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Lipid Acquisition by Apolipoprotein A-I in ER and Golgi Compartments of Primary Mouse Hepatocytes

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Lipid Acquisition by Apolipoprotein A-I in ER and Golgi Compartments of Primary Mouse Hepatocytes

Jovana Marić

Thesis submitted to the
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I. ABSTRACT

It was previously unknown what the significance and location of intracellular lipidation of newly synthesized apoA-I in hepatocytes were in plasma HDL formation. By labeling primary mouse hepatocytes with ^{3}H-choline, we showed that phospholipidation of apoA-I is most significant in endoplasmic reticulum (ER) and medial Golgi compartments with minor lipidation upon export from the cell. Intracellular LDL-cholesterol lipidation of apoA-I is absent, with rapid cholesterol accumulation at the plasma membrane. *De novo* synthesized cholesterol was able to lipidate apoA-I intracellularly to a small but significant level. In hepatocytes lacking ABCA1, phospholipidation and lipidation by *de novo* cholesterol were both reduced in Golgi, while ER lipidation remained mostly unchanged. Plasma membrane lipidation by LDL-cholesterol was also significantly reduced. This implies that HDL formation begins with apoA-I phospholipidation in the ER, followed by modest cholesterol lipidation in the Golgi, dependent on ABCA1, with the bulk of cholesterol lipidation occurring at the plasma membrane.

II. DEDICATION

I would like to dedicate this work to my husband, Mike, for loving me, supporting me and making me happy now and forever, for making me smile and smiling back at me. Mikey, you are my dream come true. Also, to my dear parents, for teaching me and guiding me, and for showing me unconditional love and patience my whole life. Also, I dedicate this paper to my wonderful brother Nikola for every smile and every hug he gave me, for every joke he told to make me smile, for being the cutest baby brother and for growing up to be such a smart, responsible and handsome young man with a big heart. I would not have been able to accomplish this work without these four important people in my life.

I would like to thank all my friends and family both here and back home in Belgrade for all the wonderful moments we spent together, and for their love and support that motivated me to keep trying and working hard.

In memory of my dear great-aunt, Mara. I will never forget the sound of your joyful laughter and the touch of your loving, gentle hands. I will miss you and love you forever.

In memory of my dear friend, Gerard. I will always remember the wonderful moments we spent together, playing soccer and tennis, going for Italian gelato and the way you always knew what to say to make me laugh. I miss you my friend. Love always.

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I would like to thank Dr. Ross Milne and Dr. Zemin Yao for their guidance as my committee members and for critical reading of my paper.

Thank you everyone, again. This has been quite a ride!

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VI. ABBREVIATIONS

9*cis* RA	9*cis* retinoic acid
ABCA1	ATP-binding cassette transporter A1
Ad-AI	adeno-apolipoprotein A-I
Ad-Luc	adeno-Luciferase
apoA-I	apolipoprotein A-I
apoB	apolipoprotein B
apoE	apolipoprotein E
APS	ammonium persulfate
BFA	brefeldin A
CAD	coronary artery disease
cAMP	cyclic AMP
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CTX	cholera toxin
dGolgi	distal Golgi
ER	Endoplasmic Reticulum
FC	free cholesterol
FHA	familial hypoalphalipoproteinemia
GFP	green fluorescent protein
HDL	high density lipoprotein
IP	immunoprecipitation
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDL-R	LDL receptor
LXR	nuclear liver X receptor
mGolgi	medial Golgi
MDM	monocyte-derived macrophages
MPM	mouse peritoneal macrophages
PC	phosphatidylcholine
PL	phospholipid
PM	plasma membrane
RCT	reverse cholesterol transport
RER	rough Endoplasmic Reticulum
RXR	nuclear retinoid X receptors
SCAP	SREBP cleavage-activating protein
SM	sphingomyelin
SR-B1	scavenger receptor class B type 1
SREBP	sterol regulatory element-binding protein
TD	Tangier disease
TG	triglycerides
TGN	trans-Golgi network
TLC	thin layer chromatography

VII. INTRODUCTION

VII. INTRODUCTION

A) *Atherosclerosis*

Atherosclerosis is the primary cause of about 50% of all deaths in the western world (1). As well, atherosclerotic cardiovascular disease has been identified as the main cause of mortality in diabetes (2). High blood concentration of low-density lipoprotein (LDL) has been linked to the increased risk of atherosclerosis. The primary initiating step of atherosclerosis involves the accumulation of LDL in the inner layer or intima of blood vessels at the future sites of the atherosclerotic lesions. Increased accumulation of lipoprotein particles in the intima sends a signal to the monocytes to migrate across the endothelial monolayer into the intima and differentiate into macrophages. The native LDL undergoes oxidation following exposure to the oxidative waste of vascular cells and the oxidized LDL is then taken up by the macrophages via scavenger receptors (1). These cholesterol-engorged macrophages or 'foam cells' act as the precursors to fatty streaks and more advanced lesions. Such fibrous lesions typically have a 'fibrous cap' consisting of smooth muscle cells and extracellular matrix that encloses a lipid-rich 'necrotic core' (1). Plaques can become increasingly instable, and ulcerations or hemorrhage can occur (1). Although advanced lesions can block blood flow, the most important clinical complication is the formation of a thrombus or blood clot, resulting in myocardial infarction or stroke (1).

B) *Reverse Cholesterol Transport*

Low plasma levels of high-density lipoprotein (HDL) are a major risk factor for cardiovascular disease, considering that HDL is thought to be involved in the clearance of accumulated cholesterol in arterial wall macrophages (3). High HDL cholesterol levels are associated with a decreased risk of atherosclerosis and coronary artery disease (CAD). Normally functioning cells have developed a mechanism to prevent the progression of atherosclerosis, termed reverse cholesterol transport (RCT).

Cholesterol is normally synthesized in the liver, packaged into LDL, which is then taken up by the LDL receptors on peripheral cells. The reverse pathway is initiated when the lipid-poor apolipoprotein A-I (apoA-I) interacts with the cell surface of peripheral cells and promotes phospholipid and cholesterol efflux (Figure 1-1). The addition of phospholipid and cholesterol to the apoA-I particle leads to the formation of a small discoidal HDL particle. Lecithin:cholesterol acyltransferase (LCAT) catalyzes the esterification of cholesterol in discoidal HDL, resulting in the movement of the neutral cholesteryl ester (CE) into the hydrophobic core as the discoidal particle turns into a spherical particle. HDL interacts with the scavenger receptor class B type 1 (SR-B1) in the liver and transfers CE into hepatocytes. Plasma HDL particle is often accompanied by cholesteryl ester transfer protein (CETP), a hydrophobic glycoprotein involved in the transfer of lipids between plasma lipoproteins (4). CETP transfers triglycerides from VLDL and LDL to HDL, and cholesteryl esters from HDL to VLDL and LDL, providing an alternate method of transporting CE to the liver (5). In hepatocytes, CE can be converted to bile acids and secreted into bile. HDL metabolism results in the efflux of cholesterol from peripheral cells and its delivery to the liver where it

can be secreted into bile (reviewed in (6)). Hence, this pathway is referred to as the reverse cholesterol transport pathway.

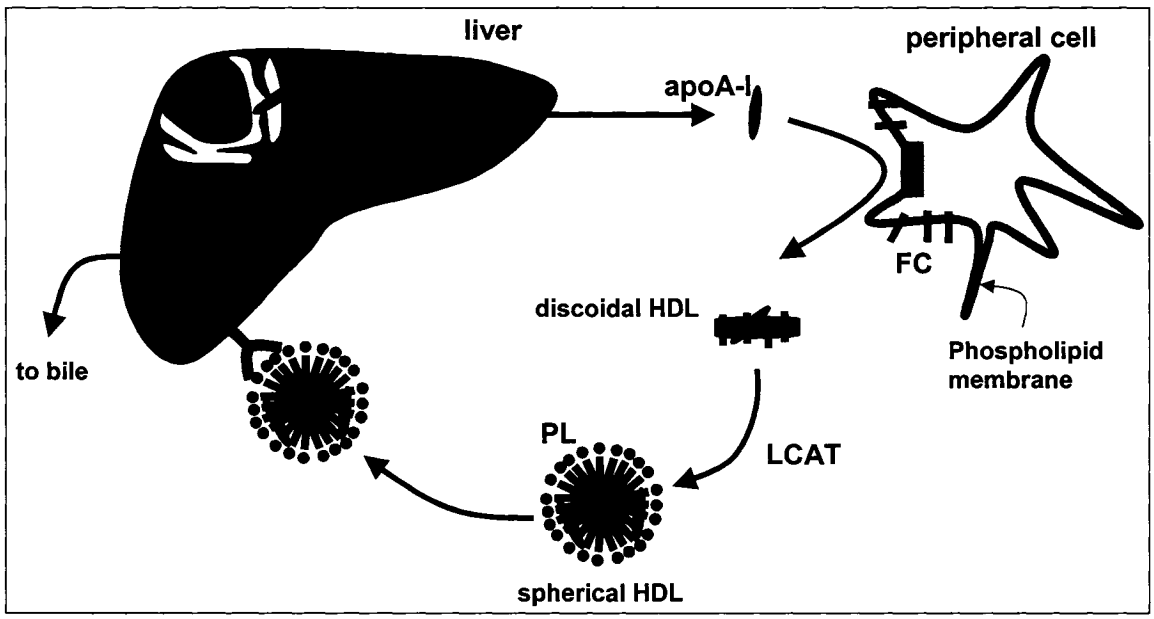

Figure 1-1: Schematic representation of the reverse cholesterol transport. ApoA-I is secreted from the liver. Lipid poor/free apoA-I interacts with peripheral cells, inducing cholesterol and phospholipid efflux. ApoA-I lipidation creates discoidal HDL. LCAT esterifies FC to CE which then gets transferred to the core of HDL, producing spherical HDL. Spherical HDL interacts with SR-B1 in the liver, where CE is converted to bile acid and secreted into bile.

C) *ABCA1*

Our knowledge of HDL metabolism has improved greatly with the finding of a new ATP-binding cassette transporter A1 molecule (ABCA1). ABCA1 is a twelve-helix transmembrane protein. It employs the mechanism of ATP hydrolysis, just like the other members of the ATP binding cassette transporter family, to transport numerous substances

across membranes (7). ABCA1 is thought to mediate the transport of cellular phospholipids to lipid-poor apoA-I.

a. Tangier Disease

It has recently been shown that the mutations in ABCA1 are the underlying cause of Tangier Disease (TD) (8). The hallmark feature of TD, a rare disorder associated with extremely low levels of HDL cholesterol (less than 5% of normal) and apoA-I (less than 1%), is the cholesteryl ester accumulation in macrophages in organs such as liver, spleen, tonsils, lymph nodes, thymus, intestine and Schwann cells (9). It follows that in TD patient fibroblasts, cholesterol and phospholipid (PL) efflux is highly reduced or completely absent. Hayden's group has shown that familial hypoalphalipoproteinemia (FHA) occurs as a consequence of a heterozygotic mutation in the ABCA1 gene and is characterized by a decreased cholesterol efflux (8).

b. ABCA1 Function

Originally, it was believed that ABCA1 was a cholesterol transporter. However, lately a large amount of evidence shows that ABCA1 acts mainly, if not only, as a phospholipid transporter. A study done by Fielding et al. suggests that PL efflux, unlike most free cholesterol (FC) efflux, is directly dependent upon ABCA1 transporter activity, and that these two lipids are most likely transferred by distinct pathways (10). This theory is the basis of a so-called two-step mechanism of cholesterol efflux (Figure 1-2). Presumably, as a first

step, apoA-I interacts with the ABCA1 protein and takes up the effluxed PL, the process in which apoA-I becomes lipidated.

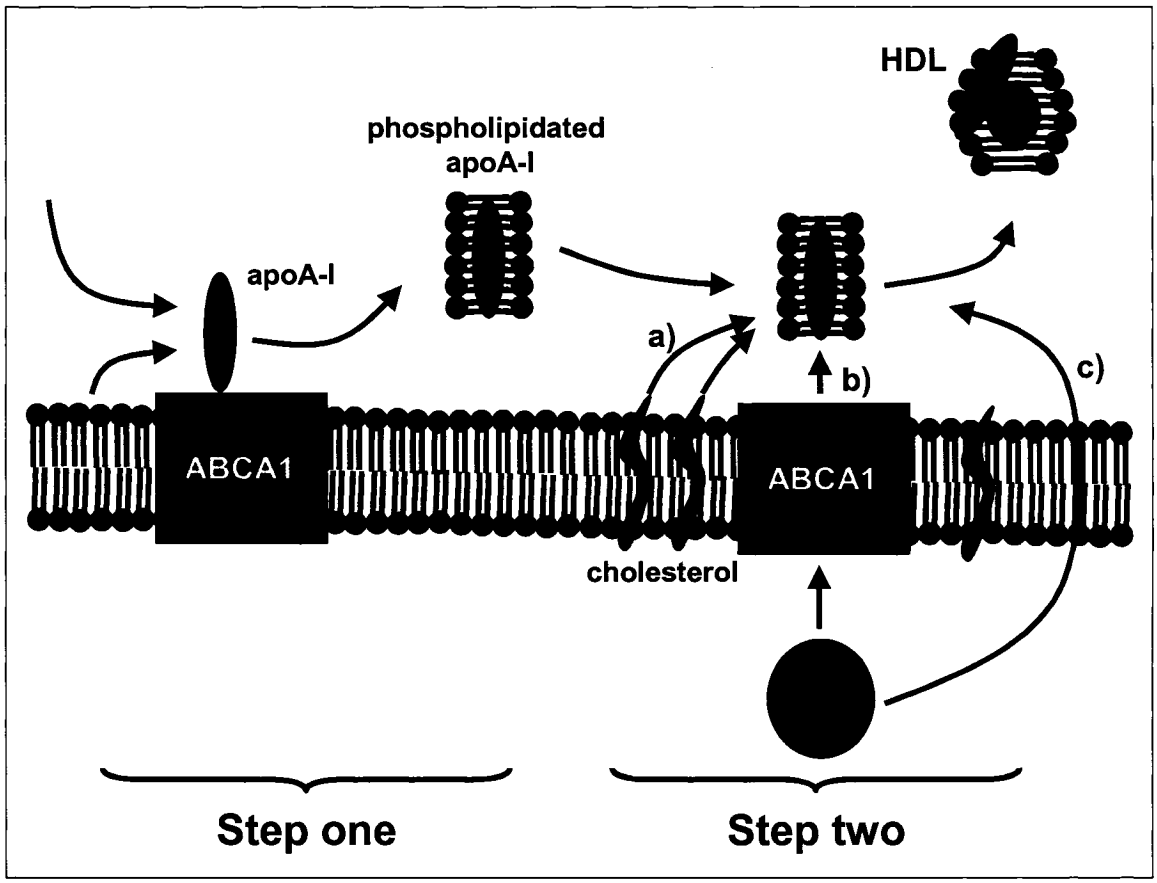

Figure 1-2: Schematic representation of the two-step mechanism of cholesterol efflux. Step one: Lipid-free apoA-I interacts with ABCA1 and acquires effluxed phospholipids. Step two: PL-apoA-I complex can then a) acquire membrane bound cholesterol; b) possibly associate with ABCA1 and pick up cholesterol; c) associate with cholesterol released by diffusion.

It is not clear yet exactly what happens from this point on inside the cell, except that the process involves a number of complex intracellular pathways, involved in the signaling and trafficking of cholesterol. However, it has been established that the newly formed

complex, PL/apoA-I or a lipidated apoA-I, is much more likely to accept the effluxed FC, than the non-lipidated complex (10). This uptake of FC is considered the second step of the two-step mechanism. Neufeld's group also believes that ABCA1 functions as a cell membrane efflux mediator whose availability at the surface can be controlled through the action of lysosomes (11). In other words, spare ABCA1 proteins can be degraded by lysosomes in an attempt to modulate its surface expression.

c. ABCA1 Expression

Cholesterol loading strongly regulates ABCA1 expression in macrophages, where it evidently increases ABCA1 mRNA abundance and protein levels (7). This gene regulation is mediated by the nuclear liver X receptors (LXR alpha and beta) and retinoid X receptors (RXR) (3). LXR and RXR can be activated by oxysterols (25-OH cholesterol) and 9-*cis*-retinoic acid (*9cis* RA), respectively. In macrophages, apoA-I specific cholesterol and phospholipid efflux can be influenced by the level of ABCA1 expression, which can in turn be controlled by either cAMP analogs or heterodimeric LXR/RXR ligands (3,12). Specifically in J774 cells, *9cis* RA, cAMP and cholera toxin (CTX), stimulator of adenylate cyclase, all increased ABCA1 expression (13). ABCA1 expression was significantly stimulated in monocyte-derived macrophages (MDM), mouse peritoneal macrophages (MPM) and THP-1 cell by *9cis* RA and only modestly by cAMP and CTX (13). In fibroblasts, ABCA1 expression is stimulated by cholesterol loading and cAMP treatment (7). Liver and intestine cells, however, are the major sites of expression for ABCA1 (7,14-18) and the main contributors to HDL synthesis and secretion. The recent targeted inactivation of

the hepatic ABCA1 demonstrated indisputably that the liver is the major site of synthesis, accounting for 83% of the circulating HDL (19).

D) ***Cellular Lipid Efflux***

Lipid efflux is the first step of reverse cholesterol transport. There are two main mechanisms for cholesterol efflux: diffusional efflux and apolipoprotein-mediated efflux. The apolipoprotein-mediated efflux, although not perfectly understood, involves the binding of apoA-I to the cell surface, which is believed to involve ABCA1. ABCA1 seems to be primarily involved in the translocation of phospholipid at the plasma membrane, but can also occasionally interact with cholesterol (20). Not only that ABCA1 expression is stimulated by cholesterol loading, but ABCA1 can also distribute cholesterol to the plasma membrane and make it available for apolipoprotein-mediated efflux (21). As PL gets transported from the interior of the cell to the outer leaflet of the cell membrane by the ABCA1 molecule, it interacts with apoA-I, which becomes lipidated in this process. These PL/apoA-I complexes may promote cholesterol efflux in a secondary step, likely in a number of distinct plasma membrane regions (20). This complex possibly triggers some receptors, which then induce downstream signals, causing the release of intracellular stores of cholesterol and cholesterol efflux from the cells. It is currently unclear what the nature of these intracellular cholesterol sites is. It is also poorly understood which cell membrane domains are responsible for donating the cholesterol to the PL-apoA-I complexes on the cell surface.

a. Cholesterol Metabolism

Cholesterol is the main sterol component of cellular membranes. The cholesterol composition of plasma membranes determines its fluidity and acts as a barrier between the cells. Cells constantly try to maintain cholesterol homeostasis by controlling its uptake, storage and efflux. When free cholesterol is taken up and the cell cannot get rid of it, the cholesterol is esterified and stored in lipid droplets (22). Although this can provide a temporary relief from the toxicity of non-degradable FC, it can lead to the formation of "foam cells" or cholesterol-engorged macrophages which contribute to the onset of heart disease.

i. Low-Density Lipoprotein (LDL)

Macrophages which accumulate cholesterol and turn into foam cells can obtain that cholesterol either from endogenous synthesis or from LDL particles that circulate in the blood (6). Since the discovery of the LDL receptor (LDL-R) by Goldstein and Brown (23), the LDL-R has become the main focus of heart disease prevention. LDL binds LDL-R which is located in clathrin-coated pits. The complex then becomes endocytosed, the LDL-R is recycled to the cell surface and LDL is degraded in the lysosomes. This process releases cholesterol for intracellular storage (23). Sterol regulatory element-binding protein (SREBP), also a membrane bound endoplasmic reticulum (ER) protein, regulates expression of LDL-R at the cell membrane. When the cellular cholesterol levels are low, SREBP is transported to the Golgi by a protein called SREBP cleavage-activating protein or SCAP. Once it reaches

the Golgi, SREBP is sequentially cleaved by two proteases, S1P and S2P. The cleaved portion of SREBP, called bHLH, is transferred to the nucleus where it activates LDL-R gene as well as the genes for *de novo* cholesterol biosynthesis. When the cholesterol concentration in cells rises, accumulated cholesterol inhibits the processing of SREBP. A protein called INSIG anchors SCAP and SREBP at the ER membrane and SREBP cannot access the nucleus. Consequently, LDL-R concentration decreases and no new cholesterol is synthesized. This is how SREBP implements negative feedback to control cholesterol accumulation in the cell (24).

ii. High-Density Lipoprotein (HDL)

Human apoA-I is a 28kDa protein responsible for decreasing the risk of heart disease. The levels of apoA-I protein and its mRNA are in direct proportion to the levels of the "good cholesterol" – HDL. Unfortunately, our current knowledge about HDL metabolism is not satisfactory. For years, it was believed that learning about LDL would provide us with a cure for heart disease, and so HDL was left behind. ApoA-I, which is synthesized by the liver and intestine cells (25,26) is the main protein component of HDL. Initial lipidation of apoA-I leads to the formation of a discoidal HDL, also referred to as pre-β-HDL. Pre-β-HDL is able to accept additional cholesterol effluxed by ABCA1-independent, aqueous diffusion. Cholesterol acts as a substrate for a plasma enzyme LCAT which esterifies HDL-bound cholesterol into CE by transferring fatty acyl chains from phosphatidylcholine to cholesterol. Hydrophobic CE moves to the core of HDL, causing discoidal HDL to become a mature, spherical HDL particle (22).

There has been much speculation as to where apoA-I comes from and how it accumulates lipids in order to become HDL. Rothblat has suggested that lipid-poor nascent HDL particles containing apoA-I bind to the plasma membrane surface through their apolipoproteins (27). Those lipoproteins then stimulate the membrane lipids to flop out from the inner leaflet to the outer leaflet of the plasma membrane, possibly causing a bulge in the outer leaflet and allowing apoA-I to acquire excess PL (Figure 1-3). A newer addition to this theory states that the binding of apoA-I to the plasma surface also stimulates the release of cholesterol and PL from their intracellular storage. The lipids are then transferred through the Golgi network, to the plasma membrane and across the membrane to the acceptor bound on

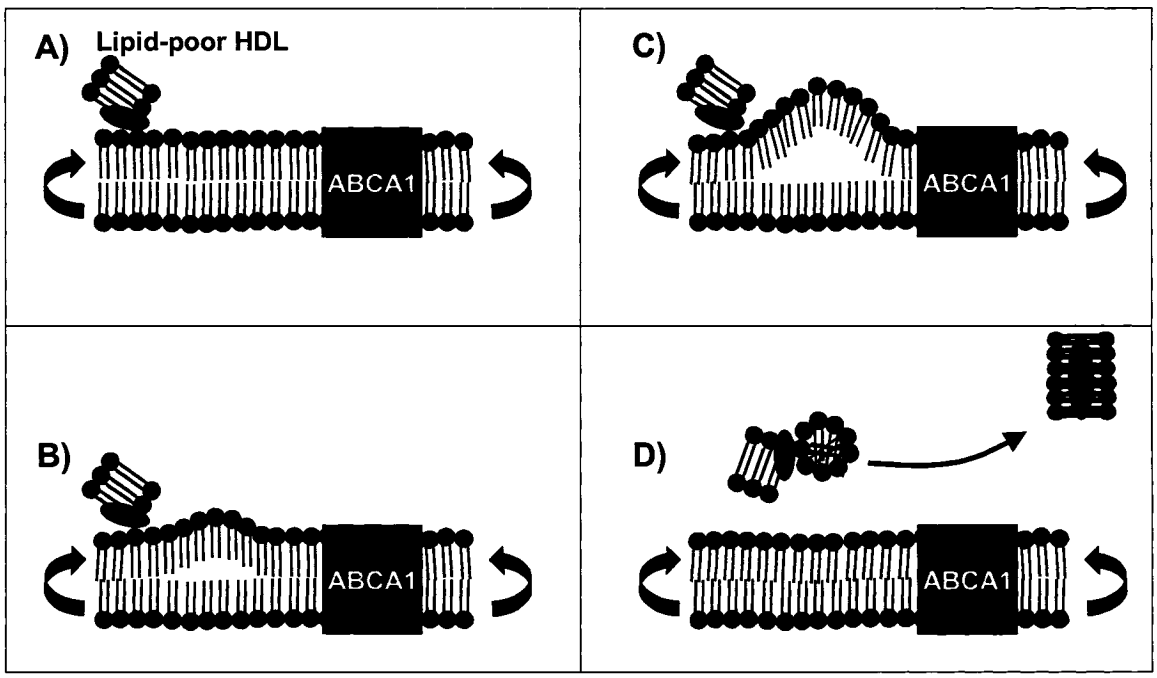

Figure 1-3: Schematic representation of phospholipid flopping and the formation of a bulge in the plasma membrane. Poorly-lipidated HDL or/and ABCA1 protein may be involved in flopping phospholipids from the inner to the outer layer of plasma membrane, creating a bulge in the outer layer. When the bulge becomes large and unstable it breaks off forming a micelle which can be picked up by a poorly-lipidated HDL.

the extracellular surface of the plasma membrane (28). There have been suggestions that apoA-I gets phospholipidated at the plasma membrane, forming a nascent, pre-β-like particle. This particle then somehow sends a message to the stored cholesterol to be released by a diffusional process (29-31). Another theory (32) suggests that apoA-I binds to the saturable sites containing accumulated cholesterol on the plasma surface. This means that apoA-I would not be recruited to the surface unless there was a need to get rid of the accumulated cholesterol. Cells that were not loaded with cholesterol had almost no ability to efflux to apoA-I (29). However, when cells were loaded with cholesterol, apoA-I was able to stimulate not only cholesterol efflux, but also phospholipid efflux, mainly phospatidylcholine (PC) and sphingomyelin (SM). The same group noticed that phospholipidation occurs quite some time before cholesterol lipidation, the idea later supported by Fielding et al. (10).

New data indicates that, although HDL formation as a consequence of lipid efflux from macrophages is clinically important, it is not the major source of plasma HDL. Recent work has shown that liver and intestine cells most significantly contribute to the formation of HDL particles (19). Although not completely understood, it is believed that these cells can secrete either native apoA-I or poorly lipidated apoA-I, which then accumulates sufficient amounts of lipid from the plasma and finally returns to the liver for degradation. These new data slightly modify the current understanding of reverse cholesterol transport, providing an alternative site and pathway of HDL synthesis. Some believe that apoA-I is secreted lipid free by liver and intestine cell and that its lipidation occurs strictly extracellularly (27,29-31,33,34). Others feel that apoA-I has to be at least poorly lipidated prior to its secretion from the cell (35-39). After many years of research, we still do not know the exact process of apoA-I lipidation, but some studies, including the early Banerjee's studies (36) and more

recently Chisholm's study done in HepG2 cells (38), show that lipidation occurs both extra- and intracellularly.

E) *Intracellular apoA-I Metabolism and Lipidation*

In 2000, Lin and colleagues proposed that lipid-free apoA-I is responsible for removing cellular phospholipid and cholesterol through some Golgi network-related mechanism, which is not yet understood. There are two likely pathways for the lipid removal. Phospholipidated apoA-I could bind to the scavenger receptor SR-B1, which facilitates the removal of previously diffused cholesterol. Alternatively, lipid-free apoA-I could interact with the cell membrane directly and pick up both phospholipid and cholesterol (28). Lin et al. suggest that apolipoproteins bind to specific "saturable" sites on the plasma membrane, referred to as protrusions, which induces a signal responsible for releasing the cholesterol located in some intracellular storage pool. This process requires a functional TGN and is responsible for preventing excess accumulation of cholesterol in macrophages and an onset of atherosclerosis.

a. ApoA-I Dependent vs. Independent Efflux

Oram's group used a Golgi disrupter Brefeldin A (BFA) and energy poison NaF, both of which inhibited apoA-I mediated efflux of cholesterol and phosphatidylcholine (28). They also noticed that following these treatments, a small detectable amount of gold-labeled apoA-I was bound to a flat plasma membrane surface and not the above mentioned protrusions.

The same effect was seen when the cells were incubated at 4°C instead of 37°C. So the formation of these protrusions was inhibited by Golgi destruction, energy depletion and low temperature, which are the three conditions that inhibit apoA-I-mediated PL and cholesterol efflux. Therefore, they concluded that these protruding structures must be involved in lipid efflux. The authors postulate that the binding of apoA-I to specific plasma membrane sites stimulates the Golgi to transport intracellular lipids from their storage site. These lipids accumulate at the cell surface, forming the protruding structures. These lipids are then available to be bound by an approaching apolipoprotein and dissociate from the membrane.

HDL is capable of inducing efflux in both apolipoprotein dependent and independent manner. If an HDL particle is depleted of apoA-I by trypsin treatment, it cannot perform the apolipoprotein dependent efflux (32). BFA and monensin are drugs used to cause a break-up of Golgi vesicles and disable any cellular transport through the Golgi network and to the plasma membrane. When the cells were exposed to either BFA or monensin or not treated at all, in the absence of any acceptors (efflux to media), the efflux was the same in all cells. However, when apoA-I or HDL were used as the acceptors in the presence of BFA, the cholesterol efflux was decreased by 85% and 40% respectively. The study concludes that the depletion of cholesterol from the intracellular sites of storage and its transport to the sites of efflux by a Golgi system is the necessary step involved in the cholesterol efflux conducted through the apolipoprotein-mediated mechanism (32). The same pathway seems to be involved in the efflux of phospholipids, namely PC and SM. The cholesterol efflux that occurs as a consequence of diffusion through the plasma membrane is not apolipoprotein-dependent and it cannot be altered or inhibited by BFA and monensin. Cholesterol efflux to the acceptors, which do not contain apolipoprotein (trypsin-HDL, phospholipid vesicles and

albumin), was not affected by BFA and monensin, proving that this diffusional and apoA-I-independent mechanism is not Golgi-mediated.

In conclusion, apoA-I-stimulated PL and cholesterol efflux are inhibited by energy depletion, low temperatures and Golgi interruption. These results show that apolipoprotein-induced efflux is conducted through the Golgi apparatus, which somehow stimulates intracellular release of stored cholesterol for either intra- or extracellular lipidation.

b. Intracellular apoA-I Localization

ApoA-I is synthesized in the ER of liver and intestine cells in a preproapoA-I form, containing 267 amino acids (40). In order for the protein to obtain its final structure consisting of 243 amino acids, two proteolytic activities need to occur. The 18-amino-acid pre-sequence is hydrolysed during the translocation of apoA-I across the ER membrane (41,42). The pro-sequence is removed in the plasma or lymph by apoA-I-specific peptidase (43). Majority of the secreted apoA-I (~95%) is in the pure apoA-I form whereas the rest might still contain the pro-sequence (44). McLeod et al. assessed the role of the pro-sequence by comparing the behaviour of a mature apoA-I protein and the proapoA-I protein in baby hamster kidney cells (45). Using immunofluorescence microscopy, they determined that most of the mature apoA-I got accumulated in the ER while the proapoA-I was mainly found in the Golgi. They concluded that the pro-sequence of apoA-I must be important in facilitating the intracellular transport of the protein from ER, through the Golgi and to the plasma membrane using the biosynthesis pathway.

ApoA-I is synthesized in the ER of hepatocytes, transferred through the trans-Golgi network (TGN) and secreted out of the cell. However, the time that apoA-I takes to go through all the intracellular compartments and the exact localization of apoA-I is still a matter of debate. The exposure of apoA-I to the cytoplasmic surface was tested by incubating isolated rough ER (RER) and Golgi vesicles with exogenous proteases (46). Chickens were intravenously injected with radioactive leucine for 5, 10 and 20min, their livers were removed, RER and Golgi isolated and then treated with proteases. In the ER, albumin, which is known to be located in the lumen of these vesicles, was completely protected from the proteases. However, only about half of the apoA-I was protected from the proteases, indicating that the other half of the apoA-I was exposed to the cytoplasmic surface. At 10 and 20min after labeling, almost 65% of the apoA-I was protected which means that with time, apoA-I migrates from the cytoplasmic surface to the lumenal side. In Golgi fractions, apoA-I was mostly protected from protease degradation and hence was contained within the lumen (46).

Many groups postulate on the exact localization of apoA-I following its synthesis, and to date, they are far from reaching an agreement. Gp 80 is the endogenous apical marker glycoprotein that is secreted in the RER similarly to apoA-I (37). However, apoA-I gets transferred to the Golgi a lot faster than gp 80. The authors suggest that if gp 80 and apoA-I are synthesized in similar locations, than the vesicles transporting apoA-I are instantly formed following apoA-I formation and apoA-I is quickly transported to the Golgi. Another possibility is that apoA-I is secreted by special polysomes. Polysomes are cytosolic ribosomes which bind to mRNA, but are not necessarily targeted to ER. If those cytoplasmic polysomes exist close to the Golgi, then the newly synthesized apoA-I has a shorter distance to cover in order to reach the Golgi and it does so in a shorter period of time. Havel and

Hamilton (47), however, predict that apoA-I is not localized in Golgi immediately following synthesis, but that it is localized in some other cellular subfraction, such as the primary or secondary endosomes, and that those endosomes subfractionate with the Golgi. Banerjee and colleagues disagree with this possibility since they see, using immunofluorescence and 3D laser scanning confocal microscopy, that apoA-I is clearly localized in the Golgi (37). They believe that apoA-I leaves the ER without any posttranslational modifications, in agreement with the idea that most lipoproteins are created in the Golgi. So transferring "naked" apoA-I quickly from ER to Golgi would prevent any lipidationof apoA-I in the ER and also prevent any permanent membrane binding and anchoring of apoA-I into ER membrane. Also, if lipoproteins are required somewhere else in the body, then the need exists for a rapid translocation of apoA-I to the Golgi, where those lipoproteins are efficiently formed.

c. Intracellular Lipid Localization

Large volumes of data support the idea that there are two major sources of cholesterol in a cell, plasma membrane and subcellular compartments. These subcellular compartments include lysosomes, endosomes, ER-associated lipid droplets, and ER-derived *de novo* synthesized cholesterol (48). Cholesterol studies have shown that between 35% and 45% of plasma membrane lipids are cholesterol molecules (49). Although ER is the main site of cholesterol synthesis and esterification, the organelle itself is very poor in cholesterol. Studies have shown that only about 2% or less of total cell cholesterol is found in the ER. In contrast, plasma membrane (PM), which has the same general surface area as ER, contains up to 80% of the total cholesterol (49-51). Transport of cholesterol from ER to the PM is still

under debate. However, many believe that it takes a vesicular pathway. Studies involving Golgi disruption, however, show that although this causes the majority of protein secretion involved in HDL formation to be inhibited, the transport of cholesterol itself is only about 20% inhibited (52,53). This finding suggests that vesicular transport is somewhat involved in cholesterol transport but is not by any means the major pathway. Other studies suggest that non-vesicular transport is more likely to take place and that SCP-2 and caveolin-1 are the two proteins involved in this process (54,55). The complete picture is still to be discovered.

It is well known that ER is the main site of lipid production and that the enzymes responsible for the lipid production are located at the cytoplasmic surface of ER membrane (56). Smooth ER (SER), RER and Golgi, which are involved in protein secretion, quickly incorporate radioactive glycerol into triglycerides (TG) and phospholipids. This incorporated radioactivity allows one to follow the pathway of triglycerides and phospholipids. The results show that triglycerides are rapidly transferred from ER to Golgi. It is also important to note that diacylglycerol acyltransferase is not found in Golgi and that any TG found in this compartment must have been transferred from the ER (35). The same group showed that phospholipid is formed in both ER and Golgi at the same time. This means that even though ER is the main site of PL synthesis, Golgi has all the necessary enzymes to produce PL on its own (57).

d. Intracellular apoA-I Lipidation

The first significant studies in the field of intracellular apoA-I lipidation were done by the Banerjee group in the early 80's. They conducted their experiments in chicken models

in vivo by perfusing chicken livers and analyzing intracellular compartments from isolated hepatocytes. Both human and chicken apoA-I are mainly secreted in liver and intestine and they are both involved in removal of cellular lipids. Human and chicken apoA-I have a 66% sequence similarity and 48% identity. Chicken HDL is a good model for studying the behavior of human HDL since it is structurally very similar but a lot simpler, containing only one lipoprotein (apoA-I) instead of the eight found in human HDL. The following is known from *in vivo* studies of HDL synthesis: a) The transport of apoA-I from ER to Golgi is very rapid (35); b) The initial lipidation of the protein occurs in Golgi fractions but the newly formed lipoprotein particles are not immediately mature. (35); c) Unlike radioactive glycerol which quickly gets incorporated into protein particles in RER and Golgi, the nascent lipids do not significantly conjugate with the proteins in ER, but the most significant binding occurs in Golgi fractions (36).

HDL particles are 8-10nm in diameter and float between 1.063 and 1.21g/ml density. (36). Newly synthesized apoA-I that was isolated from RER and SER was unable to float between those densities, unlike the apoA-I isolated from Golgi. This means that only minor levels of lipidation occur in the ER while the lipidation in Golgi is sufficient to form particles that mostly resemble the true HDL (35,39).

In another study Dixon (58) transfected COS-7 cells with a recombinant plasmid which expresses chicken apoA-I. The cells were tested for the expression of apoA-I and they have been proven competent to do so, secreting apoA-I mostly devoid of any lipids, with only a small number of exceptions. The study determined that the HDL released to the plasma does not have the same structure as the HDL found intracellularly which shows that HDL production is complete only once it leaves the cell. Although in young chicken livers, the newly secreted apoA-I accumulates lipids necessary to obtain the structure of a mature

HDL, this is not the case in primary hepatocytes and HepG2 cells where very often, lipid-poor apoA-I has been found in the plasma (59,60). In Dixon's study, some apoA-I was also only partially lipidated. However, it is possible that some lipid was lost following the release to the plasma, or during centrifugation. This study also suggests that apoA-I does not need to acquire lipids in order to be secreted. COS-7 cells are not necessarily the best model for this study because of the following: the amount of lipid that these cells produce may not be enough to be transported to the cell surface attached to the apolipoproteins, their Golgi may not be able to produce any lipid for the efflux, or the whole system required for the assembly of these apolipoprotein-lipid particles may be lacking or be dysfunctional. It is however known that apoA-I secreted by these cells is perfectly capable of binding lipids as it does so in the plasma.

Chisholm et al. performed a pulse chase study in HepG2 cells, where they immunoprecipitated apoA-I from media samples right after the secretion and 120min after (38). They noticed that ~25% of apoA-I was sufficiently lipidated to be able to float at the correct density $d > 1.25$ g/ml at time zero and ~50% was able to float after 120min. They concluded that 25% of apoA-I must have been lipidated prior to secretion, inside the cell, and that the other 25% were lipidated extracellularly. To confirm these results, Chisholm et al. assessed the lipidation of exogenous versus endogenous apoA-I. They noticed that only ~20% of exogenously introduced apoA-I was able to acquire enough lipids to become buoyant, whereas ~50% of the endogenous apoA-I acquired the same amount of lipids. So, they hypothesized the difference between these two measurements is due to the endogenous apoA-I becoming lipidated inside the cell, prior to secretion (38).

In conclusion, depending on different cell types, apoA-I is capable of being secreted out of the cell in its native state. However, a significant amount of apoA-I is also secreted

partially lipidated. Although the intracellular lipidation of a secreted apoA-I may not necessarily be complete, the amount of lipid present on these apoA-I molecules is probably sufficient to allow more favourable binding of PL and cholesterol at the plasma membrane and the formation of a mature HDL particle.

e. Intracellular ABCA1 Localization

Various studies have shown that ABCA1 resides on the plasma membrane (7,61,62). Recently, a number of investigators assessed intracellular localization of ABCA1 in cell that stably or transiently express ABCA1-green fluorescent protein (GFP) fusion constructs. They agree that, besides being localized at the plasma membrane, ABCA1 can also be found in intracellular endocytic compartments (63-65). Neufeld and colleagues performed a study using transfected HeLa cells that are able to express a functional ABCA1-GFP fusion protein. The fluorescent chimeric ABCA1 transporter was found to be localized at the cell surface, but also on intracellular vesicles, including early endosomes, late endosomes and lysosomes (11). It was suggested that this intracellular ABCA1 may play a role in apoA-I lipidation prior to secretion from the cell. A study by Orso and colleagues also concluded that ABCA1 is localized to the Golgi where it helps incorporate lipids into vesicles budding off of the Golgi. These budding vesicles would potentially be transferred to the plasma membrane where they would serve as a source of lipids for apoA-I lipidation (61). Evidence exists that exogenous apoA-I can be endocytosed by macrophages where it could accumulate lipids in the endosomal compartments (66,67). This lipidated apoA-I can then be re-secreted from the cells. As well, several groups have shown that lipids (29,68) and apoA-I (67)

involved in ABCA1-mediated efflux are trafficked through the same endocytic pathways where intracellular ABCA1 is located. Keeping this in mind, one can hypothesize that in cells which regularly synthesize lipoproteins, ABCA1 might be lipidating apoA-I prior to its secretion from the cell.

F) ***SUMMARY***

Lipid efflux can occur through either an apoA-I-dependent or -independent pathway. The apoA-I-dependent pathway, although not fully understood, seems to require a functional Golgi apparatus, which somehow signals the release of lipids from their intracellular storage compartments. Hepatocytes are known to secrete apoA-I, although the intracellular localization of apoA-I following protein synthesis is still under debate. apoA-I is likely synthesized in ER and shortly after sent to the Golgi, before being transferred to the plasma membrane. Cholesterol is synthesized in the ER and very quickly distributed throughout the cell, mainly at the plasma membrane. The ER itself is very poor in apoA-I, whereas the plasma membrane contains over two thirds of the total cellular cholesterol. Phospholipids, synthesized both in the ER and Golgi, are distributed throughout various cell membranes. Both cholesterol and phospholipids can travel to the plasma membrane by the ways of vesicular transport, although the BFA studies show that non-vesicular transport also plays an important role in cholesterol transport. Given the obvious co-localization of apoA-I, cholesterol and phospholipids in intracellular compartments, it is expected that some apoA-I protein might be able to accumulate some lipids along the way (Figure 1-4).

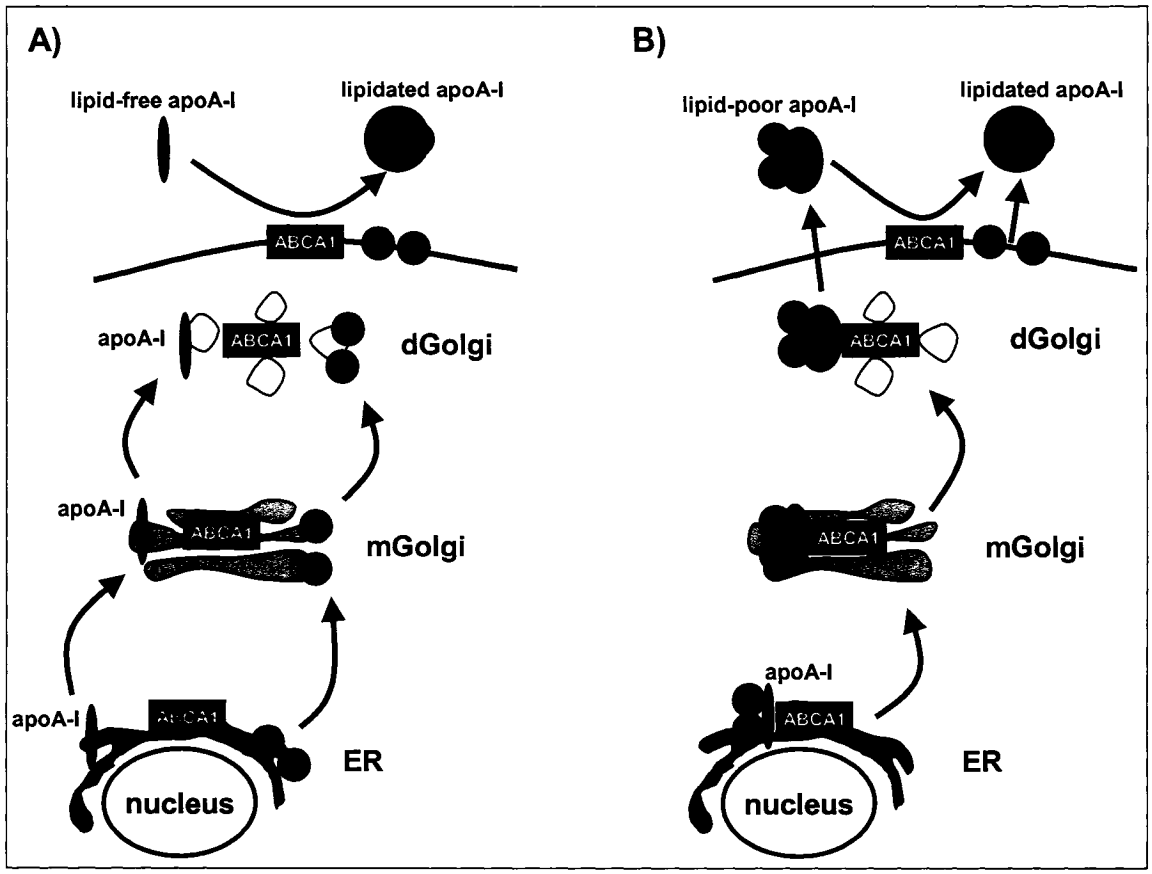

Figure 1-4: A model for possible ABCA1 involvement in intracellular apoA-I lipidation. a) apoA-I, PL (phospholipid), CH (cholesterol) and ABCA1 are synthesized in ER of hepatocytes and are all transported through the TGN to the plasma membrane. Secreted apoA-I is known to interact with ABCA1 at the plasma membrane, become lipidated and form HDL; b) we and others propose that, since all the required elements reside in the same intracellular compartments, then ABCA1 could possibly lipidate apoA-I prior to its secretion from the cell. Following secretion, this poorly lipidated apoA-I is now more likely to acquire more lipids upon interacting with the plasma membrane lipids than a lipid-free apoA-I.

Recent data indicating that ABCA1, known to be involved in apoA-I lipidation at the plasma membrane, is also localized in intracellular compartments, provides more evidence for possible intracellular lipid acquisition by apoA-I. Even though a number of studies have detected partially lipidated, endogenous apoA-I in the media outside the cell, and some investigators hypothesized that intracellular lipid acquisition by apoA-I may occur, more evidence is needed to reliably explain the events and conditions leading to the intracellular

lipidation. Our study provides detailed pulse-chase experiments carefully planned to assess protein and lipid localization following synthesis, as well as lipid acquisition by apoA-I in ER and Golgi compartments of primary mouse hepatocytes.

G) ***PURPOSE***

To test the importance of intracellular lipidation, we isolated primary mouse hepatocytes from C57BL/6 ABCA1 wt and C57BL/6 ABCA1 KO mice and labeled them with ^{3}H-choline, LDL-^{3}H-cholesterol or ^{3}H-mevalonate to track intracellular phospholipids and cholesterol. We infected the cells with Adeno-human-apoA-I (Ad-AI) and assessed the intracellular lipid acquisition by apoA-I. To better understand the flow of lipidated apoA-I, we treated the cells with cycloheximide to inhibit any new production of apoA-I and performed multiple pulse-chase experiments. Nycodenz gradient centrifugation was used to separate ER and Golgi compartments and the sodium carbonate treatment allowed for the lumen and membrane of each compartment to be isolated. We hypothesize that apoA-I acquires lipids intracellularly, in the ER and Golgi compartments, leading to the formation of lipidated HDL which is then secreted from the cell. Our study has the following objectives: 1) To determine which lipids are acquired by apoA-I and in which compartments; 2) To determine if the lipidation of apoA-I is ABCA1 dependent. By conducting these studies in a cell type thought to be the major source of HDL synthesis, a deeper understanding of apoA-I lipidation and the initial steps preceding the secretion will be attained. This study could open a new door for ABCA1 research, allowing for a better comprehension of intracellular ABCA1 function and its importance in apoA-I lipidation and HDL formation.

VIII. METHODS

VIII. METHODS

A) *Materials*

a) *Chemicals*

Nycodenz gradient maker; Fibronectin; Cycloheximide; Stigmasterol; Sil-A derivatization chemical; Collagenase were obtained from Sigma. Cholesterol-[1, 2-^{3}H], mevalono-lactone-Rs-[5-^{3}H(N)], choline chloride-[Methyl-^{3}H] were obtained from PerkinElmer. Complete protease inhibitor cocktail was obtained from Roche. Acrylamide-Bis solution, Sodium dodecyl sulfate (SDS) were obtained from Bio-Rad. Eco-Lite scintillation liquid; N,N,N',N'-Tetramethylethylenediamine (TEMED) were obtained from ICN Biomedicals. Supersignal Chemiluminescent substrate was obtained from Pierce.

b) *Cell Culture*

HepatoZYME-SFM Medium, Williams Medium E and antibiotic-antimycotic were obtained form Gibco Invitrogen Corporation.

c) *Antibodies*

Rabbit polyclonal anti-human apoA-I antibody for immunoprecipitation was obtained from Calbiochem. Polyclonal anti-mouse apoB antibody for IP was purchased from Biodesign International. Protein G- and protein A-Sepharose, Sheep anti-mouse IgG,

Horseradish Peroxidase linked whole antibodies and Donkey anti-rabbit IgG, Horseradish Peroxidase linked whole antibodies were obtained from Amersham Biosciences. Monoclonal antibodies directed against human apoA-I [a combination of 4H1 (against the extreme N terminus) and 5F6 (against the central region)] were obtained as previously described (69,70) and biotinylated with Sulfo-NHS-Biotin from Pierce. Primary anti-mouse apoB antibody was a gift from Dr. Ross Milne (University of Ottawa Heart Institute). Rabbit anti-mouse TGN38 antibody was obtained from BD Biosciences. Rabbit anti-mouse EEAI antibody was obtained from Affinity Bioreagents. Rabbit anti-mouse ManII antibody was a gift from Dr. Zemin Yao (University of Ottawa). Rabbit anti-mouse Calnexin (Cnx) antibody was obtained from Stressgen Bioreagents.

B) ***Cellular Fractionation***

a) ***Preparation of Nycodenz Gradient***

Nycodenz stock solution (27.6% Nycodenz in 10mM Tris-HCl, pH 7.4, 3mM KCl, 1mM EDTA) and saline buffer (0.75% NaCl, 10mM Tris-HCl, pH 7.4, 3mM KCl, 1mM EDTA) were used to prepare four Nycodenz solutions of increasing percent-concentrations (10%, 14.66%, 19.33% and 24%) and 2.5ml of each were loaded from the bottom of the tube (Beckman Polyallomer Centrifuge Tubes) in the increasing percentage order. The tubes were then sealed with a piece of parafilm and a linear gradient was formed by turning the tubes horizontally for 45min at room temperature. The tubes were then centrifuged at 37000rpm for 4h at 15°C (Beckman L8-70M Ultracentrifuge).

b) *Cell Culture*

Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt and C57BL/6 ABCA1 KO mice (a kind gift from Dr. Edward M. Rubin, DOE Joint Genome Institute, Berkeley, CA) by collagenase liver perfusion. The cells were plated on fibronectin-coated 10cm plates and grown in Williams media for 5h.

c) *Cell Labeling*

Five hours after the initial seeding, cells were labeled with either ^{3}H-choline (10μCi/ml), ^{3}H-cholesterol (10μCi/ml) pre-equilibrated with LDL (5μg/ml) or ^{3}H-mevalonate (15μCi/ml). The radioactive solutions were dried down with nitrogen, resuspended in a small volume of ethanol and the tubes (VWR 13x100mm Durex Borosilicate Glass) capped and vortexed. Then 2ml of the loading media (Hepatozyme media with 1% L-glutamine and 1% antimycotic antibiotic reagent) were added to the tube, capped and vortexed. This solution was incubated for 30min at 37°C. The solution was then added to the rest of the loading media, sterile filtered and 5ml of solution were added per plate. The cells were incubated for 20h at 37°C.

d) *Adenovirus Infection*

The cells were infected for 1h at 37°C with a recombinant adenovirus encoding apoA-I (Ad-AI) or control luciferase (Ad-Luc) at a multiplicity of infection of 75:1 plaque-forming units per cell in Williams' Medium E without serum (71). After the 1h infection, the adenovirus containing media was removed and the radioactive media returned for another 24h.

e) *Nycodenz Gradient Fractionation*

The subcellular fractionation protocol was essentially that described in Tran et al. (72). At the end of the labeling period, the cells were washed twice with Williams media. Cycloheximide in Hepatozyme media was administered to cells (3.55 µl/ml of 100mM stock) at time zero. The first set of cells was processed immediately while the rest of the cells were kept in Hepatozyme for the duration of a pulse chase. The Hepatozyme media was kept for the measurements of exported protein and lipids. The cells were washed twice with cold PBS. 5ml of PBS was then added to each plate and scraped with a plastic scraper. The collected cells were then spun down at 2000rpm for 5min at 15°C (MSE Mistral 2000). The pellet was resuspended in MSB buffer (10ml of MSB (10mM Tris-HCl pH 7.4, 250mM sucrose in ddH_2O), 100µl of Complete protease inhibitor cocktail (1 pill dissolved in 0.5ml of ddH_2O), 10µl of 0.5M EDTA pH8). The cells were incubated at 4°C until all time points were collected. This procedure was repeated at 20min and 40min. The resuspended samples were then homogenized using ball bearing homogenization apparatus (Figure 2-1). Using

two 5ml syringes, the cell suspension was passed through the apparatus for 10 cycles. The cell homogenate was then transferred to 15ml glass Corex tubes and centrifuged at 9000rpm for 10min at 5°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge). This centrifugation step pelletted nuclear material, while the supernatant containing sub-cellular contents was kept for further analysis. The supernatant was then layered on top of the previously created Nycodenz gradient and centrifuged at 37000rpm, for 1.5h at 15°C (Beckman L8-70M Ultracentrifuge). Following the spin, each tube was fractionated into 15 aliquots (3 tubes, one per each time point). These aliquots were incubated at 4°C for further analysis, which include western blotting and determination of radioactive content.

f) *Lumen and Membrane Separation*

In order to separate lumen from the membrane, the 15 aliquots collected following the Nycodenz gradient centrifugation were grouped into 3 microsomal fractions. 1-3 became a distal Golgi fraction, 4-8 medial Golgi and 9-15 ER. 1.5ml was removed from each fraction and 1.5ml of 0.2M Na_2CO_3 pH 12.4 was added to each condition. The samples were allowed to mix on a rotator for 30min at room temperature. The samples were transferred to open-end tubes (Beckman Polyallomer Thick Wall 3.2ml tubes) and centrifuged at 70000rpm for 30min at 15°C (TLA100.4/TLA110 rotor) (Beckman Optima TLX Ultracentrifuge). The supernatant, representing the lumenal content of each intracellular compartment, was collected and the pH adjusted to 8 with 75µl of 2.5N HCl. The pellet representing the membrane fraction was resuspended in 250µl of MSB. To resuspend, the pellet in MSB was pipetted up and down 8 times using a 1ml syringe and a 25G needle (Becton Dickinson).

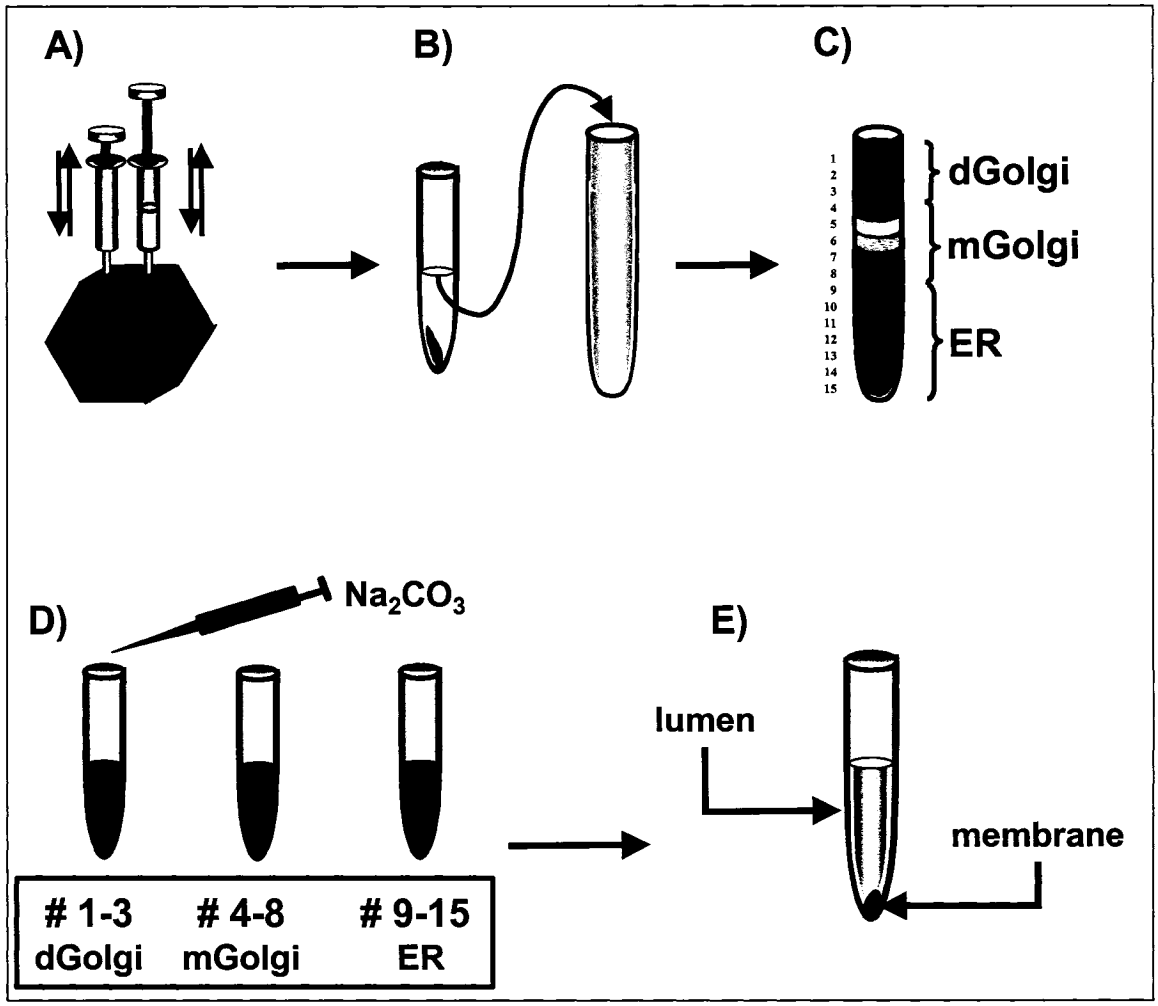

Figure 2-1: Schematic representation of Nycodenz gradient centrifugation and lumen and membrane isolation. a) Cells were scraped and homogenized in MSB buffer using ball-bearing apparatus; b) The homogenate was centrifuged and the supernatant separated from the pelletted nuclear fraction. Supernatant was loaded on a Nycodenz gradient and centrifuged at 37000 rpm for 1.5h at 15°C; c) Fifteen aliquots were obtained and grouped into dGolgi (1-3), mGolgi (4-8) and ER (9-15); d) Each fraction was treated with 0.2M Na_2CO_3 pH 12.4 and allowed to incubate for 30min with constant rocking; e) The fractions were centrifuged at 70000rpm for 30min at 15°C and membrane and lumen separated.

g) *Immunoprecipitation*

Immunoprecipitation (IP) was performed on sub-cellular and media samples collected during the fractionation procedure. 90µl of anti-apoA-I antibody (Calbiochem 178422) was added to 3ml of sample. The samples were incubated at 4°C overnight with continuous mixing. The next day, 100µl of protein G (Amersham Biosciences) were added to the samples and samples incubated overnight. The next day, samples were centrifuged at 3000rpm at 10°C for 10min (Sorvall RT 6000D). The supernatant was discarded and the pellet washed in 10ml of PBS with constant rotation for 20min at low speed followed by a centrifugation at 3000rpm for 10min at 10°C. The wash was performed 3 times. Finally, the pellet was resuspended in 1ml of PBS. When immunoprecipitating apoB with rabbit anti-apoB antibody (Biodesign) the same procedure was followed except that protein A Sepharose (Amersham Biosciences) was used. When apoA-I was immunoprecipitated from a membrane sample, the sample was treated with 1% Triton X-100 for 30min at 4°C. The solubilized sample was then centrifuged at 75000 rpm for 30min at 15°C (TLA 100.4/TLA110).

h) *Cpm Counting*

250µl of the immunoprecipitation samples were placed into scintillation vials (Sarstedt) containing 3.5ml of Eco-Lite scintillation liquid (ICN). The samples were counted (Packard Tri-Carb 2100TR Liquid Scintillation Analyzer) for 3min. The results were expressed as cpm per total protein mass in each cell lysate sample.

C) *SDS PAGE*

In order to cast two 3%-15% gradient gels, the following steps were followed. 3% gel solution was made by adding 3ml of 30% acrylamide mix (Bio-Rad), 20.8ml of ddH_2O, 6ml of 1.5M Tris-HCl (pH 8.8) and 150µl of 10% SDS (Bio-Rad) in a small flask. A 15% gel solution was made by adding 15ml of 30% acrylamide mix (Bio-Rad), 5.8ml of ddH_2O, 3ml of 100% glycerol (EMD Biosciences), 6ml of 1.5M Tris-HCl (pH 8.8) and 150µl of 10% SDS, in a large flask. 15ml of 3% gel mix was placed in the left cylinder of the gradient forming apparatus (C.B.S. Scientific Co. GM-100) and 15ml of 15% gel mix in the right cylinder. 45µl of 10% ammonium persulfate (APS) (Fisher) and 10µl of TEMED (ICN) were added to each cylinder for each gel. 1ml of butanol was added to the top of the gel and the gel allowed to polymerize for 3h. The stacking gel solution for two gels was made by adding 2ml of 30% acrylamide mix, 15ml of ddH_2O, 2.5ml of 1M Tris-HCl (pH 6.8), 200µl of 10%SDS, 200µl of APS and 20µl of TEMED. 10ml of stacking gel was poured on top of the gradient gel, with a proper comb inserted. The gel was allowed to polymerize for 1h. 4L of 1X running buffer (12.08g Tris-HCl, 75.2g glycine, 0.5% SDS, enough ddH_2O to make 4L) was poured over the cast gel, the samples loaded and the gel run overnight at 45V (Bio-Rad Protean II xi Cell running apparatus).

D) *Western Blotting*

1X Transfer buffer (2.7g boric acid, 300ml methanol, enough water to make 1L) was used to transfer proteins for 4h at maximum current and 150V. The membrane was blocked

(5% skim milk in 0.5% Tween PBS) for 30min and washed three times for 15min in 0.5% Tween PBS. Primary mouse anti-human apoA-I antibodies 5F6 and 4H1 (University of Ottawa Heart Institute) were added at 1:1000 dilution. Primary anti-mouse apoB antibody (a gift from Dr. Ross Milne) was added at 1:1000 dilution. Rabbit anti-mouse TGN38 antibody (BD Biosciences) was added at 1:1000 dilution. Rabbit anti-mouse EEAI antibody (Affinity Bioreagents) was added at 1:1000 dilution. Rabbit anti-mouse ManII antibody (a gift from Dr. Zemin Yao) was added at 1:2000 dilution. Rabbit anti-mouse Cnx antibody (Stressgen Bioreagents) was added at 1:3000 dilution. After an overnight incubation at 4°C, the membrane was washed three times for 15min in 0.5% Tween PBS. Secondary antibodies were added at 1:5000 dilution and the membrane incubated for 1h at room temperature. For apoA-I and apoB, sheep anti-mouse IgG, Horseradish Peroxidase linked whole antibodies were used (Amersham Biosciences). For all the rest of the proteins, donkey anti-rabbit IgG, Horseradish Peroxidase-linked whole antibodies were used (Amersham Biosciences). The membrane was washed again three times for 15min in 1X PBS with 0.5% Tween. The membrane was exposed to Supersignal Chemiluminescent substrate (Pierce) for 5min and exposed to film (CL-X Posure Film- Pierce).

E) *TLC Analysis*

a) *Bligh and Dyer lipid extraction*

The procedure followed is essentially that of Bligh and Dyer (73). 1ml of MeOH (methanol), 1ml of NaCl, 100µl of Glacial Acetic Acid and 4ml of $CHCl_3$ were added to 1ml

of sample. The samples were vortexed vigorously for 10sec twice and centrifuged at 3000rpm for 15min at 10°C. The bottom layer containing lipids was removed and dried under nitrogen.

b) *TLC Procedure*

In order to determine the amount of ^{3}H-choline incorporated into SM and PC, phospholipids were separated by thin layer chromatography (TLC). The polar solvent system containing 105ml of $CHCl_3$, 45ml of MeOH, 18ml of Acetic Acid, 6ml of Formic Acid and 3ml of ddH_2O was allowed to fully equilibrate. The samples were loaded onto the plate in the correct order and dried under nitrogen. The plate was lowered into the equilibrated chamber and the samples allowed to run until the solvent reached the line 1cm away from the top of the plate. The plate was removed and allowed to dry for 1h. The dried plate was placed into the iodine chamber for about 15min or until clear yellow bands appeared. After spraying the plate with water, the appropriate bands were scraped. The collected powder was added to 3.5ml of Eco-Lite scintillation liquid and the sample cpms counted.

To determine the amount of ^{3}H-mevalonate incorporated into cholesterol versus cholesteryl esters, a non-polar solvent system containing 105ml of hexane, 45ml of Diethyl Ether and 1.5 ml of Acetic Acid was used. The same procedure was followed as for phospholipid separation.

F) *Gas Chromatography Analysis*

a) *Chloroform-Methanol Lipid Extraction*

Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice, membrane and lumen samples isolated and media collected as described above. ApoA-I was immunoprecipitated from all three samples, resuspended in PBS and transferred into 2ml Teflon lid glass tubes (Kimble Glass Inc.). 1ml of $CHCl_3$ and 2ml of MeOH were added to each sample. The samples were vortexed for 2min at maximum speed and incubated at room temperature for 20min. 1ml of ddH_2O and 1ml of $CHCl_3$ were added to each sample. The samples were then vortexed for 30sec and centrifuged for 20min at 3000rpm and 10°C (Sorvall RT 6000D). The lower $CHCl_3$ phase containing lipids was removed with a glass Pasteur pipette (VWR). The sample underwent cholesteryl ester hydrolysis with KOH to allow determination of total cholesterol content.

b) *Cholesteryl Ester Hydrolysis with KOH*

1ml of alcoholic KOH (6ml of 33%KOH + 94ml EtOH) was added to a 600µl sample. The sample was vortexed and incubated at 60°C for 1h. 1ml of ddH_2O and 2ml of hexane were added and the sample vortexed for 15min. The sample was then centrifuged at 3000rpm for 20min at 10°C (Sorvall RT 6000D). The upper hexane layer was transferred into a glass vial with Teflon lid and dried under nitrogen. The sample was frozen at -20°C for gas chromatography analysis.

c) *Derivatization with Sil-A Reagent*

40µl (20µg) of stigmasterol (Sigma) internal standard was added to each dried lipid extract. Stigmasterol was also added to 20µg of dried cholesterol standard. The samples were vortexed and dried under nitrogen. 100µl of Sil-A derivatization chemical (Sigma) was added to dry lipid extracts and vortexed for 30sec. The samples were incubated at 60°C for 2h. After incubation, the samples were analyzed by gas chromatography mass spectrometry.

G) ***Isolation of LDL***

A fresh blood sample was spun at 3000 rpm for 15 min at 4°C (Sorvall RT 6000) to pellet the red blood cells. The collected plasma was spun in 39.5ml centrifuge tubes (Beckman Quick Seal Polyallomer Centrifuge Tubes) at 40000 rpm for 20 hours at 4°C (Beckman L8-70M Ultracentrifuge). The tubes were cut and the top layer containing chylomicrons removed. The density of the remaining solution was changed to 1.063 g/ml by adding a sufficient amount of KBr. New tubes were filled with the solution and spun at 40000 rpm for 20 hours at 4°C. Tubes were cut and the top layer containing LDL removed. LDL was dialyzed against 0.15 M NaCl overnight at 4°C. The concentration of LDL was calculated using the Markwell Lowry protein assay (74).

IX. RESULTS

IX. RESULTS

ApoA-I lipidation and consequent formation of HDL particles at the plasma membrane has been a subject of much discussion. Although not entirely understood, it is agreed that this process requires a partially lipidated apoA-I approaching the plasma membrane and possibly interacting with a transmembrane protein ABCA1. This interaction then allows apoA-I protein to acquire certain types of phospholipids and cholesterol, leading to the formation of HDL. Hepatocytes are known to secrete large amounts of apoA-I, which sometimes leave the cell in a lipid-free form, and other times, come out partially lipidated. Following many attempts at clarification, it is still not known which lipids could be acquired in intracellular compartments and by which method. As well, the importance of intracellular apoA-I lipidation in hepatocytes for subsequent HDL formation still needs to be determined. The main objective of this paper is to establish which lipids are acquired by apoA-I and in which compartments of primary mouse hepatocytes. Also, we want to determine if the intracellular lipidation of apoA-I is ABCA1-dependent. We labeled the cells with radioactive choline and cholesterol, fractionated the cell into ER and Golgi compartments and followed the pattern of lipid binding to apoA-I.

A) *Distribution of ER and Golgi vesicles following Nycodenz gradient centrifugation*

Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion, plated on fibronectin-coated plates and grown in the presence of Williams media. At time zero, the cells were scraped and processed as described in the Methods section above. Following the Nycodenz gradient centrifugation, fifteen aliquots were collected.

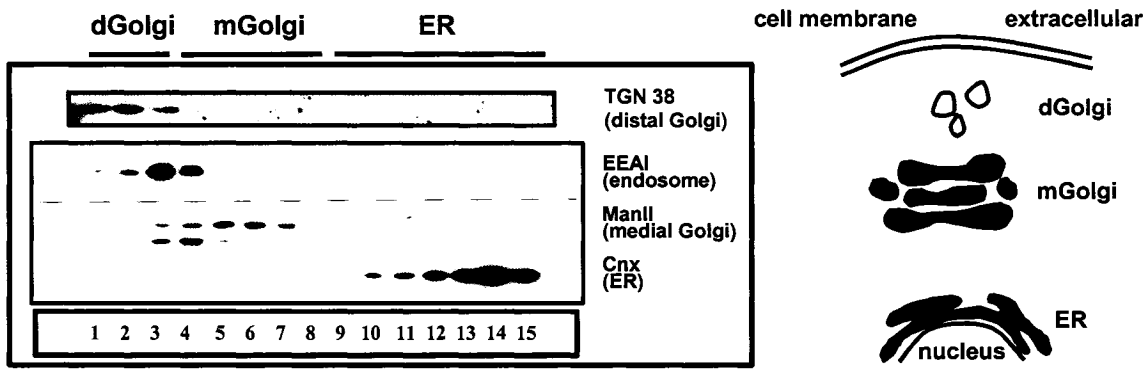

Figure 3-1: Western blot of molecular marker proteins for distal Golgi (TGN 38), early endosome (EEAI), medial Golgi (ManII) and ER (Cnx). Fifteen subcellular fractions (see methods) were run on a gel, proteins separated by SDS-PAGE, transferred to a membrane and western blots performed. Rabbit anti-TGN38 polyclonal antibody (CalBiochem) was used to detect distal Golgi marker protein, TGN38 (38kDa). Rabbit anti-EEAI polyclonal antibody (Affinity BioReagents) was used to detect early endosome marker protein, Early Endosomal Antigen 1 (160kDa). Rabbit anti-ManII polyclonal antibody (a gift from Dr. Zemin Yao) was used to detect a medial Golgi marker protein, Manosidase II (125kDa). Rabbit anti-Cnx polyclonal antibody was used to detect ER protein marker, Calnexin (90kDa). Secondary, donkey anti-rabbit antibody (Amersham) was applied to all samples.

A western blot was performed (Figure 3-1) and probed for specific intracellular marker proteins in order to determine the location of different subcellular vesicles in these fifteen aliquots. EEAI, TGN 38, ManII and Cnx are resident proteins in early endosomes, distal Golgi (dGolgi), medial Golgi (mGolgi) and ER, respectively. The results show that dGolgi vesicles can be found in the first three aliquots (1-3), mGolgi vesicles are located in the next five aliquots (4-8) and the last seven aliquots contain ER vesicles (9-15). Also, the first three aliquots contain some early endosome vesicles mixed in with the dGolgi vesicles. These aliquots were grouped accordingly to form three large fractions named ER, mGolgi and dGolgi.

B) *Steady state picture of apoA-I lipidation in the presence of ^{3}H-choline- or LDL-^{3}H-cholesterol-labeled lipids*

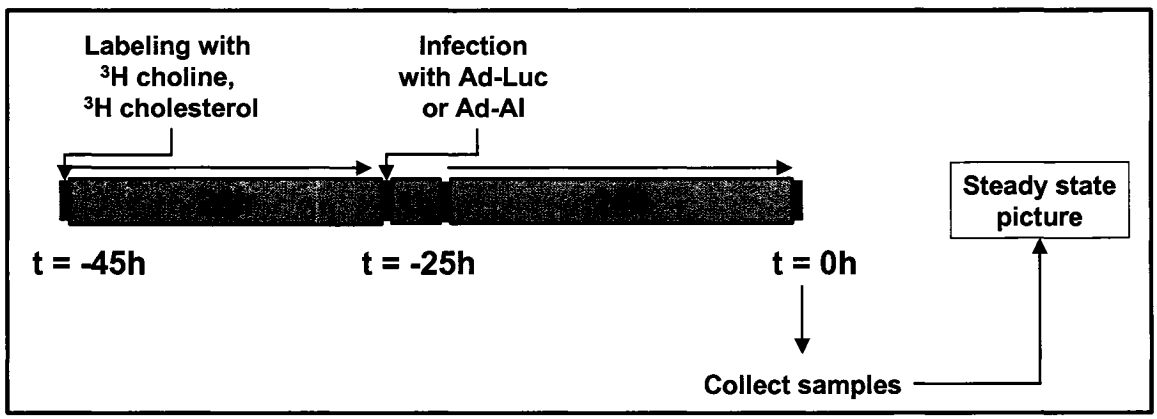

Figure 3-2: Schematic representation of the basic experimental procedure.

In order to gain initial insight into the intracellular lipidation of apoA-I, primary mouse hepatocytes were labeled with ^{3}H-choline or LDL-^{3}H-cholesterol (Figure 3-2). The cells were then infected with either Adeno-Luciferase (Ad-Luc) or Ad-AI and the amount of

radioactive lipid associated with apoA-I was measured. The purpose of this experiment was to determine whether phospholipids and cholesterol are acquired by apoA-I in ER and Golgi compartments and which type of lipid is more likely to bind. This initial experiment showed very significant acquisition of choline-labeled lipids by apoA-I in the ER compartment (Figure 3-3).

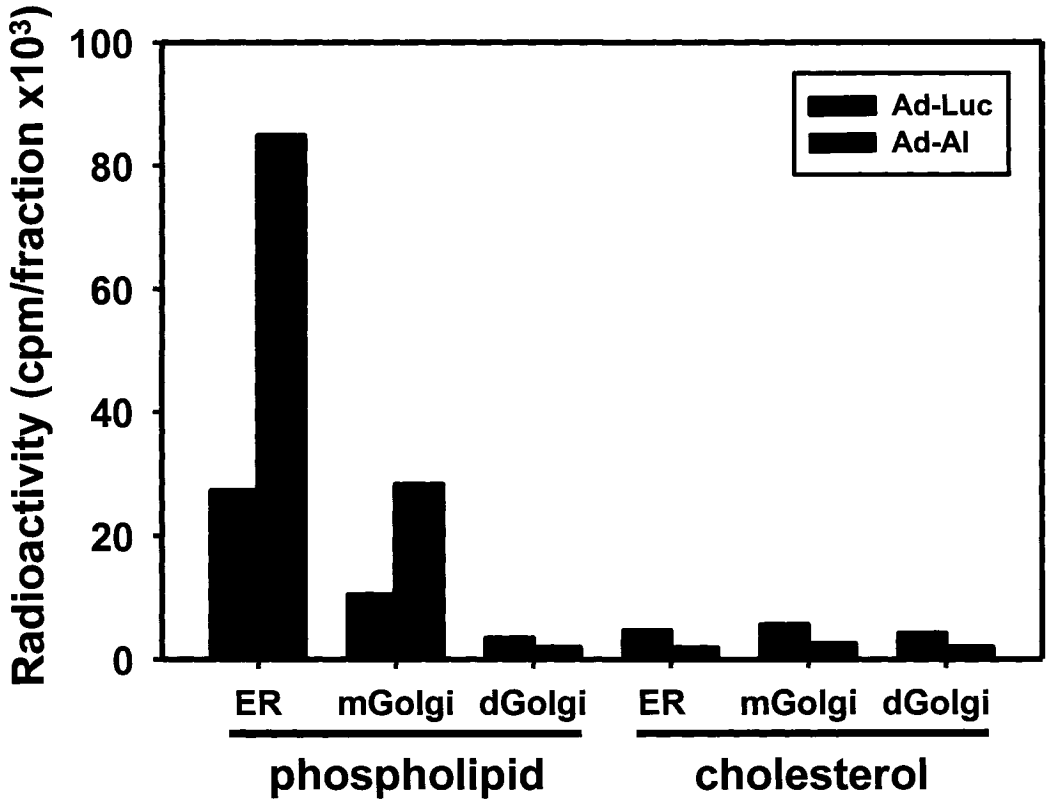

Figure 3-3: ApoAI lipidation in the lumens of intracellular fractions of ABCA1 wt cells. Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion, plated in fibronectin coated 10cm plates and grown in Williams media for 5h. Cells were then labeled with ^{3}H-choline (10μCi/ml) and ^{3}H-cholesterol (10μCi/ml) incorporated in LDL (5μg/ml) as described in methods. The lumenal apoA-I was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). The samples were washed three times in PBS and the radioactivity counted. The experiment was repeated three times and a representative experiment is shown.

Intracellularly phospholipidated apoA-I was also evident in the mGolgi, although it seems that some lipid was lost upon exiting the ER. Under the same conditions, when the cells were labeled with LDL-^{3}H-cholesterol, this early lipidation was almost completely absent. In conclusion, apoA-I is able to acquire phospholipids intracellularly, in the ER and mGolgi compartments, but LDL-derived cholesterol is not available for intracellular lipidation of apoA-I. Our laboratory has shown previously that cholesterol delivered with LDL preferentially labels the cell surface pool, including the recycling endosome compartment (75). Therefore the absence of cholesterol labeling of ER and Golgi by exogenous cholesterol explains these negative results.

C) *ApoA-I protein localization following cycloheximide treatment*

The experimental design was revised in order to examine this intriguing occurrence in more detail. A pulse-chase experiment was performed, where, at time zero, the cells were treated with cycloheximide to inhibit any new production of apoA-I (Figure 3-4). The localization of apoA-I protein following cycloheximide treatment was assessed, prior to analyzing its lipidation. The cells were scraped at 0, 20 and 40min and processed as described before. This allowed us to determine the location and the timing of apoA-I trafficking through the cell.

The samples were treated with sodium carbonate to release the proteins residing inside the ER and Golgi vesicles. Figure 3-5a shows a western blot of apoA-I located in whole samples containing both the membrane and the lumen of ER, mGolgi and dGolgi for the first hour of trafficking following cycloheximide addition.

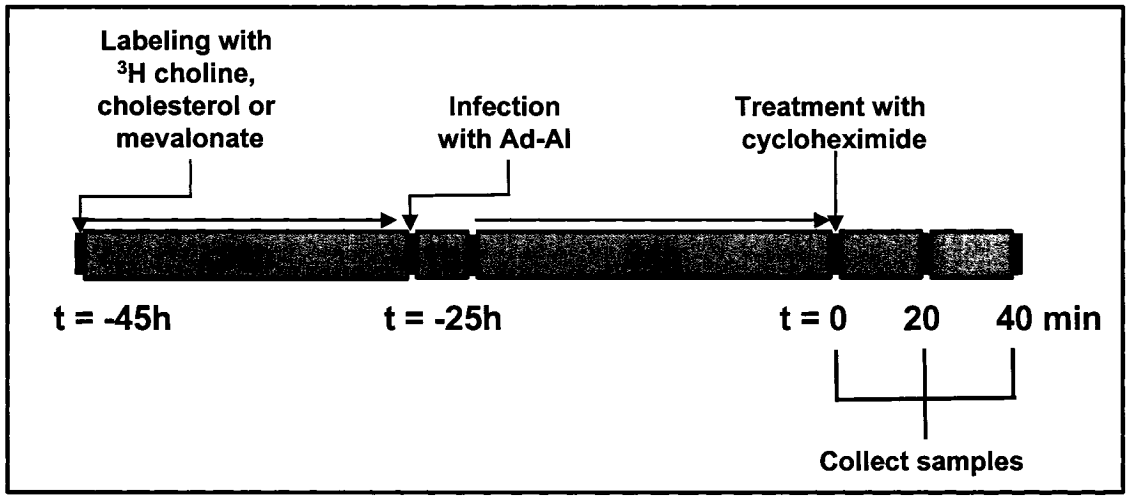

Figure 3-4: Schematic representation of the basic pulse-chase experimental procedure.

Also, the figure shows the protein being secreted into the media outside the cell. Western blot bands were analyzed and quantified using Quantity One program and the relative band density presented above the blots. The results show that initially, apoA-I is located in all three intracellular compartments, mainly in the dGolgi and mGolgi, while the ER seems to contain the smallest amount, consistent with a previous report of rapid transit of apoA-I (35). With time the ER compartment empties and the protein moves into mGolgi and dGolgi on its way out of the cell. After 40min, apoA-I leaves mGolgi and apoA-I accumulates in the media. dGolgi remains relatively abundant in apoA-I, at least for the duration of the pulse chase. This is not surprising, as some recycling is bound to occur through early endosomes, which co-exist with dGolgi in this fraction.

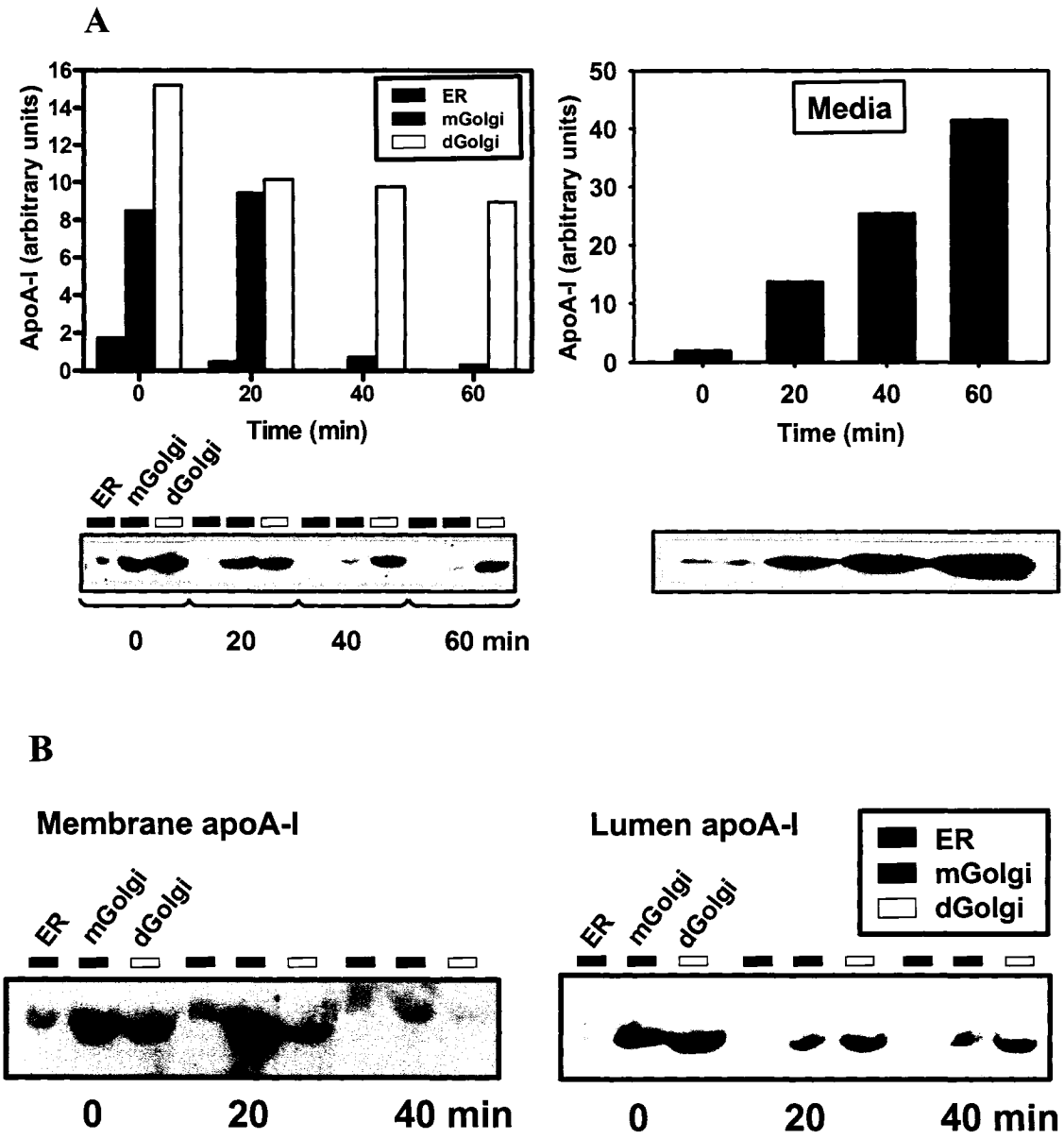

Figure 3-5: Time course of apoA-I localization in intracellular compartments and the effluxed media following cycloheximide treatment in ABCA1 wt cells. Primary mouse hepatocytes from C57BL/6 ABCA1 wt mice were isolated and treated with cycloheximide (3.55 µl/ml of 100mM stock) at time zero. A) The whole-cell and effluxed media samples were run on gels, proteins separated by SDS-PAGE, transferred to a membrane and western blots performed. B) Membrane and lumen fractions were run on gels. Anti-human apoA-I primary antibodies (5F6 and 4H1) and sheep anti-mouse apoA-I secondary antibody (Amersham) were applied. The results were quantified using Quantity One. The experiments were repeated three times and representative blots were shown.

The subcellular fractions were treated with sodium carbonate to release the proteins residing inside the ER and Golgi vesicles and centrifuged to separate lumen and membrane compartments. Western blotting (Figure 3-5b) shows at 0 min and 20 min the presence of a large amount of membrane-bound apoA-I in the mGolgi fraction, which contrasts with the low level of membrane-bound apoA-I in dGolgi. At 40 min in the dGolgi, we observed only the presence of lumenal apoA-I, which suggests that the progressive lipidation of apoA-I caused the release of apoA-I as a soluble complex.

D) ***Intracellular lipidation of apoA-I in ABCA1 wt cells***

Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion and cultured in Williams media for 5 hours. The cells were labeled with ^{3}H-choline, ^{3}H-cholesterol or ^{3}H-mevalonate and infected with Ad-AI. In this experiment, using the method described above, we assessed phospholipid and cholesterol lipidation of apoA-I with addition of cycloheximide for 0, 20 and 40min. This procedure allowed us to determine the location and the timing of apoA-I lipidation as it is being trafficked through the cell.

Assessment of apoA-I phospholipidation following ^{3}H-choline labeling in a pulse-chase experiment showed that apoA-I acquires phospholipids starting in the ER, going through all the intracellular compartments and eventually leading to the media. According to the data, phospholipids are acquired by apoA-I very early on in the ER and mGolgi (Figure 3-6a). Clearly, apoA-I was binding phospholipids during or soon after its translation in ER, and ER apoA-I, despite its low concentration, retains a high level of labelled phospholipids

throughout the chase. ApoA-I remained significantly lipidated in mGolgi, while dGolgi contained only a small fraction of phospholipidated apoA-I. After 20min, it is evident that some of the intracellularly phospholipidated apoA-I left the cell and could be found in the media, where it continued to accumulate more lipids. Finally after 40min, more lipidated apoA-I was transferred to the mGolgi from the ER, as it was being cleared from the cell.

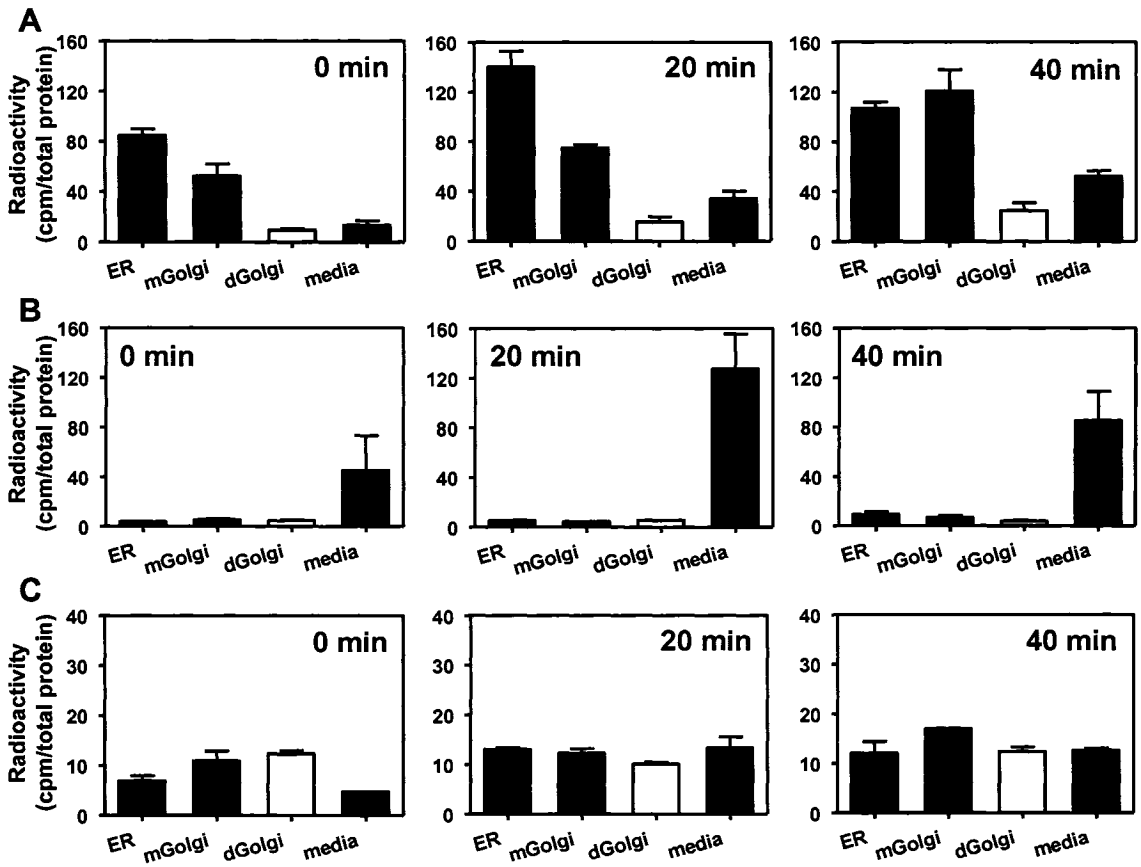

Figure 3-6: Time course of apoA-I lipidation in the lumens of intracellular fractions vs. the effluxed media following cycloheximide treatment in ABCA1 wt cells. Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion, plated in fibronectin coated 10cm plates and grown in Williams media for 5h. Cells were then labeled with A) ^{3}H-choline (10μCi/ml); B) ^{3}H-cholesterol (10μCi/ml) incorporated in LDL (5μg/ml)) and C) ^{3}H-mevalonate (15μCi/ml) as described in methods and cycloheximide was administered to cells (3.55 μl/ml of 100mM stock) at time zero. The lumenal apoA-I and the apoA-I found in the effluxed media was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). The samples were washed three times in PBS and the radioactivity counted. n = 4 for each A,B,C.

The lipidation levels in the dGolgi remained largely constant. Over the first 40min following cycloheximide treatment, the amount of lipidated apoA-I in the media kept increasing although it never reached the amount initially seen in the ER. Therefore, apoA-I binds phospholipids in ER and mGolgi compartments, travels towards the plasma membrane, sheds some lipids along the way and finally exits the cell partially phospholipidated.

The specificity of cholesterol binding by apoA-I was strikingly different when the cells were labeled with LDL-^{3}H-cholesterol (Figure 3-6b), a protocol which preferentially labels the cell surface compartment (75). At all three time points, intracellular lipidation of apoA-I was almost non-existent. However, apoA-I was able to accumulate a large amount of cholesterol at the plasma membrane (media). This lipidation was significant at time zero and continued to become even more impressive over the next 40min. The absence of cholesterol labeled apoA-I in the dGolgi fraction suggests that the secreted lipidated apoA-I does not recycle to this fraction. To establish whether or not apoA-I can acquire cholesterol in the early stages of synthesis and transport, the cells were cultured with ^{3}H-mevalonate to label *de novo* synthesized cholesterol, which originates in the ER and travels along the pathway of apoA-I transport. The results showed that *de novo* synthesized cholesterol can lipidate apoA-I as early as in ER and remain bound to apoA-I in Golgi compartments as well as after the secretion of the particle out of the cell (Figure 3-6c). The amount of *de novo* synthesized cholesterol bound to apoA-I in ER remains constant throughout the secretion pathway. It is important to note that the scale used to measure phospholipidation and LDL-cholesterol lipidation of apoA-I is different from the scale used to measure *de novo* synthesized cholesterol lipidation of apoA-I. Labeling with ^{3}H-choline and ^{3}H-cholesterol allows one to choose the amount of radioactivity that would be needed and used in an experiment.

Labelling with ^{3}H-mevalonate is a much more controlled event since mevalonate is a labeller with a lower specific activity. In order to increase the amount of label in the cell following the treatment with ^{3}H-mevalonate, one would need to use a much higher dose, which would be toxic to a cell. Another proof that *de novo* cholesterol association with apoA-I is significant is that it can be inhibited by the removal of ABCA1, as will be evident from the Figure 3-9. This lipidation of apoA-I with *de novo* cholesterol is minor but notable and it probably complements and follows the more significant apoA-I phospholipidation.

It is thus clear that hepatic apoA-I secretion is accompanied by the export of cholesterol and phospholipids. To provide a quantitative evaluation, we measured by GC/MS the net mass of cholesterol associated with the immunoprecipitated apoA-I after a 3h timepoint. In the total intracellular lumenal compartments, we found a total of 11.7ng of cholesterol associated with apoA-I per μg of total secreted apoA-I. In all the membrane compartments, we found 2.5ng of apoA-I-bound cholesterol per μg of total secreted apoA-I. In the media, we measured 6.7ng of apoA-I-assiaciated cholesterol per μg of total secreted apoA-I.

In summary, phospholipidation of apoA-I begins in the ER, it is maintained throughout the mGolgi and is still present in the media outside the cell. LDL-cholesterol lipidation is absent intracellularly but is very significant at the plasma membrane. Finally, minor apoA-I lipidation occurs in the presence of *de novo* synthesized cholesterol and is evident in the ER, mGolgi and dGolgi as well as at the plasma membrane.

E) *TLC analysis of phospholipid labeling following ^{3}H-choline treatment*

The distribution of the ^{3}H-choline label in the phospholipids associated with apoA-I was analyzed by TLC. We immunoprecipitated apoA-I from the lumenal ER and Golgi fractions and extracted the lipids by the Bligh and Dyer lipid extraction method (73). Lipid samples were loaded on a TLC plate and ran in a polar solvent. The desired bands were scraped off the plates and cpms counted. Phosphatidylcholine associated with apoA-I early in the ER lumen and peaked at 20 min (Figure 3-7a). Some sphingomyelin radioactivity started to associate with apoA-I early in the ER, with a peak in the mGolgi, where sphingomyelin is synthesized, at 40min (Figure 3-7b), consistent with Figure 3-6a. This experiment clearly shows a pulse of apoA-I from ER to mGolgi, in the process where one compartment is being cleared of phospholipidated apoA-I as the other is accepting the molecule on its way out of the cell. Very little phosphatidylcholine or sphingomyelin radioactivity was measured in dGolgi, suggesting that the lumenal apoA-I that accumulates in dGolgi is not phospholipidated in keeping with the low total radioactivity associated with apoA-I in that fraction (Figure 3-6a).

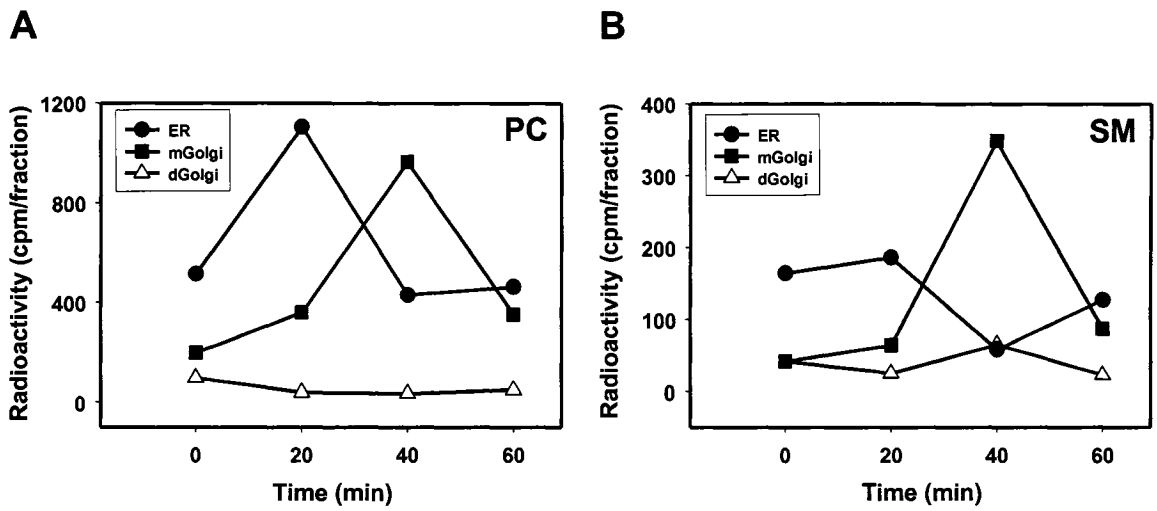

Figure 3-7: TLC analysis of the time course of apoAI phospholipidation in the lumens of intracellular fractions following cycloheximide treatment in ABCA1 wt cells. Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion, plated in fibronectin coated 10cm plates and grown in Williams media for 5h. Cells were then labeled with ^{3}H-choline (10μCi/ml) as described in methods and cycloheximide was administered to cells (3.55 μl/ml of 100mM stock) at time zero. The lumenal apoA-I was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). Lipids were extracted from these samples by Bligh and Dyer method described above and loaded on a TLC plate. The correct bands were scraped and the radioactivity counted.

F) *TLC analysis of the ^{3}H-mevalonate label distribution*

We immunoprecipitated apoA-I from the ER, Golgi and media samples containing the ^{3}H-mevalonate label. Following Bligh and Dyer lipid extraction, the samples were loaded on a TLC plate and ran in a non-polar solvent. CE and FC bands were scraped off the plate and the cpms counted in order to determine the distribution of the ^{3}H-mevalonate label. The analysis showed that ^{3}H-mevalonate did not significantly label CE, and that the label was almost exclusively found in the sterol fractions (Figure 3-8a). Interestingly, the levels of apoA-I associated with ^{3}H-mevalonate-labeled cholesterol determined by TLC analysis

(Figure 3-8a), perfectly follows the pattern of apoA-I-bound lipids determined by immunoprecipitation and simple cpm counting (Figure 3-8b). This indicates that our results with ^{3}H-mevalonate labeling represent specific sterol lipidation of apoA-I and they exclude any apoA-I-CE complexes. This information makes it easier to compare the patterns of cholesterol lipidation between LDL-^{3}H-cholesterol and ^{3}H-mevalonate labeling.

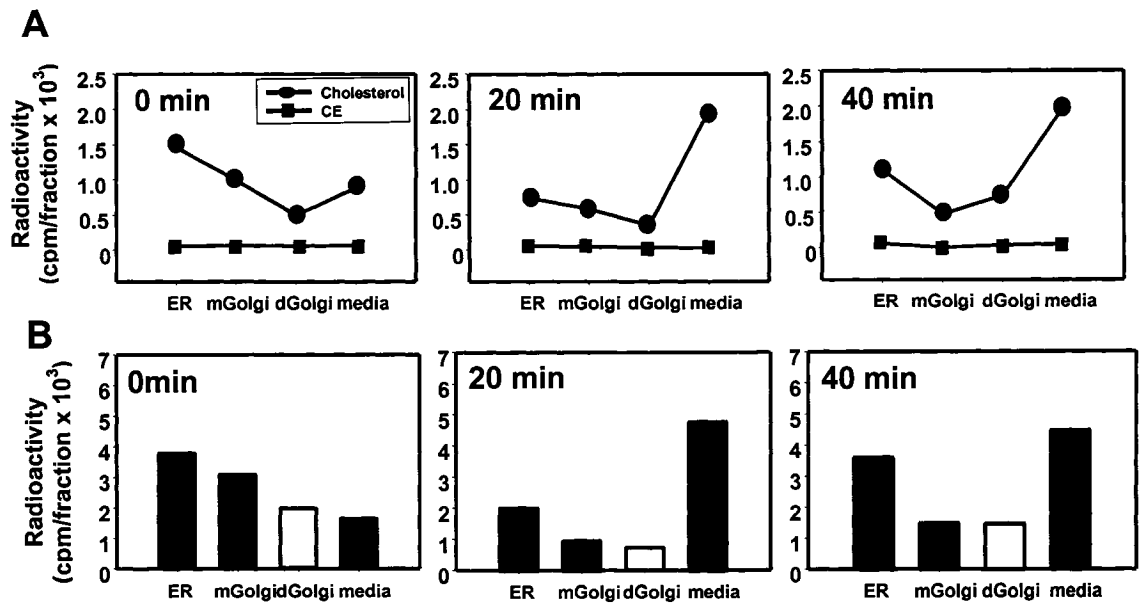

Figure 3-8: TLC analysis of the time course of apoAI lipidation by *de novo* cholesterol in the lumens of intracellular fractions following cycloheximide treatment in ABCA1 wt cells. Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion, plated in fibronectin coated 10cm plates and grown in Williams media for 5h. Cells were then labeled with ^{3}H-mevalonate (15μCi/ml) as described in methods and cycloheximide was administered to cells (3.55 μl/ml of 100mM stock) at time zero. The lumenal apoA-I was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). A) Lipids were extracted from these samples by Bligh and Dyer method described above and loaded on a TLC plate. The correct bands were scraped and the radioactivity counted. B) The lumenal and media samples containing immunoprecipitated apoA-I were washed three times in PBS and the radioactivity counted.

G) *Intracellular lipidation of apoA-I in ABCA1 KO cells*

The importance of hepatic ABCA1 in the lipidation of newly synthesized apoA-I has been documented by us and others (19,75,76) and cellular localization and traffic of ABCA1 are also well-characterized (11,61). However, we only have indirect evidence of intracellular lipidation of apoA-I by ABCA1 during secretion and no information on the sites of lipidation. We decided to establish whether ABCA1 is only being trafficked through the ER and Golgi on its way to the plasma membrane, and hence we see it in these compartments, or if it plays a significant role in intracellular lipidation.

To address these questions, primary mouse hepatocytes were isolated from C57BL/6 ABCA1 KO mice and cultured in Williams media for 5 h. During labeling with ^{3}H-choline, ^{3}H-cholesterol or ^{3}H-mevalonate, the cells were infected with Ad-AI. As described previously, phospholipid and cholesterol lipidation of apoA-I in the absence of ABCA1 was assessed in the different subcellular fractions, which were isolated following the addition of cycloheximide for 0, 20 and 40min.

Lack of ABCA1 had a strong effect on apoA-I phospholipidation in the mGolgi and at the plasma membrane (Figure 3-9a). At time zero, apoA-I was able to accumulate large amounts of phospholipid in the ER in the presence of ABCA1 (Figure 3-6a). This bulk initial lipidation in the ER was not inhibited even in the ABCA1 KO cells (Figure 3-9a). In fact, apoA-I phospholipidation in the ER is evident at least for the first 40min following cycloheximide treatment, suggesting that ER phospholipid acquisition by apoA-I is ABCA1 independent. On the contrary, phospholipidation of apoA-I was significantly inhibited in the mGolgi fraction very early on, and did not recover over time after 20 and 40min. As well, much less lipidated apoA-I accumulated in the media outside the cell. This significant

inhibition might be a consequence of decreased intracellular lipidation in the mGolgi. We must conclude that the major site of ABCA1-dependent phospholipidation for newly synthesized apoA-I is in the Golgi, in keeping with previous evidence of the presence of ABCA1 in this organelle (61).

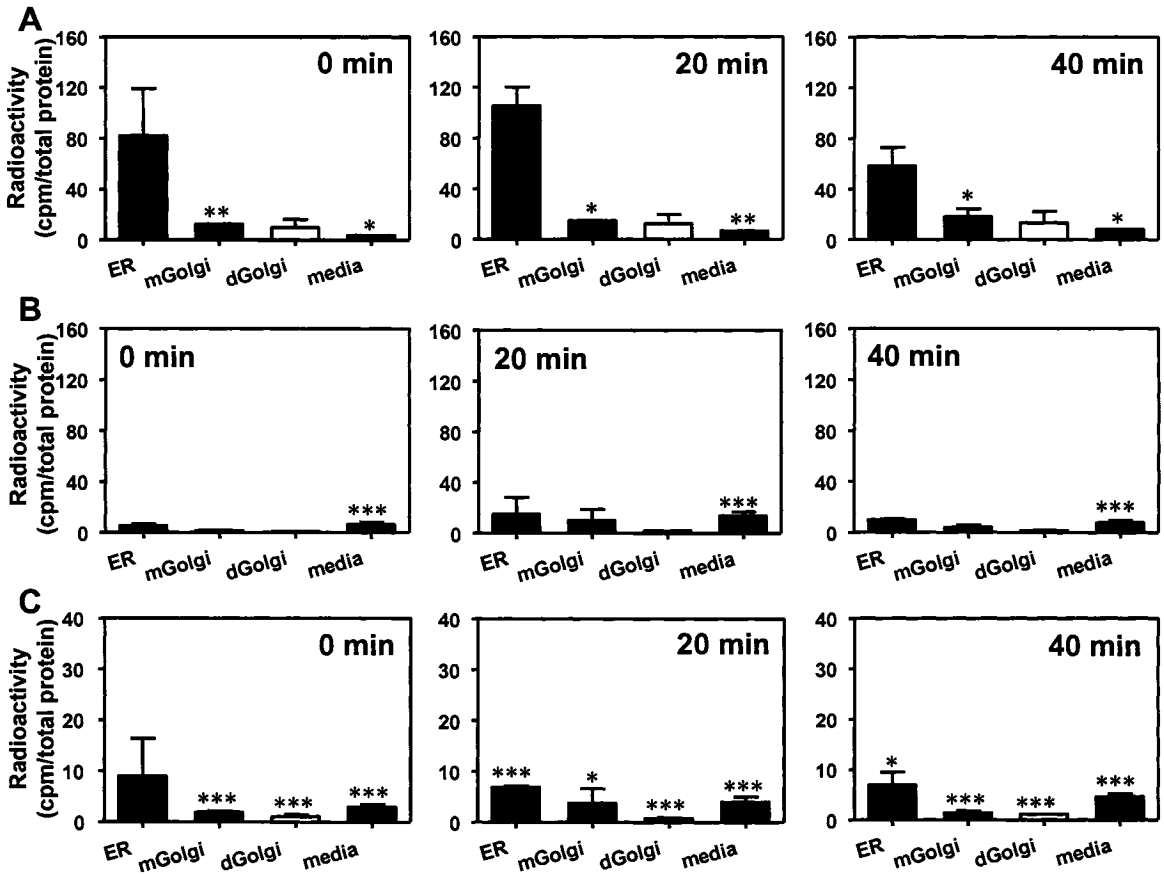

Figure 3-9: Time course of apoA-I lipidation in the lumens of intracellular fractions vs. the effluxed media following cycloheximide treatment in ABCA1 KO cells. Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 KO mice by collagenase liver perfusion, plated in fibronectin coated 10cm plates and grown in Williams media for 5h. Cells were then labeled with A) ^{3}H-choline (10μCi/ml); B) ^{3}H-cholesterol (10μCi/ml) incorporated in LDL (5μg/ml)) and C) ^{3}H-mevalonate (15μCi/ml) as described in methods and cycloheximide was administered to cells (3.55 μl/ml of 100mM stock) at time zero. The lumenal apoA-I and the apoA-I found in the effluxed media was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). The samples were washed three times in PBS and the radioactivity counted. Student t-test was performed to determine the significance of inhibition; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n = 4 for A, B, C.

LDL-cholesterol lipidation of apoA-I was almost completely absent in the intracellular compartments of ABCA1 wt cells, with the bulk lipidation happening at the plasma membrane (Figure 3-6b). As expected, intracellular cholesterol lipidation of apoA-I was not affected by the absence of ABCA1. However, lack of ABCA1 at the plasma membrane completely abolished any LDL-cholesterol accumulation in the media (Figure 3-9b). *De novo* synthesized cholesterol lipidation of apoA-I in ABCA1 wt cells was minor but noteworthy (Figure 3-6c). This lipidation decreased significantly in the absence of ABCA1 in almost all fractions, most significantly in the Golgi compartments and consequently, the amount of lipidated apoA-I accumulated in the media also decreased (Figure 3-9c).

In conclusion, intracellular phospholipidation of apoA-I was significantly inhibited in the mGolgi and at the plasma membrane of primary mouse hepatocytes lacking ABCA1. Under the same conditions, LDL-cholesterol lipidation of apoA-I was abolished at the plasma membrane, while the lipidation of apoA-I by *de novo* synthesized cholesterol decreased significantly in ER and Golgi compartments and at the plasma membrane. Therefore, a major proportion of the intracellular lipidation of apoA-I is ABCA1 dependent.

H) ***Subcellular interaction between apoA-I and ABCA1 protein***

To better understand how ABCA1 facilitates intracellular apoA-I lipidation, physical interaction between ABCA1 and apoA-I protein was investigated. apoA-I was immunoprecipitated from ER and Golgi fractions using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). The beads were washed three times in PBS and the proteins separated by SDS-PAGE. The western blot was probed with

anti-mouse ABCA1 primary antibodies (Santa Cruz) and sheep anti-mouse secondary antibodies (Amersham). Figure 3-10 clearly shows that ABCA1 interacts with apoA-I in all three intracellular compartments of ABCA1 wt cells (see lanes 1-3). Expectedly, this interaction is absent in ABCA1 KO cells (see lanes 4-6). These results show that apoA-I and ABCA1 form a physical contact with each other in ER and Golgi compartments, possibly leading to apoA-I lipidation. This data supports our finding that the intracellular lipidation of apoA-I is inhibited in the absence of ABCA1 expression.

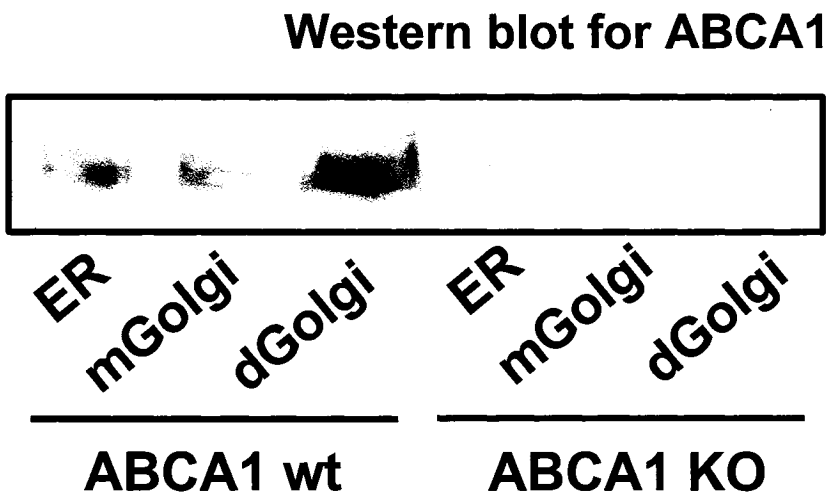

Figure 3-10: Colocalization of apoA-I and ABCA1 in membrane samples. Isolated ER, mGogli and dGolgi samples were treated with 1% Triton X-100 for 30min at 4°C and centrifuged at 75000 rpm for 30min at 15°C. Membrane apoA-I was immunoprecipitated from the supernatant using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). Proteins were separated by SDS-PAGE, transferred to a membrane and western blots performed. Anti-mouse ABCA1 primary antibodies (Santa Cruz) and sheep anti-mouse secondary antibodies (Amersham) were applied.

I) *Interaction between apoA-I and apoB protein and secretion of mixed lipoproteins*

The synthesis and assembly of VLDL in hepatocytes is well-characterized (77-79) and the acquisition of lipids by apoB begins early in the ER and continues throughout the trans-Golgi network. We have shown recently that lipoproteins containing both apoB and apoA-I are secreted by hepatocytes. To determine whether the radioactive lipids measured in apoA-I-immunoprecipitated samples are specifically bound to apoA-I or if they come from a VLDL particle as a consequence of apoB being pulled down with apoA-I, we needed to investigate if apoA-I and apoB proteins physically interact inside the cell.

Either apoA-I or apoB were immunoprecipitated from the standard samples and then probed for both apoA-I and apoB on the western blot. In support of our assumption, when apoA-I was immunoprecipitated, no apoB protein was found in any of the ER and Golgi fractions (Figure 3-11a). Also, when apoB was immunoprecipitated, no apoA-I protein was found in the fractions (Figure 3-11b). Interestingly, in the media samples containing particles that were secreted from the cell, immunoprecipitated apoA-I was able to pull down apoB (Figure 3-11a). As well, immunoprecipitated apoB from a media sample was able to pull down some apoA-I (Figure 3-11b). These results suggest that apoA-I and apoB do not interact in ER and Golgi compartments. However, once they both leave the cell, they together form larger lipoprotein particles.

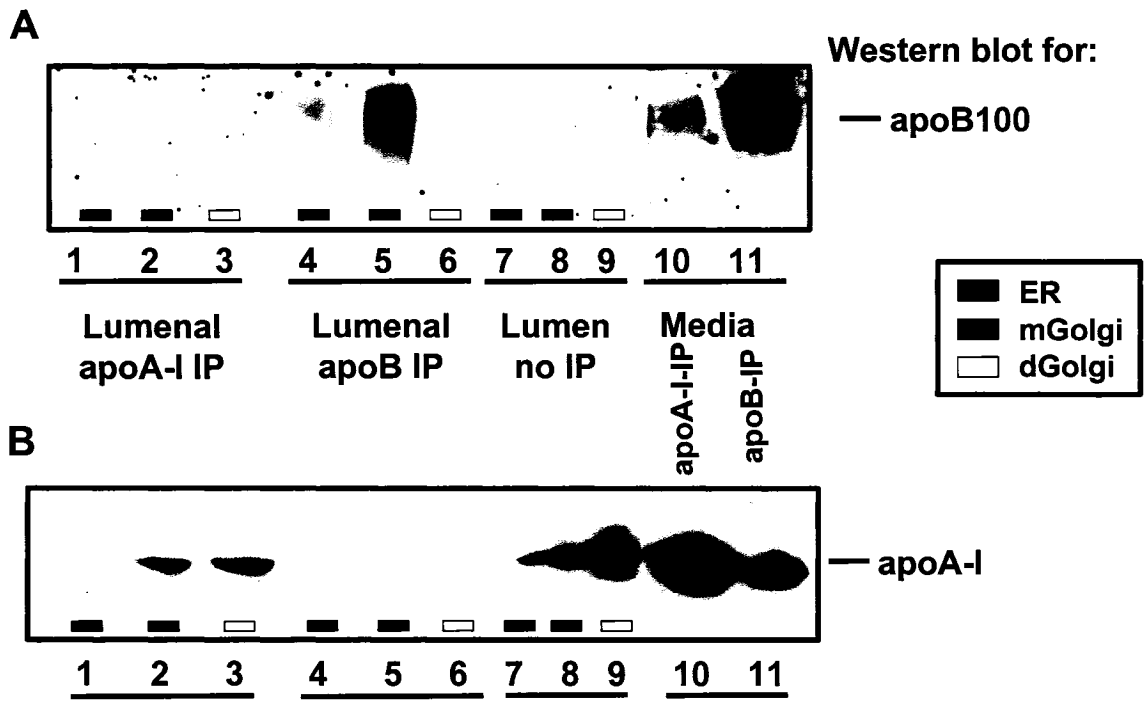

Figure 3-11: Colocalization of apoA-I and apoB in lumenal and media samples. Lumenal samples of ER, medial Golgi and distal Golgi underwent immunoprecipitation for apoA-I and apoB. The lumenal apoA-I and the apoA-I found in the effluxed media was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). The lumenal apoB and the apoB found in the effluxed media was immunoprecipitated using anti-apoB primary antibody (Biodesign) and protein A sepharose beads (Amersham). The samples were washed three times in PBS and ran on gels. Proteins were separated by SDS-PAGE, transferred to a membrane and western blots performed. Anti-human apoA-I primary antibodies (5F6 and 4H1 – our lab) and sheep anti-mouse apoA-I secondary antibody (Amersham) were applied. Anti-mouse apoB primary antibodies (gift from Dr. Milne) and sheep anti-mouse secondary antibodies (Amersham) were applied.

J) *Phospholipidation of Ad-AI vs. His-AI before and after cell death*

Cellular fractionation and separation of intracellular compartments is a complex procedure requiring a physical homogenization of a cell, followed by multiple transfer steps,

rapid centrifugations and a chemical cell break-up of microsomes by sodium carbonate treatment. Although special care is taken to make sure that each of these steps is performed in a clean and precise manner, and at 4°C and 15°C as required, a possibility always exists that some contamination may occur. Precisely, some lipids bound to apoA-I may be acquired by the protein artifactually during the fractionation process, and not prior to homogenization as a part of the normal cellular metabolism. To rule out this possibility, the following experiment was carried out.

Primary mouse hepatocytes isolated from C57BL/6 ABCA1 wt mice were labeled with ^{3}H-choline (Figure 3-12). Cells were either infected with Ad-AI (bars 2 and 4) or mock infected with Ad-Luc (bars 1 and 3). Prior to homogenization, the cells infected with Ad-Luc were treated with 5μg of His-AI, an amount equivalent to that secreted by the cell (76). Half of the cells from each condition were treated with sodium carbonate (bars 3 and 4). Lumenal apoA-I or apoA-I interacting with microsome membranes were immunoprecipitated and pulled down with Protein G-Sepharose and the radioactivity associated with precipitated apoA-I was measured.

Sodium carbonate was added to the samples to release lipidated apoA-I from the lumen of ER and Golgi vesicles. In the samples without sodium carbonate, only the membrane apoA-I is exposed, while in the samples with sodium carbonate, total lipidated apoA-I, including lumenal and membrane bound apoA-I, is presented. Our data shows that the total amount of lipids acquired after cell death by His-AI is minor compared to the total amount of lipids acquired in the cell by Ad-AI, both in the presence of sodium carbonate (Figure 3-12). Significantly more lipids were acquired by Ad-AI than by His-AI, even when sodium carbonate was not added. Importantly, the same amount of lipid was acquired by

His-AI with or without sodium carbonate, indicating that His-AI probably picks up a small amount of lipid artificially introduced during the homogenization of the cell, while the lipidation of Ad-AI which we see inside the ER and Golgi vesicles is true and specific and it occurs while the cell is still alive.

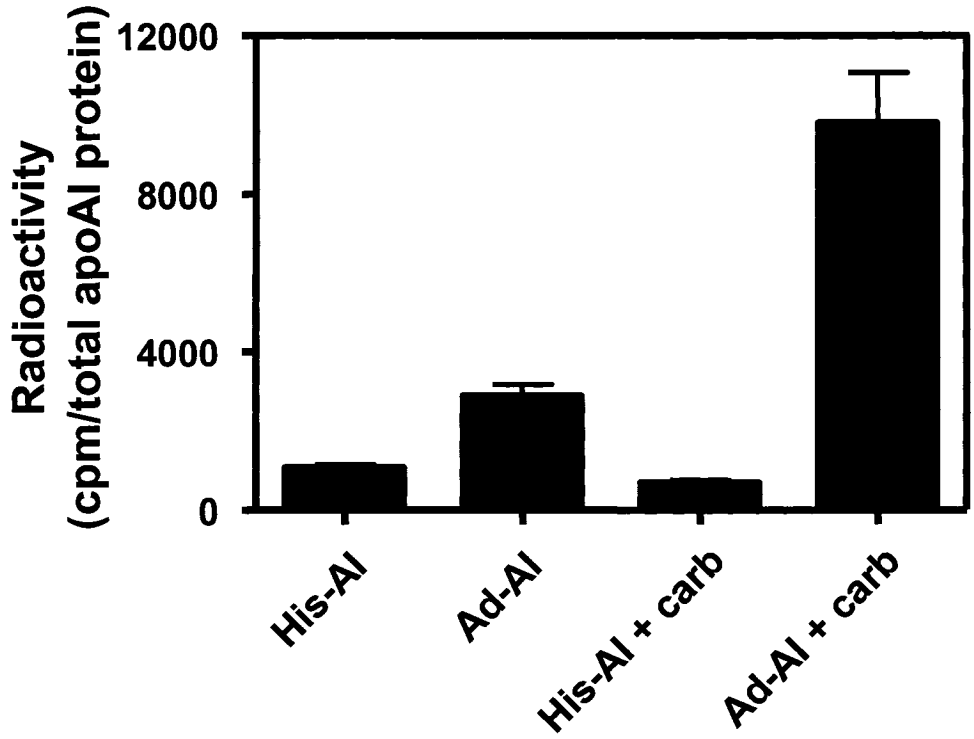

Figure 3-12: Phospholipidation of endogenously synthesized Ad-AI or exogenously added His-AI in microsomes before and after sodium carbonate treatment. Primary mouse hepatocytes were isolated from C57BL/6 ABCA1 wt mice by collagenase liver perfusion, plated in fibronectin coated 10cm plates and grown in Williams media for 5h. Cells were then labeled with ^{3}H-choline (10μCi/ml) as described in the methods. Half of cells were infected with Ad-AI (bars 2 and 4) and half were mock infected with Ad-Luc (bars 1 and 3). At the point of homogenization the mock infected cells were treated with 5μg of His-AI. Half of the cells from each condition were treated with sodium carbonate (bars 3 and 4). The lumenal apoA-I was immunoprecipitated using anti-apoA-I primary antibody (Calbiochem) and protein G sepharose beads (Amersham). The samples were washed three times in PBS, the radioactivity counted and the results expressed per total apoA-I protein. n=6.

In conclusion, the lipidation of apoA-I that has been assessed numerous times in this project is a process that occurs inside the cell, in ER and Golgi compartments, in viable cells and as a part of regular cell metabolism and it is not a product of artificial protein-lipid interactions. This important information allows us to conclude with confidence that intracellular lipidation of apoA-I is an indisputable process and partially dependent on ABCA1.

X. DISCUSSION

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The goal of the present study was to assess the importance of intracellular lipidation of apoA-I in hepatocytes. To determine the nature of lipids acquired by apoA-I and the location of intracellular lipidation, we isolated primary mouse hepatocytes from C57BL/6 ABCA1 wt mice and labeled them with ^{3}H-choline, LDL-^{3}H-cholesterol or ^{3}H-mevalonate. The cells were then infected with Ad-AI and 24h later treated with cycloheximide to inhibit any new production of apoA-I (Figure 3-6). Nycodenz gradient centrifugation was used to separate ER and Golgi compartments (Figure 3-1) and sodium carbonate treatment allowed for the lumen and membrane fractions to be isolated. ApoA-I was immunoprecipitated from the lumen samples and the lipid cpms counted. Our results show that phospholipidation of apoA-I is significant and most evident in ER and medial Golgi with only modest lipidation after export out of the cell. In cells labeled with LDL-^{3}H-cholesterol, intracellular cholesterol lipidation of apoA-I is entirely absent, but the exported apoA-I rapidly accumulates cholesterol following exit out of the cell. On the other hand, *de novo* synthesized cholesterol is able to lipidate apoA-I intracellularly. This implies that HDL formation begins with intracellular apoA-I phospholipidation in the ER and Golgi, followed by modest cholesterol lipidation in the Golgi, which later promotes significant cholesterol accumulation at the plasma membrane. To determine if the lipidation of apoA-I is ABCA1 dependent we performed the same experiment in hepatocytes isolated from C57BL/6 ABCA1 KO mice and assessed the lipidation of apoA-I in the absence of ABCA1 (Figure 3-9). In hepatocytes lacking ABCA1, lipidation of apoA-I by LDL-derived cholesterol was significantly reduced at the plasma membrane. Phospholipidation and lipidation by *de novo* cholesterol were both

reduced in Golgi compartments, while ER lipidation remained mostly unchanged, suggesting that the early lipidation in ER is ABCA1 independent while the lipidation in Golgi as well as at the plasma membrane requires ABCA1. Thus, we have demonstrated that apoA-I is lipidated intracellularly, partially dependent on ABCA1, with the bulk of cholesterol lipidation occurring at the plasma membrane.

Banerjee and colleagues performed pioneering studies in the field of intracellular lipidation. They administered ^{3}H-glycerol to chickens at a young age and assessed the amount of lipid accumulation on hepatic apoA-I (36). After fractionating the liver into RER, SER and Golgi, they used carbonate to isolate lumen and membrane fractions. They noticed a very rapid transport of lipidated apoA-I from ER to Golgi. According to their data, lipid acquisition by apoA-I in ER was done in the first 5min, by which time the lipidated apoA-I has moved to the Golgi to accumulate more lipids. The major difference between our study and all of Banerjee's studies is that they assess chicken apoA-I lipidation, whereas we use human apoA-I and determine its lipidation in mouse hepatocytes. Our western blot data agrees with Banerjee's results showing that, following cycloheximide treatment, apoA-I protein spends a limited amount of time in the ER, and within 20min the majority of apoA-I moves on to mGolgi (Figure 3-5). However, the assessment of apoA-I phospholipidation, by measuring radioactive lipids associated with apoA-I following immunoprecipitation, tells us that some apoA-I remains in the ER and is lipid associated (Figure 3-6). This discrepancy can be explained in two ways. Firstly, longer film exposure shows a band on a Western blot indicating basal levels of apoA-I in the ER compartment, which are responsible for accumulating the lipids that we see in Figure 3-6a. Secondly, immunoprecipitation method which determines the amount of lipids bound to apoA-I in the lumens is significantly more

sensitive then western blot determination. Therefore, it is not surprising that a small amount of apoA-I appears to be accumulating a large amount of lipid.

It is important to keep in mind that we labeled the cells with ^{3}H-choline allowing us to directly measure phospholipids associated with apoA-I. The Banerjee group used radioactive glycerol, and consequently, only ~4-27% of the label was incorporated into phospholipids, while the majority of label went into triglycerides. They also show that although some lipid is acquired by apoA-I in the ER, six times as much is obtained in the Golgi fraction. They suggest that apoA-I not only spends very little time in the ER, but that it also acquires only a minimal amount of lipid, whereas the Golgi is where the majority of lipidation occurs.

Our results indicate that initial phospholipidation in ER is robust and it even exceeds the phospholipidation seen in the Golgi. However, our interpretation of this result agrees significantly with the conclusions that the Banerjee's group has made. ApoA-I is synthesized on the cytosolic surface of the ER. As it is being transported through a pore on the ER membrane, apoA-I accumulates some PL. To complete the process of synthesis, the protein is then cut off to be released to the lumen of ER. Sometimes, apoA-I remains temporarily bound to the membrane for an extremely short period of time. ApoA-I is then very likely bound to a large amount of PL, some of which belongs to the membrane as well as some that truly belongs to the apoA-I protein. In the process of lumen and membrane separation, it is possible that some of this surplus lipid is still associated with apoA-I in the isolated lumen fraction. However, this lipid is only transiently bound to apoA-I and in an *in vivo* setting it would quickly be returned to the ER membrane where it belongs, while apoA-I would only retain as much phospholipid as it needs in order to be transported to the mGolgi. However, in our experiment, all of the PL that was bound to apoA-I even for a split second remains bound

to apoA-I since the membrane is removed and the lipid has nowhere else to go. Once transferred to the mGolgi, apoA-I is capable of obtaining a significant amount of PL which is permanent and remains bound to the apoA-I until it is secreted from the cell. So the initial apoA-I lipidation is very fast and transient whereas the lipidation in the mGolgi is real and lasting.

Alternatively, the synthesis of apoA-I may require a number of other chaperone proteins which bind to apoA-I as it is being released to the lumen of ER. These chaperones together with apoA-I may remove extra lipid from the ER membrane in the process of initial apoA-I lipidation. However, this lipidation may be only a facilitator for the proper folding of apoA-I. Once apoA-I is properly folded, it can shed extra lipids, which then associate back with the ER membrane and never get secreted from the cell, while apoA-I gets transferred to the mGolgi for further ABCA-dependent lipidation.

Dixon et al. expressed chicken apoA-I in a monkey kidney (COS-1) cell line and assessed HDL particle assembly (58). They noticed that apoA-I can get secreted from the cell in its native, lipid-free form and acquire lipids upon its exit. They concluded that even though some apoA-I quickly exits the ER and gets lipidated in the Golgi, this lipidation is not necessary for apoA-I to be secreted. We observe that apoA-I gets lipidated both in ER and Golgi, but we believe that it occurs by two different mechanisms. While ER lipidation seems to occur co-translationally and independently of ABCA1, the lipidation in the Golgi is more specific, dependent on ABCA1 and likely persists until secretion. Although we do not exclude the possibility that some apoA-I is secreted lipid-free, our results show that apoA-I is significantly phospholipidated and associated with cholesterol prior to secretion, while the majority of cholesterol is acquired after export out of the cell.

The ER is the major site of phospholipid synthesis (80). Also, the enzymes responsible for synthesis are located on the cytosolic side of the ER membrane (81,82), suggesting that this early phospholipidation probably occurs during or immediately after translation. However, this lipidation is transient and is likely linked to the high specific activity of newly synthesized PC in the ER membranes. As well, we postulate that the early ER lipidation provides sufficient lipids to apoA-I to assist the proper protein folding and transport to the Golgi. Dixon et al. (58) suggest that some lipid associated with apoA-I intracellularly may dissociate from apoA-I after being secreted in the media and therefore give the impression of being secreted lipid-free. Presumably, not all of the intracellularly lipidated apoA-I needs to be secreted as such. During the transport from ER to Golgi, some excess lipids associated with apoA-I can shed along the way. Some phospholipidated particles may dissociate into lipid-poor apoA-I and free phospholipids which may then be incorporated into the membrane of the dGolgi. In the dGolgi, where the decrease in apoA-I lipidation is evident, the remaining phospholipids associated with apoA-I might be exchanged with the phospholipids of lower specific activity, located in the membranes of the dGolgi. As dGolgi particles co-exist with recycling endosomes, some apoA-I proteins which have lost their lipids may still be re-secreted in their lipid-free/poor form. They might then acquire lipids in the media by interacting with the plasma surface, or be recycled into the cell.

In hepatocytes lacking ABCA1, the phospholipidation occurring in the medial Golgi was significantly decreased, indicating that this process is at least partially ABCA1 dependent (Figure 3-9). However, the early bulk apoA-I phospholipidation in the ER was not affected in ABCA1 KO cells, demonstrating that ABCA1 is not involved in phospholipid acquisition at this step. Nevertheless, our co-immunoprecipitation data shows that apoA-I

and ABCA1 are able to interact in this compartment (Figure 3-10), which indicates that both apoA-I and ABCA1 are normally folded and able to interact. It is possible that ABCA1 is not fully activated at this stage, presumably due to lack of phosphorylation (83-86) and glycosylation (63). The relatively low level of ABCA1 seen in the ER is in agreement with previous observations that only some fluorescently tagged ABCA1 resides in the ER, while the main pools are in early and late endosomes-lysosomes (11) as well as in the Golgi (61).

We showed that apoA-I is located in all three intracellular compartments (Figure 3-5a), mainly in the mGolgi and dGolgi, while the ER level remained low, in keeping with its reported rapid transit out of ER (35). In all compartments, apoA-I could be found in both membrane bound form and in the lumenal fractions (Figure 3-5b). After 40 min of the chase only lumenal apoA-I was seen in mGolgi and dGolgi, which demonstrates that at that stage apoA-I is sufficiently lipidated to be released from the membranes and form stable soluble lipoproteins. With time the ER compartment emptied and apoA-I transits into mGolgi and dGolgi. After 40min of chase, apoA-I leaves the mGolgi to accumulate in the media.

In control hepatocytes, most phospholipids (Figure 3-6a) and *de novo* synthesized cholesterol (labeled with ^{3}H-mevalonate, Figure 3-6c) are transferred to apoA-I in ER and mGolgi. However, when LDL-^{3}H-cholesterol is used to label plasma membrane and recycling endosome, newly synthesized apoA-I does not interact with any of this labeled cholesterol until it is secreted (Figure 3-6b). These results suggest that a separate mechanism exists for phospholipids vs. cholesterol transfer to apoA-I during secretion, which is in agreement with previous work (75). In ABCA1 KO hepatocytes, the residual cholesterol-lipidation of apoA-I by LDL-derived cholesterol at the plasma membrane is probably mediated by diffusional transfer from the cell surface compartment to the phospholipidated apoA-I, originating from ER and accumulating in the media. (Figure 3-9b).

The idea of HDL formation inside the cell has been discussed previously. Howell and Palade labeled newly synthesized phospholipids by treating primary rat cells with ^{14}C-choline, ^{3}H-myo-inositol, or ^{33}P-phosphate (39). They isolated Golgi fractions and looked for HDL and VLDL particles in Golgi membrane and lumen. They noticed that all the apoproteins incorporated into lipoproteins found in the plasma of these rats were also present in both the membrane and lumen fractions of the isolated Golgi. Even more importantly, they discovered that almost a half of the Golgi particles were very similar to the VLDL particles in the plasma. The rest of the particles, although not identical to HDL and VLDL found in the plasma, contained the same apoproteins and similar lipid composition. This led them to believe that these Golgi particles could be the precursors of the mature VLDL and HDL particles. Our study very much supports their findings. Although we do not show that the particles in the Golgi fractions of primary mouse hepatocytes are HDL-like particles, we prove that both phospholipids, and to some extent cholesterol, are acquired by apoA-I (Figure 3-6), the main HDL apoprotein, and that this lipidation is ABCA1 dependent (Figure 3-9). As discussed earlier, ABCA1 plays an important role in HDL formation at the plasma membrane, and the fact that we determined that the lipidation of apoA-I in the Golgi is ABCA1-dependent as well, confirms the idea that the HDL formation begins early in the secretory pathway.

Significantly different results were obtained by Hamilton et al. (87). They isolated Golgi fractions from primary rat hepatocytes and compared the structure of VLDL found in the Golgi with the VLDL in the plasma. Protein found in the VLDL isolated from the Golgi were separated by SDS gel electrophoresis and viewed with Coomassie Blue. Among the expected apoB48 and apoB100 proteins, Golgi VLDL was found to contain apoA-I and apoE as well. Also, using radioimmunoassay, they determined that apoE and apoA-I are found in

the Golgi either as a part of VLDL or as Golgi membrane proteins. This finding strongly disagrees with our results where we clearly show that apoA-I and apoB do not at all interact in any of the intracellular compartments (Figure 3-11). We immunoprecipitated either apoA-I or apoB from our samples and then probed the western blot for apoB and apoA-I respectively. Although no intracellular interaction was observed, we did notice that apoA-I and apoB interact after being secreted from the cell, in agreement with our previous results (75). This suggests that the surface of newly secreted VLDL either is not saturated with apolipoproteins or that apoA-I displaces other exchangeable apolipoproteins. Our observations that apoA-I and apoB only associate after secretion could reflect the existence of separate pathways for secretion of these lipoproteins. After performing electron microscopy studies, Hamilton and colleagues concluded that Golgi vesicles do not contain any particles that resemble HDL. This statement, however, cannot exclude the possibility that apoA-I gets lipidated intracellularly, but it merely states that the lipidation is not large enough to form a mature, spherical particle which fully resembles HDL.

The most recent study dealing with intracellular lipidation of apoA-I was performed by Chisholm et al. in HepG2 cells (38). Initially, they radiolabeled apoA-I with ^{35}S and tested the density at which it floats following secretion. They concluded that since 25% of apoA-I was able to float at d >1.25g/ml right after secretion and 50% was able to float after 120min, the first 25% of apoA-I must have acquired sufficient lipids intracellularly and the other 25% extracellularly. Since only apoA-I was labeled and not the lipids, it is impossible to say which lipids were bound to apoA-I. Our results show that intracellular LDL-cholesterol lipidation of apoA-I is practically non-existent (Figure 3-6b). Also, the lipidation by *de novo* synthesized cholesterol is minor but notable and it could not contribute sufficiently to apoA-I lipidation to float as an HDL (Figure 3-6c). So the lipids that Chisholm

et al. observed bound to apoA-I must have been of a phospholipid nature. We have separated the microsomes into three intracellular compartments providing more detail about localization of apoA-I and the localization of the lipidation steps. We used labeled lipids to determine the exact nature of lipids binding to apoA-I but with no description of the extent of buoyancy. To further their understanding of this concept, Chisholm and colleagues assessed the floatation of endogenous apoA-I vs. exogenously added apoA-I. They observed that ~20-30% more endogenous apoA-I floats at d >1.25 g/ml compared to exogenous apoA-I, indicating intracellular lipidation of apoA-I (38). They conclude simply that there is intracellular cholesterol lipidation of apoA-I; we show the specific site of phospholipid and cholesterol lipidation in a relevant primary hepatocyte model.

Cellular fractionation experiments are complex, multi-step procedures, requiring cell rupture and gradient centrifugation. These steps could introduce artificial mixing of proteins and lipids, causing lipidation to occur outside the cell and not in true intracellular compartments. To avoid such an occurrence, Chisholm and colleagues assessed lipid acquisition by endogenous apoA-I and compared it to the acquisition of lipids by apoA-I exogenously added during microsomal isolation (38). They determined that only about 8% of extracellular apoA-I was non-specifically labeled during the process of cell homogenization. 27% of specifically lipidated endogenous apoA-I isolated from microsomal samples were buoyant. This experiment indicated that the lipidation they were seeing was real and it could be quantified to 19% of total endogenous apoA-I.

We performed a similar experiment where we tested how much of the phospholipid associated with apoA-I was acquired intracellularly and how much was added to apoA-I artificially during the isolation of ER and Golgi fractions (Fractions 3-12). We labeled the cells with ^{3}H-choline and infected them with either Ad-AI or Ad-Luc. We exogenously

added His-tagged apoA-I to the Ad-Luc infected cells and performed cellular fractionation. We immunoprecipitated both Ad-AI and His-AI and measured the amount of radiolabeled lipid bound to it. Our results indicate that in samples which were treated with carbonate, and therefore the total cellular content measured, ~10 times more lipids were acquired by Ad-AI intracellularly compared to by His-AI artificially. This result provides strong evidence that the intracellular lipidation seen in pulse-chase experiments is real (ie. not artificial) and the lipidation occurs in the ER and Golgi compartments.

A) Revised Model for Reverse Cholesterol Transport

We have made remarkable progress in understanding intracellular apoA-I lipidation which will forever change the way we view HDL formation and reverse cholesterol transport (RCT). The current opinion about the role of RCT is that it transfers cholesterol from peripheral cells to the liver where cholesteryl esters can be sent to the bile for degradation (22,88). Cholesterol and phospholipid efflux from peripheral cells is viewed as the first step of RCT. Specifically, lipid free/poor apoA-I secreted by hepatocytes or dissociated from the surface of circulating HDL particles initiates cholesterol and phospholipid efflux from peripheral, non-hepatic cells, creating a discoidal HDL particle. Bielicki et al. suggest that lipid-free apoA-I can remove cholesterol and phospolipid from the membranes of fibroblasts and extracellularly generate HDL particles as long as the cells are cholesterol enriched (29). Oram and Yokoyama propose that apoA-I can bind to the cell surface and stimulate cholesterol release from the intracellular sites. Presumably, cholesteryl ester hydrolase acts on the stored CE and forms FC which is trafficked to the plasma membrane and accepted by

lipid free/poor apoA-I (34). Alternatively, phospholipidated HDL interacts with the cell surface and removes membrane-incorporated cholesterol (34). Both of these pathways involve the transport of lipids of non-hepatic cells where lipid-poor or lipid-free apoA-I acts as an acceptor of lipids at the plasma membrane. However, the new pathway that we propose in this paper involves the secretion of lipids from hepatocytes in association with apoA-I, indicating hepatic intracellular lipidation of apoA-I as the preliminary step in lipid efflux and HDL formation. Other authors have recently suggested that hepatocytes might be involved in RCT. Sahoo et al. showed that both endogenous and exogenous apoA-I were able to stimulate cholesterol efflux in primary mouse hepatocytes, but only in the presence of ABCA1 (89). They conclude that hepatocytes provide significant levels of HDL precursor particles involved in the formation of mature HDL. A number of studies provide evidence that in ABCA1 KO cells, HDL cholesterol is almost completely absent from the circulation and cholesterol tends to accumulate in various macrophages (61,90,91). Basso and colleagues performed *in vivo* studies where they expressed ABCA1 in the liver using adeno virus construct and assessed the changes in various cholesterol efflux components (92). They noticed that cholesterol efflux from hepatocytes isolated from the liver one day following the infection almost tripled compared to the controls. As well, the expression of hepatic ABCA1 was able to increase the basal levels of PL, FC, HDL-cholesterol and apoA-I by 150 - 300%. Recently, targeted inactivation of the hepatic ABCA1 demonstrated with certainty that the liver is the major site of synthesis, representing 83% of the circulating HDL (19). All these data suggest that hepatic cholesterol efflux plays a major role in HDL formation and therefore contributes significantly to the process of reverse cholesterol transport. Although all of these studies provide convincing evidence that apoA-I lipidation occurs as a concequence of lipid efflux from the hepatocytes, they all derive their conclusions only by

assessing the amount of lipidated apoA-I found in the media following its export out of the cell. However, none of these studies evaluated the hepatic contribution to apoA-I lipidation intracellularly and the exact timing and location of lipid acquisition inside the cell. Our study, which is based on the separation of ER and Golgi fractions, provides a detailed and comprehensive explanation of apoA-I transport, cholesterol and phospholipid acquisition and secretion from the cell.

Our results show that apoA-I becomes lipidated intracellularly in hepatocytes prior to secretion from the cell. The lipidation begins with early apoA-I phospholipidation in the ER and Golgi compartments. Although both compartments contribute to the lipidation, it is evident that in the Golgi compartments, ABCA1 plays a major role in phospholipidation. This phospholipidated apoA-I can accumulate small amounts of *de novo* synthesized cholesterol in ER and Golgi compartments. Our results show that hepatocytes can secrete sufficient amounts of phospholipid and cholesterol to lipidate *de novo* synthesized apoA-I, both intracellularly and at the plasma membrane. This indicates that cholesterol efflux from peripheral cells, often viewed as the first step of reverse cholesterol transport, is not the primary source of cholesterol efflux and is really the old way of understanding this significant cellular mechanism. Here we propose a modern, revised model of reverse cholesterol transport, which places the liver at the center of cholesterol efflux events and intracellularly lipidated apoA-I as the main precursor of HDL formation (Figure 4-1). The role of the liver in early lipidation of *de novo* synthesized apoA-I is to provide the best substrate lipoprotein that can mediate cholesterol efflux by both the diffusional and ABCA1-dependent process for subsequent reverse cholesterol transport from peripheral tissues. Although macrophages still remain the most significant cell for cholesterol efflux clinically as they are very important in atherosclerosis, it is now obvious that the initial hepatic

lipidation of apoA-I is crucial for providing the appropriate HDL substrate which would prevent the macrophages from inducing the onset of atherosclerosis. Therefore, the early apoA-I lipidation in hepatocytes, studied in this paper, is the primary and most significant step of reverse cholesterol transport and the prevention of heart disease.

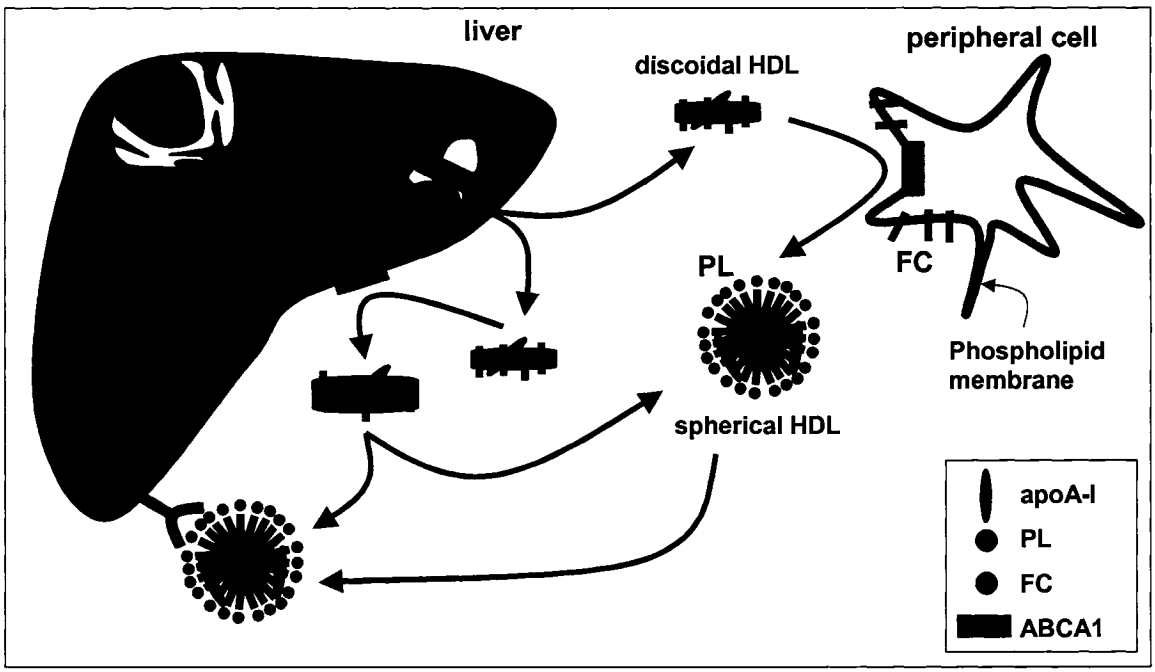

Figure 4-1: Revised Model of Reverse Cholesterol Transport. Hepatocytes secrete partially lipidated apoA-I, which acts as a substrate for cholesterol effluxed from both the hepatocytes and the peripheral cells.

XI. CONCLUSION

This paper shows that apoA-I, newly synthesized in hepatocytes, undergoes an early ABCA1-independent phospholipidation in the ER that is followed by significant phospholipidation in the Golgi. ApoA-I acquires some cholesterol in the ER and Golgi but the major transfer occurs at the cell surface. These pathways result in the net export of hepatic cholesterol in the HDL fraction. Finally, we have established the formation of apoA-I and apoB containing lipoproteins that are assembled after secretion.

XII. FUTURE WORK

This project has answered many important questions regarding intracellular lipidation of apoA-I and the initial steps of HDL formation. However, a number of crucial experiments need to be performed in order to have a more complete picture of these processes.

In this project, we determined the mass of cholesterol associated with apoA-I in the lumen and membrane fractions of hepatocytes as well as in the media secreted from the cells. It would be beneficial to determine the mass phospholipid bound to apoA-I in each of these compartments. Currently, our understanding of apoA-I phospholipidation is limited to the measurement of the amount of radiolabeled PL accumulated by apoA-I. PL mass determination might explain the seemingly low efflux of apoA-I bound PL from the cell. Although we believe that most lipids bound to apoA-I intracellularly, which do not get secreted from the cell as such are probably incorporated back into the membranes, a possibility exists that radiolabeled PL become replaced by non-labeled PL with higher

specific activity. The knowledge about the actual mass of total PL bound to apoA-I would provide a better understanding about the true mechanism of apoA-I phospholipidation.

This project has provided evidence that apoA-I is constantly exchanged from the lumen of intracellular compartments to the membranes, as well as that apoA-I becomes lipidated in the lumens of ER and Golgi. Further experiments are needed to solidify our understanding of lipidated apoA-I transfer from one fraction to another. Since membranes are rich in the lipids which also associate with apoA-I, a careful procedure needs to be derived which would allow us to determine with certainty which lipids in the membrane are apoA-I bound and which lipids are membrane bound but in the close proximity of apoA-I. Triton X-100 was the choice of detergent in this project for the removal of apoA-I from the membranes. Triton X-100, as well as other detergents including Tween, digitonin and CHAPS could be used to derive a concentration curve for the removal of membrane-bound lipids.

Finally, we have assessed the interaction between apoA-I and apoB in the ER and Golgi compartments as well as in the secreted media. Our results convincingly demonstrate that apoA-I and apoB do not interact inside the cell, whereas they seem to form a larger molecule upon exit from the cell. In the future, it would be important to evaluate the involvement of apoE in this process and to test a possible protein-protein interaction between apoA-I and apoE inside the cell and in the media.

These additional experiments would significantly improve our current understanding of intracellular apoA-I lipidation.

XII. REFERENCES

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JOVANA MARIC

WORK EXPERIENCE

TUTORING **Feb 1996- Present time**

- tutor in the fields of math and science
- tutored grade 9-OAC students for a minimum of 10 hours a week in math, physics and chemistry in the last six years

OTTAWA UNIVERSITY HEART INSTITUTE **May 2001- Aug 2005**

- researcher/graduate student/summer student in the Lipoproteins and Atherosclerosis Research Group
- conducted research work in the field of lipid efflux and prevention of heart disease
- obtained significant experience in the following techniques:
    - cell culture
    - cell labeling
    - gradient fractionation
    - immunoprecipitation
    - SDS-PAGE
    - western blotting
    - TLC analysis
    - lipid extraction
    - gas chromatography
    - lipid isolation

LESLIE PARK - PUBLIC SCHOOL **Nov 2000 - June 2001**

- Tutor in Classroom - Program organized by University of Ottawa and Ottawa-Carleton Board of Education for university students in the field of Science
- taught math and science to elementary school children as an assistant teacher
- shared my knowledge, skills and learning experience with the students
- received glowing reviews from the staff members

CHEZ 106 - OTTAWA **May 1997 - Aug 1997**

- member of the "Road Crew", organized creative play activities for children and worked cooperatively as a team member

EDUCATION

UNIVERSITY OF OTTAWA – TEACHERS COLLEGE **Sept 2005 - Present Time**

- Intermediate-Senior Division: Chemistry and Biology
- first practicum completed successfully at Bell High School
- obtained experience in teaching grade 9 academic science and grade 11 university chemistry
- prepared creative lesson plans, wrote quizzes and tests, conducted labs and helped out with fieldtrips

UNIVERSITY OF OTTAWA – M.Sc. **Sept 2003 - Aug 2005**

- successfully defended a masters thesis in Biochemistry
- Thesis title: Lipid acquisition by ApoA-I in ER and Golgi Compartments of Primary Mouse Hepatocytes

UNIVERSITY OF OTTAWA - B. Sc. **Sept 1998 - May 2002**

- obtained a Bachelor degree in Science - Honours Biochemistry
- self-financed 100% of education with summer and school-year jobs
- obtained laboratory experience in organic and analytical chemistry, biochemistry, microbiology, genetics and animal dissection

SIR ROBERT BORDEN HIGH SCHOOL **Feb 1996 - June1998**

- Honour Roll student throughout high school, received Award of Excellence for Physical Education (grade 10, 11, 12)

VOLUNTEERING

QUEENSWAY-CARLETON HOSPITAL **Dec 1998 - Dec 2000**

- volunteered in Geriatric-Day Hospital and Mother-Baby Unit: served meals and beverages, assisted the patients in walking, socialized with the patients and organized fun group activities

HOTSPURS SOCCER CLUB **Summer 1998**

- assistant coach for a boys under 9 team

OTHER

- fluently bilingual (English/Serbian) and basic conversational French capabilities
- extremely dependable and reliable
- have the initiative, energy and persistence to get the job done