Medium (DMEM) growth medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Confluent cultures were plated 1:4 into 6 (35mm) or 12 well (20mm) or 100mm plates from Corning and grown to 90% confluence for individual experiments.

2.2.2 *Preparation of Phospholipid Vesicles*

PI, POPC, DOPA and DLPC vesicles in PBS (1 mg/ml) were prepared by sonication as previously described (107). In brief, 1mg of phospholipid was dried down under nitrogen. 1 mL of PBS was added to the dry lipids and sonicated with a probe sonicator for 1 minute at 100% duty cycle. Vesicles were then incubated for 30 minutes at 37°C. Finally, the vesicles were sonicated for 5 minutes at 95% duty cycle. Vesicles were prepared for immediate use.

2.2.3 *PI-dependent ApoA-I secretion from HepG2 Cells*

HepG2 cells grown in 20mm, 35mm, or 100mm plates were incubated in serum free media with or without 10 ug/ml sonicated phospholipid vesicles, unless otherwise noted. HepG2 cells were incubated with phospholipid vesicles for 24 hours unless otherwise noted. In select cases, glucose levels in the media were adjusted. The two cases are defined as high (25mM glucose) and low (5.5mM glucose) glucose. At the end of the time point, cell media was collected from each well and apoA-I mass was

measured via ELISA. Cell lysate was also collected from each well and analyzed for total protein content by the BCA assay from Pierce (Rockford, IL, USA).

2.2.4 *Quantification of ApoA-I mass using an ApoA-I ELISA*

Protein was analyzed by ELISA on a 96 well plate. The Nunc Immuno-maxisorp 96 well plate was coated overnight with the capture antibody mouse anti-human apoA-I monoclonal antibody (1:1000 dilution) in PBS. The plate was washed with PBS-0.05%Tween-20. Human apoA-I protein was used as the standard. The standard curve contained 8 points in triplicate ranging from 0.25ug/100ul – 0.005ug/100ul apoA-I (diluted with serum free DMEM). 100ul of media samples (1:2 dilution in serum free DMEM) and standards were incubated in the wells for 2 hours. The plate was washed as previously stated, followed by a 1 hour incubation with a horseradish peroxidase-linked goat anti-human apoA-I antibody (1:1000 dilution) in PBS.

The plate was washed and 100ul of K-blue Max TMB substrate was added to each well and allowed to incubate for 5 minutes. The reaction was stopped using 50ul of 1N HCl solution. The absorbance was read at 450nm.

2.2.5 *Quantification of ApoA-I mass using Western Blot analysis*

After incubation with 10ug/mL PI and DLPC for 24h, cells were washed twice with ice-cold PBS-T on ice. Cells were lysed by adding buffer [NaF 1 mmol/L, NaCl 5 mmol/L,EDTA 1 mmol/L, NP40 1 mmol/L (Roche Diagnostics, Indianapolis, IN, USA), HEPES 10 mmol/L, pepstatin A 1 mg/mL, leupeptin 1 mg/mL, aprotinin 1

mg/mL, Na_3VO_4 1 mmol/L, PMSF 1 mmol/L] and total protein was extracted. An equal amount of cell proteins were separated by 12% SDS-PAGE and were analyzed by Western blot using specific mouse anti-human apoA-I antibodies 4H1 and 5F6.

2.2.6 *Treatment with Signaling Inhibitors*

HepG2 Cells were grown to 90% confluency in 35mm culture dishes. Cells were washed with PBS and fresh serum free media was added to the cells. The cells were then pre-incubated for 30 minutes with various chemical inhibitors: U73122 (PI-PLC inhibitor), D-609 (PC-PLC inhibitor), Wortmannin (PI3K inhibitor), U0126 (MEK1/2 inhibitor), U0124 (inactive form of U0126), SB203580 (p38MAPK inhibitor), SB202474 (inactive form of SB203580), SP600125 (JNK inhibitor), Sulindac Sulfide (RAS inhibitor). All inhibitors were used at concentration of 10uM except for wortmannin which was used at a concentration of 1uM. Inhibitors were solubilized in DMSO and diluted with serum free media. Cells were then incubated with or without 10ug/mL PI for 23.5h. The media and cell lysate were collected and analyzed as previously described (2.2.3).

2.2.7 *ERK1/2 Immunoblotting*

HepG2 cells were grown to 90% confluency in 35mm culture dishes and incubated with or without PI (10ug/mL) for 15, 30, and 60 minutes. At each time point the cells were collected using Laemmli sample buffer. Cell extracts were electrophoresed on 12% Tris-glycine SDS-PAGE gels under denaturing conditions. Protein was transferred to nitrocellulose. The membrane was blocked for 1 hour in

blocking buffer (TBS containing 0.1% Tween-20 and 5% skim milk). The membrane was then incubated overnight at 4 degrees Celsius with a phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody (1:1000 dilution) in primary dilution buffer (TBS containing 0.1% Tween-20 and 5% BSA). Membranes were washed 3 times with TBS-0.1%Tween-20. An anti-rabbit IgG, HRP-linked antibody was used as a secondary antibody and diluted (1:2000) in blocking buffer. After a 1 hour incubation with secondary antibody, the membrane was washed 3 times with TBS-0.1%Tween-20. The membrane was incubated with the Super Signal West Pico Chemiluminescent Substrate (Pierce) to visualize the phosho-ERK1/2. Membranes were developed on FluorChemHD from Alpha Innotech (San Leandro, CA, USA) and band density was determined using Quantity One software. Membrane was then stripped at 70 degrees Celsius using a stripping buffer (62.5 mM Tris HCl pH 6.8, 2% SDS and 100mM 2-mercaptoethanol) for 30 minutes. The membrane was then blocked and re-probed using the p44/42 MAP Kinase antibody. The membrane was probed, developed and quantified as previously described.

2.2.8 *RNA Preparation*

HepG2 cells were cultured in 35mm well plates and incubated with PI and DLPC as previously described (2.2.3). At the end of the treatment period, total RNA was extracted using 0.5mL TRI-Reagent according to the manufacturer's instructions. In brief, 50ul 1-Bromo-3-Chloro-Propane (BCP) was added to each sample forcing phase separation followed by centrifugation at 12000g. The aqueous phase was transferred and 250ul of isopropanol was added to precipitate total RNA followed by centrifugation at 12000g. The RNA pellet was washed with 75% ethanol/DEPC and

centrifuged as before. Finally, the 75% ethanol/DEPC solution was removed and the RNA pellet was air dried for 10 minutes on ice. The RNA was dissolved in 50ul DEPC treated water and quantified on a ND-1000 spectrophotometer from NanoDrop Technologies (Wilmington, DE, USA). One microgram of RNA was then run on a 1% TAE (tris-acetate buffer, 40mM Tris acetate, 1mM EDTA pH 8.0) agarose gel to access the integrity of the RNA.

2.2.9 *Quantification of ApoA-I mRNA Levels by Real-Time Polymerase Chain Reaction (PCR)*

cDNA was reverse-transcribed from the total RNA using a Superscript II RNAse H- RT Kit. Random primers from Invitrogen (Burlington, ON, CAN) were incubated with the total RNA samples for 3 minutes at 70°C. The RT reaction buffer (8ul 5X First Strand buffer, 10mM dNTPs, 100mM DTT, 2ul RNAsin, 2ul reverse transciptase) was incubated with the sample for 2 hours at 42°C. Finally, cDNA was hydrolyzed using 50mM EDTA and 10N NaOH. cDNA samples were then purified using the Promega Wizard PCR preps DNA Purification System from Promega (Madison, WI, USA).

The purified cDNA was quantified using the OliGreen ssDNA Quantitation kit purchased from Invitrogen (Burlington, ON, CAN). ssDNA standard was prepared from calf Thymus Single strand DNA from Sigma (St.Louis, MO, USA). The calf thymus single stranded DNA was diluted to 0, 10, 30, 50, 100, 150, and 200 ng/mL to make a standard curve. Samples were diluted (1:10) in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). 100ul of sample or standard was added to each well and then each well had 100ul of OliGreen dye added to it. All samples were incubated in the fluorescent dye for 5 minutes and the fluorescence was measured using the Fluorostar

purchased from BMG Labtechnologies (Durham, NC, USA). Excitation filter was set at 480nm and the emission filter was set at 560-12nm.

Oligonucleotide primers were designed from the GenBank cDNA sequence of the human apoA-I (accession no.NM000039) gene using Lightcycler LC Probe Design software. Real-time PCR of apoA-I was performed using LightCycler Fast Start DNA Master SYBR Green I kit in the Lightcycler (Roche), with the following set of primers: Forward Primer – 5'-GATGAAAGCTGCGGTG-3' and Reverse Primer – 5'-CTGCCGCTGTCTTTGA-3'. This primer set results in a PCR product of 153bp. The amplification reaction was performed in a final volume of 20ul (9.4ul water, 1.6ul of 25mM Mg, 1ul of 10uM Forward primer, 1ul of 10uM Reverse primer, 2ul Master SYBR Green I, 5ul diluted (1:5) cDNA sample or standard); with thermal cycling conditions of 10 minutes at 95°C, and 55 cycles of 15 seconds at 95°C, 15 seconds at 50°C, and 15 seconds at 72°C. The standard curve method was used to quantitate the apoA-I mRNA levels. The standard curve used a plasmid with an insert of the full length cDNA of apoA-I (pDNR-LIB vector). The plasmid was serial diluted to make a standard curve of 100, 10, 1, 0.1, and 0.01 pg/ul.

2.2.10 *Quantification of ApoA-I mRNA Levels by Northern Blot*

HepG2 Cells were grown to 90% confluency in 100mm culture dishes and incubated with or without 25ug/mL PI for 24 hours. Total RNA was isolated using the method previously described (2.2.8). Fifteen micrograms of RNA was fractionated by electrophoresis through a 1.5% agarose gel containing 2.2M formaldehyde, and transferred to a nylon membrane. The apoA-I cDNA probe was labeled with [α-^{32}P]-dCTP by oligolabeling, hybridized to the RNA on the membrane, and washed under

high stringency conditions (1xSSC [SSC is 0.15M NaCl, 15M sodium citrate, pH 7.0], 0.1% SDS) for 20 minutes followed by 3 washes at 60°C in 0.2xSSC, 0.1% SDS. The membrane was exposed to a white surface casing for 24 hours at 4°C to create an autoradiograph. Autoradiograph was developed in a phosphoimager and the amount of signal was quantified by densitometry using Quantity One software.

2.2.11 *Centrifugation – HDL separation*

Plasma samples were collected from random patients at the Ottawa Hospital Civic Campus and received from transfusion medicine. Plasma samples were collected and HDL was isolated by sequential ultracentrifugation. Plasma density was adjusted to $\rho = 1.063$ g/mL and the VLDL/LDL fraction was spun up at 40000rpm for 20h. The remaining fraction was adjusted to a density of $\rho = 1.21$ g/mL and the HDL fraction was spun up at 44000rpm for 40h (108). The HDL fraction was extensively dialyzed in 1XPBS and then the protein concentration of the HDL fraction was determined by the Lowry method as modified by Markwell et al (109).

2.2.12 *Iodination of ApoA-I*

Iodo-gen Pre-coated Iodination tubes were rinsed with 300ul of sodium phosphate buffer. 200ug of apoA-I was added to each tube followed by the addition of 350uCi of Iodine^{125}. Tubes were stirred constantly for 1 minute. This mixture was loaded onto PD-10 Sephadex columns which had been pre-equilibrated with PBS. 250ul fractions were collected and the four most radioactive ^{125}IApoA-I fractions were pooled.

2.2.13 *Production of [^{125}IApoA-I]-HDL*

The entire volume of ^{125}IApoA-I that was pooled in 2.2.12 was incubated with 5mg of cold HDL overnight at 37°C. [^{125}IApoA-I]-HDL was isolated by density centrifugation. The density was raised to 1.21g/mL and spun up at 60000rpm for 20h. Isolated [^{125}IApoA-I]-HDL was dialyzed extensively in 1XPBS and quantified as previously described (2.2.11).

2.2.14 *ApoA-I Degradation*

HepG2 cells were grown to 90% confluency in 20mm plates and incubated in serum free media with or without 10 ug/ml PI for 24 hours. The media was removed and the cells were washed with PBS and fresh serum free media was put back on the cells. Cells were incubated with 50ug [^{125}IApoA-I]-HDL for 4 hours. The media and cell lysate from each well was collected. Media was analyzed using the TCA assay which determines the amount of free ^{125}I in the media. This assay precipitates all the protein and the supernatant is counted for ^{125}I which correlates with the amount of apoA-I degradation.

2.2.15 *Statistical analysis*

All statistical analyses were performed using SigmaStat software (version 3.0). A one-way analysis of variance (ANOVA) was performed in order to determine the significance of difference between multiple group means. If the p value was found to be less than 0.05, post-test analyses were performed using the Holm-Sidak method for all pairwise comparisons. In select cases where there were only two groups, the

significance of difference was calculated using a two-tailed Student's t-test. All experiments were completed three separate times in triplicate. The only exception was the degradation experiment (figure 3.6.1, 3.6.2) which was completed two separate times in triplicate. All presented data is representative of one experiment.

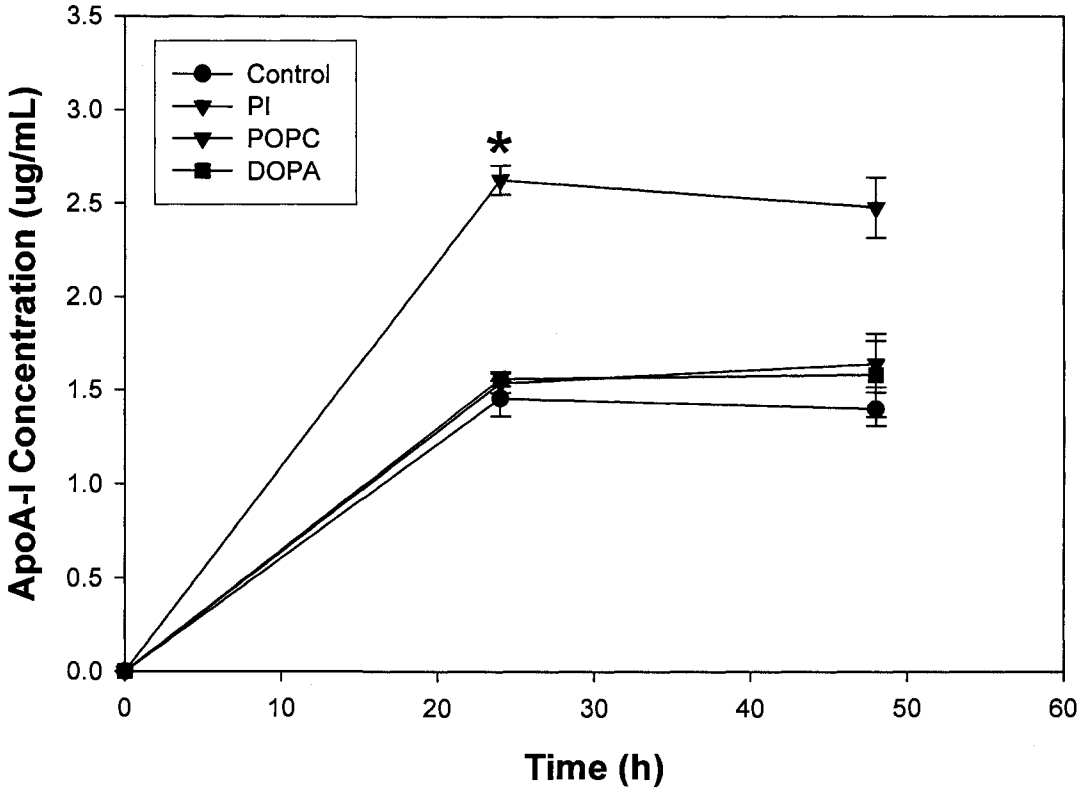
Chapter 3 – Results

3.1 Phosphatidylinositol uniquely regulates ApoA-I secretion in a HepG2 cell model

3.1.1 *PI increases ApoA-I secretion in HepG2 cells*

The major site of apoA-I production is known to be in the liver. As such, we used a liver hepatoma cell line (HepG2) to evaluate the mechanism of action of PI with respect to the elevation of apoA-I seen in rats and humans (85). It was first identified that the action of PI was unique compared with the other common phospholipids found in vivo. The phospholipid PI was compared with POPC, and DOPA with respect to apoA-I secretion stimulation in HepG2 cells. It was found that PI significantly increased the secretion of apoA-I by 86% at 24 hours compared with controls ($P<0.001$). PI increased secretion by 73% over POPC and DOPA ($P<0.001$) (figure 3.1.1). These results suggest that there is a unique property pertaining to PI which allows it to stimulate apoA-I secretion compared with POPC and DOPA. In subsequent studies, it was found that intracellular levels of apoA-I remain constant between PI treated cells and control cells (figure 3.5.1b). In light of this discovery a broad protein and phospho-protein array was used to probe the pathways that may be affected by PI and cause the elevation in apoA-I secretion. This work provided early evidence that PI may be acting through the mitogen-activated protein kinase (MAPK) pathways.

Figure 3.1.1: PI increases ApoA-I secretion in HepG2 Cells
HepG2 cell media was analysed for apoA-I mass by ELISA. HepG2 cells were grown in 6-well plates and incubated with 10 ug/ml of soy PI, POPC, or DOPA, for 24 and 48 hours. Control cells were given an equivalent volume of PBS. Values shown are the mean $\pm$ SD for each group. ANOVA was performed to determine significance of difference between groups. Cells treated with PI showed increased apoA-I secretion compared to control while intracellular apoA-I levels remained the same in all groups. *$p<0.001$ compared with control, DOPA, and POPC.


ApoA-I Concentration (ug/mL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Time (h)
Control
PI
POPC
DOPA
*

3.2 Signaling cascades through which PI acts in the HepG2 cell model

3.2.1 *MEK/ERK MAPK pathway is affected by PI*

The data from the proteomic array revealed that many proteins and phosphorylated proteins of the MAPK pathway were affected in the HepG2 cells which were exposed to PI (Kinexus Inc. generated the data). Several proteins in the MEK/ERK MAPK and JNK MAPK pathways were stimulated by PI compared to both control and POPC. This knowledge allowed us to pursue these pathways using different techniques to ensure the involvement of the MAPK pathways. All of the following inhibitor concentrations were optimized using a concentration curve. The concentration used was determined based on cell viability and ability to inhibit its target protein. Firstly, a variety of inhibitor studies were carried out to evaluate the role of both MEK1/2 and p38MAPK in the mechanism of action of PI. The inhibitor for p38MAPK (SB203580) and its inactive homologue (SB202474) gave the same result of having no effect on PI (figure 3.2.1). In contrast, it was found that the inhibition of MEK1/2 using U0126 caused a significant decrease in PI mediated apoA-I secretion. U0126 almost completely blocked the PI effect ($P<0.005$ compared to PI alone) while the inactive MEK1/2 inhibitor U0124 exerted no effect on PI stimulation of apoA-I (figure 3.2.2).

3.2.2 *PI increases phosphorylation of ERK1/2*

To further this finding, we examined a time course of ERK1/2 phosphorylation in HepG2 cells treated with or without PI. HepG2 cells were incubated for 15, 30, and 60 minutes with PI (10ug/mL) and the phosphorylation of ERK1/2 relative to total

Figure 3.2.1: Effect of p38MAPK inhibition on PI dependent ApoAI secretion in HepG2 cells

HepG2 cells were grown to confluency in 6-well plates. Cells were incubated for 30 minutes with either SB203580 (p38MAPK inhibitor) or SB202474 (inactive form of SB203580) and then incubated with 10 ug/ml of PI for 24 hours. All inhibitors were administered at a concentration of 10uM. Control cells were given an equivalent volume of PBS. ApoA-I concentration in the cell media was determined by ELISA. Values indicated are means $\pm$ SD for each group. The p38MAPK inhibitor SB203580 had no impact on the PI dependent secretion of apoA-I by HepG2 cells. This figure represents part of a large experiment which includes figure 3.2.2 and 3.2.7.

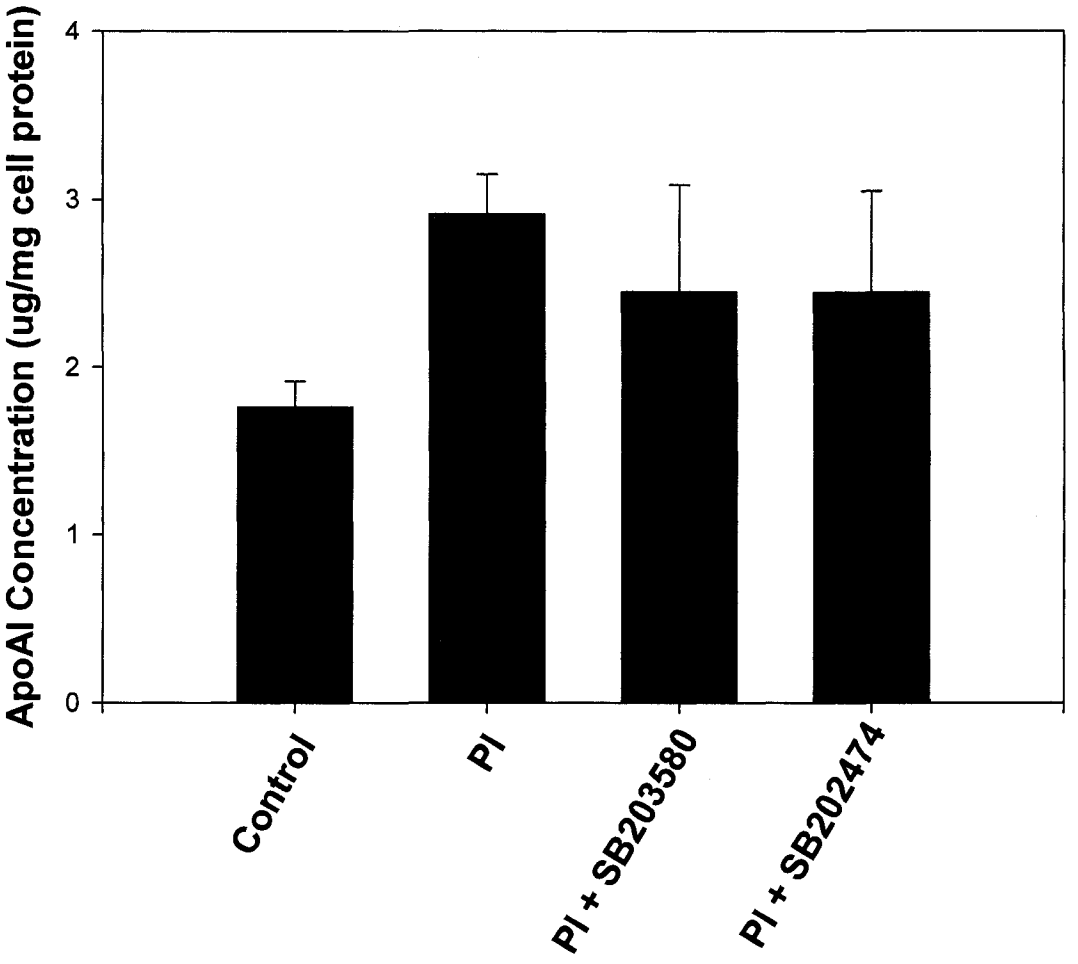
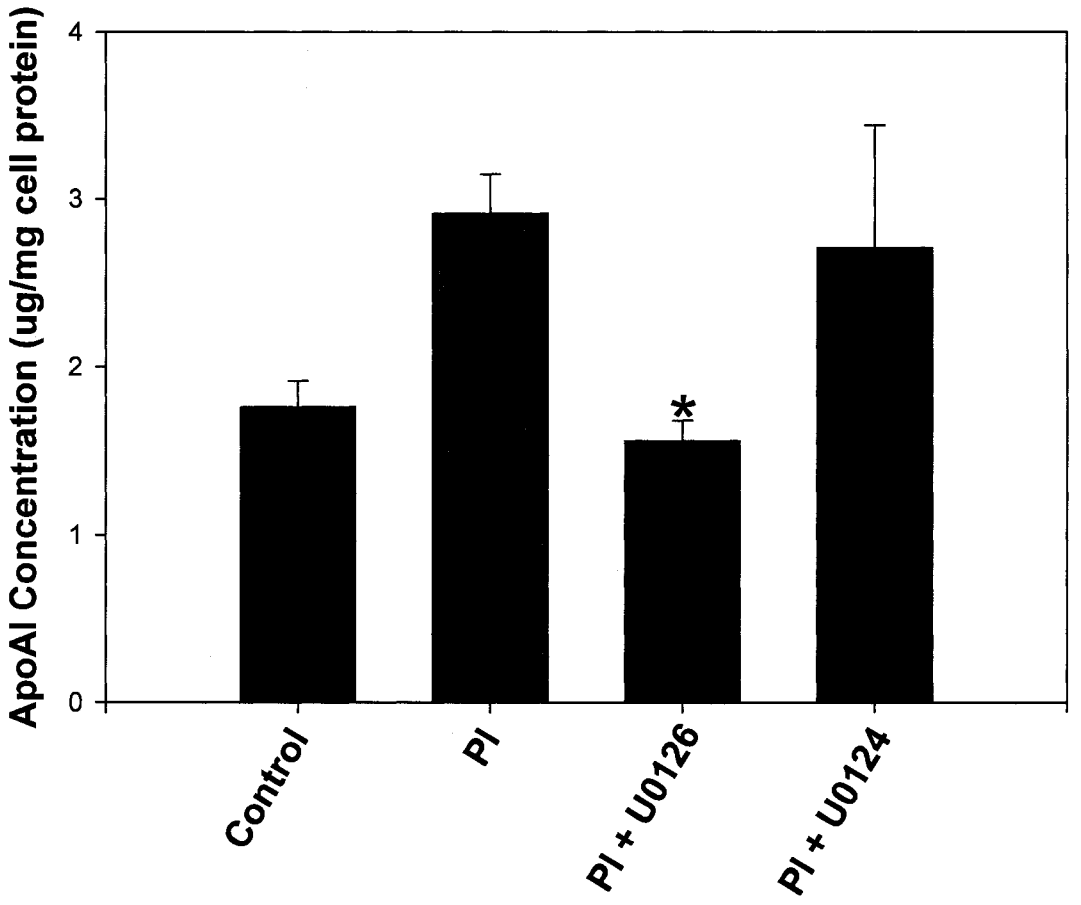

ApoAI Concentration (ug/mg cell protein)
4
3
2
1
0
Control
PI
PI + SB203580
PI + SB202474

Figure 3.2.2: Effect of MEK1/2 inhibition on PI dependent ApoAI secretion in HepG2 cells

HepG2 cells were grown to confluency in 6-well plates. Cells were incubated for 30 minutes with either U0126 (MEK1/2 inhibitor) or U0124 (inactive form of U0126) and then incubated with 10 ug/ml of PI for 24 hours. All inhibitors were administered at a concentration of 10uM. Control cells were given an equivalent volume of PBS. ApoA-I concentration in the cell media was determined by ELISA. Values indicated are means $\pm$ SD for each group. ANOVA was performed to determine significance of difference between groups. The MEK inhibitor U0126 blocked the PI dependent secretion of apoA-I by HepG2 cells. * $p<0.005$ vs PI. This figure represents part of a large experiment which includes figure 3.2.1 and 3.2.7.


ApoAI Concentration (ug/mg cell protein)
4
3
2
1
0
*
Control
PI
PI + U0126
PI + U0124

ERK1/2 protein levels were compared to controls. PI had an immediate impact at the 30 minute time point ($P<0.05$) (figure 3.2.3, 3.2.4). This type of oscillation is common in phosphorylation signaling, much like insulin which also increased ERK1/2 phosphorylation by 5 minutes (97).

3.2.3 *JNK MAPK pathway is affected by PI*

In another set of experiments, the inhibition of JNK was tested to see if it would impact secretion of apoA-I in HepG2 cells. Cells were preincubated with the JNK inhibitor SP600125 for 30 minutes prior to a 23.5 hour incubation with PI. The inhibition of JNK decreased the effect of PI from a 7.3ug/mg protein of secreted apoA-I to 4.5ug/mg protein compared to control levels of 3.6ug/mg protein ($P<0.001$) (figure 3.2.5).

3.2.4 *Investigating upstream contributors to the MAPK pathways*

As an upstream contributor to the MAPK pathway, Ras inhibition was also studied. This study was done as seen in the previous HepG2 inhibition studies. This also concluded that Ras plays a role in PI signaling. Inhibiting Ras with sulindac sulfide resulted in a 22% decrease in PI efficacy with respect to apoA-I secretion in HepG2 cells ($P<0.05$) (figure 3.2.6).

3.2.5 *Intracellular signaling Inhibitors of PI-PLC impact PI mediated apoA-I secretion*

In order to evaluate the mechanism of PI mediated apoA-I secretion at the level of the plasma membrane, several inhibitors were used which were known to impact signaling molecules at the plasma membrane. The effects of inhibiting phosphatidylinositol-phospholipase C (PI-PLC), phosphatidylcholine-phospholipase C

Figure 3.2.3: PI promotes the phosphorylation of ERK1/2 in HepG2 Cells

HepG2 cells were grown to confluency in 6-well plates. Cells were incubated with PI for 15, 30, and 60 minutes. After the incubation period, the cell extracts were electrophoresed using SDS-PAGE and probed with a phospho-p44/42 MAP Kinase antibody and a p44/42 MAP Kinase (ERK1/2) antibody.

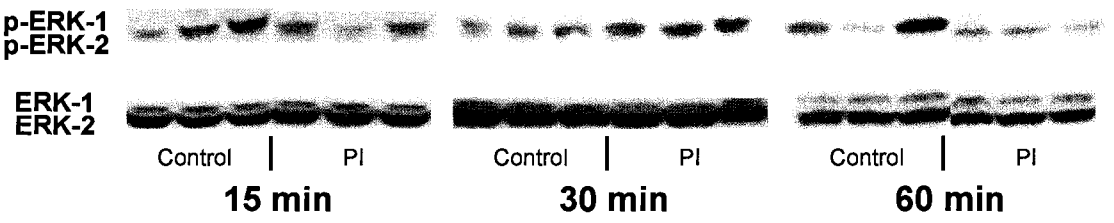

p-ERK-1
p-ERK-2
ERK-1
ERK-2
Control
PI
Control
PI
Control
PI
15 min
30 min
60 min

Figure 3.2.4: Quantification of ERK1/2 results using Quantity One densitometry software

Western blot from figure 3.2.3 was quantified using the Quantity One software. Values shown are the mean $\pm$ SD for each group. ANOVA was performed to determine significance of difference between groups.

* $p<0.05$ vs control.

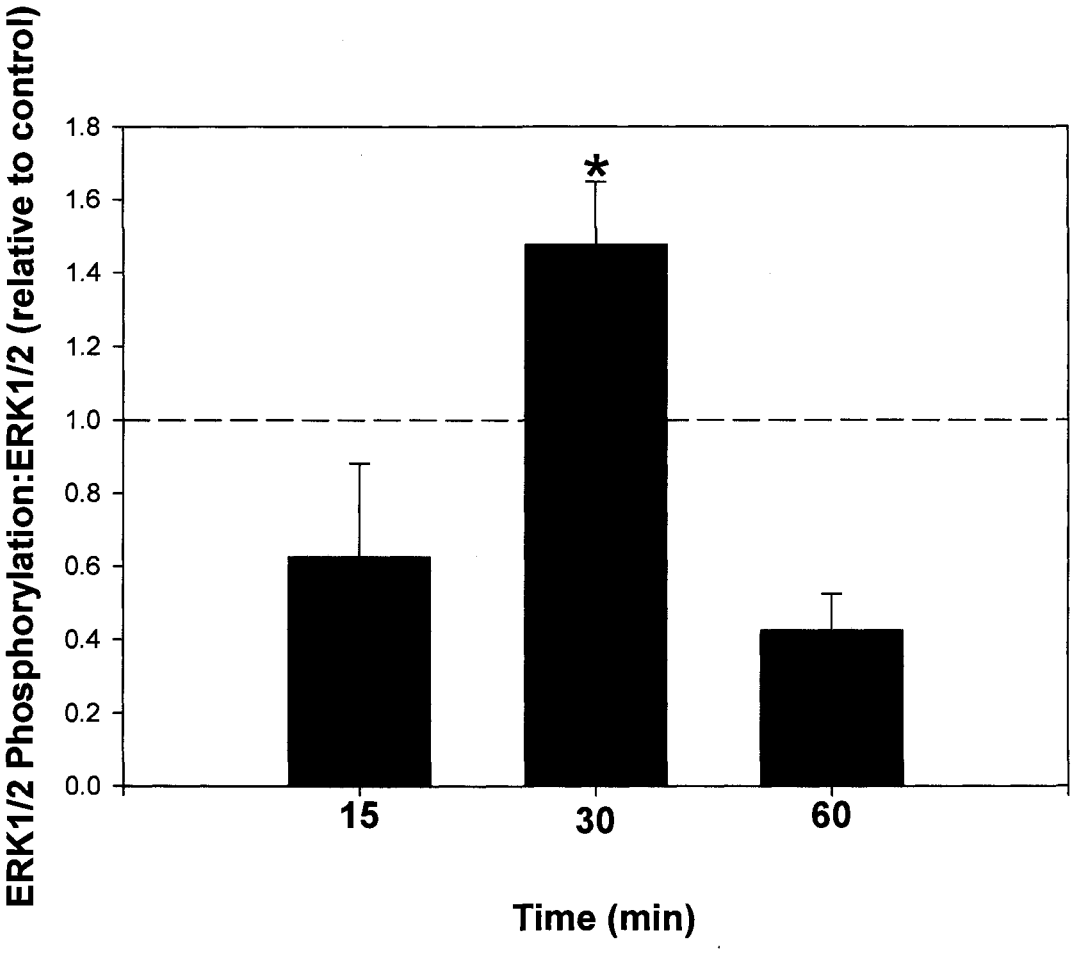

ERK1/2 Phosphorylation:ERK1/2 (relative to control)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
15
30
60
Time (min)

Figure 3.2.5: Effect of JNK inhibition on PI dependent ApoAI secretion in HepG2 cells

HepG2 cells were grown to confluency in 6-well plates. Cells were incubated for 30 minutes with the inhibitor SP600125 (JNK inhibitor) and then incubated with 10 ug/ml of PI for 24 hours. The inhibitor was administered at a concentration of 10uM. Control cells were given an equivalent volume of PBS. ApoA-I concentration in the cell media was determined by ELISA. Values indicated are presented as means $\pm$ SD. ANOVA was performed to determine significance of difference between groups. Inhibiting JNK partially blocked the PI dependent secretion of apoA-I by HepG2 cells. *$p<0.001$ compared to PI. This figure represents part of a large experiment which includes figure 3.2.6.

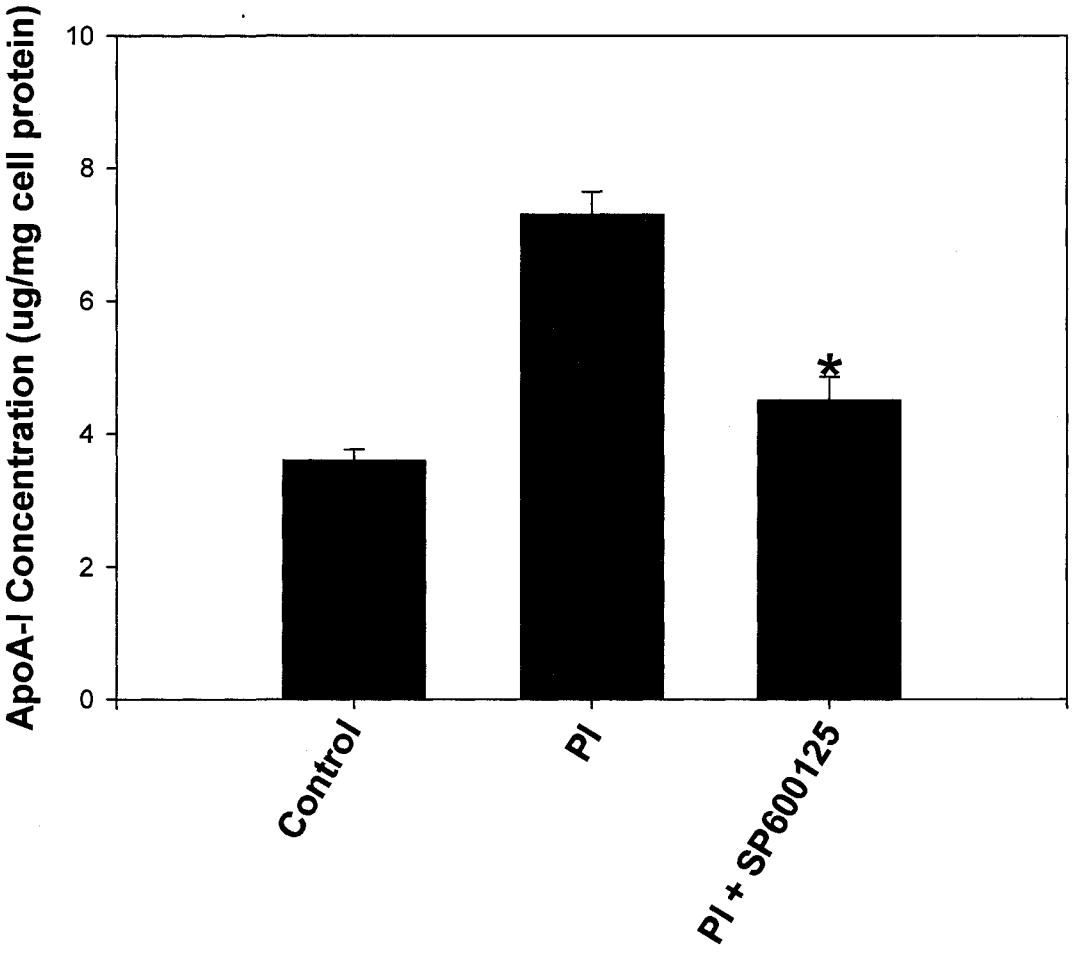
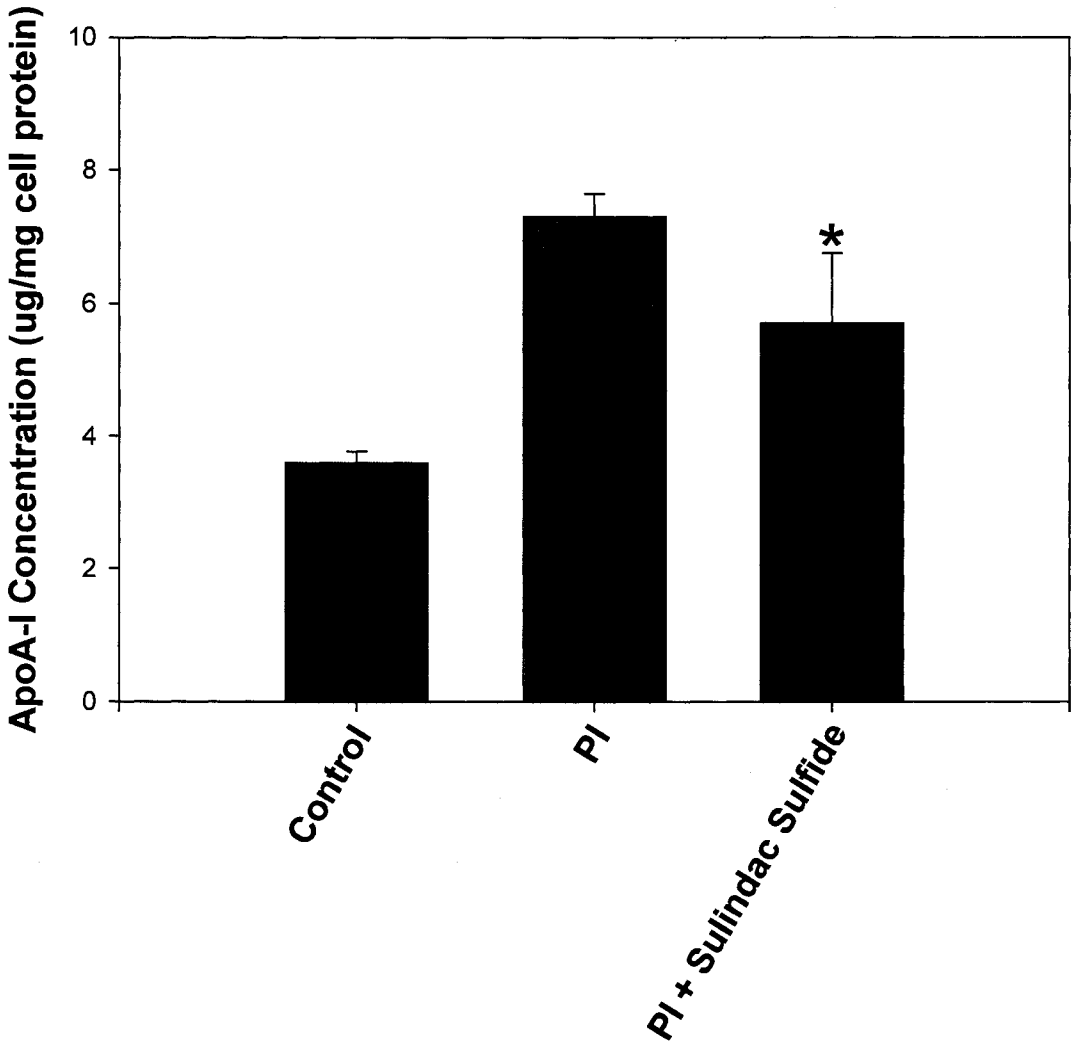

ApoA-I Concentration (ug/mg cell protein)
10
8
6
4
2
0
*
Control
PI
PI + SP600125

Figure 3.2.6: Effect of Ras inhibition on PI dependent ApoAI secretion in HepG2 cells

HepG2 cells were grown to confluency in 6-well plates. Cells were incubated for 30 minutes with the inhibitor Sulindac Sulfide (Ras inhibitor) and then incubated with 10 ug/ml of PI for 24 hours. The inhibitor was administered at a concentration of 10uM. Control cells were given an equivalent volume of PBS. ApoA-I concentration in the cell media was determined by ELISA. Values indicated are presented as means $\pm$ SD. ANOVA was performed to determine significance of difference between groups. Inhibiting Ras partially blocked the PI dependent secretion of apoA-I by HepG2 cells. *$p<0.05$ compared to PI. This figure represents part of a large experiment which includes figure 3.2.5.


ApoA-I Concentration (ug/mg cell protein)
10
8
6
4
2
0
*
Control
PI
PI + Sulindac Sulfide

(PC-PLC), and phosphatidylinositol-3-kinase (PI3K) were examined. The inhibitors U73122 (PI-PLC inhibitor), D-609 (PC-PLC inhibitor), and wortmannin (PI3K inhibitor) were used in the analysis. The inhibitors were incubated with cells for 30 minutes prior to a 23.5h incubation with PI. PI3K inhibition had no significant effect on the PI mediated secretion of apoA-I (figure 3.2.7). In contrast, both the PLC inhibitors significantly blocked the PI mediated effect. Both the PLC inhibitors reduced the PI effect from 1.7 to 1.1 fold compared to untreated control cells ($P<0.001$) (figure 3.2.8).

3.3 Effects of PI on ApoA-I Synthesis

3.3.1 *Effect of PI on ApoA-I mRNA levels*

In order to determine if the increase in apoA-I levels was due to an increase in apoA-I mRNA, HepG2 were treated with 10ug/mL PI for 24 hours. Both Northern blot and real-time PCR were used to quantify the levels of apoA-I mRNA. Northern and real-time PCR using total RNA and cDNA, respectively, showed no significant differences between PI or POPC treated groups compared to control. In fact, real-time PCR revealed a trend towards less mRNA in the PI treated groups (Figure 3.3.1, 3.3.2).

3.3.2 *Effect of PI on ApoA-I mRNA levels at early time points*

To determine if the transcriptional flux of mRNA was earlier than 25 hours, many early time points were investigated. As before, HepG2 cells were incubated with 10ug/mL PI for 0.5, 1, 2, and 8 hours. Total RNA was collected from each individual

Figure 3.2.7: Effect of PI-3-Kinase signaling inhibitor Wortmannin on PI dependent stimulation of apoA-I secretion by HepG2 cells
HepG2 cells were grown to confluence in 6-well plates. Cells were incubated for 30 minutes with the inhibitor wortmannin (PI3K inhibitor) and then incubated with 10 ug/ml of PI for 24 hours. Wortmannin was administered at a concentration of 1uM. Control cells were given an equivalent volume of PBS. ApoA-I concentration in the cell media was determined by ELISA. Values indicated are means $\pm$ SD. Inhibition of PI3K had no effect on PI mediated apoA-I secretion. This figure represents part of a large experiment which includes figure 3.2.1 and 3.2.2.

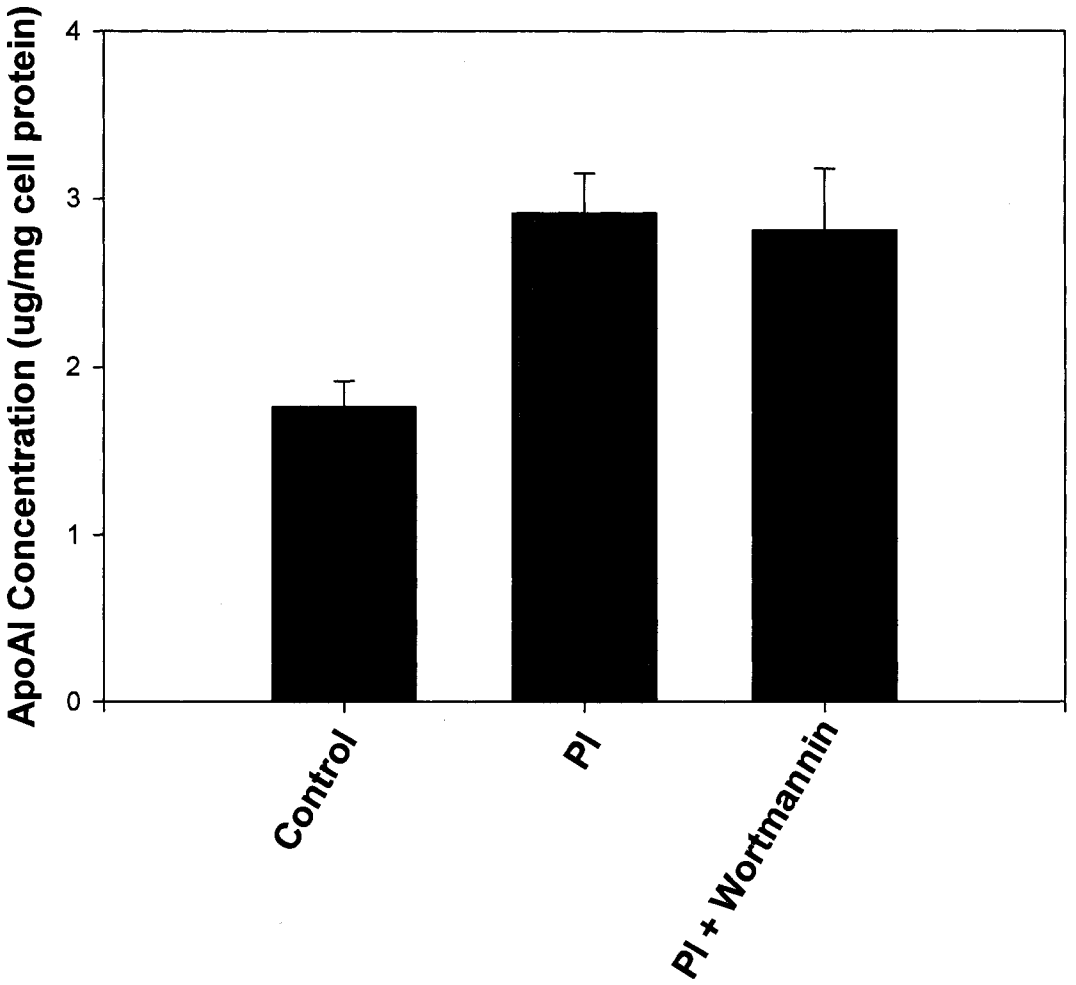

ApoAI Concentration (ug/mg cell protein)
4
3
2
1
0
Control
PI
PI + Wortmannin

Figure 3.2.8: Effect of intracellular signaling inhibitors U73122 and D-609 on PI dependent stimulation of apoA-I secretion by HepG2 cells
HepG2 cells were grown to confluence in 6-well plates. Cells were incubated for 30 minutes with either U73122 (PI-PLC inhibitor) or D-609 (PC-PLC inhibitor) and then incubated with 10 ug/ml of PI for 24 hours. Both inhibitors were administered at a concentration of 10uM. Control cells were given an equivalent volume of PBS. ApoA-I concentration in the cell media was determined by ELISA. Values indicated are average increase (ug/mg cell protein) relative to control $\pm$ SD. ANOVA was performed to determine significance of difference between groups. Inhibitors of PI-PLC and PC-PLC prevented the PI dependent stimulation in apoA-I secretion.
*$p<0.001$ compared to PI.

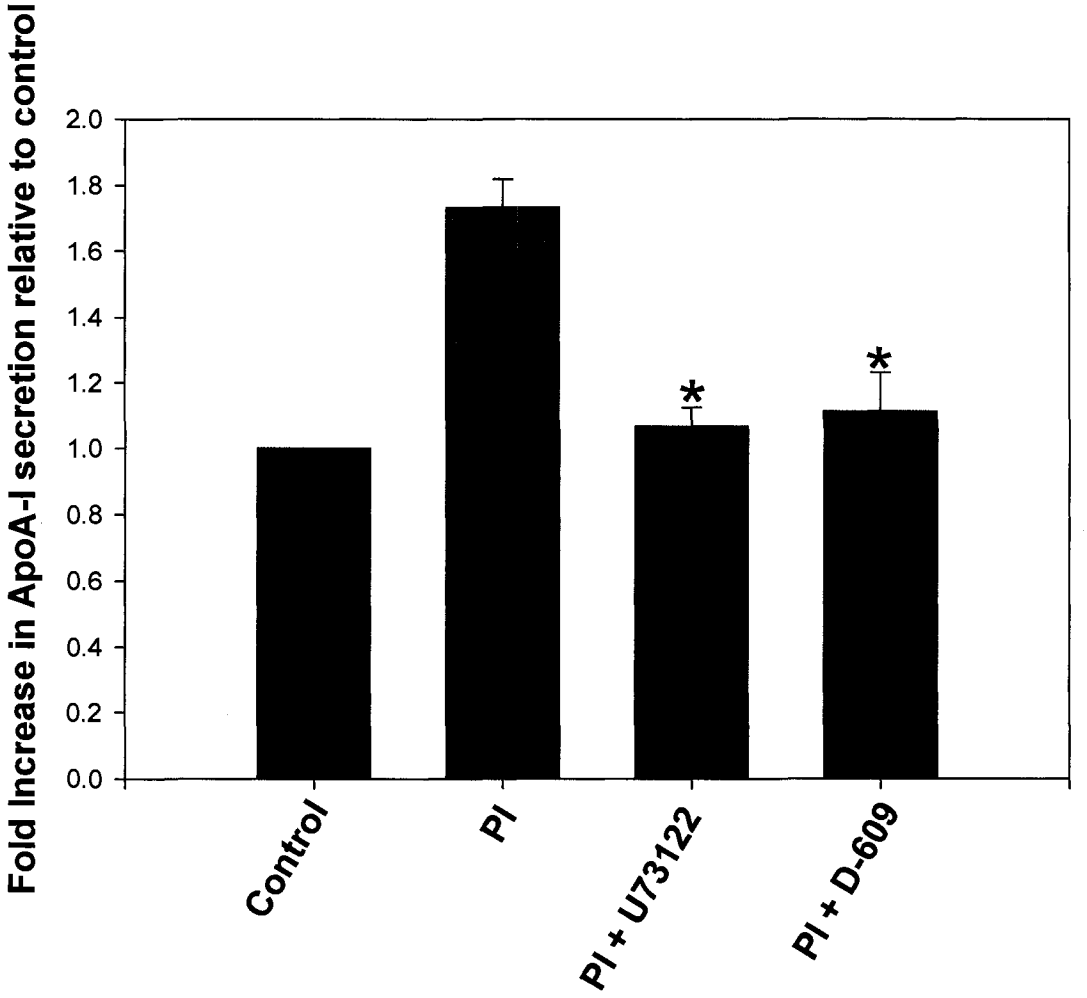

Fold Increase in ApoA-I secretion relative to control
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
PI
PI + U73122
PI + D-609

Figure 3.3.1: PI does not significantly impact the transcription of ApoA-I in HepG2 cells

HepG2 cells were grown to confluence in 6-well plates. Cells were incubated with 10 ug/ml of PI for 24 hours. Control cells were given an equivalent volume of PBS. Cells were collected using TRI-reagent and total RNA was isolated, converted to cDNA and ApoA-I mRNA was quantified using specific primers and Real Time PCR. Values indicated are means $\pm$ SD for each group. PI did not significantly impact apoA-I transcription at 24 hours.

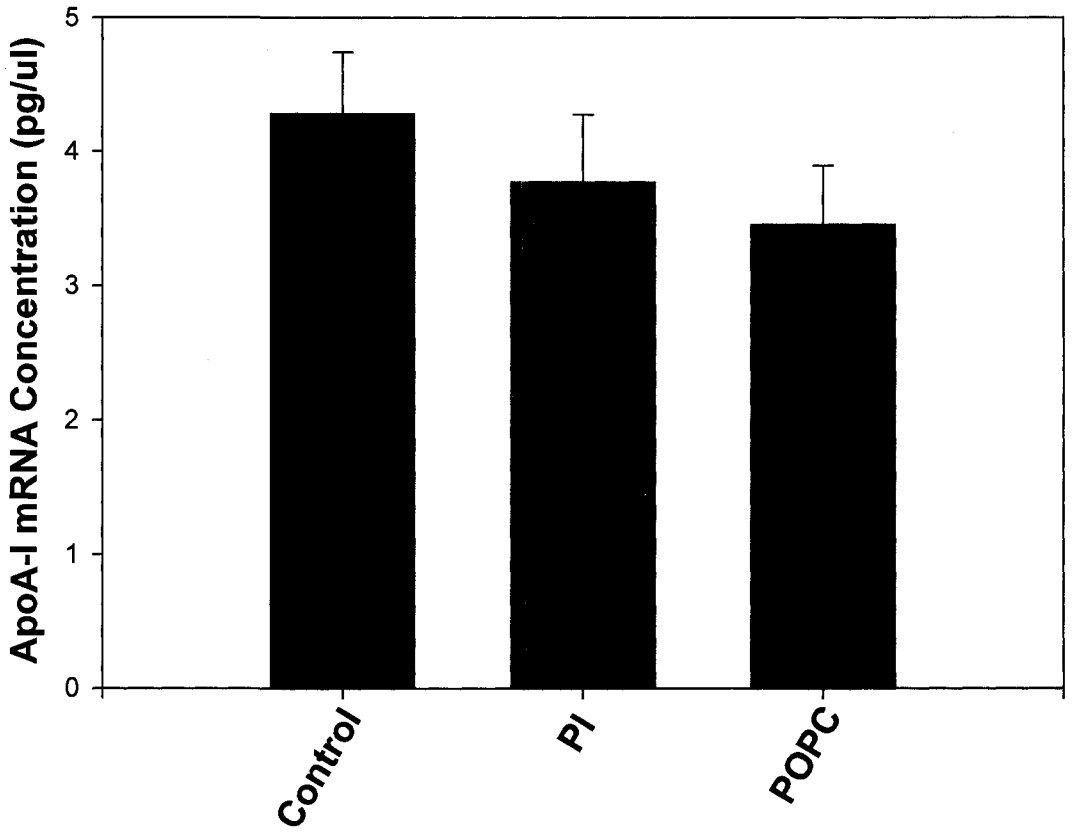
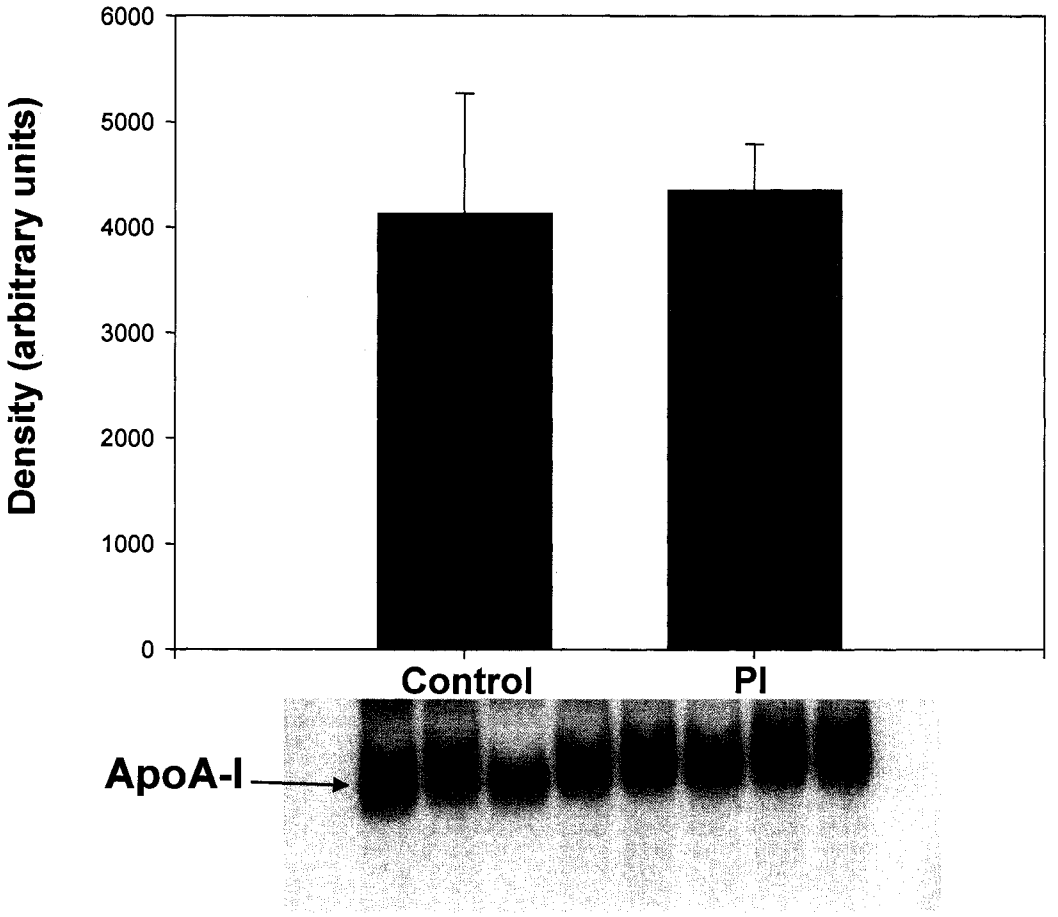

ApoA-I mRNA Concentration (pg/ul)
5
4
3
2
1
0
Control
PI
POPC

Figure 3.3.2: PI does not significantly impact the transcription of ApoA-I in HepG2 cells

HepG2 cells were grown to confluence in 10cm plates. Cells were incubated with 25 ug/ml of PI for 24 hours. Control cells were given an equivalent volume of PBS. Cells were collected using TRI-reagent and total RNA was isolated. ApoA-I mRNA was quantified using specific [α-32P]-dCTP probes in a Northern blot. Band densities were calculated using Quantity One software. Values indicated are means $\pm$ SD for each group. PI did not significantly impact apoA-I transcription at 24 hours.


Density (arbitrary units)
6000
5000
4000
3000
2000
1000
0
Control
PI
ApoA-I

35mm well and real-time PCR was performed as previously described. It was shown that there was a consistent increase in apoA-I mRNA levels in both control and PI treated groups. However, there was still no significant difference between the two groups and the trend towards less apoA-I mRNA in the PI treated groups was still evident (figure 3.3.3).

3.4 Effects of Glucose on PI mediated ApoA-I Synthesis and Secretion

To this point, all the experiments have been conducted at 25mM glucose. In some cases this could be referred to as physiological glucose levels close to the liver, however it can be argued that 5mM glucose is physiologically relevant. Also, studies have shown that glucose itself can have a significant impact on apoA-I mRNA levels (98). Therefore, in order to determine if glucose was impacting the ability of the assay to pick up small increases in apoA-I mRNA in PI treated cells and to make a more physiologically relevant model some experiments at 5.5mM glucose were done. It was determined from these experiments that glucose does not affect the outcome of the assay. HepG2 cells were grown in low glucose (5.5mM) media until confluent. This was followed by incubation with PI for 24h. Cells and media were collected to assess the apoA-I secretion and mRNA levels. PI stimulated cells continue to produce more apoA-I protein (figure 3.4.1) and tend to produce slightly less apoA-I mRNA (figure 3.4.2).

Figure 3.3.3: PI does not affect ApoA-I transcription at early time points

HepG2 cells were grown to confluence in 6-well plates. Cells were incubated with 10 ug/ml of PI for 0.5, 1, 2, 8 hours. Control cells were given an equivalent volume of PBS. Cells were collected using TRI-reagent and total RNA was isolated, converted to cDNA and ApoA-I mRNA was quantified using specific primer and Real Time PCR. Values indicated are means $\pm$ SD for each group. PI did not significantly impact apoA-I transcription at any of the early time points.

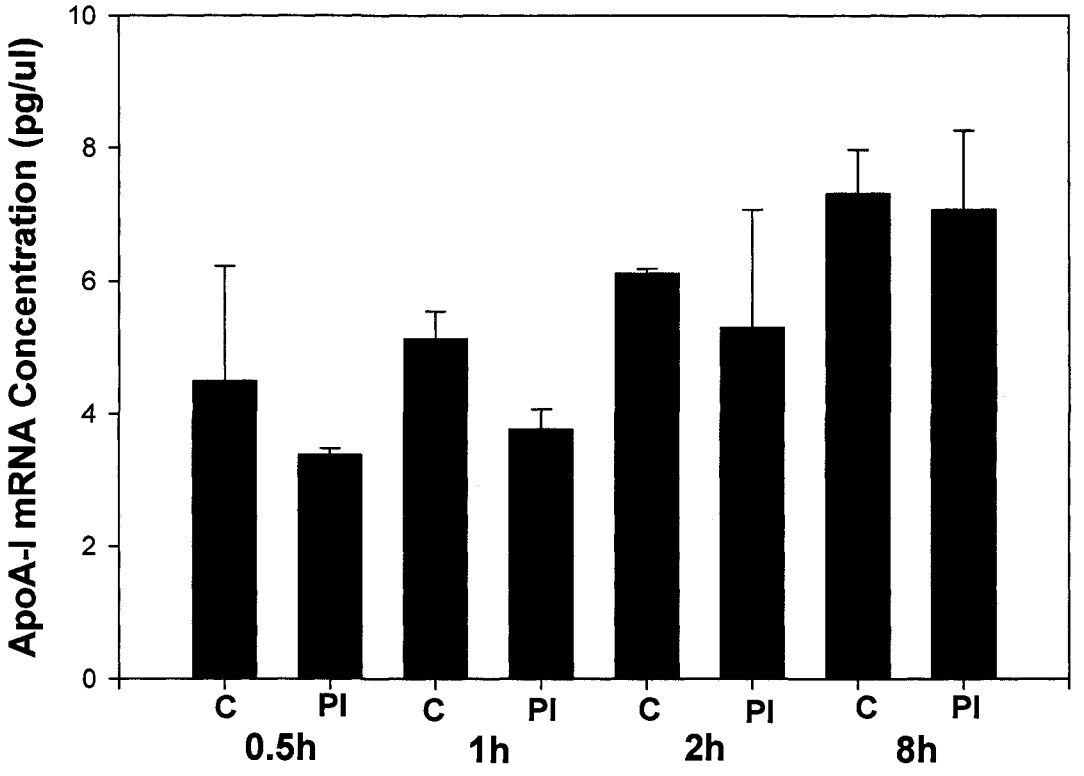

ApoA-I mRNA Concentration (pg/ul)
10
8
6
4
2
0
C
Pl
C
Pl
C
Pl
C
Pl
0.5h
1h
2h
8h

Figure 3.4.1: PI increases ApoA-I secretion in HepG2 cells in both 5.5mM and 25mM glucose media

HepG2 cells were grown in 6-well plates and incubated with 10 ug/ml PI 24 hours in both 5.5mM and 25mM (low and high) glucose media. Control cells were given an equivalent volume of PBS. HepG2 cell media was analyzed for apoA-I mass by ELISA. Values shown are the mean $\pm$ SD for each group. ANOVA was performed to determine significance of difference between groups. Cells treated with PI showed increased apoA-I secretion compared to control in both glucose conditions.

*$p<0.001$ compared to control

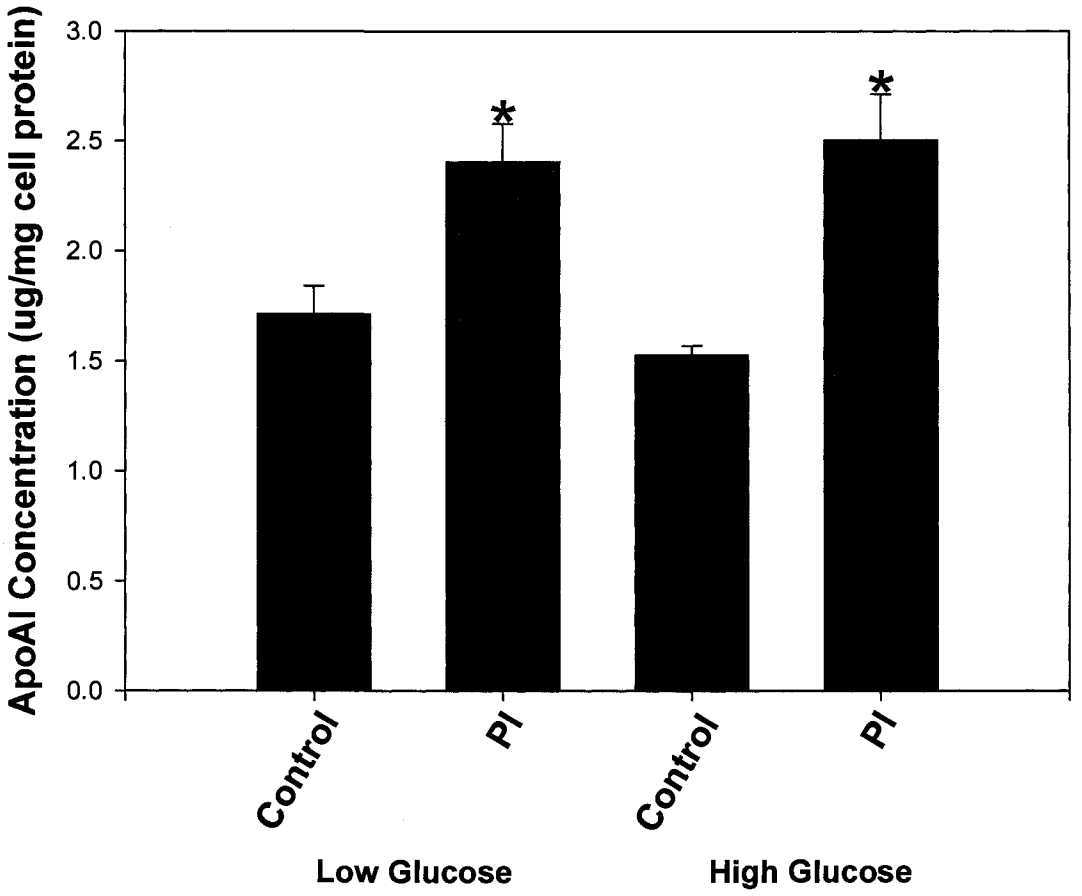
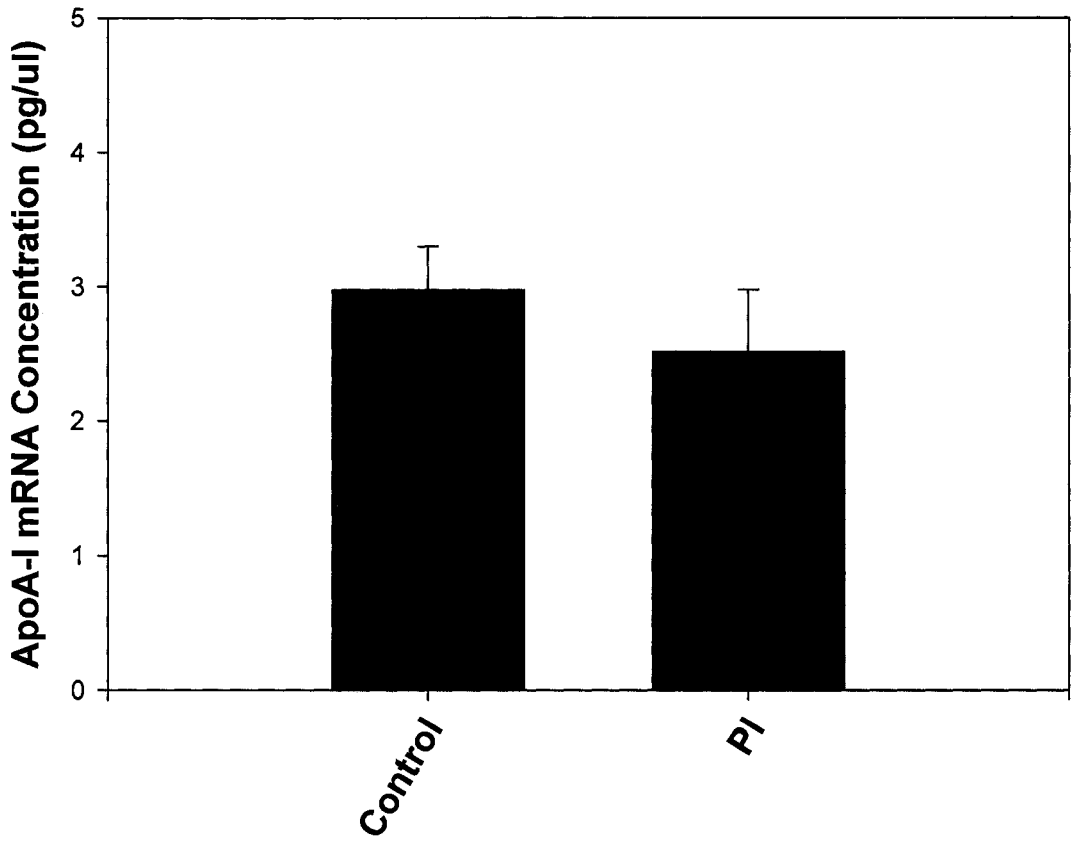

ApoAI Concentration (ug/mg cell protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
PI
Control
PI
Low Glucose
High Glucose

Figure 3.4.2: PI does not effect transcription at 5.5mM glucose conditions

HepG2 cells were grown to confluence in 6-well plates in 5.5mM and 25mM glucose media. Cells were incubated with 10 ug/ml of PI for 24 hours. Control cells were given an equivalent volume of PBS. Cells were collected using TRI-reagent and total RNA was isolated, converted to cDNA and ApoA-I mRNA was quantified using specific primers and Real Time PCR. Values indicated are means $\pm$ SD for each group. PI did not significantly impact apoA-I transcription at 24 hours, however glucose was found to elevate apoA-I transcription and was used as a positive control.


ApoA-I mRNA Concentration (pg/ul)
5
4
3
2
1
0
Control
PI

3.5 Effects of DLPC on ApoA-I Synthesis and Secretion

3.5.1 *DLPC increases ApoA-I secretion in HepG2 cells*

All the experiments with in this thesis used a soybean lecithin derived PI. Soy PI is known to have some linoleic acid content whereas animal derived PI tends to have arachidonic acid as its acyl chains. In an attempt to discover if the inositol ring or the acyl chains of PI were of equal importance in the mechanism of action, we used DLPC which only contains linoleic acid. The results showed that although POPC had no effect on apoA-I secretion (figure 3.1.1), DLPC had a far greater effect on apoA-I secretion compared with PI and control. DLPC was found to increase apoA-I secretion by 95% relative to control and by 38% relative to PI (figure 3.5.1a). Again, intracellular levels of apoA-I remain constant (3.5.1b).

3.5.2 *DLPC tends to decrease transcription*

HepG2 cells were incubated with DLPC for 24h, followed by RT-PCR analysis to determine if the addition of DLPC to the media would affect apoA-I mRNA levels. As seen with PI, DLPC tends to decrease the amount of apoA-I mRNA (figure 3.5.2) even though it mediates an increase in protein levels in the media.

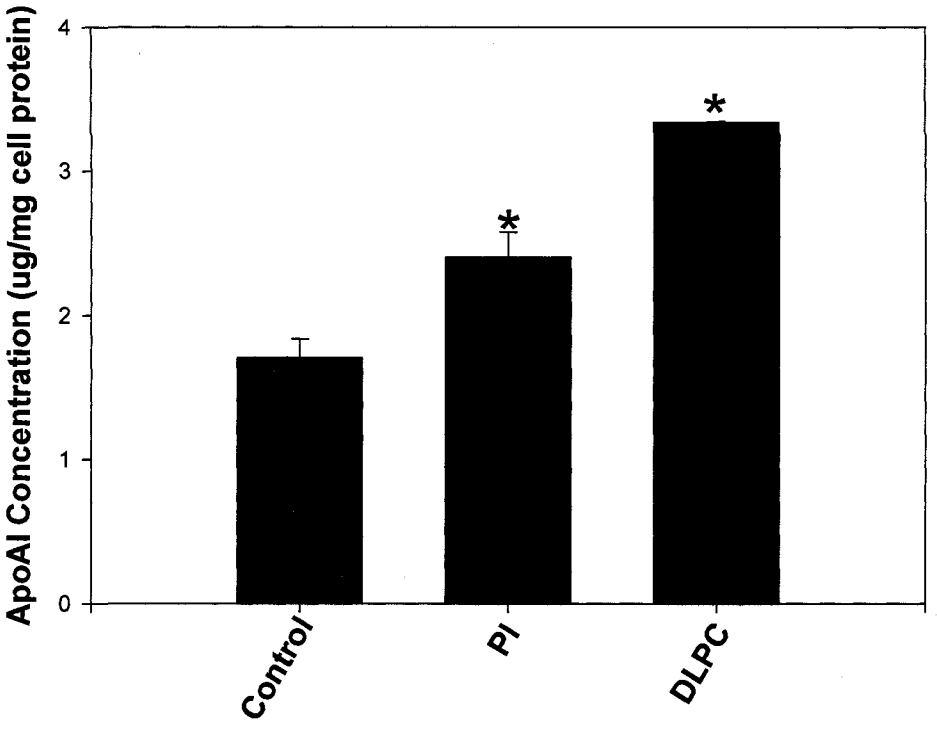
3.6 Effects of PI on Degradation

3.6.1 *Effect of PI on ApoA-I Degradation*

Since there was no significant changes in apoA-I mRNA levels to account for the increase in apoA-I protein, further experiments were performed to assess the effect of PI on apoA-I degradation. ApoA-I degradation was measured using apoA-I protein

Figure 3.5.1: DLPC also increases ApoA-I secretion in 5.5mM glucose
A. HepG2 cells were grown in 6-well plates and incubated with 10 ug/ml PI or DLPC for 24 hours in 5.5mM (low) glucose media. Control cells were given an equivalent volume of PBS. HepG2 cell media was analysed for apoA-I mass by ELISA. **B.** Intracellular apoA-I levels in HepG2 cells treated with PI and DLPC at 24h was assessed using western blot. Values shown are the mean $\pm$ SD for each group. ANOVA was performed to determine significance of difference between groups. Cells treated with PI and DLPC showed increased apoA-I secretion compared to control in low glucose conditions.
*$p<0.001$ compared to control

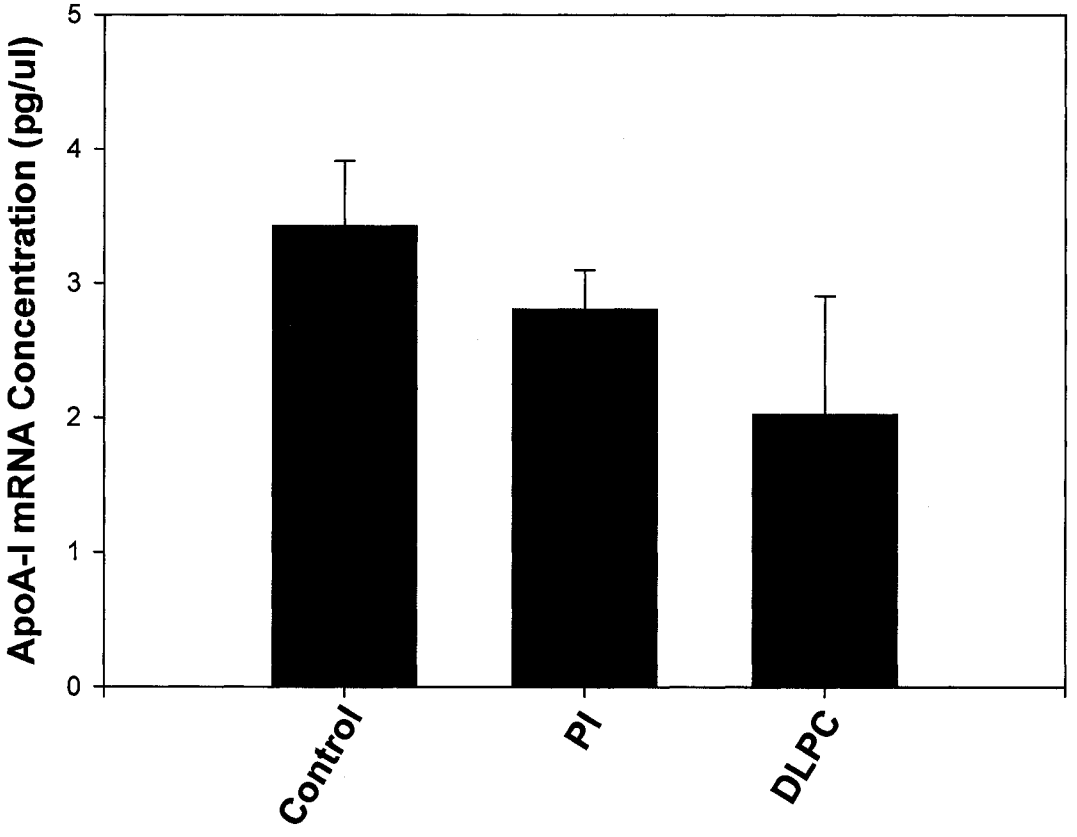
A.


ApoAI Concentration (ug/mg cell protein)
0
1
2
3
4
*
*
Control
PI
DLPC

B.


28 kDa
Control
PI
DLPC

Figure 3.5.2: DLPC has no significant impact on ApoA-I transcription in low (5.5mM) glucose media
HepG2 cells were grown to confluence in 6-well plates in 5.5mM glucose media. Cells were incubated with 10 ug/ml of PI or DLPC for 24 hours. Control cells were given an equivalent volume of PBS. Cells were collected using TRI-reagent and total RNA was isolated, converted to cDNA and ApoA-I mRNA was quantified using specific primers and Real Time PCR. Values indicated are means $\pm$ SD for each group. PI did not significantly impact apoA-I transcription at 24 hours and DLPC trended towards decreasing apoA-I transcription at 24 hours.


ApoA-I mRNA Concentration (pg/ul)
5
4
3
2
1
0
Control
PI
DLPC

labeled with ^{125}I. Upon degradation of apoA-I, the radioactive tag will be cleaved and remain free in the media. Therefore, the amount of free ^{125}I was calculated as an indirect measure of apoA-I degradation. HepG2 cells were treated for 24 hours with PI followed by a 4 hour incubation with 50ug [^{125}IApoA-I]-HDL. These results showed that PI significantly ($p<0.05$) reduces the amount of apoA-I degradation by 22% (figure 3.6.1).

This experiment was also done at a 16 hour time point. At this time point, apoA-I degradation was even further protected in PI treated cells. Results from this experiment showed a 27% decrease in apoA-I degradation in PI treated cells compared with control cells (figure 3.6.2). This study also examined cell association and cell surface binding of [^{125}I-apoA-I]-HDL. However, no significant differences in cell association or cell surface binding between control cells and PI treated cells were observed (data not shown). These results suggest that the increase in apoA-I protein secretion observed in PI treated HepG2 media is due, at least in part, to a decrease in apoA-I degradation.

Figure 3.6.1: PI decreases the degradation of ApoA-I in HepG2 Cells at 4h

HepG2 cells were grown to confluence in 12-well plates. Cells were incubated with 10ug/ml of PI for 24 hours, followed by a four hour incubation with [^{125}IApoA-I]-HDL (50ug). Cell media was collected and a TCA precipitation was performed. Supernatant from the precipitation was counted for ^{125}I as an indirect measure of apoA-I degradation. Data is presented as means $\pm$ SD for each group. A two-tailed Student's t-test was used to calculate significance of difference.

*$p<0.05$ compared to control

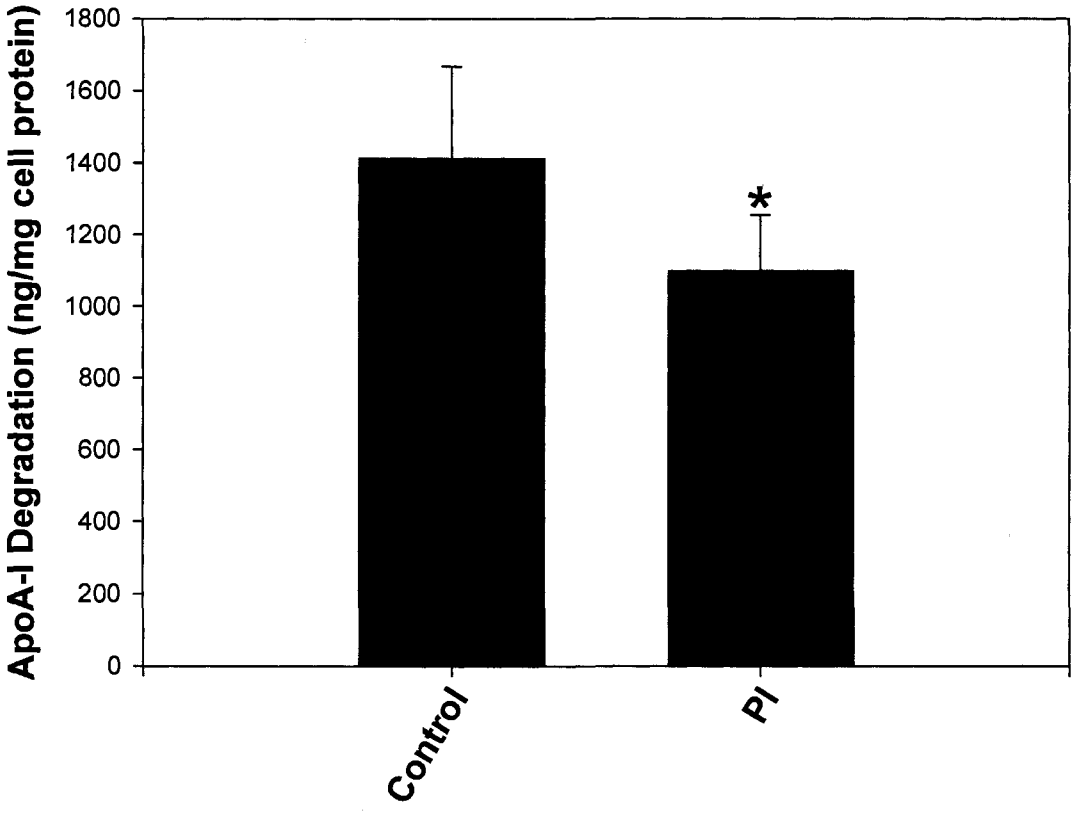
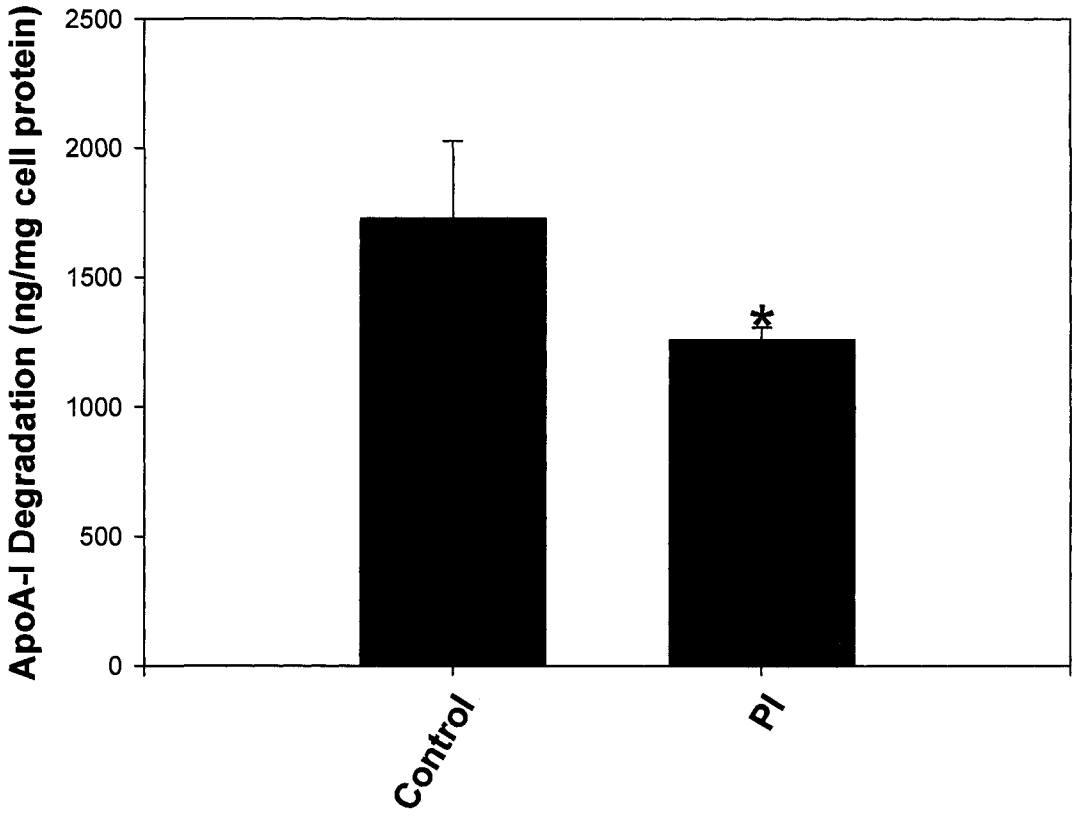

ApoA-I Degradation (ng/mg cell protein)
1800
1600
1400
1200
1000
800
600
400
200
0
*
Control
PI

Figure 3.6.2: PI decreases the degradation of ApoA-I in HepG2 Cells at 16h

HepG2 cells were grown to confluence in 12-well plates. Cells were incubated with 10ug/ml of PI for 24 hours, followed by a 16 hour incubation with [^{125}I-ApoA-I]-HDL (50ug). Cell media was collected and a TCA precipitation was performed. Supernatant from the precipitation was counted for ^{125}I as an indirect measure of apoA-I degradation. Data is presented as means $\pm$ SD for each group. A two-tailed Student's t-test was used to calculate significance of difference.

*$p<0.05$ compared to control


ApoA-I Degradation (ng/mg cell protein)
2500
2000
1500
1000
500
0
*
Control
PI

Chapter 4 – Discussion

4.1 Introduction

Cardiovascular disease is one of the major causes of mortality and morbidity in the industrialized world. It has been shown that levels of plasma HDL are inversely correlated to risk of cardiovascular disease (86). Epidemiological studies indicate that a 1mg/dl increase in the HDL-cholesterol concentration is associated with a 2-3% decrease in cardiovascular risk (87-89). Currently it is accepted that nicotinic acid is the most effective agent for raising HDL cholesterol. Niacin has been shown to increase HDL 15-20% in human trials (90), while other drugs such as fibrates have been shown to raise HDL to a lesser extent (81). However, recent human trials have shown that PI is a potent HDL raising agent as well (85). We now extend those findings and show for the first time that PI can stimulate apoA-I secretion in a HepG2 cell model system (2-fold increase).

4.2 Development of an in vitro cell model – The HepG2 Cell

Due to the fact that most apoA-I is produced in the liver in humans, we decided to use a HepG2 cell culture system to investigate the role of PI in apoA-I metabolism. This cell line has been used extensively to study HDL metabolism and apoA-I metabolism. The HepG2 cell is a human hepatoblastoma cell line. HepG2 cells exhibit considerably higher levels of the ERK1/2 mass compared to primary hepatocytes (113), however both cell lines produce similar levels of apoA-I when dosed with PI. The HepG2 cell line is easily grown and passaged, which allows for

very reproducible results. The initial experiments using the HepG2 cells treated with PI showed an excellent effect on the elevation of apoA-I into the media. Initial experiments showed a 1.5 fold increase over control cells (figure 3.1.1a). This compared well with the increase in HDL seen in recent human trials using PI. Preliminary studies with PI in primary human hepatocytes yielded similar results (97), and therefore it was determined that the HepG2 cells would be an accurate representation of a human model. The HepG2 model was then used to elucidate the impact that PI was having on the molecular processes of the cell.

4.3 Investigation of the signaling cascades affected by PI

In previous studies, HDL has been shown to impact the ERK1/2 MAPK pathway (91). We had also collected preliminary data from a phospho-protein array that suggested that the ERK1/2 MAPK and JNK MAPK pathways were being affected by PI in HepG2 cells. Here, we show that PI mediates apoA-I levels in the media via the ERK1/2 and JNK MAPK pathways. It has been clearly shown by this research that U0126 (inhibitor of MEK1/2) completely inhibits the effects usually seen when PI is incubated with HepG2 cells (figure 3.2.2). The same sort of inhibition is seen when the JNK MAPK pathway is inhibited by SP600125 (figure 3.2.5). This may be a direct effect of PI or a secondary effect of the increased amount of apoA-I/HDL in the media. As mentioned earlier, there is a potential that the increase in HDL (91) could be impacting these pathways. However, the immediate impact of PI on ERK1/2 (figure 3.2.3, 3.2.4) would suggest that PI is the primary stimulant of the ERK1/2 MAPK pathway. Inhibition of PI3K and p38MAPK showed no impact on the PI stimulation of

apoA-I (figure 3.2.1, 3.2.7). This showed that PI has a specific effect in the cell through both the ERK1/2 and JNK MAPK pathways. Recently, the ERK1/2 and JNK pathways have been shown to impact cellular processes outside of there typical mitogenic and apoptotic roles. In many cases ERK and JNK pathways have been shown to work in tandem as is the case with apoA-I gene expression (92). While the MEK/ERK MAPK pathway has been implicated in apoA-I regulation for many years, this is a novel finding for the JNK signaling pathway to stimulate apoA-I secretion. Insulin has been commonly known to mediate apoA-I expression through the ERK1/2 MAPK cascade (93). Further investigation into the proteins that have a role upstream of the ERK1/2 and JNK pathways solidified our findings. Inhibition of Ras, a common GTPase which initiates both MAPK pathways, was shown to inhibit the effects of PI (figure 3.2.6).

In general, such a process would be initiated via binding to a receptor and propagating a signal into the cell. At this time, it is uncertain as to whether or not PI binds to a receptor or is transported into the cell. However, upon inhibition of PI-PLC, the PI mediated effects on apoA-I were abolished (figure 3.2.8). PC-PLC also had a similar effect. It is possible in this case that the inhibitors were not specific enough. Potentially, the PC-PLC inhibitor impacted the function of PI-PLC. The involvement of PC-PLC is of interest and suggests that a diglyceride cascade stimulation of PKC may be central to the actions of PI. Also, inhibition of PLC suggests that a receptor, perhaps receptor tyrosine kinase (RTK) or G-coupled protein receptor, is involved in the PI mediated effects.

4.4 PI Structure – The Linoleic Acid and DLPC Discovery

At this stage, it was important to determine which of the components of soy PI were active. The main components of PI are an inositol head group and two acyl chains. Soybean derived PI contains a linoleic acid acyl chain as oppose to human derived PI which contains arachidonic acid. In early studies POPC was compared to PI and POPC showed no effects. This lead to the belief that the inositol head group was responsible for the PI mediated effect on apoA-I secretion. This was a natural conclusion as the inositol head group has been implicated in many pathways of signal transduction (94). However, upon further research on the various species of PL molecules, it was discovered that PL rich in linoleic acid had activity. DLPC has a more potent effect (2-fold), compared to PI, in regards to stimulating apoA-I secretion from HepG2 cells. The major difference in this PC molecule is that it contains two linoleic acid chains. As seen in figure 3.5.1, DLPC has the capacity to mediate a much larger secretion of apoA-I compared to PI treated cells. This finding lead to the thought that it is the acyl chains of PI that are responsible for the significant impact on apoA-I metabolism. This phenomenon has been shown by decreasing the saturation of the acyl chains of PC in reconstituted HDL. Decreasing the saturation of the acyl chains of PC had a beneficial effect on reverse cholesterol transport in HepG2 cells (95). In this thesis, we see an increased secretion of apoA-I which would have a beneficial effect on reverse cholesterol transport and could be due to the same reasons. Unfortunately, DLPI can not be produced and therefore can not be compared to DLPC. Linoleic acid is present on PI and could play a key role in its affects on apoA-I secretion. PI only has one linoleic acid acyl chain whereas DLPC has two which

promotes nearly twice the effect on apoA-I secretion. However, the power of the inositol head group can not be completely dismissed. It has been shown by many studies including some personal preliminary studies (96), that unsaturated fatty acids such as linoleic and linolenic acid have no effect on apoA-I promoter activity or secreted protein levels. There is an apparent need for the inositol head group for the acyl chains to have there effect on apoA-I secretion. The head group may provide the anchor needed to bind to a receptor or allow it to gain access to the cell so that the acyl chains can exert there affects on the MAPK pathways. The effect of inhibiting PI-PLC suggests that the acyl chains of PI play a vital role in the activation of the ERK1/2 and JNK MAPK pathways which lead to the increase in apoA-I secretion in HepG2 cells. Classic PI signaling would suggest that PKC is also involved as it can be activated by the bi-products of PI-PLC cleavage and then itself, activate Ras. In its entirety, these data suggest that both the inositol head group and acyl chain components contribute to the PI mediated apoA-I secretion. However, with the recent data on di-linoleic compounds (DLPC), it suggests that the acyl chains may play a more significant role.

4.5 Transcription vs. Degradation

Although the MAPK pathway usually impacts transcription of mRNA, it does not seem to do so in this case. The MEK/ERK and JNK pathways do not increase apoA-I mRNA levels when stimulated by PI (figure 3.3.1, 3.3.2, 3.3.3). However, such a large increase in apoA-I protein would suggest such a mechanism. The effect of MAPK could indirectly affect apoA-I levels by regulating other genes related to its secretion such as ABCAI. Data suggests that PI increases cellular levels of PPARα.

PPARs have been shown to be catalyzed by the MAPK pathway. Also, PI induced hepatic apoA-I secretion was attenuated by PPARα inhibition with the specific inhibitor MK886. The acyl chains of PI, specifically linoleic acid, appear to be the ligand for PPARα and may play a direct role in up regulating the apoA-I gene and apoA-I secretion. Although PPARs are transcription factors, increasing PPARα protein expression with linoleic acid had no impact on apoA-I secretion. This suggests that stimulation of apoA-I secretion requires more than just PPARα expression (97).

In many of the initial experiments, a high glucose (25mM) media was used in the experiments. It has been shown that glucose itself can elevate apoA-I transcription and increase mRNA levels (98). For this reason, both low (5.5mM) and high (25mM) glucose media was tested in both the secretion and transcription experiments. The low glucose media yielded similar results to the high glucose media with respect to apoA-I secretion in PI treated cells (figure 3.4.1). Even though the high glucose media did have an impact on apoA-I transcription it was not masking the effect of PI on transcription of apoA-I mRNA. In a low glucose environment, the PI treated HepG2 cells did not show a significant difference between control cells at the 24 hour time point (figure 3.4.2). As mentioned earlier, DLPC appears to impact apoA-I secretion more than PI. DLPC was tested to see if it was impacting transcription of apoA-I mRNA. Similarly to PI, DLPC had no significant impact on apoA-I mRNA levels. However, PI and DLPC shared a similar trend to decreasing the amount of apoA-I mRNA (figure 3.5.2). These values are almost inversely correlated with the increase in apoA-I levels. DLPC creates a larger secretion of apoA-I into the HepG2 media compared with PI and therefore has a larger trend of reducing the transcription of

apoA-I. This linkage is not conclusive, but may suggest that apoA-I levels in the media can down regulate the amount of apoA-I mRNA being produced.

Niacin has been shown to increase HDL levels in humans (90) and it has also been proven to increase apoA-I by ways other than transcription. Niacin has been shown to increase apoA-I by inhibiting the cellular association of apoA-I with HepG2 cells (99). Limiting association with the cell prevents the cell from taking up the protein for recycling or degradation. This increases the circulation time of apoA-I allowing it to up regulate processes such as reverse cholesterol transport. This thesis also examined cell association and cell surface binding of exogenous [^{125}I-apoA-I]-HDL. However, no significant difference in cell association or cell surface binding was observed between control cells and PI treated cells (data not shown).

Catabolism of apoA-I appears to play an important role in this model. Although exogenous apoA-I association with HepG2 cells was the same in PI treated cells compared to control cells, the amount of exogenous apoA-I degradation was increased in PI treated cells. In fact, in vivo studies of HDL metabolism in human populations have shown that clearance of apoA-I, rather than its production rate, is the most important determinant of the variability of plasma HDL cholesterol and apoA-I concentrations. It appears that variation in plasma HDL and apoA-I concentrations in the general population is primarily a function of variation in clearance rather than production rates (100-103).

Our studies correspond with this data, showing a 22% reduction in exogenous apoA-I degradation in PI treated cells at 4 hours (figure 3.6.1). This reduction is further increased when looking at the 16 hour time point (figure 3.6.2). The reduction

in exogenous apoA-I degradation explains, in part, the elevation of apoA-I in the media. Exogenous apoA-I was studied because of the precedence set by the niacin study (99).

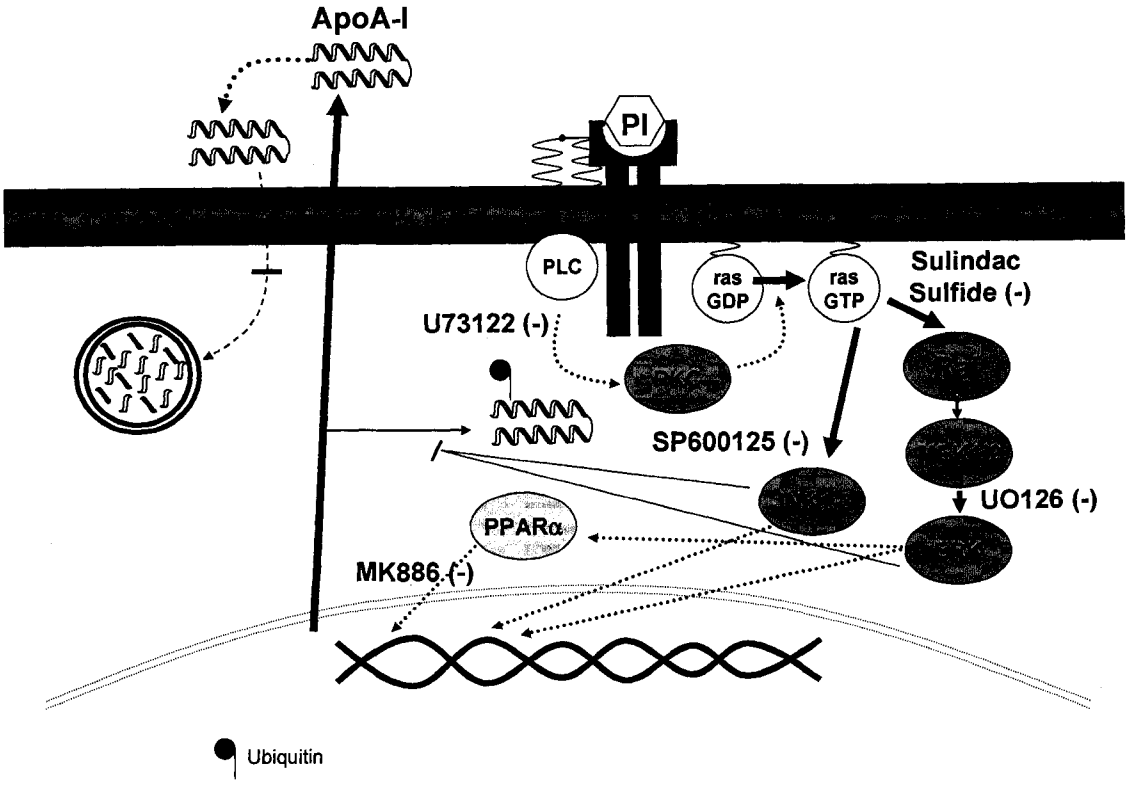
While these studies need to be interpreted with caution, as they do not track endogenous apoA-I metabolism, the data suggests that the binding and uptake of HDL is affected by PI. It was also important to know the impact PI was having on mature HDL particles as it pertains to RCT. Recent studies have shown that MAPK plays a role in monocytes to raise the expression of ABCAI (117). This elevation would potentially increase the production of mature HDL. Recent findings in our lab have shown that PI decreases the expression of ABCAI in HEPG2 cells (118). Together, these findings suggest that PI has the potential to raise ABCAI in the periphery allowing for the production of HDL, while decreasing ABCAI at the liver preventing the uptake of apoA-I (104). Both mechanisms helping to raise apoA-I levels. The two main cellular pathways for protein degradation in a cell are lysosomal and proteasomal. It is proposed that both these pathways have been inhibited indirectly by PI. It has been shown that apoA-I uptake and degradation in the lysosome can be mediated by the ABCAI transporter. It is known that apoA-I bind to ABCAI to create HDL through lipidation. ApoA-I has been suggested to be internalized with ABCAI in what was thought to be an internalization process. However, it has been shown that most of the apoA-I is targeted for lysosomal degradation and therefore does not significantly contribute to the biogenesis of HDL (104). It is therefore hypothesized that PI protects apoA-I from this sort of degradation in order to increase circulating levels of apoA-I.

Proteasomal degradation is another major pathway for cellular protein degradation. Both the ERK1/2 and JNK MAPK pathways have been implicated in this type of degradation. It has been shown that the ERK1/2 pathway can inhibit the degradation of GATA3 protein through the inhibition of the ubiquitin-proteasome pathway (105). The JNK MAPK pathway has also been implicated in this type of protein stability. The JNK pathway has previously been shown to stabilize p21, a negative regulator of the cell cycle, through inhibiting the ubiquitination of p21 (106). It is hypothesized that the decrease in degradation of apoA-I is related to the inhibition of its ubiquitination in the cell via both the ERK1/2 and JNK MAPK pathways.

4.6 The Mechanism of Action

We hypothesize that PI may act through a cell surface RTK to stimulate MEK/ERK and JNK pathways (figure 4.6.1). Receptor kinases catalyze cellular signaling pathways and regulate key cell functions such as proliferation and differentiation. Signaling proteins that bind to the intracellular domain of receptor tyrosine kinases include phospholipase C (PLC) and adaptor proteins such as Shc and Grb2. PI appears to act through a PLC pathway, as we showed that inhibition of either PI-PLC or PC-PLC directly affects the ability of PI to increase apoA-I secretion. PLC production of diglyceride activates PKC and together with other adaptor proteins (Shc, Grb2) may activate a Ras GTPase signaling cascade and directly impact the activation of the ERK1/2 and JNK MAPK pathways. Both proteomic arrays and chemical inhibitor studies suggest that the expression and phosphorylation status of several components of the ERK1/2 and JNK MAPK pathways are affected by treatment of HepG2 cells with

Figure 4.6.1: Model of PI mediated ApoA-I secretion
We hypothesize that PI may act through a cell surface receptor tyrosine kinase (RTK) to stimulate MEK/ERK and JNK pathways. Receptor kinases catalyze cellular signaling pathways and regulate key cell functions such as proliferation and differentiation. Signaling proteins that bind to the intracellular domain of receptor tyrosine kinases include phospholipase C (PLC). PI appears to act through a PLC pathway. PLC production of diglyceride activates PKC and then activates a Ras GTPase signaling cascade. This directly impacts the activation of the ERK and JNK MAP kinase pathways. We hypothesize that through this cascade, PI increases the amount of secreted apoA-I by limiting the amount of apoA-I degradation by perhaps downregulating important transporters involved in apoA-I uptake and degradation, such as ABCAI, or by inhibiting the ubiquitination and lysosomal degradation of the protein.


ApoA-I
PI
PLC
ras GDP
ras GTP
Sulindac Sulfide (-)
U73122 (-)
SP600125 (-)
UO126 (-)
PPARα
MK886 (-)
Ubiquitin

PI. The ERK1/2 MAPK pathway also stimulates PPAR□. We hypothesize that through this cascade, PI increases the amount of secreted apoA-I by limiting the amount of apoA-I degradation. This potentially occurs through down regulating important transporters involved in apoA-I uptake and degradation, such as ABCAI, which would limit lysosomal degradation of apoA-I, or by inhibiting the ubiquitin-proteasomal degradation of the protein.

4.7 Physiological Relevance

PI raises HDL in humans and in this HepG2 cell culture system. Raising HDL would have a positive impact on the risk of developing CHD. This potential therapeutic appears to act through PPARα much like the fibrate drugs, but with greater potency. It is also much safer to use compared to most synthetic drugs. Large amounts of PL are regularly ingested in food products and there exists well documented prior human experience showing that orally administered mixtures of PL are safe and well tolerated. Soy lecithin and all of it constituents (including PI) are on the US FDA GRAS (Generally Regarded as Safe) list. The HepG2 model employed in this thesis represents as accurately as possible the in vivo cellular events in the liver. The biggest difference in the model and in vivo is the digestive tract. Dietary PL is degraded in both the stomach and intestinal track. They are sensitive to acid degradation in the stomach and can be broken down into diacylglycerol by an acid pH dependent head group hydrolysis (110-112). At present, studies are underway to attempt to prevent gastric degradation of PL (enteric coating) and enhance transport of intact PI to the

liver. Much of the dietary PL is quickly transported to the liver via the lymphatics where PI would be expected to impact hepatocyte cellular metabolism.

Due to the unique mechanism of PI, it would be a candidate for combination therapy with statins. PI has been shown to not impact CYP P450 enzymes (97) which are inhibited by fibrate drugs. The inhibition of these enzymes exacerbates the toxicity of statin drugs. It is also note worthy that PI increased apoA-I secretion in a hyperglycemic (high glucose) state. This may suggest that the HDL raising efficacy of PI may be unaffected in the hyperglycemia of diabetic patients. Therefore, PI may provide a novel therapeutic alternative to treat a number of metabolic disorders safely and effectively.

4.8 Concluding remarks and future experiments

HDL and apoA-I play an extremely important role in lipid metabolism. They have been shown to be inversely correlated with the risk of developing CHD and therefore play a significant role in the prevention of this disease. The studies presented in this thesis provide a novel mechanism by which apoA-I can be increased. This knowledge will hopefully create the foundation for research which will uncover the vast potential of PI and consequently uncover new insight into apoA-I metabolism. Although this thesis presents a mechanism of action for PI, much work needs to be done in order to fill in the knowledge gaps. Experiments are on going to determine the exact mechanism of PI at the cell membrane level. Experiments to elucidate the entry mechanism or receptor mediated signaling of PI are ongoing. As well, inhibitor experiments of the various degradative pathways are ongoing in order to specify the

effects of PI with regards to apoA-I degradation. The transcriptional effects of PI on apoA-I are also being revisited. Future plans to evaluate the effect of PI on the transcription of transporter proteins such as ABCAI and SR-BI are on going. PI creates a large increase in apoA-I protein levels, and degradation alone can not account for this entire effect. PI stimulates an 86% increase in apoA-I secretion whereas it only decreases the degradation of apoA-I by 27%. With the new insight of PI mediated PPARα stimulation, transcription may become a factor in the future.

In conclusion, PI increases apoA-I secretion in HepG2 cells which is mediated by the MEK/ERK and JNK MAPK cascades. These pathways appear to play a role in limiting the degradation of apoA-I.

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Publications

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Hopewell S., Sinclair P., Burgess J., Sparks DL. Phosphatidylinositol Acts Through Mitogen-activated Protein Kinase Pathways to Stimulate Secretion of ApoA-I. Canadian Lipoprotein Conference, Gimli, Manitoba, October 2006.