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Apolipoprotein A-1 Lipidation in Primary Mouse Hepatocytes: Separate Controls for Phospholipid and Cholesterol Transfers

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Apolipoprotein A-I Lipidation in Primary Mouse Hepatocytes: Separate Controls for Phospholipid and Cholesterol Transfers

by

Hui Zheng

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Masters in Science

University of Ottawa
Ottawa, Ontario, Canada

Your file Votre référence
ISBN: 0-494-11476-2
Our file Notre référence
ISBN: 0-494-11476-2

ABSTRACT

The liver is the site for both apolipoprotein A-I (apoA-I) synthesis and ATP-binding cassette transporter A1 (ABCA1) expression. To investigate the function of hepatic lipidation of apoA-I by cholesterol and phospholipid, and delineate the role of hepatic ABCA1 in these lipidation pathways, I have expressed human apoA-I by adenovector-mediated gene transfer into primary mouse hepatocytes. Kinetic studies demonstrate that newly synthesized apoA-I acquires both cholesterol and phospholipid during secretion and at the cell surface. Approximately 80% of the phospholipidation, and only 40-60% of the cholesterol lipidation of apoA-I is ABCA1-dependent. Hepatic apoA-I cholesterol lipidation depends on the active transfer from intracellular compartments to the cell surface. Under ABCA1 deficiency, both apoA-I and cholesterol associated with apoA-I in high-density lipoprotein (HDL) fraction decrease, but the nature of the lipoprotein particles is not affected. Furthermore, this study demonstrates the different regulation and/or controls are required for apoA-I cholesterol lipidation and phospholipidation.

DEDICATION

I dedicate my thesis to my parents, my lovely son, Richard for their constant understanding and support; in particular, to my wonderful husband, Timao who has provided a guiding light and encouragement to make this work possible. Furthermore, I dedicate this work to all of my relatives and friends whose effort made my life joyful and fruitful.

ACKNOWLEDGEMENTS

This work would never be possible without help of many people. First I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Yves L. Marcel for his guidance, support and encouragement in academic and other aspects throughout the entire study.

I would also like to express my appreciation to Dr. Robert S. Kiss who has provided invaluable advice and support for completing this study. Dr. Ming-Dong Wang, Dr. Bassam Haidar for their knowledgeable suggestions. The expert assistance and timely advice of Vivian Franklin made my work more efficient. My thanks to everyone in the labotory, Andrea MacKenzie, Jovina Maric for their constant friendship and help made my work much enjoyable.

My sincere thanks are extended to the members of my Thesis Advisory Committee: Dr. Ruth Mcpherson, Dr. Heidi McBride, for their support, help and advice in completing this work. Special thank to Dr. Ross Milne for his invaluable advice and help in preparing for the manuscript. Furthermore, my thanks to all members of the Lipoprotein and Atherosclerosis Group at the University of Ottawa Heart Institute made contributions to this work.

Finally, I would also like to express my thanks to Dr. Vasek A. Mezl, Ms. Carolann Kelly for their support and help to finish my study. Traineeship from the Ontario Graduate Scholarships in Science and Technology (OGSST) are highly appreciated.

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ABBREVIATIONS

ABCA1	ATP-Binding Cassette Transporter A1
ApoA-I	Apolipoprotein A-I
ApoB	Apolipoprotein B
ApoB-48	Apolipoprotein B-48
ApoB-100	Apolipoprotein B-100
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
BFA	Brefeldin A
CAD	Coronary Artery Disease
CAM	Cell Adhesion Molecule
cAMP	cyclic AMP
CE	Cholesteryl Ester
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
EC	Endothelial Cell
EE	Early Endosome
EL	Endothelial Lipase
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FC	Free Cholesterol
FPLC	Fast Protein Liquid Chromatography
HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein-Cholesterol
HL	Hepatic Lipase
HRP	Horse Radish Peroxidase
IDL	Intermediate Density Lipoprotein
IgG	Immunoglobulin G
INFγ	Interferon-γ
LCAT	Lecithin:Cholesterol Acyltransferase
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein Receptor
LE	Late Endosome
LpA-I	Apolipoprotein A-I containing Lipoprotein
LpA-II	Apolipoprotein A-II containing Lipoprotein
LPL	Lipoprotein Lipase
LXRα	Liver X Receptor α
LXRβ	Liver X Receptor β
mβCD	Methy-beta Cyclodextrin
NO	Nitric Oxide
NPC	Niemann Pick Type C
PAF-AH	Platelet Activating Factor Acetylhydrolase
PL	Phospholipid
PM	Plasma Membrane
RCT	Reverse Cholesterol Transport

RE	Recycling Endosome
ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
SMase	Sphingomyelinase
SMC	Smooth Muscle Cell
SR-BI	Scavenger Receptor class B type I
TD	Tangier Disease
TG	Triacylglycerol

CHAPTER 1: INTRODUCTION

1.1 Clinical Significance of the Study

1.1.1 Pathogenesis of Atherosclerosis

Atherosclerosis, the cause underlying most heart attacks and strokes, is one of the major killers in the world. According to the World Health Organization (WHO) statistics, it will be the most common cause of morbidity and mortality in both the industrialized and developing countries by year 2020. Atherosclerosis is defined as a lesion developing slowly over years of large- and medium-sized arteries, with deposits in the intima of yellowish plaques containing predominantly lipids and fibrous elements [1;2]. There is now compelling evidence indicating that the development of atherosclerosis is a healing response to vascular wall injury. Among all the risk factors identified, the most prominent ones are elevated levels of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) that are enriched in cholesterol [3;4].

Both cholesterol and triglyceride (TG) absorbed from the diet, are packaged into chylomicron particles containing apolipoprotein B (apoB) as its core protein and secreted by intestine. In the circulation, these particles deliver fatty acids to peripheral tissues giving rise to chylomicron remnants, which circulate back to the liver and are cleared by interaction between apolipoprotein E (apoE) and members of the LDL receptor (LDLr) gene family. Endogenously synthesized cholesterol and some cholesterol acquired from remnant lipoproteins is packaged by the liver in the larger form of apoB, apoB100, which serves the similar function of delivering TG to the periphery. The lipolysis process shrinks the particles that become the intermediate density lipoprotein (IDL) and then low density lipoprotein (LDL). LDL is enriched in cholesterol through transfer of cholesteryl esters by cholesterol

ester transfer protein (CETP), is eventually cleared by the LDLr pathway. Slow clearance of LDL leads to the pathogenesis of atherosclerosis.

LDL in the blood passively diffuses from the circulation to the intima through endothelial cell (EC) tight junctions and/or transcytosis and aggregates in the intima via direct interaction between apoB and extracellular matrix proteoglycans [4;5]. LDL must be extensively modified before it can be taken up sufficiently by macrophages to produce foam cells and early atherosclerotic lesions. This modification involves reactive oxygen species (ROS) produced by ECs and macrophages. Limited oxidation of LDL occurs as ROS, derived from vascular cells, modify the phospholipid protein components of LDL to extensively oxidized LDL. The oxidation of LDL produces a variety of peroxidized lipids that induce the production of monocyte chemotactic protein-1 (MCP-1) and monocyte adhesion to the endothelium [6]. Adhesion molecules and proinflammatory cytokines are induced. Adherent monocytes migrate into the intima and differentiate into macrophages. Oxidized LDL is constitutively endocytosed by the macrophage via scavenger receptors, and this process leads to lipid accumulation within the atherosclerotic lesion and formation of macrophage foam cells [7].

Smooth muscle cell (SMC) migration and proliferation from the media to intima are induced by inflammatory cytokines and growth factors secreted from macrophages and T cells. SMC then produces extracellular matrix to provide structural support and fibrous cap to the growing mass of extracellular lipids derived from the death of foam cells, which eventually becomes a 'necrotic plaque'. Further calcification and ulceration of the plaque at the luminal surface increase its complexity. Lesions will grow sufficiently large to block blood flow. The thrombosis is usually associated with the rupture of the lesion, and an acute

occlusion due to the formation of a thrombus or blood clot can result in myocardial infarction or stroke.

1.1.2 High Density Lipoprotein (HDL) and Atherosclerosis

Both epidemiological [8] [9;10]and clinical studies [11;12]have shown the inverse relationship between HDL-cholesterol (HDL-C) and the risk of cardiovascular diseases. The high levels of HDL-C are closely associated with a decreased risk of Coronary Heart Disease (CHD). This relationship is attributed to the potential antiatherogenic properties of HDL through the following mechanisms.

1.1.2.1 HDL Promotes Reverse Cholesterol Transport (RCT)

The cardioprotective effect of HDL has been largely attributed to its role in RCT [13;14]. Both lipid-poor preβ-HDL and larger apolipoprotein A-I containing lipoprotein (LpA-I) particles secreted from hepatocytes, or derived from lipolysis of TG-rich lipoprotein particles promote phospholipid and cholesterol efflux from peripheral tissue, predominantly macrophages, and transport lipids to the liver for excretion which otherwise will accumulate within the body to induce foam cells formation. Experiments with transgenic animals suggest that disruption of one or more steps in RCT results in accelerated atherosclerosis, whereas overexpression of important proteins in RCT, including apolipoprotein A-I (apoA-I), which is the predominant protein of HDL, exerts atheroprotective effects [14]. The mechanism of HDL in RCT will be discussed later.

1.1.2.2 HDL Protects Endothelial Function

In addition to its role in RCT to remove cholesterol accumulation in macrophages and to prevent foam cell formation, other antiatherogenic functions of HDL include inhibition of endothelial dysfunction during the development of atherosclerosis. Early stages of atherosclerosis is often associated with endothelial dysfunction, which is characterized by decreasing bioavailability of a potent vasodilator, nitric oxide (NO), and increasing affinity of the endothelial surface for leukocytes. *In vivo* studies support the role of HDL in restoring and protecting endothelial dysfunction [15]. In hypercholesterolemic patients, intravenous infusion with cholesterol-free reconstituted HDL rapidly normalized endothelium-dependent vasodilation by increasing NO bioavailability [16]. It has been also reported that the effects of HDL-induced increasing of NO bioavailability are at least in part due to the stimulation of endothelial NO synthase expression by HDL [17].

Injury to vascular endothelium induces the expression of cell adhesion molecules (CAMs), which attract monocytes and other leukocytes to hold on to the endothelial surface and migrate to the intima tissue. HDL downregulates tumor necrosis factor-α (TNF-α)-induced CAM expression in cultured human endothelial cells [18]. The expression of vascular cell adhesion molecule (VCAM)-1, which binds leukocytes in early atheroma, and the formation of neointima were also inhibited by reconstituted HDL in a mouse model of artery injury [19].

1.1.2.3 HDL Inhibits LDL Oxidation

As described previously, the production of oxidized LDL is required for their uptake by macrophages and the formation of foam cells during the development of atherosclerosis.

Studies have provided evidence that the antioxidative properties of HDL, predominantly apoA-I in this lipoprotein, inhibits LDL oxidation [20]. It has been reported that platelet activating factor acetylhydrolase (PAF-AH) is responsible for the degradation of oxidized phospholipids, and overexpression of human apoA-I in apoE knockout mice increases PAF-AH activity and simultaneously reduces oxidative stress, furthermore, reduces monocyte recruitment into the arterial wall by decreasing CAM expression [21].

1.2 Human Plasma Lipoproteins

Plasma lipoproteins are lipid-protein particles composed of a neutral lipid core of hydrophobic lipids, predominantly cholesteryl ester (CE) and triacylglycerol (TG), and an amphipathic surface layer of polar lipids primarily phospholipid (PL), as well as free cholesterol (FC) [22]. All lipids on the surface layer are oriented with their hydrophobic moiety directed towards the lipoprotein core and hydrophilic moiety exposed to the aqueous environment. The molecular structure of a lipoprotein particle is maintained by its unique proteins, called apolipoproteins (Fig. 1-1). The apolipoproteins are also amphipathic and are inserted within the PL monolayer by which hydrophobic domains interact with surface lipid and polar residues are exposed to the aqueous environment. The plasma lipoproteins contain exchangeable and/or non-exchangeable apolipoproteins. Exchangeable apolipoproteins, such as apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III and apoE are water soluble in the absence of lipids while non-exchangeable apolipoproteins, like apoB-48 and apoB-100 are insoluble. In addition, exchangeable apoliproteins can associate with, or dissociate from the lipid particle during its intravascular metabolism whereas non-exchangeable apolipoprotein remains with the lipid particle until its ultimate catabolism.

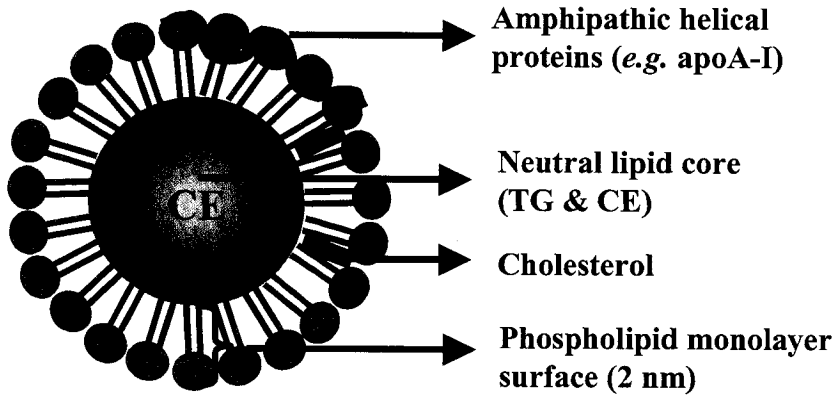

Figure 1-1 Schematic Representation of Plasma Lipoprotein

Plasma lipoproteins are classified into 5 classes according to their heterogeneity in density (ultracentrifugation), size (gel filtration), surface electrical charge (agarose electrophoresis), and lipid and protein composition (Tables 1-1 and 1-2). Since lipids have lower buoyant densities than proteins, plasma lipoproteins with a higher ratio of lipid to protein have a lower density than lipoproteins with a lower ratio of lipid to protein. Chylomicrons, the largest and most lipid-rich particles, are secreted from intestinal enterocytes and transport dietary TG to sites of storage and utilization in the body, and are abundant in plasma only after a meal. Chylomicrons are approximately 75-1200 nm in diameter, and float at the density of 0.930 g/mL due to their lowest ratio of protein to lipids. The second largest particles are VLDL. They are secreted from hepatocytes and responsible for transporting TG and cholesterol from liver to sites of utilization and storage in the body. VLDLs range in diameter from 30-80 nm and float at the density of 0.930-1.006 g/mL. Hydrolysis of VLDL and chylomicrons TG core, principally by lipoprotein lipase in the circulation generates IDL. IDL are smaller in diameter ranging from 25-35 nm, and are denser with the density of 1.006-1.019 g/mL. The subsequent class, LDL are generated from hydrolysis of IDL, and as well are secreted initially from the liver in the form of VLDL. The

diameter of LDL particles ranges from 18-25nm. LDL particles have a density of 1.019-1.063 g/mL. These particles transport cholesterol from liver to peripheral tissues where they can be utilized and/or stored. Lipoprotein particles with the density corresponding to 1.063-1.210 g/mL are called HDL. They are the smallest particles and range from 5-12 nm in diameter. HDL is a diverse population of particles both in structure and route of formation.

Table 1-1: Physical Properties of Human Lipoproteins

Class	Density g/ml	Diameter nm	Electrophoretic mobility	Molecular weight
Chylomicron	0.93	75-1200	Remains at origin	400 000 000
VLDL	0.930-1.006	30-80	Preβ-migration	10-80 000 000
IDL	1.006-1.019	25-35	Slow preβ-migration	5-10 000 000
LDL	1.019-1.063	18-25	β-migration	2300 000
HDL_2	1.063-1.125	9-12	α-migration	360 000
HDL_3	1.125-1.210	5-9	α-migration	175 000

Adapted from Vance DE, Vance J, Biochemistry of Lipids, Lipoproteins and Membranes, 1996 [22].

Table 1-2: Chemical Composition of Human Lipoproteins

	Surface components % of dry mass			Core lipids % of dry mass	
	FC	**PL**	**Apolipoprotein**	**TG**	**CE**
Chylomicron	2	7	2	86	3
VLDL	7	18	8	55	12
IDL	9	19	19	23	29
LDL	8	22	22	6	42
HDL_2	5	33	40	5	17
HDL_3	4	35	55	3	13

Adapted from Vance DE, Vance J, Biochemistry of Lipids, Lipoproteins and Membranes, 1996 [22].
FC: free cholesterol; PL: phospholipid; TG: triacylglycerol; CE: cholesteryl ester.

1.3 HDL Metabolism

Plasma HDLs are smallest among of lipoprotein classes that function in the transport of water-insoluble lipid molecules. There is an inverse relationship between plasma HDL levels and the prevalence of CHD. Therefore, HDLs are believed to be antiatherogenic [8;23;24] by several mechanisms. In addition to its major function in promoting reverse cholesterol transport [25], inhibition of cytokine-induced expression of adhesion molecules by endothelial cells [26], protection of LDL from oxidation [27]and other potential antiatherogenic activities have also been reported [28]

1.3.1 HDL Structure and Subclasses

Plasma HDL particles range from 5-12 nm with a molecular mass of 200-400 kDa. HDL is classically defined to exist in the density range of 1.063 to 1.21 g/ ml (see reviews [29]and [30]. The molecular structure of HDL is similar to other plasma lipoproteins as described previously. ApoA-I is the major protein of HDL, comprising about 70% of the total HDL protein. While chylomicron and VLDL mass is predominantly TG, 80% of HDL mass is apolipoprotein and PL. It is believed that apoA-I is mainly responsible for the subspecification of HDL due to the fact that greater than 90% of HDL particles contain apoA-I.

1.3.1.1 Lipoprotein A-I (LpA-I) and Lipoprotein A-I: A-II (LpA-I:A-II)

On the basis of apoliporotein composition of HDL particles, two predominant classes of HDL are now recognized and can be isolated by sequential immunoaffinity chromatography: Those that contain only apolipoprotein A-I, designated lipoprotein A-I

(LpA-I), but the majority of HDL contains both apolipoprotein A-I and A-II, and is designated lipoprotein A-I:A-II (LpA-I:A-II) [31;32]. Both LpA-I and LpA-I:A-II include HDL particles across the entire size range of HDL [33-36]with small, medium and large particles, as separated on nondenaturing gradient gel electrophoresis [37 38]. There is only a minor, about 10% subset of HDL that contains apoA-II but no apoA-I designated LpA-II [39;40].

It has been reported that LpA-I is more protective against atherosclerosis than lipoprotein A-I:A-II because elevated levels of LpA-I are associated with decreased risk of atherosclerosis while no correlation is reported for LpA-I:A-II [41;42]. ApoA-II appears to modulate the activity of factors involved in HDL metabolism. Studies in apoA-II and hepatic lipase (HL) double knockout mice suggested apoA-II maintains HDL levels in part by inhibition of HL [43;43]. Other studies also support that the effects of HL on HDL metabolism is inhibited by apoA-II [44;45].

1.3.1.2 $HDL_1/HDL_2/HDL_3$

Earlier studies by Gofman *et al.* separated plasma HDL into three subclasses in the order of increasing density and decreasing size[46], defined as HDL_1, HDL_2, and HDL_3. HDL_2 are 360 kDa in size, 9-12 nm in diameter and have a density of 1.063-1.125 g/ml. HDL_3 are 175 kDa in size, 5-9 nm in diameter and with a density of 1.125-1.210 g/ml. HDL_2 and HDL_3 are the most abundant in normolipidemic humans. Due to the smaller size, HDL_3 readily accept PL and FC from lipolyzed VLDL and cell membranes to generate intermediate HDL_2 particles [47-49], defined as HDL_{2a}. HDL_{2a} further accepts FC and leads to the enrichment of CE core through the action of Lecithin:Cholesterol Acyltransferase (LCAT) to become more mature HDL_{2b} and ultimate conversion to HDL_2 particles.

HDL_1 are less abundant in human plasma [50] comparing to HDL_2 and HDL_3, but predominantly present in rodents [51-53]. It has been suggested that incubation of human plasma in the test tube at 37°C resulted in an increasing in HDL_1 levels due to the accumulation of LCAT derived CE core [54]. Therefore, HDL_1 particles represent a further maturation of HDL, predominantly from HDL_2 particles.

1.3.1.3 Preβ- and α-HDL

Based on the lipoprotein surface charge that gives different electrophoretic mobility on agarose gel, HDL particles are separated into preβ- and α-HDL subclasses, which reflect their migration relative to β and α globulins, respectively [55;56]. Lipid-poor HDL has a characteristic preβ- electrophoretic migration on agarose gel that distinguishes them from the bulk of α-migrating HDL. The molecular basis for the difference in charge between preβ- and α-migrating HDL particles was investigated by Davidson *et al.* [57]who proposed that an alteration in the conformation of the resident apolipoproteins, primarily apoA-I (due to a particle shape change from disk to sphere) was responsible for the higher net negative surface charge of α-HDL.

Preβ-HDL is a protein-rich particle containing very little lipids. The subsets referred as $preβ_1$, $preβ_2$, and $preβ_3$ differ in size, with molecular weights of 70kDa, 325 kDa, and greater than 325kDa, respectively [56;56]. Their lipid components are predominantly PL, and with various amount of FC without CE core in general. $Preβ_1$-HDL is the smallest particle. As $preβ_1$-HDL particles mature by increasing the number of FC and PL, they become $preβ_2$-HDL, and subsequently $preβ_3$-HDL particles.

There is compelling data to indicate that preβ-HDL plays an important role in delivering cholesterol from cultured cells for Lecithin:Cholesterol Acyltransferase (LCAT)-mediated esterification in plasma [55;58]. Preβ$_1$-HDL are the most effective acceptors of FC from cells due to their lower ratio of FC to PL relative to larger preβ$_2$- and preβ$_3$-HDL particles [25]. Studies have shown that when plasma is incubated at 37°C for 90 min, total preβ-HDL decreases by 33%, and when LCAT activity is inhibited, this decrease does not occur, indicating conversion of FC to CE is necessary for the conversion of preβ- to α-migrating HDL [59]. Other studies by Hennessy *et al.* [60] have reported that isolated LpA-I and LpA-I:A-II may be converted to preβ-HDL when CEs are removed from HDL. Therefore, FC availability and subsequent esterification by LCAT is the key factor in determining the size of HDL particles.

1.3.1.4 Discoidal and Spheroidal HDL

During the maturation of HDL, native apoA-I assumes two quite different conformations, which can be distinguished by monoclonal antibodies directed to different regions of the protein, referred as discoidal and spheroidal HDL[22]. Continued transfer of FC to lipid-poor-apoA-I (preβ-HDL) leads to the formation of discoidal HDL. These discs then become substrates for further metabolism by LCAT. CE synthesis by LCAT activity leads to the appearance of CE core within the lipid bilayer of the disc, and this formation of CE is associated with the maturation of the precursor HDL to spheroidal HDL particle.

The optimal substrate for LCAT is the discoidal HDL. Discoidal HDL is likely the intermediate between lipid-poor apoA-I and spheroidal HDL. In support of this hypothesis, incubation of both plasma from LCAT-deficient patients and reconstituted discoidal HDL

with purified human LCAT *in vitro* results in the formation of spheroidal HDL and the shift to an α-electrophoretic migration [61;62]. Thus discoidal and spheroidal HDL is synonymous with preβ- and α-HDL, respectively.

1.3.2 Reverse Cholesterol Transport Concept

As mentioned earlier, a major known function of HDL is to act as a receptacle for excess PL and FC, derived from cells or as by-products of lipolysis. These lipids are normally recycled from HDL to the liver in a process called reverse cholesterol transport (RCT) [63;64] (Fig. 1-2).

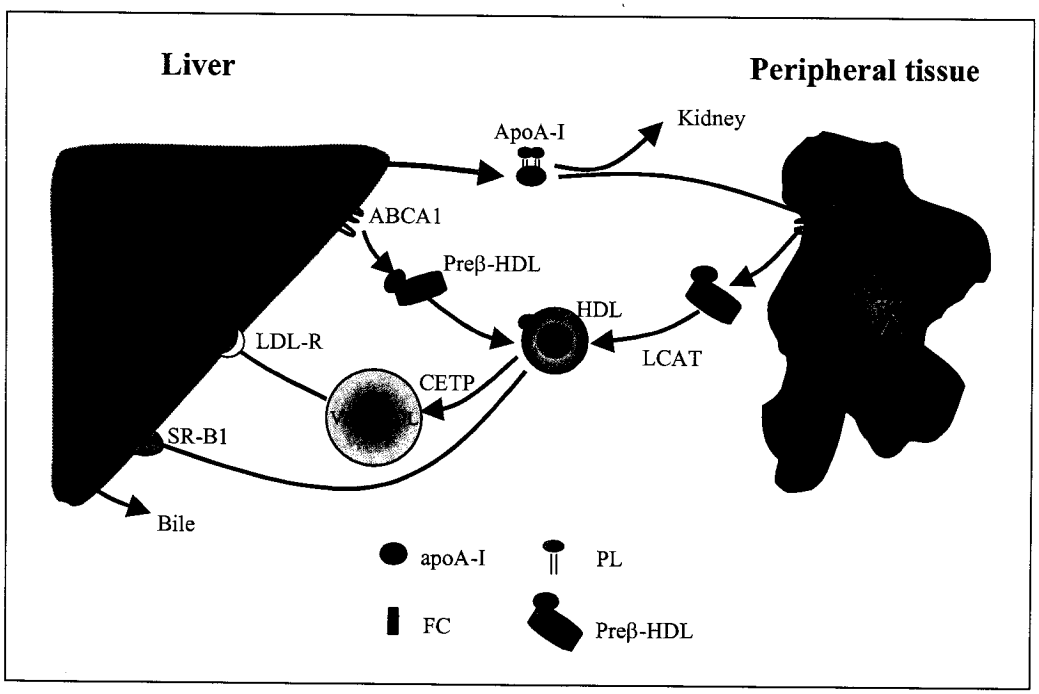

Figure 1-2: Reverse Cholesterol Transport

ApoA-I is produced by the liver and acquires FC and PL from macrophages and hepatocytes to form nascent HDL particles (disks). These particles mature into spherical HDL through the action of the enzyme LCAT, and the CE are transferred to the liver by scarvenger receptor class B type I (SR-BI) and to other lipoproteins by CETP. LDL-CE are delivered into hepatocytes by the LDL receptor (LDL-R). Adapted from Oram *et al.* [65].

This proposed model of RCT involves a direct pathway where cholesterol is transferred into HDL, then taken up by the liver, and also an indirect pathway, involving transfer of CE from HDL to VLDL, followed by uptake of VLDL in liver, or conversion of VLDL to LDL, and uptake of LDL by the liver. The negative correlation between HDL levels and the incidence of CHD is generally thought to be due primarily to its role of mediating reverse cholesterol transport [66]. This hypothesis states that the level of HDL cholesterol is a marker of the efficiency of a system for removal of excess lipids from circulation and/or peripheral tissues to the liver for excretion. Lipid removal from tissue sites and the mechanism for transport of lipid from peripheral tissues to the liver for ultimate catabolism and excretion depend on the function of HDL in multiple steps.

1.3.2.1 Synthesis and Secretion of Plasma HDL

The major human HDL apolipoprotein, apoA-I is synthesized in the liver and the small intestine. However, HDL lipid constituents have more complex and multiple origins that include secretion as nascent lipoproteins containing apoA-I. This will generate small lipid-poor particles considered as preβ-HDL or larger LpA-I particles [67]. These newly secreted lipid-poor HDL particles can further acquire more lipids from catabolism of TG-rich particles, like VLDL or chylomicrons, or acquire lipids at the surface of hepatocytes and other peripheral cells, such as macrophages defined as lipid efflux [67](Fig. 1-3). In deed, a large portion of the apolipoprotein and phospholipid destined to become HDL is initially secreted on large, lipid-rich VLDL (liver) or chylomicrons (intestine). During lipolysis of these particles surface lipids (PL and FC) and proteins (apoA-I, A-II, and Cs) are transferred into HDL.

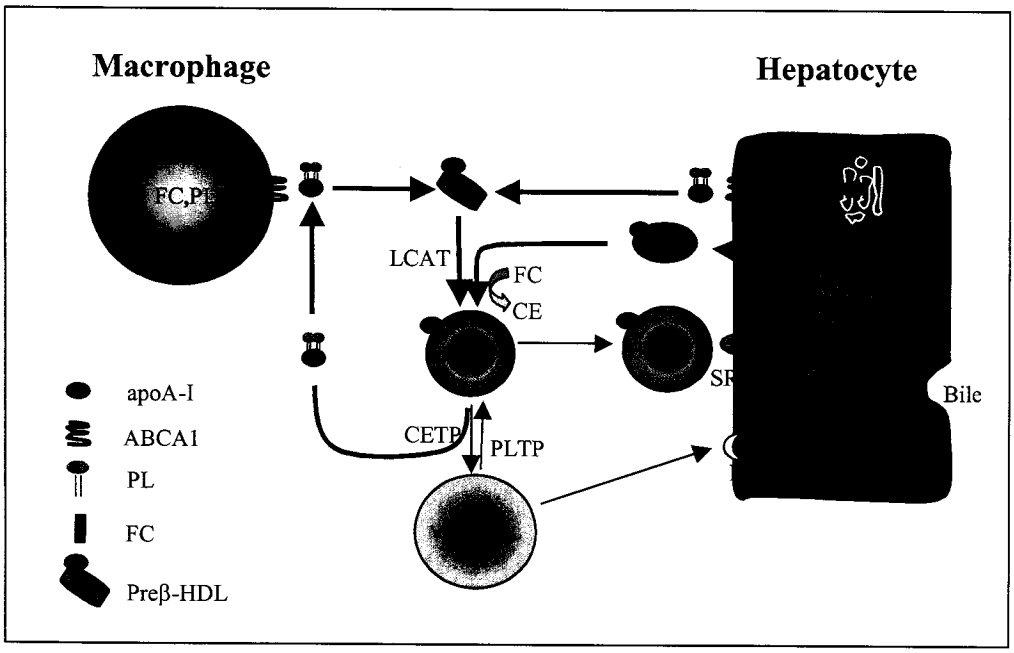

Figure 1-3: Synthesis and Secretion of HDL

Liver is the site of both HDL synthesis and excess lipids excretion from the body as bile (see detail in the text).

1.3.2.2 HDL Maturation

Lecithin:Cholesterol Acyltransferase (LCAT), first identified by Glomset *et al* [63], is a plasma enzyme that catalyzes the hydrolysis and transfer of an acyl chain from phosphatidylcholine (PC) to cholesterol forming cholesteryl ester [68;69]. CE is the predominant storage form of cholesterol both in cells and in lipoproteins. CE, unlike FC, is not amphipathic and therefore partitions into the core of a discoidal particle [63]. The CE-enriched HDL particle that results from LCAT action is called "α-HDL". This action enables small HDL particles to accept more cholesterol from any cholesterol donor.

Although apolipoproteins can not bind to cholesterol directly, apolipoprotein/PL particles are cholesterol sinks when they are in the presence of a cholesterol-rich membrane.

Deletion of LCAT in transgenic mice results in more than 90% loss of HDL, due to the inability to create mature, stable particles [70]. In human, LCAT deficiency also results in HDL deficiency. It was first evidenced by the aberrant HDL metabolism in familial LCAT deficiency (FLD) and fish-eye disease (FED) [71]. Both diseases are characterized by extremely low or non-detectable plasma LCAT activity, significantly decreased lipoprotein CE content, abnormal lipoprotein profiles and morphologically abnormal erythrocytes. Incubation of plasma from both patients with purified human LCAT induced a transformation from discoidal to spherical morphology, suggesting plasma LCAT activity is required for the proper conversion of discoidal to spherical HDL *in vivo.*

Overexpression of human LCAT in mice and rabbits results in high plasma LCAT activity. When fed a high cholesterol diet, LCAT transgenic rabbits were significantly protected from atherosclerosis as compared to controls not expressing LCAT, while LCAT transgenic mice are more susceptible to diet induced atherogenesis [72;73]. This is due to the presence of CETP in rabbits and its absence in mice. The larger, cholesterol-rich HDL generated in the absence of CETP is cleared much slower by the liver and is proatherogenic.

1.3.2.3 HDL Remodelling

"Remodelling" refers to the process that alters the biochemical and physical properties of CE-containing HDL in the circulation. This involves the combined actions of cholesterol ester transfer protein (CETP), phospholipid transfer protein (PLTP), hepatic lipase (HL), and endothelial lipase (EL), which modulate the lipid composition and plasma

levels of HDL. These processes serve to transfer LCAT-derived CE from HDL to apoB-containing lipoproteins allowing subsequent uptake by the liver and regeneration of HDL precursors.

The Role of PLTP: The phospholipid on HDL particles are acquired from cellular efflux and during lipolysis of TG-rich lipoproteins (chylomicrons and VLDL), much of the phospholipid monolayer surface becomes redundant relative to the depleted neutral lipid core [74]. This surface becomes available to form HDL precursor particles. PLTP facilitates the formation of preβ-HDL particles from this phospholipid source [75]. Studies from Jiang *et al* group [76] have demonstrated that loss of PLTP results in 60-70% HDL deficiency in mice.

The Role of CETP: CETP is a member of the lipid transfer protein gene family that also includes PLTP. CETP reversibly associates with lipoproteins within the circulation and mediates the exchange of CE in HDL for TG in apoB-containing particles, like VLDL, and LDL [75]. Two models of CETP-mediated lipid transfer between lipoproteins have been proposed (Fig. 1-4). CETP contributes to reverse cholesterol transport in a pathway that involves CE transport from HDL to VLDL and then to the liver for clearance through VLDL remnant in human, secondary to the scavenger receptor class B type I (SR-B1)-mediated cholesterol removal pathway [77;78].

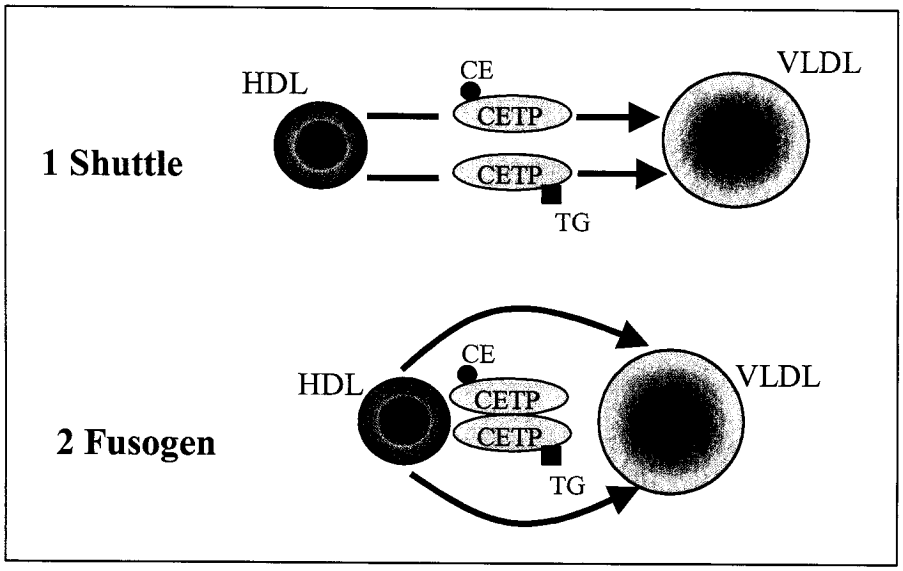

Figure 1-4: CETP Functions to Transfer Lipid Between Lipoproteins

CETP is thought to function either as a shuttle protein that transports lipid between lipoprotein particles, or in a fusogenic fashion, bridging interfacial surfaces and accessing lipid domains.

Recent data indicate that complete CETP deficiency is associated with a marked increase in HDL-C levels and the formation of very large HDL particles [79-81]. These large particles may not be as atheroprotective as the normal size HDL particles are due to the presence of apoA-II or apoE in these apoA-I containing particles, thereby decreasing the SR-BI-mediated selective cholesterol uptake from HDL [82]. By contrast, heterozygous CETP-deficient subjects, with approximately 30 to 60% of normal CETP mass/activity, have significantly increased levels of the regular α-HDL, with normal size and composition, and decreased levels of preβ-HDL, representing an improved antiatherogenic property [81]. Studies from overexpressing human CETP in transgenic mice have been found to reduce HDL-C levels [83]. This occurs because the CE-enriched HDL particles are more stable than smaller particles [73]. To date, the optimal amount of CETP mass or activity needed for an atheroprotective lipoprotein and HDL subpopulation profile are debatable [84;85]. There is

increasing acceptance that it is not the static concentration of HDL particles, but the dynamic function of RCT they initiate, that is probably more important in the atheroprotective role of HDL. CETP catalyzes the exchange of VLDL-TG for HDL-CE, resulting in HDL particles with a TG core that can then be a substrate for hepatic lipase (HL) and/or endothelial lipase (EL) [86-88].

The Role of HL: HL is a cell surface-bound (and in mouse, secreted) enzyme capable of hydrolysing PL and TG in both HDL and apoB-containing plasma lipoproteins [89;90]. HL has been shown responsible for converting VLDL remnants and IDL to LDL and larger TG-rich HDL_2 to smaller HDL_3 [89]. Through its depletion of HDL phospholipid, HL facilitates the removal of CE from HDL through CETP [91] and through selective HDL-CE uptake by SR-BI into cells [92;93].

Transgenic animals have been generated to study HL in lipoprotein metabolism. HL-deficient mice showed elevated levels of large PL-rich HDL particles in plasma [94]. On the other hand, overexpressing HL in rabbits and mice have decreased HDL-C levels in plasma and HDL particle size[95;96]. Furthermore, clinical studies showed that patients with HL deficiency have elevated plasma cholesterol and TG levels and the presence of large TG- and PL-rich HDL and LDL particles compared with unaffected subjects[97-100]. All these results suggest an important role of HL in HDL metabolism.

The Role of EL: EL is highly homologous to HL, forming another member of the triacylglycerol lipase synthesized by ECs [101;102]. Compared with HL, EL is more HDL specific, and more active in hydrolyzing PL than TG on HDL particles [103]. Independent to their lipolytic properties, both HL and EL have been shown to mediate the binding of

lipoproteins to the cell surface heparan sulfate proteoglycans (HSPGs) [104]. EL however has higher activity of mediating the bridging between HDL and HSPGs, whereas HL is more active for the binding of VLDL and LDL to HSPGs [105].

Jaye *et al.* have generated hepatic overexpression of EL by adenoviral gene transfer and showed a dramatic decrease (90%) in HDL-C and PL levels [106]. Studies by transgenic overexpression of human EL in mice resulted in a 19% reduction in HDL cholesterol levels compared with wild type mice [107]. Another study shown by Broedl *et al.* [108] group demonstrated that EL could compensate the defect of HL activity. In their studies, they over-expressed catalytically inactive EL or wild type EL in wild type mice, they found no effect on HDL-C levels in mice over-expressing catalytically inactive EL in contrast to wild type EL. However, when they performed a similar experiment in HL-deficient mice, they found a modest but significant decrease in HDL-C levels in mice over-expressing catalytically inactive EL in comparison with wild type EL and control mice. Loss-of-function studies performed by Jin *et al.* [109] showed that inhibition of mouse EL activity by antibody resulted in significantly increased HDL-C and PL levels associated with increased HDL particle size, indicating a possible strategy for raising HDL-C levels by inhibition of EL activity.

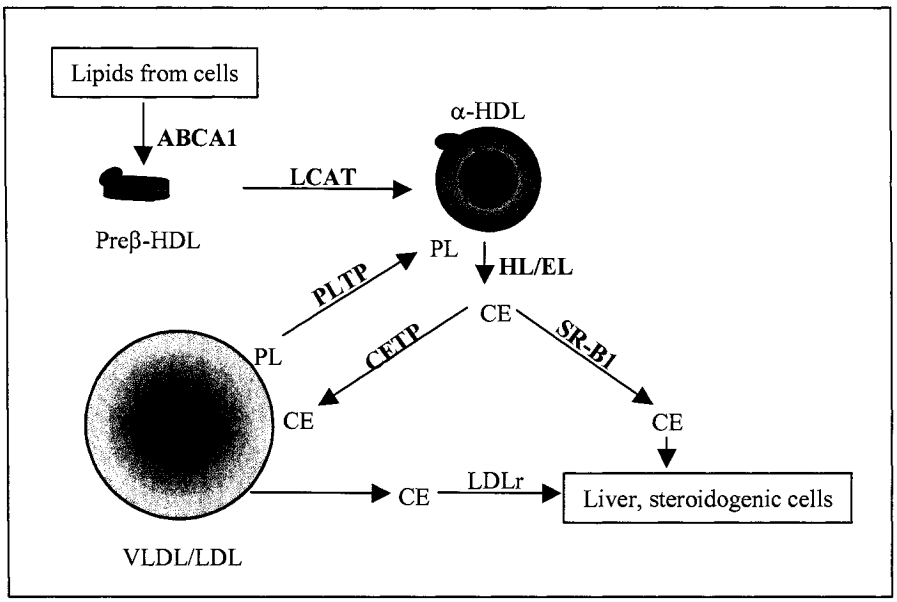

Figure 1-5: Combined Actions of LCAT, PLTP, CETP, HL, EL and SR-BI in HDL Metabolism

ATP-binding cassette transporter A1 (ABCA1) is necessary for the lipid efflux from cells to form preβ-HDL. Surface phspholipid is derived from the surface of triglyceride-rich lipoproteins through the action of PLTP. The gain or loss of CE from these particles determines their residence time in the circulation. CE is derived from the LCAT reaction, which promotes the conversion of HDL disks to spherical HDL. CE is removed from HDL particles by two processes: 1) CETP catalyzed transfer of CE to VLDL and LDL, and then CE is transported to the liver by LDL receptor (LDLr) pathway, and 2) the selective uptake of CE into hepatocytes and steroidogenic cells via the SR-BI. The hydrolysis of surface phospholipid by HL and/or EL enhances the loss of CE from HDL by both CETP- and the SR-BI-mediated pathways.

The concerted action of CETP-mediated CE transfer and HL mediated hydrolysis of TG and PL helps to form the smaller HDL particles that are the preferred binding partners for SR-BI, the major HDL receptor on hepatocytes to remove cholesterol from the body. Figure 1-5 summarizes the combined actions of the factors affecting the size and stability of HDL particles.

1.3.3 SR-B1 Mediated Selective Uptake of HDL Cholesterol

Earlier studies by Glass *et al* showed that the uptake of CE in HDL by hepatocytes and steroidogenic cells was much higher than that of apoA-I, a process termed "selective uptake" of cholesterol [110]. The discovery of the SR-B1 protein confirmed their studies showing that this receptor binds to HDL and mediates selective uptake of CE from HDL to hepatocytes and steroidogenic cells [111-113](Fig. 1-6). The work by Schwartz *et al* [114] has suggested that HDL-C is the primary source of bile production compared to LDL cholesterol. The studies with mice overexpressing SR-BI have significantly reduced HDL cholesterol and increased transport of HDL cholesterol to the bile [112;115]. Genetic ablation of SR-BI resulted in increased plasma HDL cholesterol associated with larger HDL particles relative to wild type animals [116]. Moreover, SR-BI-deficient mice exhibited reduction in the cholesterol content in steroidogenic tissues [116]. These results all indicate that SR-BI plays an important role in HDL metabolism and determining the plasma lipoprotein cholesterol levels. Thus selective uptake of HDL cholesterol by SR-BI may be a major route for cholesterol clearance by the liver with respect to reverse cholesterol transport.

In addition to mediating selective uptake of HDL-CE, SR-BI also promotes cholesterol bi-directional flux of FC between cells and HDL by a mechanism still poorly understood[117;118]. It is apparently controversial whether HDL must physically bind to SR-BI in order to mediate cholesterol efflux from cells. In Studies from Llera-Moya *et al* [119], they overexpressed SR-BI and CD36, another receptor structurally related to SR-BI in COS-7 cells, and found that only SR-BI promoted significant cholesterol efflux, although HDL bound with high affinity to CD36 compared to SB-BI. Furthermore, SR-BI stimulated cholesterol efflux to phospholipid vesicles, even though such vesicles do not bind to surface receptors in SR-BI expressing cells. In the later studies the same group [120] reported that SR-

BI expression altered the distribution of membrane FC to a cholesterol-rich domain or altered the accessibility of this membrane fraction to exogenous cholesterol oxidase. They therefore concluded that SR-BI expression leads to a redistribution of cholesterol to membrane domains that serves to mediate the efflux of FC between cells and HDL rather than the binding of HDL. However, another study by Gu *et al* [121] showed that HDL binding to SR-BI was required for mediating cholesterol efflux. Moreover, Liadaki *et al* [122] had demonstrated that SR-BI could bind both apolipoproteins and HDL particles, but binding affinity was greater for large, spherical HDL particles. Their studies therefore suggest that these larger HDL particles may be the predominant substrates for SR-BI *in vivo*. Clearly, more studies are required to delineate the precise mechanism of SR-BI mediated cholesterol efflux from cells.

1.3.4 HDL Catabolism

Early studies demonstrated that increased catabolism rate is the principal metabolic determinant of plasma HDL levels rather than production rate [123;124]. The lipidation state of apoA-I affects its metabolic rate *in vivo*. The rate of HDL clearance from plasma is inversely correlated to the particle size. Smaller HDL containing fewer lipids are cleared faster than larger HDL particles.

TG-enriched HDL particles have been shown to increase lipolysis of HDL by HL, reduce HDL size and increase catabolism of the particles [125;126]. Hypertriglyceridemia is often associated with low plasma HDL levels and a greater fractional catabolic rate of apoA-I [127]. As described earlier, the enzyme responsible for TG enrichment of HDL is CETP, which mediates the exchange of HDL-CE for TG from VLDL and LDL. Subsequent hydrolysis by HL results in the production of smaller HDL particles and the generation of

nascent preβ-HDL. *In vitro* studies from Clay *et al* [128] had demonstrated that hydrolysis of HDL-TG promotes the dissociation of apoA-I, suggesting that HDL in hypertriglyceridemic individuals may contain a greater proportion of loosely associated apoA-I, which is more rapidly cleared from the circulation through renal filtration [129].

1.3.5 ATP-Binding Cassette Transporter A1 (ABCA1) and Cellular Lipids Removal

The dynamic modifications of HDL subfractions in the circulation by several enzymes, including LCAT, CETP, PLTP, HL and EL have led the investigation of HDL formation and metabolism. However, the discovery of a protein, named ATP-binding cassette transporter A1 (ABCA1) has accelerated the research on HDL formation and metabolism.

ABCA1 was first discovered in 1999 by several groups [130-132]. It was the gene product defective in Tangier Disease (TD), a severe HDL deficiency characterized by impaired ability to transport cholesterol and phospholipid to apoA-I [133;134], and deposition of CE in tissues, including tonsils, thymus, lymph, node, bone marrow, spleen, liver, gall bladder, and intestine [130;131]. Cells isolated from TD homozygotes were shown to have a severely impaired ability to transport cholesterol and phospholipid to purified apolipoproteins [133;134], apoA-I remained lipid-poor and was removed rapidly by the kidney, preventing the formation of HDL. These results indicate that the participation of ABCA1 in cellular lipids removal is required for normal HDL metabolism.

1.3.5.1 Structure of the ABCA1 Gene

ABCA1 is an integral membrane protein consisting of 2261 amino acids. It is a member of a large family of ABC transporters that utilize ATP as an energy source to pump lipids and other metabolites across membranes [135]. ABCA1 protein is composed of 2 halves of similar structure that are linked by covalent bond. Each half has a nucleotide-binding domain (NBD) containing two conserved peptide motifs known as Walker A and Walker B, and a transmembrane domain containing 6 helixes (Fig 1-6). The N-terminus of the ABCA1 is predicted to be in the cytosol and 2 large extracellular loops that are highly glycosylated and linked by one or more cysteine bonds [136;137].

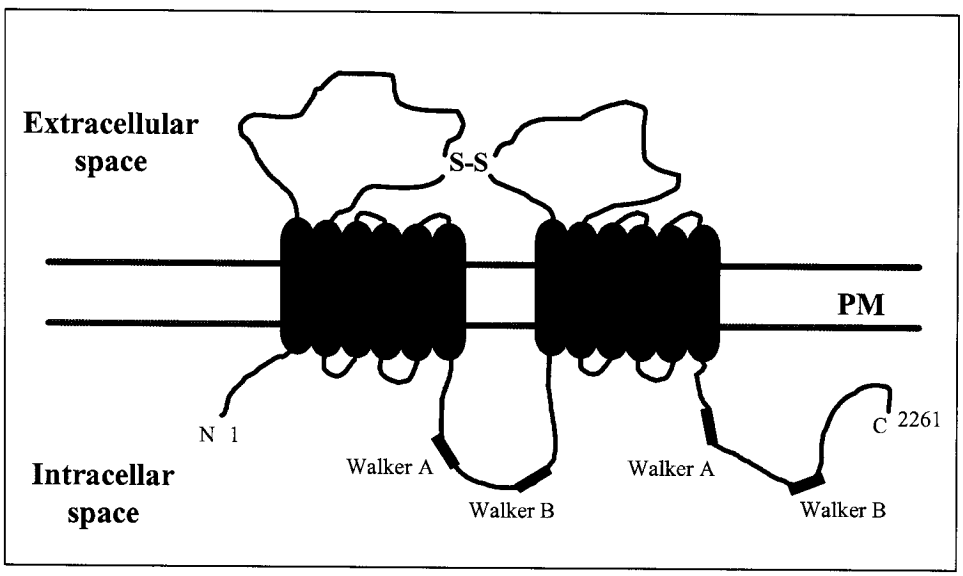

Figure 1-6: Topological Model of ATP-Binding Cassette Transporter

Adapted from Tall *et al.* [138].

1.3.5.2 Regulation and Tissue Expression of ABCA1 Gene

As expected, transcription of ABCA1 is highly upregulated upon cholesterol loading, since it controls the levels of cholesterol in the cells [139;140]. This upregulation occurs through

the activation of the nuclear receptors liver X receptor (LXRα and/or LXRβ) and retinoid X receptor (RXR) [141;142]. LXR and RXR form obligate heterodimers that preferentially bind to response elements within the ABCA1 gene promoter and the first intron. LXR and RXR bind to and are activated by oxysterols and 9-cis-retinoic acid, respectively (Figure 1-7) [143].

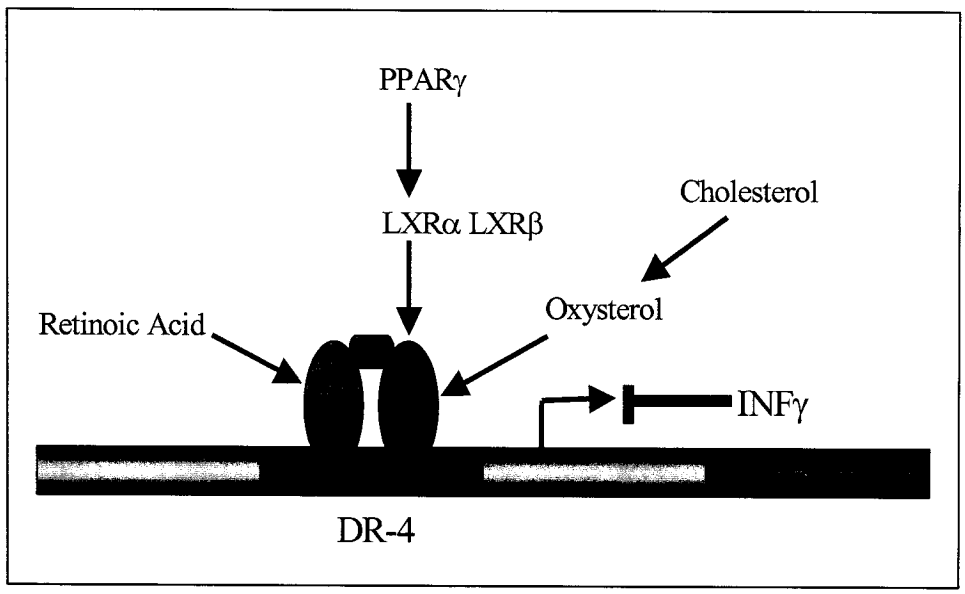

Figure 1-7: Regulation of ABCA1 Gene Transcription.

DR-4: direct repeat response element, sequence which binds the heterodimer receptor pair. Adapted from Oram *et al* [144].

Binding of either one or both ligands to LXR or/and RXR heterodimers can activate gene transcription, but their combined treatment has significant synergic effect [141;142]. Activators of peroxisome proliferator-activating receptors (PPARs) also enhance ABCA1 transcription in some cells. PPARγ activators stimulate cholesterol efflux from cells by activating transcription of LXRα gene, which then enhance ABCA1 transcription [145;146]. ABCA1 transcription is also stimulated by membrane permeable analogs of cyclic AMP (cAMP) [147;148] in some macrophage lines by unknown mechanisms. In addition, cytokines

also appear to modulate ABCA1 transcription in some cells. Interferon-γ (INFγ) inhibits ABCA1 expression in macrophages, which is antagonized by transforming growth factor-β (TGF-β) [149], and oncostatin M induces ABCA1 expression in cultured hepatoma cells [150].

Post-transcriptional regulation of ABCA1 is also important for maintaining this protein level. It has been shown that binding of apoA-I to ABCA1 retarded degradation of ABCA1 protein in THP-1 cells by protecting ABCA1 from being degraded by a thiol protease [151]. Studies from Wang *et al.* [152] identified a sequence rich in proline, glutamic acid, serine and threonine (PEST sequence) present in the cytoplasmic region of ABCA1, which increased the degradation of ABCA1 protein by calpain protease. Binding of apoA-I to ABCA1 abolished the effect of the calpain protease and increased efflux of PL and FC. The same group also reported that phosphorylation of the ABCA1 PEST sequence directed ABCA1 to calpain proteolysis and extracellular apoA-I diminished the phosphorylation of the PEST sequence, resulting in increased cell surface expression of ABCA1 [153]. Therefore, blocking the phosphorylation of the ABCA1 PEST sequence has been proposed to be the therapeutic target to increase cellular lipid efflux.

ABCA1 is widely expressed throughout animal tissues. ABCA1 mRNA was shown to be abundant in tissues containing inflammatory cells and lymphocytes, but it was also detected in noninflammatory cells, which has high expression in the liver, kidney, brain, adrenal, heart, bladder and testis [154;155].

1.4 Intracellular Cholesterol Movement and Roles ApoA-I and ABCA1

Population studies have consistently shown an inverse relation between plasma HDL levels and risk of cardiovascular disease, implying that factors associated with HDL are atheroprotective. It is widely believed that HDL components inhibit atherosclerotic lesion formation by removing excess cholesterol from peripheral cells and prevent foam cell formation [13;14].

1.4.1 Cholesterol Metabolism

Cholesterol is one of the essential components of cell membranes. It functions to maintain the barrier between the cell and its environment, and modulate membrane fluidity. Cholesterol also provides the raw material for the production of bile acids in hepatocytes, which the body uses to eliminate cholesterol. Maintenance of cellular cholesterol homeostasis therefore requires cholesterol losses from the body to balance cholesterol input from the food and by synthesis. If cells are unable to eliminate excess non-degradable free cholesterol, the cholesterol will be then esterified and stored as CE droplets within the cytoplasm [13]. As described earlier, these CE-enriched foam cells arise when blood monocyte-macrophage infiltrate the endothelium and contribute to the pathology of CHD.

1.4.2 Cellular Cholesterol Distribution and Transport

Cells maintain distinct protein and lipid composition that are essential for their various functions. Although cholesterol is synthesized in the endoplasmic reticulum (ER), the composition of cholesterol however in the ER is very low, and accounts for only 0.1-2%

of cellular cholesterol, whereas 60-90% of cellular cholesterol presents on the plasma membrane depending on the cell types and the methodologies used [156-158]. The recycling endosome (RE) compartments, which contain recycling membrane proteins and lipids, also contain high levels of cholesterol [159;160]. The cholesterol content in the late endosomes and lysosomes has been shown to be lower than in the RE [158;160]. Intracellular transport of cholesterol between cellular compartments is required for its metabolism and functions. Understanding intracellular cholesterol transport is therefore important for study of apoA-I cholesterol lipidation and HDL formation.

There are two pathways whereby cholesterol can move intracellularly: vesicular and nonvesicular [156]. Vesicular traffic requires an intact cytoskeleton, and ATP, which provides energy. Non-vesicular transport can be mediated by diffusible carrier proteins that have hydrophobic cavities to bind cholesterol and transport it across the aqueous cytosol. One example is the steroidogenic acute regulatory protein (STAR protein), which stimulates the conversion of cholesterol to steroids [161]. Another form of non-vesicular transport may be mediated by spontaneous desorption of cholesterol from one membrane and diffusion to another juxtaposed membrane [156;157]. Specific proteins may be required to bring the two membranes together at contact sites.

1.4.2.1 Cholesterol Transport from ER to PM

Since the ER is the site of cholesterol synthesis but contains a low concentration of cholesterol, efficient transport mechanisms must exist to export cholesterol from ER to PM against a concentration gradient. Vesicular transport through the Golgi is one pathway via which cholesterol achieves ER to PM transport. Consistent with this mechanism, DeGrella

et al. showed that the transport of cholesterol from ER to PM is inhibited when energy is depleted or the cells are incubated at low temperature [162;163]. In addition, a non-vesicular transport mechanism also exists. Studies have suggested that both caveolin-1, the structural protein of caveolae and sterol-carrier protein 2 (SCP-2) are involved in intracellular cholesterol transport [164;165]. However, cultured cells lacking either caveolin-1 or SCP-2 showed a decreased rate of cholesterol appearance on the PM, but no defects of cholesterol transport to the PM after longer incubation [165;166], indicating neither caveolin-1 nor SCP-2 satisfactorily accounts for nonvesicular movement of nascent cholesterol from ER to PM.

1.4.2.2 Cholesterol Transport from PM to ER

Excess cellular cholesterol from other compartments goes to the ER for esterification, while newly synthesized cholesterol leaves the ER. As described earlier, cholesterol transport from PM to ER following vesicular, via endosomes and/or Golgi, and nonvesicular pathways. Studies by Zha *et al* [167] have demonstrated that cholesterol-containing vesicles remain in the cell periphery, and addition of ATP results in the transport of these vesicles from PM to endosomes and then reach the ER for cholesterol esterification. However, another study has shown that cholesterol movement to ER even in the ATP-depleted condition when PM-derived vesicles remain in the cell periphery [168].

1.4.2.3 Cholesterol Transport in Early and Recycling Endosomes

Cholesterol-containing LDL is endocytosed through LDLr, and these vesicles fuse with early endosomes (EE). The lower pH in EE promotes dissociation of LDL from LDLr.

The LDLr and other recycling proteins then localize to early endosomal tubular extensions, which bud off vesicles that fuse with the RE compartment. Recent studies have suggested that the acid lipase enzyme was localized to an early acidic compartment, so LDL-CE-derived FC may be generated soon after endocytosis [169]. LDLr from these vesicles returns to the PM. The RE is a cholesterol-rich compartment [170;171], and cholesterol is transported from RE to PM through the same vesicles that carry recycling proteins [160].

1.4.2.4 Cholesterol Transport from Late Endosomes/Lysosomes

The non-recycled contents of EE proceed to late endosome (LE), by a process involving vesicular transport or the evolution of early to late endosome. LE fuses with Golgi-derived vesicles containing hydrolytic enzymes and then mature into lysosomes. Under normal condition, LDL-derived cholesterol leaves late endosomes/lysosomes to reach other compartments like ER and PM. Cholesterol is thought to follow at least 2 pathways from endosomes to ER: one pathway involves the PM as an intermediate because study has demonstrated that cyclodextrin-mediated cholesterol extraction from the PM inhibits esterification of LDL-derived cholesterol by 70% [158]. Another minor pathway that bypasses the PM has been proposed by Neufeld *et al.* as 30% of esterification is not inhibited by cyclodextrin [172].

The fatal neurodegenerative disease Niemann Pick Type C (NPC) is an autosomal recessive lipid storage disorder caused by the defective NPC protein located in late endosomes and/or lysosomes, and is characterized by a defective transport of cholesterol out of late endosomes and/or lysosomes, and subsequently causes accumulation of intracellular FC in these compartments [173;174]. Whereas LDL is the primary source of this intracellular

cholesterol, newly synthesized cholesterol also accumulates at a slower rate [175;176]. This may represent the PM cholesterol that is derived from ER and is endocytosed but not sorted to the RE, instead reaching late endosomes/lysosomes for accumulation. To study the intracellular cholesterol trafficking, the NPC phenotype can also be reproduced by treatment of normal cells with sterols like progesterone or with hydrophobic amines like U18666A [177;178].

There are currently many methods for studying cholesterol distribution and transport. Newly synthesized cholesterol can be determined by introducing ^{3}H-mevalonate or ^{3}H-acetate into living cells, and measuring the amount of labeled cholesterol in isolated membranes or vesicles [179]. Radiolabeled cholesterol can also be delivered by lipoproteins or via specific cyclodextrin carriers, such as methyl-β-cyclodextrin (mβCD) [179]. Total cholesterol can be measured by direct chemical methods such as gas chromatography-mass spectrometry or by indirect methods such as assays based on cholesterol oxidase [158;180].

1.4.3 Cellular Cholesterol Efflux

Efflux of cellular cholesterol is a complex process, which occurs at the plasma membrane level and involves multiple mechanisms. Three distinct mechanisms of cellular cholesterol efflux involving HDL and its apolipoproteins have been proposed (Fig. 1-8). First, plasma HDL particles can promote cholesterol efflux by a process of passive aqueous diffusion [181]. Free cholesterol molecules spontaneously desorb from the plasma membrane, and are incorporated into HDL particles by collision. This type of efflux is driven by the phospholipid content of lipoprotein acceptors and the cholesterol concentration gradient [182].

The HDL receptor, SR-BI is involved in the second mechanism of efflux. SR-BI is a multi-ligand receptor that interacts with a wide range of acceptors, including HDL, LDL, and

modified LDL [183]. As described earlier, in addition to its role in selective uptake of HDL-CE, SR-B1 can also mediate cellular cholesterol efflux to large, mature HDL particles [117;121]. However, there is an absolute requirement for phospholipid in the acceptor [184]. Therefore, as long as apoA-I acquires a sufficient amount of phospholipid, SR-B1 can mediate cholesterol efflux by a diffusional mechanism when there is a favourable cholesterol gradient.

The third mechanism of efflux involves ABCA1-mediated active transport of cellular cholesterol to lipid-poor apolipoproteins, in particular, apoA-I. Lipid efflux to secreted and/or circulating apoA-I generates preβ-HDL and/or LpA-I particles. Subsequent esterification of FC by LCAT promotes the formation of mature, spherical α-HDL particles that deliver CE to steroidogenic tissues and hepatocytes via SR-B1 as described earlier. Since HDL biogenesis contributes significantly to RCT, this mechanism of lipid efflux is critical to cellular cholesterol homeostasis (See the following section).

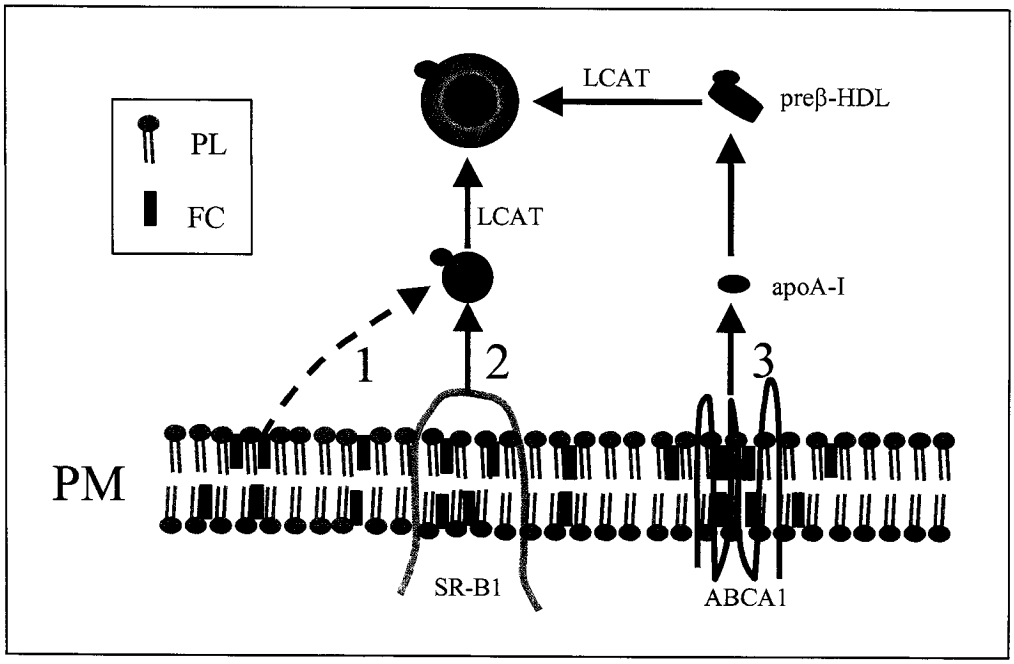

Figure 1-8: Lipid Efflux from Cell Membrane

Three mechanisms of cellular cholesterol efflux: 1) diffusion efflux; 2) SR-B1 mediated efflux; 3) ABCA1/apoA-I mediated efflux.

In addition to ABCA1-mediated cholesterol efflux, other members of the ABC transporter family also have been shown to participate in lipid efflux. ABCA7 is highly homologous to ABCA1 and has been shown to bind and mediate PL efflux, but not FC efflux to apoA-I in cell culture [185]. Inconsistent with these results, studies from Yokoyama *et al* [186] showed that in addition to ABCA1, human ABCA7 promoted both PL and FC efflux to apoA-I and proposed that ABCA7 might compensate the lack of ABCA1 function to mediate cellular cholesterol efflux in a certain condition(s), but their post-transcriptional regulation is different. However, neither genetic knockdown of ABCA7 in mouse macrophages nor ABCA7-null mice showed a defective lipid efflux to apoA-I [187;188]. Clearly, whether ABCA7 functions to promote lipid efflux *in vivo* remains to be identified.

ABC transporters of human ABCG subfamily are half transporters [189], and they form either homo- or heterodimers in order to become functionally active transporters [190]. Both elevated gene expression of ABCG1 and ABCG4 have been shown upon cholesterol loading, or LXR and RXR agonists treatments [191-193]. Whereas ABCA1 predominantly binds to and mediates cholesterol efflux to poorly lipidated apoA-I, ABCG1 and G4 have been shown to mediate cholesterol efflux to larger HDL_2 and HDL_3 particles [194] in macrophages. Furthermore, ABCG4 is highly expressed in brain, where HDL-like particles are present in cerebrospinal fluids, ABCG4 therefore has been proposed to mediate cholesterol efflux to these HDL particles in brain [195;196].

Two other members of the ABCG subfamily, ABCG5 and ABCG8 are highly expressed in liver and intestine [197]. They form functional heterodimers, and have been shown to be responsible for the efflux of dietary sterols from intestinal epithelial cells back into the gut lumen and from the liver to the bile duct [197;198]. Mutations in either ABCG5 or ABCG8 were found in patients with β-sitosterolemia, also known as phytosterolemia, the disease characterized by increased trapping of cholesterol and other sterols in the intestinal cells and inability to convert these sterols in the bile [199;200]. Thus ABCG5 and ABCG8 play an important role in reducing intestinal cholesterol absorption and promoting biliary sterols excretion.

1.4.4 Mechanisms of ApoA-I and ABCA1 Mediated Cellular Lipid Removal

Studies have shown that individuals with TD have almost no HDL, however, apoA-I secreted from these patients were normal, but poorly lipidated due to the defective cholesterol efflux mediated by ABCA1 and their apoA-I is thus rapidly catabolized by

kidney [145;146] (Fig. 1-9). Thus, in mediating the efflux of lipid from cells, ABCA1 also facilitates the lipidation of apoA-I and the formation of HDL [133;134], as well maintaining the proper HDL levels in the circulation.

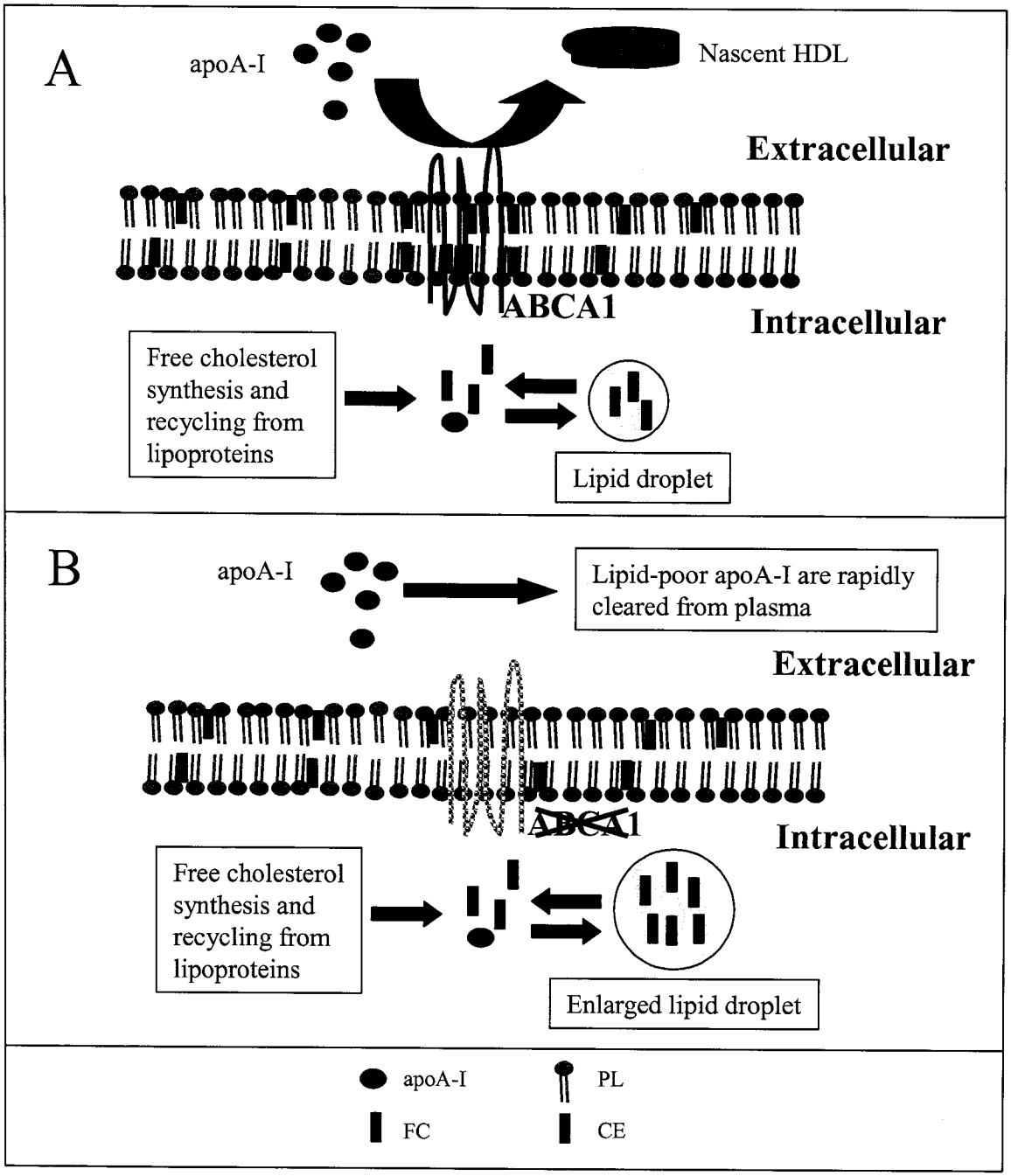

Figure 1-9: Defective Cholesterol Efflux in Tangier Disease Condition

Model for the apoA-I mediated cholesterol transport in normal subjects (A) and patients with Tangier disease (B).

Unlike passive diffusion and SR-B1-mediated efflux, which involve bi-directional exchange of cholesterol between cells and HDL. ABCA1/apoA-I-mediated cholesterol and

phospholipid efflux is a unidirectional net transfer process [201]. ABCA1 binds and cross-links lipid-poor apoA-I, while minor interaction with smaller HDL_3 and no interaction with large HDL_2 subspecies [202], indicating the importance of a small pool of lipid apolipoproteins either secreted by cells or generated by lipid exchange and lipolysis of HDL particles in the circulation.

1.4.4.1 ApoA-I/ABCA1 Binding and Lipid Removal

There are several lines of evidence showing the direct interaction of apoA-I with ABCA1. Studies have shown that ABCA1 expression substantially increased apoA-I binding at the cell surface, and chemical cross-linking and immunoprecipitation analysis showed a direct binding of ABCA1 to apoA-I, but not to HDL [201]. Other studies showed that treatment of cells with cAMP increased cellular FC and PL efflux to apoA-I, and that the enhanced lipid efflux is associated with increased binding of apoA-I to ABCA1 on the cell surface [148;203;204]. It has been proposed that the interactions of apoA-I with residues in extracellular loops of the ABCA1 are important for mediating lipid efflux; mutations in these loops result in the impairment of both the lipid transport and binding of apoA-I [205].

Binding of apoA-I to ABCA1 is proposed to play one or more important roles in mediating lipid transport and efflux. First, it may serve to target apolipoprotein to lipid domains formed by ABCA1 and facilitate the assembly of apolipoprotein/lipid particles. Second, apoA-I binding may stabilize ABCA1, increasing its membrane content and enhancing the lipid efflux. In support of this idea is a study showing that incubating cells with apoA-I inhibits proteolytic degradation of ABCA1 protein[151]. Lastly, binding of apoA-I to ABCA1 may activate intracellular signalling pathways that modulate lipid trafficking.

Study has shown that the interaction of lipid-poor apoA-I with cells elicits signals, which involve activation of phospholipases and protein kinase C [65].

1.4.4.2 Proposed Mechanisms of ABCA1 Mediated Lipid Removal

Two mechanisms of ABCA1-mediated PL and FC transfer to apoA-I have been suggested: (i) "simultaneous" (one-step) [206;207], in which PL and FC are mobilized simultaneously as discrete units, and (ii) "sequential" (two-step) [208;209], in which PL are transferred to apoA-I followed by acquisition of FC. The simultaneous mechanism describes that PL and FC are released together. In the later two-step mechanism, ABCA1 first promotes the lipidation of apoA-I with PL, generating the classic preβ-, phospholipid-rich particles and then acquires cholesterol secondarily by a passive diffusion mechanism [210]. In support to this mechanism, ABCA1 has been proposed to be a PL translocase/flippase, whereas no evidence has shown that cholesterol is the substrate for ABCA1 [130;211]. When FC efflux from ABCA1-expressing cells was inhibited, PL efflux to apoA-I still occurred, and when this conditioned media containing PL/apoA-I complexes was transferred to ABCA1-deficient cells, it stimulated FC efflux, but not PL efflux [209]. Another study has demonstrated that ABCA1 functions as a cholesterol efflux regulatory protein rather than a direct transporter [202]. Their data suggested that ABCA1 acts primarily as a PL translocase, an activity that is closely associated with the binding of apoA-I to ABCA1, and cholesterol efflux is mediated indirectly by ABCA1.

In addition to apoA-I, ABCA1-mediated cholesterol removal can also be stimulated by other exchangeable apolipoproteins, including apoA-II, apoC, and apoE [212;213]. The amphipathic helices of the apolipoproteins have been demonstrated to act as structural

recognition motifs for lipid efflux, facilitating the first and most critical step of lipid flux interaction of apolipoproteins with modified lipid microdomains in the plasma membrane [214].

Initial studies have suggested that endogenously expressed ABCA1 was localized on the plasma membrane [201;215;216]. Studies by Neufeld *et al* using stably and transiently transfected HeLa cells expressing a functional human ABCA1-GFP have demonstrated that ABCA1 also resided in intracellular vesicles, including early endosomes, late endosomes/lysosomes, and moves between PM and these intracellular compartments [217]. This intracellular trafficking of ABCA1 transporter may also play an important role in mediating lipid removal and apoA-I lipidation [217]. The functional significance of the trafficking of ABCA1 along the intracellular endocytic pathways is not well established. Several studies have provided evidence suggesting that ABCA1-mediated lipid efflux involves intracellular trafficking of substrate lipids [218;219] as well as the apoA-I [220]. Studies have also provided evidence suggesting that extracellular apoA-I may be endocytosed and recycled back to the PM before their release from the cells[220;221]. Studies by Neufeld *et al* also suggested that the delivery of ABCA1 to lysosomes might serve as a degradation mechanism to control the cell surface expression of the protein, and therefore modulate apoA-I mediated lipid efflux. However, the significance of the intracellular ABCA1 trafficking and recycling for lipidation of newly synthesized apoA-I by hepatocytes remains to be identified.

1.5 Current Understanding of Hepatic ApoA-I Lipidation

ApoA-I is the structural protein component of HDL particle. The levels of apoA-I protein and its mRNA are in direct proportion to the plasma HDL levels. The state of the apoA-I lipidation is closely related to its catabolism as in TD where lipid-poor apoA-I is rapidly catabolized by kidney even though apoA-I secretion remains unchanged compared to normal condition [145;146]. Therefore, the capacity of the liver to secrete a certain level of lipidated-apoA-I, but not of lipid-poor apoA-I plays a crucial role for maintaining proper plasma HDL levels.

1.5.1 Intracellular and Extracellular Hepatic Lipidation of ApoA-I

Early studies of the synthesis and secretion of apoA-I in the liver and hepatocytes provided evidence for both the intracellular and extracellular lipidation of apoA-I. Studies with hepatocytes from chicken [222;223] or rat [224] have suggested that apoA-I was lipidated intracellularly. However, Hamilton *et al.* [225], using electron microscopy failed to identify any lipidated apoA-I particles in hepatocytes. Lately, Chisholm *et al.* [226] investigated the secretion and lipidation of apoA-I from HepG2 cells. They concluded that some apoA-I acquired lipid intracellularly and was then secreted along with lipid-poor apoA-I. Subsequently, the secreted apoA-I could acquire lipids extracellularly to form buoyant HDL particles. However, we have overall a limited knowledge of the process and pathways of HDL assembly in hepatocytes and of the nature of lipoproteins secreted. More recently, studies from our laboratory had demonstrated the intracellular and extracellular phospholipidation of newly sythesized apoA-I by primary mouse hepatocytes, and

intracellular lipidation is greater than extracellular lipidation by the efflux pathway. We also demonstrated the existence of a limited ABCA1-independent phospholipidation pathway for apoA-I [67]. Cholesterol lipidation of exogenous apoA-I in hepatocytes was studied by Sahoo *et al.* [227], and their study showed the lipidation of exogenous apoA-I. However the cholesterol lipidation of endogenously synthesized apoA-I has not been evaluated.

1.5.2 Role of Hepatic ABCA1 in Mediating ApoA-I Lipidation

ABCA1 is the major determinant of the phospholipid and cholesterol lipidation of apoA-I that enables formation of HDL [228]. ABCA1 has been shown to regulate lipid efflux, and a deficiency in ABCA1, as in Tangier disease, results in nearly undetectable levels of HDL [130-132;211;215]. Macrophages in various tissues are predominantly affected by this disease. This suggests that ABCA1 function in macrophages is critical for the maintenance of HDL levels in the plasma, as part of the reverse cholesterol transport. However, Haghpassand, *et al.* [229] transplanted bone marrow from wild-type mice into ABCA1 KO mice and from ABCA1 KO mice into wild type mice. They found that in the former experiment, apoA-I and HDL cholesterol were slightly but significantly increased; in the latter experiment, they could detect no contribution at all, HDL levels were not changed. They concluded that macrophages account for only 20% of the generation of circulating HDL. Since ABCA1 [155;230-232] is highly expressed and apoA-I [233;234] is synthesized in the liver, suggesting that the hepatic ABCA1 contributes to the lipidation of newly secreted apoA-I in those tissues and the liver is responsible for generating the plasma HDL. Since ABCA1 binds and lipidates lipid-poor-apoA-I, but not large HDL [201], these results imply that hepatic ABCA1 gives rise to bulk HDL particles, independent of direct interaction with macrophage ABCA1, and the

cause of the plasma HDL deficiency in TD patients may be due to an impaired liver ABCA1 pathway.

1.6 Rational and Specific Aims

The goal of this study is to identify the function of hepatocytes for lipidation of newly synthesized apoA-I. Specific aims are: 1) to characterize the pathways involved and the origins of the pools used in lipidation of apoA-I by cholesterol, (and compare to that with phospholipids); and 2) to evaluate the role of hepatic ABCA1 in mediating apoA-I cholesterol lipidation, as well as HDL formation.

Epidemiological studies have consistently shown an inverse relation between plasma HDL levels and the risk for cardiovascular disease. However, the diverse action of HDL observed *in vivo* and *in vitro* can be explained by the heterogeneity of HDL particles. Patients with CHD have significantly altered HDL subpopulation profiles compared with control participants. CHD patients have significantly lower concentrations of the large cholesterol ester-rich particles and significantly higher concentrations of the small and less lipidated HDL particles, including preβ-HDL. Therefore, the assignment of various cellular effects to HDL subclasses and the clarification of their role in the prevention of CHD is a great challenge left to be explored in lipid research.

Apparently, modification of HDL metabolism has a great potential in antiatherogenic drug therapy. The major question is how to modify specific HDL subpopulations to reach the maximum atheroprotective effects under various pathologic conditions. There is evidence that it is involved not only in removing cholesterol from peripheral tissues but also the potential of the production of lipidated apoA-I, and generation of HDL by the liver. Thus pharmacological control of hepatic ABCA1 activity, increasing hepatic secretion of lipidated apoA-I and therefore decreasing its degradation could be the potential targets for regulating

the proper plasma HDL levels and promoting regression of atherosclerosis by mediating cholesterol efflux from peripheral tissues.

CHAPTER 2: KINETIC STUDIES OF CHOLESTEROL AND PHOSPHOLIPID ACQUISITION BY HUMAN APO A-I IN ABCA1-WILD TYPE AND -NULL HEPATOCYTES

2.1 Summary

ABCA1 mediates phospholipid and cholesterol efflux from cells to contribute to apoA-I lipidation, and through this pathway to maintain the plasma HDL levels. Since liver is the site for both apoA-I synthesis and ABCA1 expression, we hypothesized that liver plays an important role, and hepatic ABCA1 is active in mediating cholesterol lipidation of newly synthesized apoA-I. Our previous studies had demonstrated that apoA-I is phospholipidated both during secretion and at the cell surface of hepatocytes, and most of this phospholipidation is ABCA1-dependent [67]. Here I compare the kinetics of cholesterol and phospholipid acquisition by newly synthesized human apoA-I (hapoA-I) using adenoviral vector-mediated endogenous expression (endo-apoA-I) or exogenously added (exo-apoA-I) apoA-I in wild type and ABCA1-null primary mouse hepatocytes.

The experiments were conducted by labelling cells with ^{3}H-choline and ^{3}H-mevalonate for *de novo* synthesized phospholipid and cholesterol, respectively; or ^{3}H-cholesterol delivered with LDL (^{3}H-cholesterol-LDL) or methyl-β-cyclodextran (mβCD) (^{3}H-cholesterol-mβCD) for exogenously supplied, or plasma membrane derived cholesterol, respectively. The apoA-I lipidation was measured by immunoprecipitation with anti-human apoA-I antibody. In wide type hepatocytes, cholesterol and phospholipid association with apoA-I increased in parallel with time for both endogenous and exogenous apoA-I. The kinetics of cholesterol lipidation of apoA-I was similar to its phospholipidation, indicating simultaneous acquisition of both lipids. However, ABCA1-deficiency decreased apoA-I phospholipidation by 80%, but acquisition of *de novo* synthesized and exogenous cholesterol only decreased by 40-60%. The transfer of *de novo* synthesized cholesterol to apoA-I was

decreased at all time points, but that of exogenously delivered cholesterol was independent of ABCA1 activity at the early time points, suggesting heterogeneity of the cell surface cholesterol pools accessible to ABCA1. FPLC analysis of hepatocyte media lipoproteins confirmed that with ABCA1 deficiency the proportion of secreted HDL-associated apoA-I and cholesterol decreased by about 50%. The VLDL/LDL size fraction also contained a significant amount of cholesterol in ABCA1 deficiency consistent with the result of immunoprecipitation showing the presence of lipoproteins with both apoA-I and murine apoB, indicating the presence of apoA-I/apoB complexes secreted from hepatocytes.

These studies demonstrate the important role of liver in mediating apoA-I lipidation. In primary mouse hepatocytes, newly synthesized apoA-I acquires cholesterol and phospholipid during secretion and at the cell surface. Most hepatic phospholipidation is mediated by ABCA1, but acquisition of cholesterol operates by both ABCA1-dependent and -independent pathways [179;235].

2.2 Experimental Procedures

2.2.1 Materials

Cholesterol-[1, 2-^{3}H], mevalonolactone-Rs-[5-^{3}H(N)], choline chloride-[Methyl-^{3}H] were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA) . Williams' Medium E, HepatoZYME-SFM, antibiotic-antimycotic, Polyacrylamide gel electrophoresis were purchased from Invitrogen Corporation (Burlington, ON). Rabbit polyclonal anti-human apoA-I antibody was purchased from Calbiochem (Mississaga, ON). Monoclonal antibodies directed against human apoA-I [a combination of 4H1 (against the extreme N terminus) and 5F6 (against the central region)] were biotinylated with Sulfo-NHS-Biotin from Pierce [236;237]. Polyclonal anti-murine apoB and anti-murine apoE antibodies were purchased from Biodesign International (Brockville, ON). Streptavindin-horseradish peroxidase conjugate and protein G-Sepharose were obtained from Amersham Biosciences (Baie d'Urfé, Québec). Centrifugal Filters were purchased from Millipore Corporation (Nepean, ON). Pure nitrocellulose membrane was purchased from Bio-rad Laboratories (Philadelphia, PA).

2.2.2 Animal Models

Wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). ABCA1-deficient mice (a kind gift from Dr. Edward M. Rubin, DOE Joint Genome Institute, Berkeley, CA) were generated as follows: A 1.65-kb PCR-generated fragment containing abca1 exon 16 was inserted between the PGK terminator of the PGKNeo cassette and the double PGKtk cassettes of pPN2T at EcoRI and BamHI sites. A 12-kb PCR-

generated fragment from Intron 22 to Exon 30 was then inserted on the PGK promoter side of the PGKneo at blunted-XhoI and NotI sites to generate the final targeting vector pAbc1SALA. Stratagenes TaqPlus Long PCR System was used for amplification of 12 kb fragments. ESVJ ES cells from Genomesystems were electroporated with NotI linearized pAbc1SALA and subjected to G418 and FIAU selection. Correctly targeted clones were screened by PCR, and then confirmed by Southern blotting. Chimeric mice were generated using standard procedures. Chimeric males were bred with C57BL/6J (Charles River) to create mice heterozygous for the targeted allele on the 129Sv/C57 mixed background. 129SV/C57 mice have higher baseline HDL levels, and are less suceptible to atherosclerosis than the C57BL/6J (30) and, as a result, provide an easier phenotype to detect differences in HDL synthesis and secretion. All mice were maintained on a normal chow diet in a 12 h light/12 h dark schedule and used between the ages of 4-6 months. All experiments performed were in accordance with protocols approved by the University of Ottawa Animal Care Committee.

2.2.3 Primary Hepatocyte Cultures

Primary hepatocytes were isolated from these mice by liver collagenase perfusion according to the established protocols [238;239]. Briefly, the cells were plated in fibronectin pre-coated (25 μg/well) 6-well plates at an initial density of 1.5×10^6 cells per well in Williams' Medium E containing penicillin (100 units/ml), streptomycin sulfate (100 units/ml), Fungizone® (250 ng/ml) and 10% fetal bovine serum (FBS).

2.2.4 *Cell Labeling and Adenovirus Infection*

Five hour following the initial seeding, the cells were washed in Williams' Medium E without serum (2 × 2 ml) and incubated with HepatoZYME-SFM containing antibiotic-antimycotic, 10 μCi/ml of ^{3}H-mevalonate and 5 μCi/ml of ^{3}H-choline for labeling *de novo* synthesized cholesterol and phospholipid, respectively, or 5 μCi/ml of ^{3}H-cholesterol delivered with 10μg/ml LDL for exogenously supplied cholesterol. Mevalonate label is incorporated predominantly into cholesterol [240]. The following day (24 h) the labeled medium was removed and the cells were infected for 1 h with either the recombinant adenovector expressing human apoA-I (Ad AI) or as control adeno-luciferase (Ad Luc) at a multiplicity of infection of 75:1 plaque-forming units per cell in Williams' Medium E without serum [236;237]. After 1 h infection, hepatocytes were incubated for an additional 24 h with original labeling medium as described above. In order to label the plasma membrane cholesterol pool, hepatocytes were cooled down at 4°C for 1 h on the third day. The medium was replaced by 5 mM mβCD : cholesterol (^{3}H-cholesterol, 5μCi / ml) at molar ration 8:1 for 3 h at 4°C [241;242]. After washing 3 times, the cells were immediately used for efflux assays.

2.2.5 *Time Course of ^{3}H-Cholesterol and ^{3}H-Choline-Phospholipid Efflux Assay*

For cells that were labeled with ^{3}H-mevalonate, ^{3}H-choline and ^{3}H-cholesterol-LDL, on the third day, hepatocytes were equilibrated in non-labeled HepatoZYME-SFM for 1 h at 37°C. Following 2 × 2 ml washes in Williams' Medium E, cells were incubated with 1 ml per well of non-labeled HepatoZYME-SFM in the absence (for endo-apoA-I) or presence of 5 μg of hapoA-I (for exo-apoA-I). For cells that were labeled with ^{3}H-cholesterol-mβCD, after washing 3 times, cells were immediately incubated with 1 ml per well of non-labeled

HepatoZYME-SFM in the absence or presence of 5 μg of hapoA-I. The cells were returned to the 37 °C incubator (5% CO_2) for the various times, and the efflux medium was subsequently collected at different time points and spun down to pellet any cell debris. All media were analyzed as described below.

2.2.6 FPLC Separation of Different Lipoprotein Fractions

The medium from five 6-well plates (30 wells) were pooled and concentrated down to 2 ml with Amicon 10K filter units. The samples were immediately loaded on calibrated Superdex 200 columns connected in series with a total bed volume of approximately 400 ml and void volume of 100 ml. The columns were standardized with a mixture of high and low molecular weight markers of known Stokes' diameters (Amersham Pharmacia Biotech). Samples were passed down the columns at a flow rate of 0.1 ml per min, and 5-ml fractions were collected. The VLDL and LDL component of the samples appeared in the void volume (fractions 9-12) on these columns. Large size $HDL_{2/3}$ particles were localized in fractions 15-23 (12.1-8.2 nm), and smaller VHDL fractions containing albumin were in fractions 24-28 (≤ 7.1 nm diameter) [236]. The concentration of newly secreted apoA-I and their associated ^3H-cholesterol content in the different lipoprotein fractions were analyzed as described below.

2.2.7 Immunoblot Analysis for ApoA-I in the Different Lipoprotein Fractions

Aliquots (100 μl) from each fraction separated by FPLC were analyzed for apoA-I by slot blot apparatus (BioRad Bio-Dot SF unit) analysis. The nitrocellulose was probed with the biotinylated monoclonal antibodies directed against human apoA-I [a combination of 4H1 (against the extreme N terminus) and 5F6 (against the central region)]. The presence of

the apoA-I proteins in each of the FPLC fractions was detected by chemiluminescence following incubation with streptavidin-conjugated horseradish peroxidase. In other experiments, the presence of secreted apoB in apoA-I-containing complex was analyzed by slot blot as with apoA-I after immunoprecipitation with anti-human apoA-I. The protein of the immunoprecipitate was separated from the beads by 0.5mM citric acid (pH 3.5) and neutralized with 10N NaOH, and loaded directly to slot blot apparatus. The nitrocellulose membrane was probed with anti-murine apoB from mouse (generous gift from Dr. Ross Mile, University of Ottawa Heart Institute, Ottawa, ON). Proteins were visualized by chemiluminescence following incubation with anti-mouse IgG coupled to HRP. All proteins were quantified by densitometric scanning (BioRad software, Quantity One, version 4.11).

2.2.8 Immunoprecipitation of HapoA-I Associated Cholesterol and Choline-Phospholipid

ApoA-I from hepatocytes was immunoprecipitated under conditions either directly from the efflux media or from lipoprotein fractions separated by FPLC. The immunoprecipitations were carried out with a polyclonal anti-human apoA-I antiserum from sheep followed by protein G-Sepharose. An equal volume of an anti-human apoB antiserum from sheep, which does not cross-react with murine apoB, or the efflux media from time 0 that immunoprecipitated with anti-human apoA-I antiserum from sheep, were used as a background control. The immunoprecipitates were collected following centrifugation (10 min at 3000 x g) and washed three times with 10 ml phosphate-buffered saline (PBS) without detergents and resuspended in a final volume of 1 ml of PBS. These immunoprecipitates were subjected directly to scintillation counting to quantify the specific association of radio labeled lipids with apoA-I. For analysis of secreted murine apoB- and apoE-associated cholesterol, efflux media was immunoprecipitated either with anti-murine apoB or anti-

murine apoE polyclonal antibodies from rabbit, an equal volume of efflux media that was immunoprecipitated with anti-rabbit serum (a generous gift from Dr. Zemin Yao, University of Ottawa Heart Institute, Ottawa, ON) was used as a background control. The specific association of cholesterol with apoB or apoE was quantified by scintillation counting. All results were obtained after subtraction by the background control counting.

2.3 Results

Previous work from our laboratory had provided evidence for the phospholipidation of apoA-I during secretion and post-secretion at the cell surface, and shown that most but not all (80%) of this phospholipidation is ABCA1-dependent [67]. Here, my first objective was to compare the kinetics of cholesterol acquisition by apoA-I in relation to that of phospholipid acquisition. Whereas ^{3}H-choline labeling allows uniform labeling of phosphatidylcholine and sphingomyelin in cellular membranes, labeling of cholesterol pools varies with methods used to deliver the labeled cholesterol or its precursors. Therefore, I have first evaluated the distribution of ^{3}H-cholesterol in several models of cellular cholesterol labeling.

2.3.1 Quantification of Different Cellular Pools of ^{3}H-Cholesterol by Accessibility of Cholesterol to mβCD Extraction at Different Temperatures

At present, estimates of the percentage of cellular cholesterol distribution have not been very well characterized. The precise lipid composition of the plasma membrane and intracellular compartments has been a source of controversy because not only the methodologies used were different, but also the cell types studied were not the same. In order to quantify the distribution of ^{3}H-cholesterol in the different cellular compartments as a function of labeling method, hepatocytes were labeled with ^{3}H-mevalonate or ^{3}H-cholesterol delivered by LDL or ^{3}H-cholesterol delivered by mβCD. Using the accessibility of ^{3}H-cholesterol to mβCD extraction (20mM) at 37^{o}C and 4^{o}C, I estimated the labeling of different pools: plasma membrane, defined by accessibility at 4^{o}C; recycling endosome

compartments represented by the difference between accessibility at 4°C and at 37°C; and other intracellular pools represented by ^{3}H-cholesterol that is not accessible at 37°C as shown in Figure 2-1.

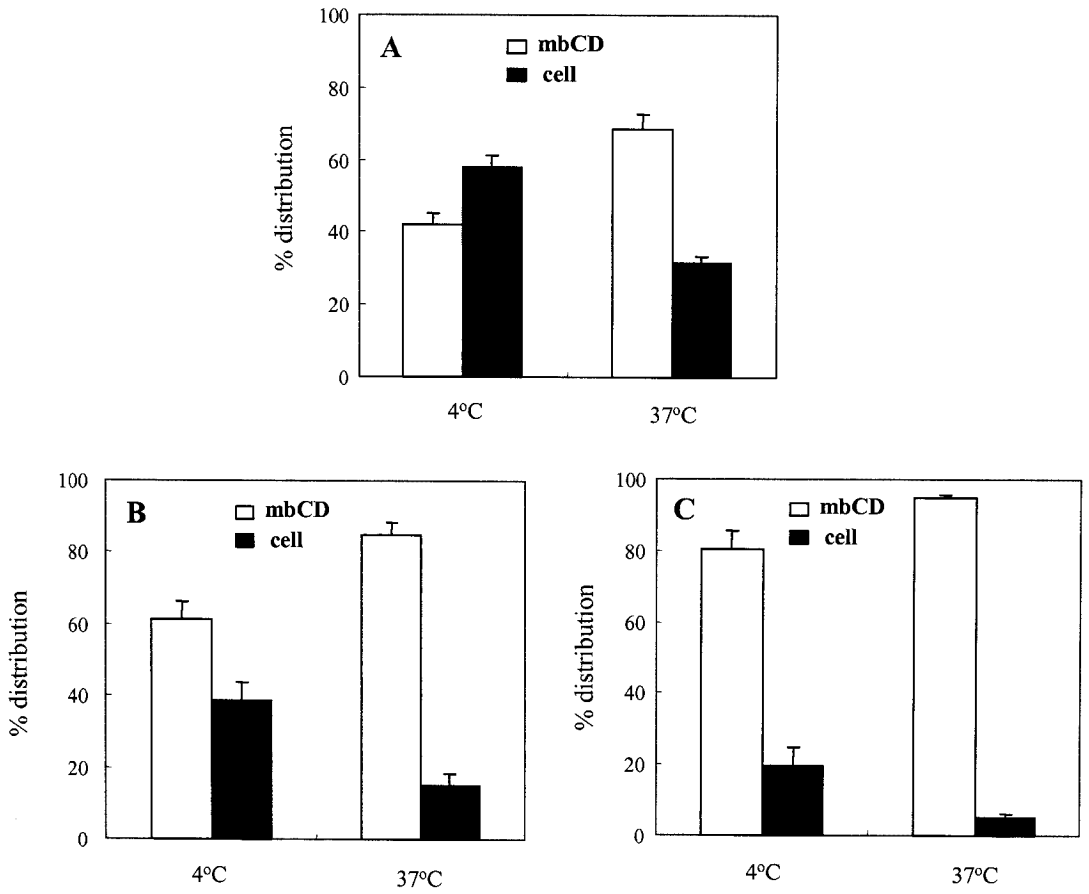

Figure 2-1: Accessibility of ^{3}H-cholesterol to mbCD Extraction at Different Temperatures

Hepatocytes from C57B mice were isolated and labeled with ^{3}H-mevalonate or ^{3}H-cholesterol-LDL for 40 h or ^{3}H-cholesterol-mβCD for 3 h in HepatoZYME-SFM as described in detail under Experimental Procedures. Cells were treated with 20 mM mβCD at either 37°C for 15 min, or 4°C for 15 min following cooling down at 4°C for 1 h. The radioactivity associated with mβCD in the media (mβCD) and the cells (cell) were quantified by scintillation counting. Panel A: cells were labeled with ^{3}H-mevalonate, Panel B: cells were labeled with ^{3}H-LDL-cholesterol, Panel C: cells were labeled with ^{3}H-mβCD-cholesterol. Results are presented as the mean values for three separated experiments (± SD).

Lower concentration of mβCD has been used as a carrier to deliver cholesterol to the cells [243;244], whereas higher concentration extracts cholesterol from the cells [241;245]. Table 2-1 summarized the results of this analysis. MβCD preferentially labeled plasma membrane cholesterol pool at an efficiency of 85%, and delivering only 10 and 5% of ^{3}H-cholesterol in the recycling compartments and other intracellular pools, respectively. In contrast, newly synthesized cholesterol derived from ^{3}H-mevalonate preferentially and predominantly labeled intracellular compartments (58% of the ^{3}H-cholesterol). ^{3}H-cholesterol derived from LDL labeled 61% on the plasma membrane and 39% in the intracellular compartments.

	Plasma membrane	**Recycling endosome compartments**	**Other intracellular pools**
^{3}H-mevalonate	42±3%	27±2%	31±2%
^{3}H-cholesterol-LDL	61±5%	24±3%	15±4%
^{3}H-cholesterol-mβCD	85±5%	10±3%	5±1%

Table 2-1: Quantification of Different Cellular Pools of ^{3}H-cholesterol in C57B Control Hepatocytes

Hepatocytes from C57B mice were isolated, labelled and treated as described in Figure 2-1. Results from three separate experiments are summarized in this table.

2.3.2 Time Course of Acquisition of de novo Synthesized Cholesterol and Phospholipid by Newly Synthesized HapoA-I in ABCA1+/+ and ABCA1-/- Hepatocytes

To compare the kinetics of cholesterol and phospholipid acquisition by apoA-I during secretion, primary hepatocytes from ABCA1-wild type and -null mice were isolated and

incubated with ^{3}H-mevalonate or ^{3}H-choline for labeling of endogenously synthesized cholesterol and phospholipid, respectively. ApoA-I was expressed in the cells by adenoviral-vector gene transfer for 24 h and secretion was monitored for various times after an 1 h equilibration period in non-labeled HepatoZYME-SFM. Media collected at each time point were immunoprecipitated with anti-human apoA-I to quantify the radio labeled lipids specifically associated with apoA-I as showing in Figure 2-2.

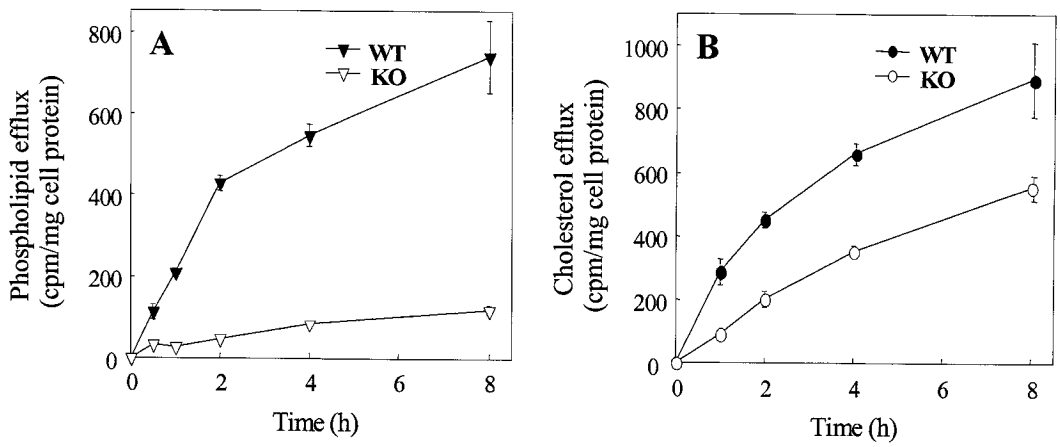

Figure 2-2 :Comparison of Acquisition of *de novo* Synthesized Phospholipid and Cholesterol by Newly Secreted apoA-I in ABCA1 +/+ and ABCA1 -/- Hepatocytes

Hepatocytes from ABCA1-wild type and ABCA1-null mice were labeled with either 10 μCi / ml ^{3}H-mevalonate or 5 μCi / ml ^{3}H-choline after 5 h initial plating, and infected with Ad AI as described in detail under Experimental Procedures. Cells were equilibrated in non-labeled media for 1 h, efflux was then carried out in non-labeled HepatoZYME-SFM. Media containing newly secreted apoA-I was collected at each time point and immunoprecipitated with anti-human apoA-I, then subjected to scintillation counting. All of the values shown were determined by subtracting the background value of time 0 or the value from immunoprecipitation with anti-human apoB. Newly secreted apoA-I associated with ^{3}H-choline-phospholipid (A), ^{3}H-mevalonate derived cholesterol (B) in ABCA1-wild type hepatocytes was compared to ABCA1-null hepatocytes. Results presented here were representatives of at least three separate experiments (± SD).

In ABCA1 wild type hepatocytes, the acquisition by apoA-I of newly synthesized phospholipid (Fig. 2-2A) and sterols (Fig. 2-2B) increased in parallel with time, indicating that apoA-I was secreted with both lipids, even though the results did not define where apoA-I acquires these lipids, intracellularly or at the cell surface. Consistent with our previous finding, ABCA1 deficiency resulted in the loss of approximately 80% of phospholipid acquisition by apoA-I at both 4 h and 8 h time points. In contrast, ABCA1 deficiency caused only a loss of 45% and 40% in the acquisition of newly synthesized cholesterol by apoA-I at the respective 4 h and 8 h time points.

2.3.3 Time Course of Acquisition of Exogenous Supplied Cholesterol by Newly Synthesized HapoA-I in ABCA1+/+ and ABCA1-/- Hepatocytes

The hepatocytes were then labeled with exogenously supplied cholesterol derived from LDL and mβCD as showing in Figure 2-3. As what was observed with *de novo* synthesized cholesterol, in ABCA1 wild type hepatocytes, there was a time-dependent increase of exogenously supplied cholesterol transfers to apoA-I. The data also indicated that with all labeling procedures, the acquisition of cholesterol by newly synthesized apoA-I was only decreased by 40 to 60% in ABCA1 deficiency as showed in Figure 2-2B, Figure 2-3A and 2-3B. However, with exogenous cholesterol labeling delivered by LDL and mβCD, which preferentially label plasma membrane, 61% and 85% respectively (Table 2-1), ABCA1 deficiency had no effect on the early time points of apoA-I cholesterol acquisition (Fig. 2-3), a result not seen with mevalonate-derived cholesterol (Fig. 2-2B). This suggests the existence of a pool of cholesterol at the plasma membrane whose transfer to apoA-I is

mostly independent of ABCA1. However, long term incubation showed the existence of a pathway for cholesterol acquisition by secreted apoA-I that is independent of ABCA1 activity. The discrepancy between the rates of cholesterol and phospholipid association with apoA-I (Fig. 2-2 and 2-3) first suggested the possibility of differential access to lipid pools and second of heterogeneity in labeling of cholesterol pools.

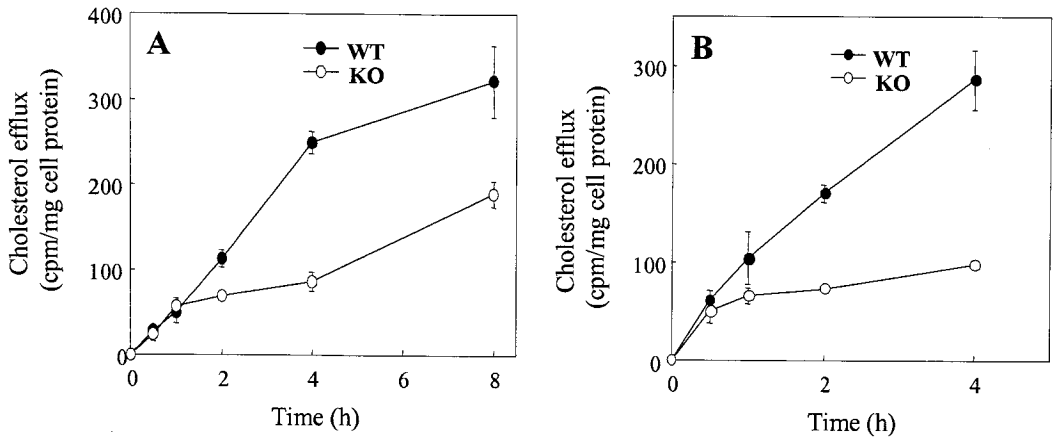

Figure 2-3: Comparison of Acquisition of Exogenously Supplied Cholesterol by Newly Secreted HapoA-I in ABCA1 +/+ and ABCA1 -/- Hepatocytes

Hepatocytes from ABCA1-wild type and ABCA1-null mice were labeled with either 5 μCi/ml ^{3}H-cholesterol-LDL or 5 μCi/ml ^{3}H-cholesterol-mβCD after 5 h initial plating, and infected with Ad AI. Cells were equilibrated for 1 h, efflux was then carried out in non-labeled HepatoZYME-SFM. Media containing newly secreted apoA-I was collected at each time point and immunoprecipitated with anti-human apoA-I, then subjected to scintillation counting. All of the values shown were determined by subtracting the background value of time 0 or the value from immunoprecipitation with anti-human apoB. Newly secreted apoA-I associated with ^{3}H-cholesterol-LDL (A), with ^{3}H-cholesterol-mβCD (B) in ABCA1-wild type hepatocytes were compared to ABCA1-null hepatocytes. Results presented here were representatives of at least three separate experiments (± SD).

2.3.4 *Time Course of Acquisition of Cholesterol and Phospholipid by Exogenous HapoA-I (exo-apoA-I) in ABCA1 +/+ and ABCA1-/- Hepatocytes*

To further evaluate the cholesterol acquisition by apoA-I after its secretion at the cell surface by interaction with the plasma membrane and the recycling compartments, apoA-I was added exogenously to the cells. As above, the hepatocytes from ABCA1+/+ and ABCA1-/- mice were labeled with ^{3}H-choline, ^{3}H-mevalonate, ^{3}H-cholesterol–LDL, or ^{3}H-cholesterol-mβCD. The cells were infected with Ad Luc to correct for independent effects of adenovectors and allow comparison with the endogenous apoA-I synthesis experiments. Exogenous apoA-I was added during the efflux period and the specific association of lipids was determined by immunoprecipitation as showing in Figure 2-4. The data clearly showed that the hepatocytes also contribute to apoA-I cholesterol acquisition at the cell surface: there was a similar time-dependent transfer of cholesterol derived from all labeling methods (Fig. 2-4B, 2-4C and 2-4D) to exogenous apoA-I in wild type hepatocytes. Consistent with our previous observations [67] and in parallel to cholesterol, exogenous apoA-I similarly acquired phospholipid in a time dependent manner in ABCA1 wild type hepatocytes (Figure 2-4A).

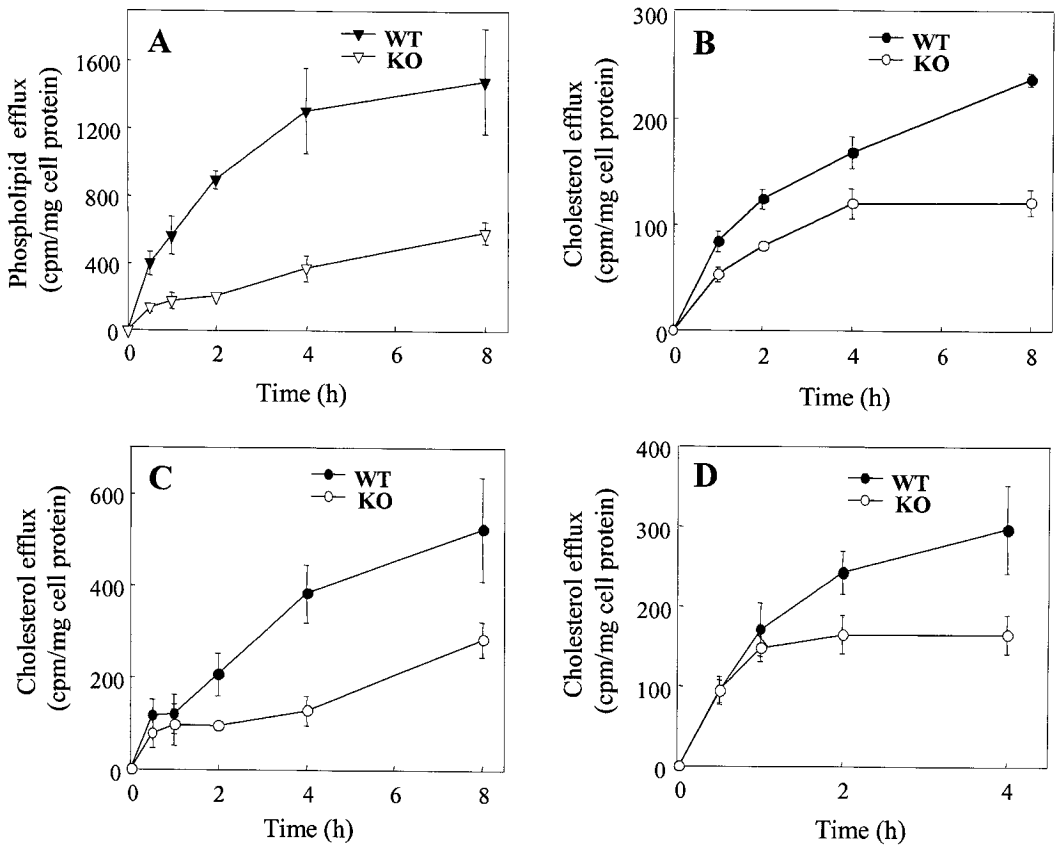

Figure 2-4 Comparison of Acquisition of Phospholipid and Cholesterol by Exogenous HapoA-I in ABCA1 +/+ and ABCA1 -/- Hepatocytes

ABCA1-wild type and -null hepatocytes were isolated and labeled as with Figure 2-2 and Figure 2-3. Cells were infected with Ad Luc for 1 h on the second day, and then switched to the original labeling media for another 24 h. The third day, after 1 h equilibration, efflux was monitored in the presence of 5 μg of hapoA-I in non-labeled HepatoZYME-SFM. Media was collected at each time point and analyzed in the same manner. All of the values shown were determined by subtracting the background value of time 0 or the value from immunoprecipitation with anti-human apoB. Exogenous apoA-I associated with ^{3}H-choline-phospholipid (A), ^{3}H-mevalonate derived cholesterol(B), ^{3}H-cholesterol-LDL (C), and ^{3}H-cholesterol-mβCD (D) in ABCA1-wild type hepatocytes were compared to ABCA1-null hepatocytes. The results presented are representative of three independent experiments (± SD).

As with newly synthesized apoA-I, ABCA1 deficiency had no effect on the early time points of exogenous apoA-I acquisition of exogenously supplied cholesterol (Fig. 2-4C and 2-4D). This was not seen for the endogenously synthesized cholesterol that was derived from mevalonate (Fig. 2-4B) or for the endogenously synthesized phospholipid (Fig. 2-4A).

Again, ABCA1 deficiency had significantly less effect on apoA-I lipidation by cholesterol, which was decreased by 25 to 50 % depending on the labeling methods used, whereas in the case of phospholipid, the decrease was by 75%.

2.3.5 *Effect of ABCA1 Deficiency on Distribution of HapoA-I and Cholesterol in the Lipoprotein Fractions Separated by FPLC*

To evaluate the size distribution of apoA-I-containing lipoproteins secreted by the hepatocytes expressing hapoA-I, 4 h efflux media were collected, concentrated and fractionated by FPLC. Fractions corresponding to VLDL/LDL, HDL and VHDL were collected based on the distribution of markers (murine apoB and human apoA-I). The concentration of immunoreactive human apoA-I in these fractions was quantified by slot blot as shown in Figure 2-5A. In ABCA1 +/+ hepatocytes, there was a large amount of secreted apoA-I associated with the HDL fraction and a smaller but significant proportion was found in the VLDL/LDL fraction. These results are consistent with previous observations in monkey hepatocytes [246]. In ABCA1 -/- hepatocytes, there was a significant decrease of apoA-I associated with the HDL fraction, and a concomitant increase in the VHDL fraction compared with control hepatocytes. However, large proportions of the total apoA-I were still associated with the VLDL/LDL and HDL fractions in ABCA1 deficiency.

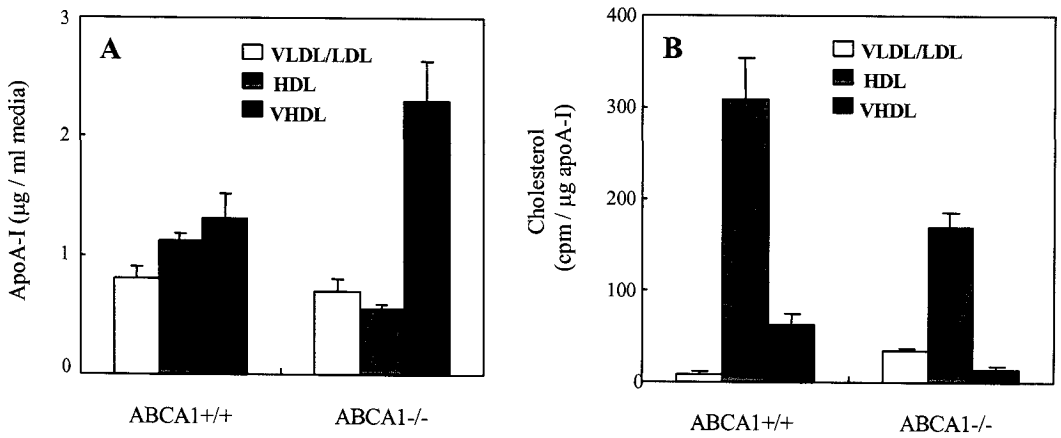

Figure 2-5: HapoA-I and ^{3}H-cholesterol Content in HDL Fractions are Reduced in ABCA1 Deficiency

ABCA1-wild type and -null hepatocytes were labeled with ^{3}H-cholesterol-LDL. Cells were infected with Ad AI. Four-hour efflux was carried out in non-labeled HepatoZYME-SFM. Panel A. The efflux media was pooled and concentrated down to 2 ml and immediately loaded on calibrated Superdex 200 columns for separation of different lipoprotein fractions. Aliquots from each fraction were analyzed for apoA-I by slot blot following transfer to nitrocellulose and quantified by densitometric scanning. The absolute concentration (µg apoA-I/mL media) of apoA-I in the VLDL/LDL, HDL, and VHDL lipoprotein fractions is compared between ABCA1-wild type and -null hepatocytes. Panel B. Aliquots from each fraction separated by FPLC were immunoprecipitated with anti-human apoA-I and quantified by scintillation counting. The association of ^{3}H-cholesterol with apoA-I in the VLDL/LDL, HDL, and VHDL lipoprotein fractions (cpm/µg apoA-I) is compared between ABCA1-wild type and -null hepatocytes. Results are presented as the mean value for three separate experiments (± SD).

Quantification of ^{3}H-cholesterol associated with immunoprecipitated apoA-I in the different lipoprotein fractions (Fig. 2-5B) showed that most cholesterol was found in the HDL size fraction, demonstrating the secretion of cholesterol-containing and large apoA-I-lipoproteins from wild type hepatocytes. This result provides further support for the secretion of cholesterol lipidated apoA-I from hepatocytes. Noteworthy was the significant proportion of labeled cholesterol associated with VHDL apoA-I in ABCA1 +/+ hepatocytes, a fraction representing the small preβ-apoA-I lipoproteins. Although the ^{3}H-cholesterol content

decreased significantly in both HDL and VHDL fractions in ABCA1-deficient hepatocytes, it is important to note that the HDL secreted by ABCA1 -/- hepatocytes still contained a significant amount of labeled cholesterol (Fig. 2-5B). This result suggests that in ABCA1 deficiency, the net levels of HDL formed by apoA-I decreased, but the nature of lipoproteins and/or the full spectrum of the lipoprotein particles formed is not affected by ABCA1 deficiency.

2.3.6 Respective Contributions of ApoA-I, ApoB and ApoE to Export of Cholesterol

The increase in apoA-I-associated ^{3}H-cholesterol in the VLDL/LDL fraction in ABCA1 -/- compared with control hepatocytes (Fig. 2-5B) is in agreement with the recent reports of Sahoo *et al*, [227] and Sniderman *et al.* [247], which demonstrate a regulatory pool of cholesterol in hepatocytes that is shared for the formation of both VLDL and HDL. In order to evaluate the respective contribution of apoB to cholesterol export and delineate the phenomenon of increased apoA-I association with ^{3}H-cholesterol in VLDL/LDL fraction by ABCA1 deficiency, I labeled control hepatocytes with ^{3}H-cholesterol-LDL, and infected cells with Ad AI. Immunoprecipitations of the total 4 h efflux media with either anti-human apoA-I or anti-murine apoB or anti-murine apoE showed as expected that apoB-containing lipoproteins were major exporters of cholesterol followed by apoA-I and apoE as shown in Figure 2-6A. The immunoprecipitates obtained with anti-apoA-I were analyzed by slot blot assays with anti-murine apoB (Fig. 2-6B), which demonstrated the presence of murine apoB in the anti-apoA-I immunoprecipitate. This reflected the secretion of lipoproteins containing both apolipoproteins in both control and ABCA1-null hepatocytes.

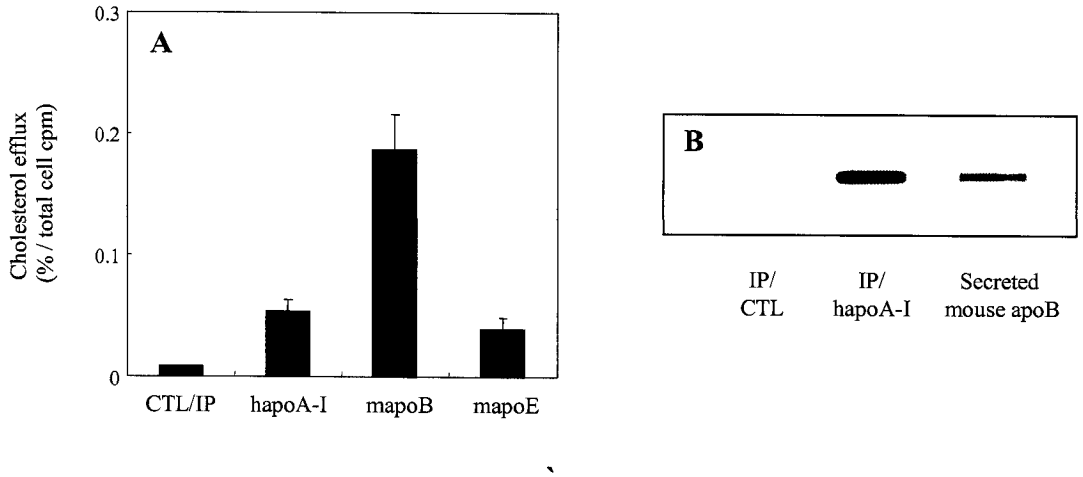

Figure 2-6: Respective Contributions of ApoA-I, ApoB and ApoE to Export of Cholesterol

ABCA1-wild type hepatocytes were isolated and labeled with ^{3}H-cholesterol-LDL. Cells were infected with Ad AI. Panel A. ApoA-I, apoB and apoE acquisition of cholesterol. Four-hour efflux media were immunoprecipitated with anti-human apoA-I, anti-murine apoB or apoE, and counted. Panel B. Immunoblot analysis of murine apoB. Four-hour efflux media was immunoprecipitated with control, non-immune antibody or anti-human apoA-I antibody. As a positive control, the media was probed for secretion of mouse apoB.

Taken together, all results demonstrated here agree with a function of hepatocytes in secretion of cholesterol lipidated apoA-I and the major role of hepatic ABCA1 in the cholesterol lipidation of apoA-I. However, the results also support the existence of an ABCA1-independent apoA-I cholesterol acquisition pathway.

2.4 Discussion

The liver is the major site of both apoA-I synthesis and ABCA1 expression. Our previous studies have provided evidence for the acquisition of phospholipid by apoA-I during secretion and post-secretion at the hepatocyte surface, and 80% of this phospholipidation is ABCA1-dependent. My first objective was to evaluate cholesterol acquisition by newly synthesized and post-secreted-apoA-I in relation to phospholipid acquisition, and to identify the role of hepatic ABCA1 in apoA-I cholesterol acquisition.

At present, the working model for cellular cholesterol distribution has been classified into plasma membrane, recycling endosomes, and other intracellular (including newly synthesized cholesterol) compartments. Estimates of the distribution of cellular cholesterol in these compartments however have not been well characterized. Similarly, after many years of intracellular cholesterol trafficking studies, establishment of precise methodologies for labeling specific cellular cholesterol pools have not been well documented. This is largely due to the different cell types used and the specific objectives proposed in the studies. To allow a complete study of cellular cholesterol acquisition by apoA-I, the distribution of the cellular pools of labeled cholesterol in relation to the methods for labeling has been carefully analyzed. A simplified model for cellular cholesterol distribution classified into plasma membrane, recycling endosomes, and other intracellular pools has been estimated by differential accessibility to mβCD (Fig. 2-1, Table 2-1). Whereas newly synthesized sterols predominantly associate (58%) with intracellular compartments that are not accessible to mβCD at 4°C, a majority of the exogenously delivered cholesterol remains associated with the plasma membrane (61% and 85% for ^{3}H-cholesterol-LDL and ^{3}H-cholesterol-mβCD,

respectively). These results are in good general agreement with the studies from van Meer and Lange et al. [248;249] demonstrating cellular cholesterol in the plasma membrane range from 40% to 90% depended on the methodologies used.

I have shown here that hepatocytes secrete a lipidated apoA-I complex or particle that contains both phospholipid and cholesterol. In wild type hepatocytes, cholesterol and phospholipid association with endo- and exo-apoA-I increases in parallel with time (Fig. 2-2, 2-3 and 2-4). However, the results also infer that newly synthesized apoA-I acquires mostly intracellular cholesterol, because the endogenous apoA-I acquires more newly synthesized cholesterol than exogenously added apoA-I (comparing Fig. 2-2B and 2-4B), whereas endogenous and exogenous apoA-I acquire the same level of exogenously delivered cholesterol (comparing Fig. 2-3A and 2-4C, and Fig. 2-3B and 2-4D). Therefore, in the hepatocyte, newly synthesized apoA-I acquires cholesterol molecules during secretion, primarily intracellularly and secondarily at the cell surface. This contrasts with exogenous apoA-I, which as expected acquires cholesterol mostly at the cell surface. Thus, the same pool of cell surface cholesterol (located at the plasma membrane and the recycling endosome compartments), whose efflux is mediated by ABCA1 [217;250;251] appears to be used by both endogenous and exogenous apoA-I.

In ABCA1 deficiency, the hepatocytes also secrete lipidated apoA-I particles that contain both phospholipid and cholesterol, but the proportion of lipidated apoA-I is decreased (Fig. 2-5). In keeping with our previous observations [67], the ABCA1 -/- hepatocytes secrete apoA-I with about 80% less phospholipid (Fig. 2-2A), but only 40% less newly synthesized cholesterol (Fig. 2-2B). This surprising result is corroborated by the similar decreases observed with exogenously delivered cholesterol, which range from 50 to 60% (Fig. 2-3A and 2-3B). A similar picture also merges for the cholesterol acquisition by

exogenous apoA-I (Fig. 2-4). It must be therefore concluded that cholesterol transfer to apoA-I can occur independent of ABCA1 activity. In the absence of ABCA1, the remaining basal phospholipid transfer activity at about 20% of control is sufficient to elicit 50% or more of the normal cholesterol transfer. The mechanisms for cholesterol transfer under these conditions remain unclear, but diffusional transfer and/or microsolubillization may take place driven by the secretion and formation of an apoA-I/phospholipid complex [181;252;253].

Studies of ABCA1 function in transgenic mice generated by Vaisman *et al.* group had demonstrated overexpression of human ABCA1 in only the liver and macrophages significantly raised plasma HDL-C level [254]. Since the liver is a major site of ABCA1 gene expression [155;231] and macrophage ABCA1 has been recently shown to make only a minor contribution to plasma HDL level, these data implied that hepatic ABCA1 may be an important source for plasma HDL-C levels. These current, as well previous studies agreed with this observation that hepatic ABCA1 does contribute significantly to apoA-I lipidation and HDL formation. This assertion was reinforced by FPLC analysis of the effect of ABCA1 on the distribution of apoA-I in different lipoprotein fractions and on their respective content of ^{3}H-cholesterol as shown in Fig. 2-5. In consistent with other studies [145;255-258], my results show that in our study model, ABCA1 deficiency did not affect the net secretion of the total apoA-I expressed by the adenoviral construct compared to control hepatocytes (Fig. 2-5A). ABCA1 deficiency reduced the proportion of apoA-I in HDL fraction significantly and concomitantly increased the proportion of apoA-I in VHDL fraction, and reduced cholesterol content in both HDL, VHDL fractions (Fig. 2-5B), as well the total ^{3}H-cholesterol released from hepatocytes. However, in support to the results from the kinetic studies, the data also indicated the existence of an ABCA1-independent apoA-I cholesterol acquisition pathway as shown in ABCA1 deficiency, a large proportion of apoA-I

was still associated with HDL fraction, in addition, both HDL and VHDL fraction still contained a significant amount of ^{3}H-cholesterol. These results suggest that in ABCA1 deficiency, the net levels of HDL formed by apoA-I decreased, but the nature of lipoproteins and/or the full spectrum of the lipoprotein particles formed is not affected by ABCA1 deficiency.

It is well defined that in addition to secrete apoA-I, hepatocytes also secrete apoB-containing particles, such as VLDL/LDL. In this study, I also observed a significant amount of apoA-I was associated with VLDL/LDL fraction in control hepatocytes, in keeping with earlier reports of apoA-I association with newly synthesized VLDL [259;260]. I corroborated these observations with immunoprecipitation of newly secreted lipoproteins with antibodies to human apoA-I, and with slot blot analysis of the immunoprecipitates with anti-murine apoB (Fig. 2-6). Furthermore, in ABCA1 deficiency, the amount of ^{3}H-cholesterol associated with VLDL/LDL fraction was increased compared to wild type hepatocytes (Fig, 2-5B). This is in agreement with recent reports from Sahoo *et al*, [227] and Sniderman *et al.* [247], demonstrating a regulatory pool of cholesterol in hepatocytes that is shared for formation of both VLDL and HDL. Previous studies have shown that inhibition of HMG-CoA reductase also inhibited VLDL secretion in cultured hepatocytes [261;262], suggesting that cholesterol availability is a key determinant of VLDL secretion, and the depletion of the regulatory pool of cholesterol by apoA-I made less cholesterol available for VLDL synthesis and secretion [227;247]. It is apparently that results from the effects of ABCA1 activity on the metabolism of apoB-containing lipoproteins are controversy (see review by Charles Joyce *et al.* [263]. McNeish *et al.* and Schaefer *et al.* reported that VLDL-C and LDL-C are reduced in ABCA1 deficiency [264;265]. However, in the Schaefer *et al.* study [265], they concluded that the reduced concentrations of LDL in TD patients are due to hypercatabolism of abnormal LDL

particles, secondary to the inability to efflux cholesterol to HDL, the lack of HDL in the plasma prohibits the normal transfer of CE from HDL to LDL via CETP, generating cholesterol-poor, triglyceride-rich LDL that are rapidly catabolized. In contrast, other studies from Orso *et al.* [216] and Poernama *et al* [266] did not observe the decrease of VLDL-C and LDL-C in ABCA1 deficiency. Further more, Wellington *et al.* concluded from their study that ABCA1-dependent HDL formation by the liver might decrease VLDL particle secretion [267]. These results all provided evidence that in hepatocytes, where apoB-containing particles are also secreted, both apoA-I-containing HDL and apoB-containing VLDL/LDL share the same regulatory pool of cholesterol. Therefore, secretion of cholesterol in apoB-containing particles would compensate the lack of ABCA1-dependent apoA-I cholesterol removal. In addition to apoB, hepatocytes also secrete apoE, but the mechanism of apoE lipid acquisition remains unclear.

CHAPTER 3: IDENTIFICATION OF THE ORIGIN OF CHOLESTEROL POOLS ACQUISITION BY HUMAN APOA-I

3.1 Summary

The difference in kinetics of apoA-I lipidation by cholesterol derived from endogenous synthesis or from exogenous delivery suggested heterogeneity in origin and transfer from cholesterol pools. To identify the contribution of other pathways to the cholesterol lipidation of apoA-I, and to define the pools of cholesterol transferred to apoA-I during secretion, hepatocytes were labeled with either ^{3}H-cholesterol-LDL or ^{3}H-mevalonate or ^{3}H-choline, lipid association with apoA-I was analyzed under conditions where specific pathways are inhibited.

When progesterone was added to the cells during 4 h efflux period, it did not affect apoA-I synthesis and secretion. However, both *de novo* synthesized and exogenously supplied cholesterol acquisition by apoA-I was decreased significantly in wild type and ABCA1-null hepatocytes, indicating the existence of an ABCA1-independent but progesterone-sensitive pathway for apoA-I cholesterol acquisition. When plasma membrane cholesterol was depleted by treatment of mβCD prior to progesterone addition, cholesterol acquisition by apoA-I was further decreased in control hepatocytes, suggesting a significant amount of cholesterol is acquired by apoA-I at the plasma membrane level. Consistent with kinetic studies, progesterone pre-treatment (1 h immediately after removal of labelling media and before the start of the efflux assay) experiments indicate the heterogeneity of intracellular cholesterol acquisition by apoA-I. Progesterone however did not inhibit apoA-I phospholipidation neither in wild type nor ABCA1-null hepatocytes. Furthermore, both brefeldin A and monensin inhibited newly synthesized cholesterol acquisition by exo-apoA-I

significantly, suggesting vesicular transport of cholesterol plays an important role in mediating apoA-I cholesterol lipidation.

In conclusion, the results indicate that the active transport of intracellular cholesterol is crucial for mediating apoA-I acquisition. It occurs by both ABCA1-dependent and -independent pathways, sensitive to progesterone. My studies also demonstrate the functional separation of phospholipidation and cholesterol lipidation of apoA-I in mouse primary hepatocytes [179;235].

3.2 Experimental Procedures

3.2.1 Materials

In addition to the Materials described in Chapter 2, progesterone (4-pregnene-3, 20-dione), brefeldin A, monensin and other reagents were obtained from Sigma (Oakville, ON).

3.2.2 Animals and Primary Hepatocyte Cultures

Mice that were used in this section were C57BL/6J control, ABCA1-wild type and -null mice. Primary hepatocyte cultures were prepared same as described in Chapter 2.

3.2.3 Cell Labeling and Adenovirus Infection

Five hour following the initial seeding, the cells were washed in Williams' Medium E without serum (2 × 2 ml) and subsequently labeled with ^{3}H-choline (5 μCi/ml), ^{3}H-mevalonate (10 μCi/ml), or ^{3}H-cholesterol (5 μCi/ml) delivered with 10 μg/ml LDL, and infected with either Ad AI or as control Ad Luc as indicated previously in Chapter 2.

3.2.4 Cholesterol and Phospholipid Efflux Assays

Efflux of cholesterol and phospholipid from hepatocytes were measured over 4 hour after equilibration for an 1 hour period in non-labelled HepatoZYME-SFM on the third day following the labeling and infection as described in Chapter 2. In some experiments, plasma membrane cholesterol was extracted by treatment with 20 mM of mβCD at 4°C for 15 min after an 1 hour cooling down at 4°C for stopping cellular cholesterol trafficking. Ten microgram per ml of progesterone, an inhibitor of cholesterol transport from intracellular to

the plasma membrane [177;178;268;269] or 5 μM brefeldin A (BFA), or 20 μM monensin were added 15 min before and during the efflux period. In other experiments, Progesterone was added during the entire equilibration and the efflux periods. Media was collected and immunoprecipitated with anti-human apoA-I. An equal volume of media from cells infected with Ad Luc was also analyzed as background and was subtracted. The immunoprecipitates were collected and quantified for specific association of labeled lipid with apoA-I same as described in Chapter 2.

3.2.5 Immunoblot and Western Blot Analysis

The concentration of protein was determined by Lowry assay. Equal volume from each sample was separated by 12% SDS-PAGE and transferred to nitrocellulose at 100V for 1 hour. Membranes were probed with biotinylated monoclonal antibodies directed against human apoA-I. The presence of apoA-I was detected by chemiluminescence following incubation with streptavidin-conjugated horseradish peroxidase. Proteins were quantified by densitometric scanning (BioRad software, Quantity One, version 4.11).

3.3 Results

Progesterone has been shown to inhibit cholesterol transport from intracellular compartments to the plasma membrane and in particular, the transport from late endosome and/or lysosome to plasma membrane, and subsequently causes cholesterol accumulation in the late endosome and/or lysosome [177;270]. Therefore, it has been widely used to mimic the Niemann-Pick Type C phenotype, which characterized as defective transport of intracellular cholesterol.

3.3.1 Progesterone did not Affect the Expression and Secretion of HapoA-I from Hepatocytes

Hepatocytes were infected with Ad AI for 1 h and incubated for 24 h as indicated under Experimental Procedures. Progesterone (10 μg/ml) was added 15 min before and during 4 h efflux period. Then the expression and secretion of hapoA-I were analyzed by immunoblot analysis as shown in Figure 3-1.

Immunoblots of cell lysate and media indicated that progesterone inhibited neither apoA-I expression nor its secretion from hepatocytes.

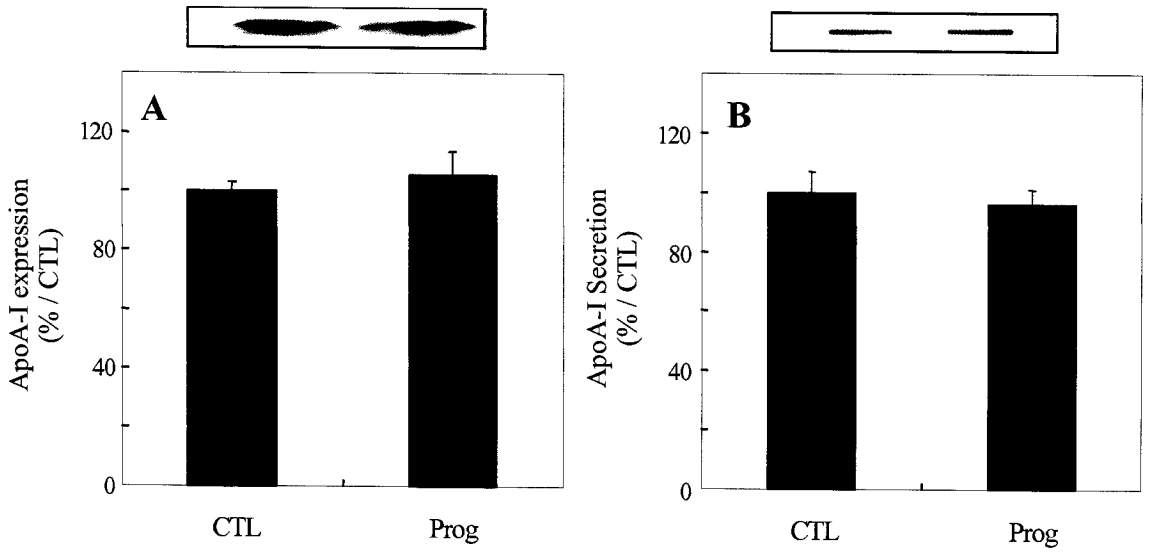

Figure 3-1: Progesterone does not Affect Expression and Secretion of ApoA-I

Hepatocytes from C57B mice were isolated and infected with Ad AI. The 4 h efflux was carried out in the presence (Prog) or absence (CTL) of progesterone (10 μg/ml). Panel A. Effect of progesterone on newly synthesized apoA-I. Hepatocyte cell lysate was analyzed by western blot following transfer to nitrocellulose and quantified. Panel B. Effect of progesterone on the secretion of newly synthesized apoA-I. ApoA-I containing media was analyzed by slot blot and quantified by densitometric scanning.

3.3.2 Progesterone Caused Significant Inhibition of HapoA-I Cholesterol-LDL Acquisition, but not Phospholipid Acquisition in C57B Hepatocytes

C57B hepatocytes were labeled with ^{3}H-cholesterol-LDL or ^{3}H-choline, infected with either Ad A-I or Ad Luc and treated with progesterone as indicated above. Four-hour efflux media was collected and immunoprecipitated for apoA-I. ApoA-I associated cholesterol, corrected for the Ad Luc non-specific effect was significantly decreased after progesterone treatment as showing in Figure 3-2A. When plasma membrane cholesterol was depleted by mβCD prior to progesterone treatment, the cholesterol acquisition with apoA-I was further decreased significantly. With the single mβCD treatment, there was less inhibition effect

compared to the double treatment. These results suggest a significant amount of cholesterol was acquired by apoA-I at the plasma membrane level and perhaps late endosome and/or lysosomal cholesterol is the major source for apoA-I lipidation. In contrast, progesterone did not inhibit apoA-I phospholipid acquisition as indicated in Figure 3-2B. Taken together, these results indicate that acquisition of cholesterol by apoA-I depends on the active transfer from intracellular compartments to plasma membrane via a process apparently dissociable from phospholipid transport.

To test the reversibility of progesterone-caused cholesterol accumulation and subsequent inhibition of apoA-I lipidation, hepatocytes were infected with Ad AI and treated with progesterone for 4 h same as in other experiments. Then cells were washed 3 times and allowed to efflux for another 4 h in the absence of progesterone. I found that apoA-I cholesterol acquisition was totally recovered as shown in Figure 3-2C. This result was consistent with other studies [177], indicating that the effect of progesterone-caused inhibition of cholesterol transport is reversible following the removal of progesterone from cells.

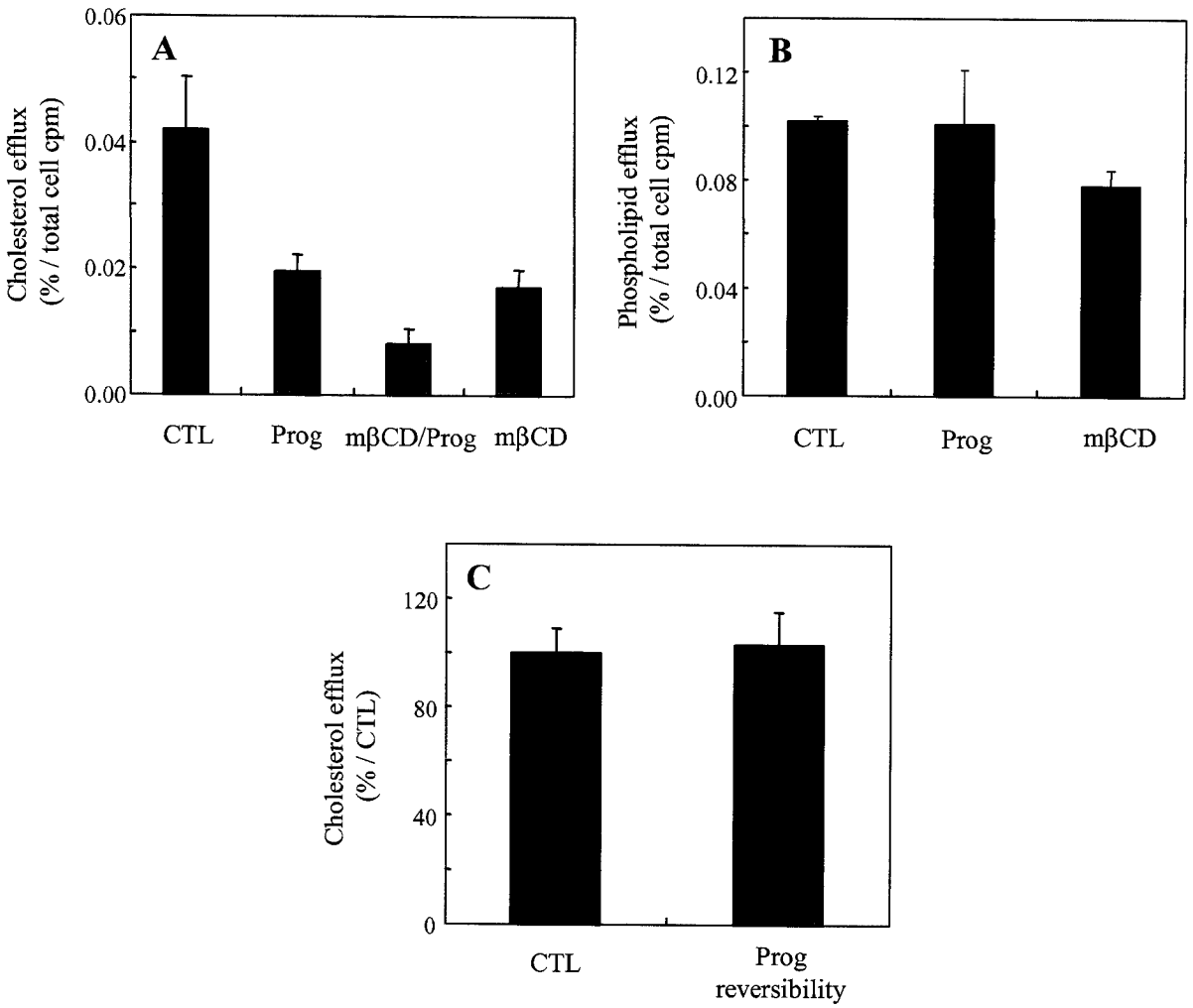

Figure 3-2: Progesterone Significantly Inhibits Cholesterol Acquisition, but not Phospholipidation in C57B Hepatocytes

C57B hepatocytes were labeled with either ^{3}H-cholesterol-LDL or ^{3}H-choline-phospholipid, and infected with Ad AI (or Ad Luc as a control). The 4 h efflux was carried out in the presence or absence of progesterone (10 μg/ml). To determine apoA-I specific lipid association, the background value from cells infected with Ad Luc were subtracted. In some conditions in Panel A and B, cells were pretreated with 20 mM mβCD at 4°C for 15 min before the efflux. Panel A. Effect of progesterone on apoA-I ^{3}H-cholesterol acquisition. Panel B. Effect of progesterone on apoA-I ^{3}H-phospholipid acquisition. Panel C. The reversibility of progesterone induced inhibition of apoA-I cholesterol acquisition was analyzed by removing the progesterone and allowing cells to efflux for another 4 h. Results are presented as the mean values for three separate experiments (± SD).

3.3.3 *Progesterone Inhibited HapoA-I Cholesterol Acquisition in Both ABCA1 +/+ and ABCA1 -/- Hepatocytes*

To define the origin of the cholesterol pool acquisition by apoA-I in the ABCA1-independent pathway, I labeled both ABCA1 +/+ and ABCA1 -/- hepatocytes with either ^{3}H-cholesterol-LDL or ^{3}H-mevalonate. The cells were infected with Ad AI or Ad Luc. The effect of progesterone on the apoA-I cholesterol acquisition was analyzed as described above and the results are showed in Figure 3-3. Progesterone significantly inhibited apoA-I cholesterol acquisition in both wild type and ABCA1-deficient hepatocytes, and this effect was observed with both LDL and mevalonate-derived cholesterol. However it is clear that progesterone had a greater effect on newly synthesized cholesterol secretion (Fig. 3-3B). These results further support the existence of an ABCA1-independent, but progesterone-sensitive pathway for apoA-I cholesterol acquisition in hepatocytes. Again, progesterone did not affect apoA-I phospholipid acquisition in either ABCA1 +/+ or ABCA1 -/- hepatocytes (Figure 3-3C).

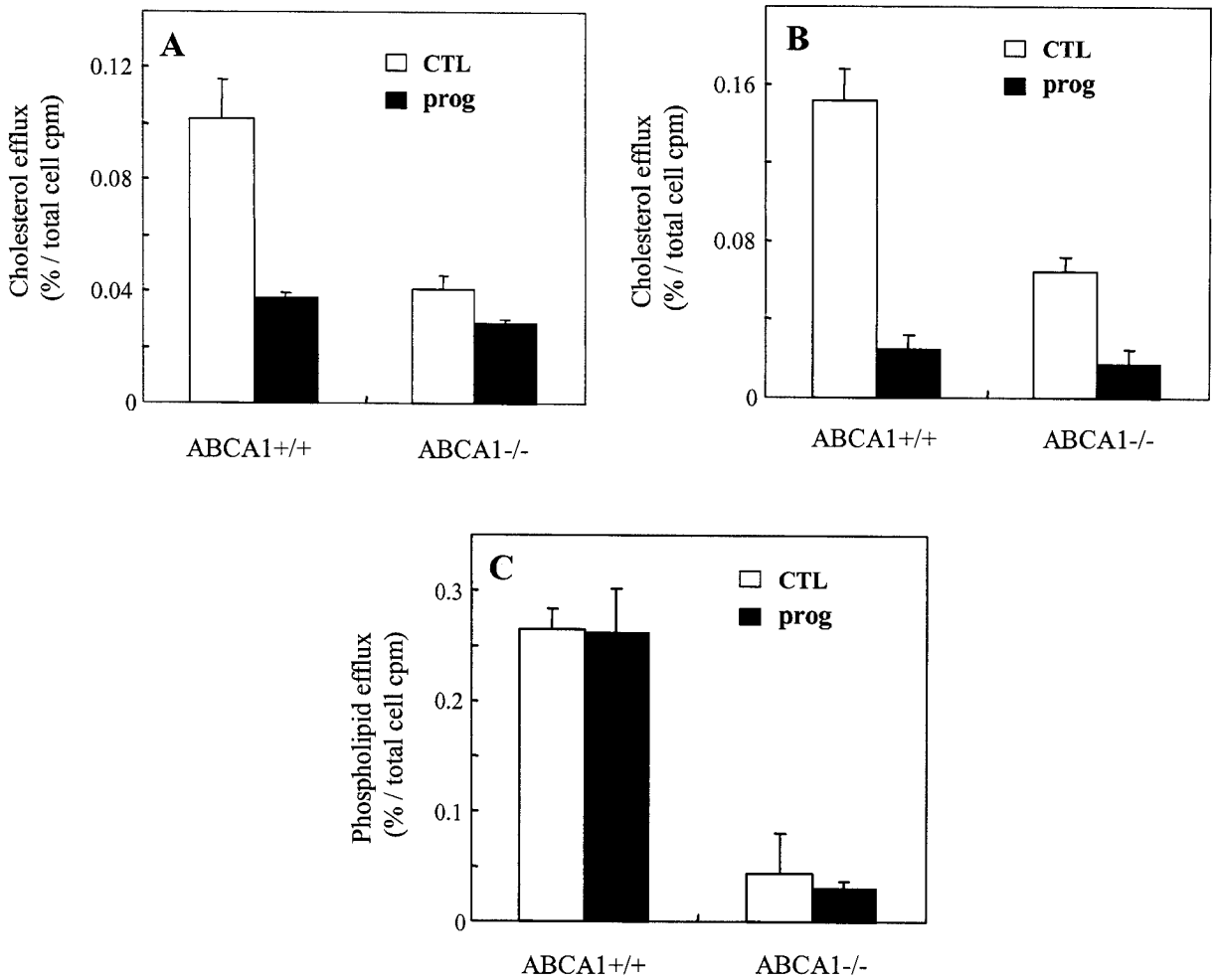

Figure 3-3: Progesterone Inhibits apoA-I Cholesterol Acquisition in Both ABCA1 +/+ and ABCA1 -/- Hepatocytes

Hepatocytes from ABCA1-wild type and -null mice were labeled with ^{3}H-cholesterol-LDL, ^{3}H-mevalonate, or ^{3}H-choline and infected with either Ad AI or Ad Luc. Cells were treated with progesterone and efflux was carried out as described previously. All values shown were determined by subtracting the background value from cells infected with Ad Luc. Panel A. apoA-I acquisition of ^{3}H-cholesterol-LDL. Panel B. apoA-I acquisition of ^{3}H-mevalonate-derived cholesterol. Panel C. apoA-I acquisition of ^{3}H-choline-phospholipid. Results are presented as the mean values for three separate experiments (± SD).

3.3.4 *Effects of Progesterone on the Early and Late Phases of Cholesterol Acquisition by ApoA-I*

To evaluate the sensitivity of early and late phases of cholesterol acquisition by apoA-I to progesterone, I included a progesterone pre-treatment (1h) immediately after the removal of the labeling medium and before the start of the efflux assay (zero time). Under these conditions, the early phase of cholesterol acquisition from ABCA1 -/- cells was markedly reduced by progesterone, and, most interestingly, the residual acquisition (ABCA1 independent) was the same as that from control cells treated with progesterone (Fig. 3-4). At 4 hours, the acquisition from control cells treated with progesterone, but not that from ABCA1 -/- cells increased significantly, demonstrating that ABCA1 acts as a cholesterol transporter independently of a progesterone block. At 8 hours, acquisition from ABCA1 -/- cells started to increase, an effect, which we attribute to the leakiness of the progesterone block with time (Fig. 3-4).

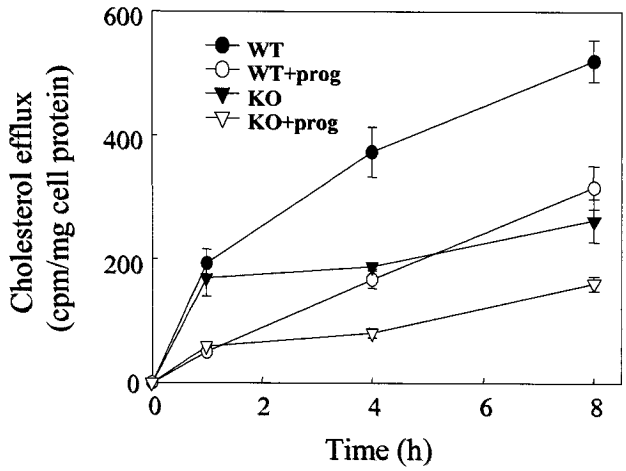

Figure 3-4: Effects of Progesterone on the Early and Late Phases of Cholesterol Acquisition by ApoA-I in ABCA1 +/+ and ABCA1 -/- Hepatocytes

Hepatocytes were labeled with ^{3}H-cholesterol-LDL and infected with Ad AI. Cells were equilibrated for 1 h in the presence of 10 μg / ml of progesterone immediately after removal of labelling media, efflux was then carried out in the presence of progesterone. Media containing apoA-I was collected at each time point and analysized in the same manner. All of the values shown were determined by subtracting the background value.

3.3.5 Both Brefeldin A and Monensin Inhibited Exo-apoA-I Acquisition of ^{3}H-mevalonate in ABCA1 -/- Hepatocytes

Brefeldin A, an antibiotic drug has been shown to inhibit vesicle formation both *in vivo* [271] and *in vitro* [272] by reversibly disassembling of the Golgi complex. Monensin is a sodium ionophore known to alter the structure of the Golgi apparatus [273] and has been shown to be an inhibitor of trans Golgi apparatus function. BFA and monensin have been reported to block both ABCA1-dependent [217] and diffusional efflux [274]. Since I could not evaluate their effects on the lipidation of newly synthesized apoA-I because they would interfere with

the apoA-I secretion, I therefore, tested only their effect on exo-apoA-I cholesterol acquisition in ABCA1 deficient hepatocytes.

Hepatocytes were preincubated with 5 μM BFA and 20 μM monensin for 15 min prior to efflux, and then efflux was conducted in the presence of BFA and monensin, and 5 μg/ml exo-apoA-I for 4 h. The media was collected and analyzed in the same manner as described previously and shown in Figure 3-5. The result clearly demonstrated that both BFA and monensin significantly inhibited exo-apoA-I cholesterol acquisition in ABCA1-deficient hepatocytes. As expected from the literature, the inhibition caused by BFA was reversible, after BFA removal, apoA-I cholesterol acquisition was recovered significantly. This result indicates that vesicular transport of intracellular cholesterol plays an important role in mediating the ABCA1-independent pathway for apoA-I cholesterol acquisition.

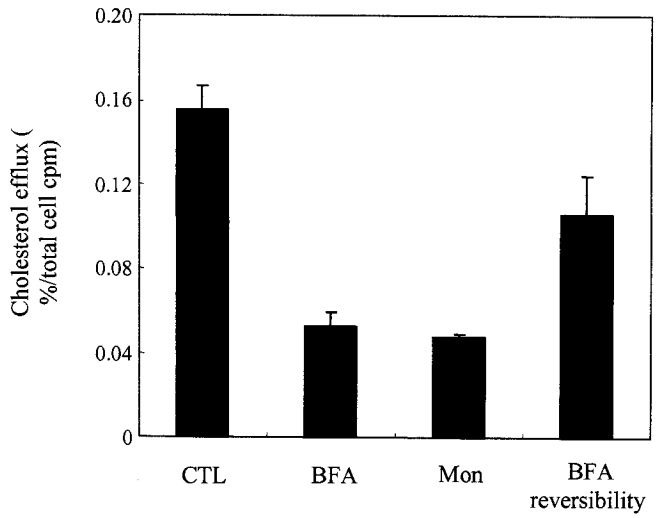

Figure 3-5: Brefeldin A and Monensin Inhibit Exo-apoA-I Acquisition of ^{3}H-mevalonate Cholesterol

ABCA1 -/- hepatocytes were labeled with ^{3}H-mevalonate and infected with Ad Luc. Cells were preincubated with 5 μM brefeldin A or 20 μM monensin for 15 min, 4 h efflux was carried out in the presence of brefeldin A or monensin and 5 μg/ml apoA-I. Efflux media was collected and analyzed. Results are presented as the mean values for three separate experiments (± SD).

3.4 Discussion

One of my objectives was to identify the origin of cholesterol transferred to apoA-I and understand the relationship between cholesterol and phospholipid transfers. For that purpose, I designed the experiment by labeling hepatocytes with ^{3}H-mevalonate, ^{3}H-cholesterol-LDL, or ^{3}H-choline, and looking for apoA-I cholesterol and phospholipid acquisition under conditions where specific pathways were inhibited.

Progesterone has been shown to interrupt cholesterol transport from the endoplasmic reticulum [165] and from the plasma membrane to the ER [178], and transport from late endosomes/lysosomes to the plasma membrane [177;268]. In our study, progesterone affects most apoA-I acquisition of *de novo* synthesized cholesterol (from mevalonate) and somewhat less LDL-derived cholesterol in both wild type and ABCA1-deficient hepatocytes (Fig. 3-3A, B), indicating the existence of an ABCA1-independent but progesterone sensitive pathway for apoA-I cholesterol acquisition. This defines the origin of cholesterol translocated to the cell surface for eventual transfer to apoA-I. However the progesterone effect is partial (about 60% inhibition), and additive to the effect of mβCD, which is compatible with the observation that secreted apoA-I, is lipidated with cholesterol derived from both intracellular and cell surface pools. When the cells are labeled with mevalonate, progesterone inhibits cholesterol transfer to apoA-I by 80% in keeping with its block on transport of newly synthesized cholesterol from ER to plasma membrane [165;275]. Longer pre-treatment of cells with progesterone identifies three control levels for cholesterol acquisition by apoA-I, one dependent on a progesterone-sensitive transport, one progesterone-insensitive but ABCA1 dependent, and finally, one independent of both ABCA1 and progesterone (Fig

3-4). However, Progesterone neither affects phospholipidation of apoA-I in ABCA1-wild type nor ABCA1-null hepatocytes. BFA, as well as monensin, significantly affect exo-apoA-I mevalonate-derived cholesterol acquisition in ABCA1-deficient hepatocytes (Figure 3-5), indicating that vesicular transport of intracellular cholesterol plays an important role in mediating the ABCA1-independent pathway for apoA-I cholesterol lipidation.

My study highlighted the difference between cholesterol and phospholipid acquisition by apoA-I. Previous studies have provided evidence for different transport of cholesterol and phospholipid from the ER to the plasma membrane [276;277]. Cholesterol transport is energy dependent and temperature sensitive, and requires vesicular transport [276], whereas phospholipid transport is not mediated by vesicles and independent of energy and temperature [277]. Other studies from DeGrella *et al.* [162] and Urbani *et al.* [163] also reported that ATP depletion or low temperature inhibit rapid ER to plasma membrane cholesterol transport. Therefore, it is not surprising that apoA-I acquires cholesterol and phospholipid by different mechanisms and/or pathways. Vesicular traffic typically requires an intact cytoskeleton, the tracks for vesicles to move. A recent study also reported the existence of apoA-I-inducible, ABCA1-independent but cytoskeleton-dependent cholesterol removal pathway in Tangier Disease cell lines [278].

CHAPTER 4: CONCLUSIONS

Compelling evidence has approved the inverse relationship between HDL-C levels and the risk of cardiovascular diseases. The high levels of HDL-C are closely associated with a decreased risk of CHD. The liver is the major site of both apoA-I synthesis [233;234] and ABCA1 expression [215;230;232] making it also important in controlling of HDL-C levels. However, to date most studies have only addressed the lipidation of exogenously added apoA-I, which delineates the post secretion apoA-I lipidation, most at the cell surface level and neglects the characterization of *de novo* synthesized apoA-I. In addition, most hepatocyte lipid efflux studies did not use immunoprecipitations, therefore, ignored the fact that hepatocytes also export cholesterol with other lipoproteins. The study of adenovector delivered human apoA-I gene transfer into mouse primary hepatocytes allowed me to indeed, evaluate the role of hepatocyte in mediating newly synthesized apoA-I lipidation and characterize the acquisition of both cholesterol and phospholipid by apoA-I [179;235].

We have previously shown that hepatic apoA-I acquires phospholipid during secretion and post secretion at cell surface, and most of this phospholipidation is ABCA1 dependent [67]. In this work I compared the activity of wild type and ABCA1-null hepatocytes in phospholipid and cholesterol lipidation for both endogenously synthesized and exogenously added apoA-I. I used different cellular cholesterol labeling methods to derive the precise cellular cholesterol pool used for lipidation of apoA-I, and followed the acquisition of lipids as a function of time, analyzing the early and late phases of lipidation. By immunoprecipitation with apoA-I to assure only apoA-I associated lipids were being quantified. My study showed that in wild type hepatocytes, cholesterol and phospholipid acquisition increased in parallel with time by both endogenous and exogenous apoA-I, suggesting simultaneous acquisition of both lipids. However, ABCA1 deficiency decreased apoA-I phospholipidation by 80%, but acquisition of *de novo* synthesized and exogenous

cholesterol only decreased by 40-60%. The transfer of *de novo* synthesized cholesterol to apoA-I was decreased at all time points, but that of exogenously delivered cholesterol was independent of ABCA1 activity at the early time points, suggesting heterogeneity of the cell surface cholesterol pools accessible to ABCA1. FPLC analysis of medium lipoproteins confirmed that with ABCA1 deficiency the proportion of secreted HDL-associated apoA-I and cholesterol decreased by about 50%. The VLDL/LDL size fraction also contained a significant level of cholesterol in ABCA1 deficiency, consistent with the result of immunoprecipitations showing the presence of lipoproteins with both apoA-I and murine apoB. Progesterone did not affect apoA-I synthesis and secretion nor its phospholipidation, but significantly inhibited apoA-I acquisition of cholesterol in both wild type and ABCA1-null hepatocytes, indicating the different controls of phospholipid and cholesterol transfers to apoA-I. ApoA-I lipidation with newly synthesized cholesterol was also significantly decreased by brefeldin A and monensin, suggesting that vesicular transport of cholesterol plays an important role in mediating hepatic apoA-I cholesterol lipidation.

Our previously studies have shown that compared to wild type, ABCA1-deficient hepatocytes secrete fewer but qualitatively similar apoA-I-containing lipoproteins that include large particles of about 17 nm, HDL size particles of 8-10 nm and small particles of about 7 nm [67]. Here, I demonstrate that most of the cholesterol associated with these particles resides in the HDL size fraction (Fig. 2-5). Consistent with previous studies in chicken [222;223] or rat [224] hepatocytes, and with HepG2 cells [226], the current study demonstrates that both the intracellular and extracellular lipidation of apoA-I occur to form buoyant HDL particles [179;235].

The current study challenges the traditional concept of reverse cholesterol transport which defined as cholesterol from peripheral tissues and metabolism of apoB-containing

lipoproteins are the major source of plasma HDL, cholesterol is then transported to the liver to be removed either by HDL/SR-B1 pathway or through the apoB-containing lipoproteins [63;279;280]. The lipidation of both newly synthesized and exogenously added apoA-I indicates that the liver may serve as a source of lipids for nascent HDL particles as well as plasma HDL acceptors. My results clearly demonstrate that the contribution of the hepatocytes to the secretion of cholesterol-containing apoA-I particles, and thus its contribution to the genesis of the HDL-cholesterol pool.

Both the one-step and two-step models of cholesterol efflux have been proposed in fibroblasts, macrophages and smooth muscle cells, but the results remain controversial [202;209;281]. My results support the two-step model and suggest that cholesterol transfer may be secondary to phospholipid transfer, but not necessarily linked to ABCA1 activity. This is also compatible with the independent regulation of cholesterol and phospholipid efflux [282-284] as shown with stimulators and inhibitors of cholesterol efflux that have no effect on phospholipid efflux. My current study provides the first evidence for the independent regulation of phospholipid and cholesterol lipidation of apoA-I in hepatocytes. Because hepatocytes can both synthesize and secrete endogenous apoA-I and lipidate exogenous apoA-I (similar to the other cell types), it represents a unique model that has not been evaluated

The current studies suggest that both ABCA1-dependent and -independent transfer of cholesterol are occurring in excess of phospholipid transfer. I observed an even more significant level of cholesterol lipidation in the absence of ABCA1. Therefore, I further evaluated this ABCA1-independent lipidation pathway by using progesterone to dissect it into a progesterone-sensitive pool (i.e. requiring intracellular transport) and an ABCA1-

independent, progesterone-insensitive pool (likely cell surface associated) as shown in Figure 3-4, suggesting the heterogeneity of cholesterol transfers to apoA-I.

In conclusion, my study demonstrated that in primary mouse hepatocytes, newly synthesized apoA-I acquires cholesterol and phospholipid during secretion and as well at the plasma membrane. ApoA-I cholesterol acquisition operates by both ABCA1-dependent and -independent pathways, both depending on the active transfer from intracellular compartments. There is more cholesterol than phospholipid acquired by apoA-I independent of ABCA1 activity. Cholesterol is acquired by apoA-I, mainly from intracellular pools for endogenously expressed apoA-I, and mainly at the cell surface for exogenous apoA-I. Finally, the lipidation of apoA-I with cholesterol and phospholipid is controlled through different pathways and/or occurs through independent mechanisms (summarized in Fig. 4-1). Finally, my study highlighted the contribution of hepatocytes in apoA-I lipidation and HDL metabolism as shown in Figure 4-2.

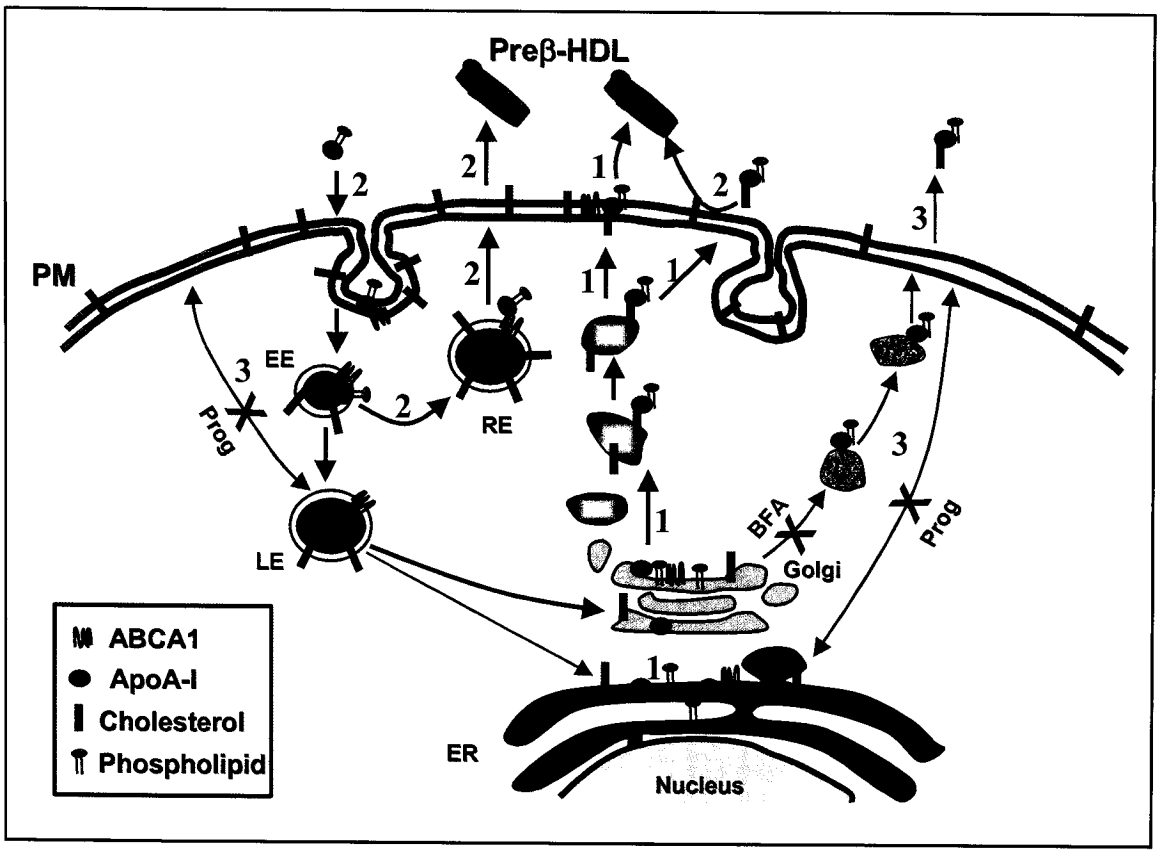

Figure 4-1: Proposed Model for ApoA-I Lipidation in Mouse Hepatocytes

1) ApoA-I is synthesized in the ER and secreted along with phospholipid and cholesterol from hepatocytes; 2) Secreted apoA-I or circulating apoA-I can acquire more cholesterol at the plasma membrane to become preβ-HDL or LpA-I particles, ABCA1 involved uptaking and resecretion of apoA-I may be required for this process; 3) Progesterone partially decreased apoA-I cholesterol acquisition by inhibition of intracellular cholesterol transport. PM: plasma membrane; EE: early endosome; RE: recycling endosome; LE: late endosome; ER: endoplasmic reticulum; Prog: progesterone; BFA: brefeldin A.

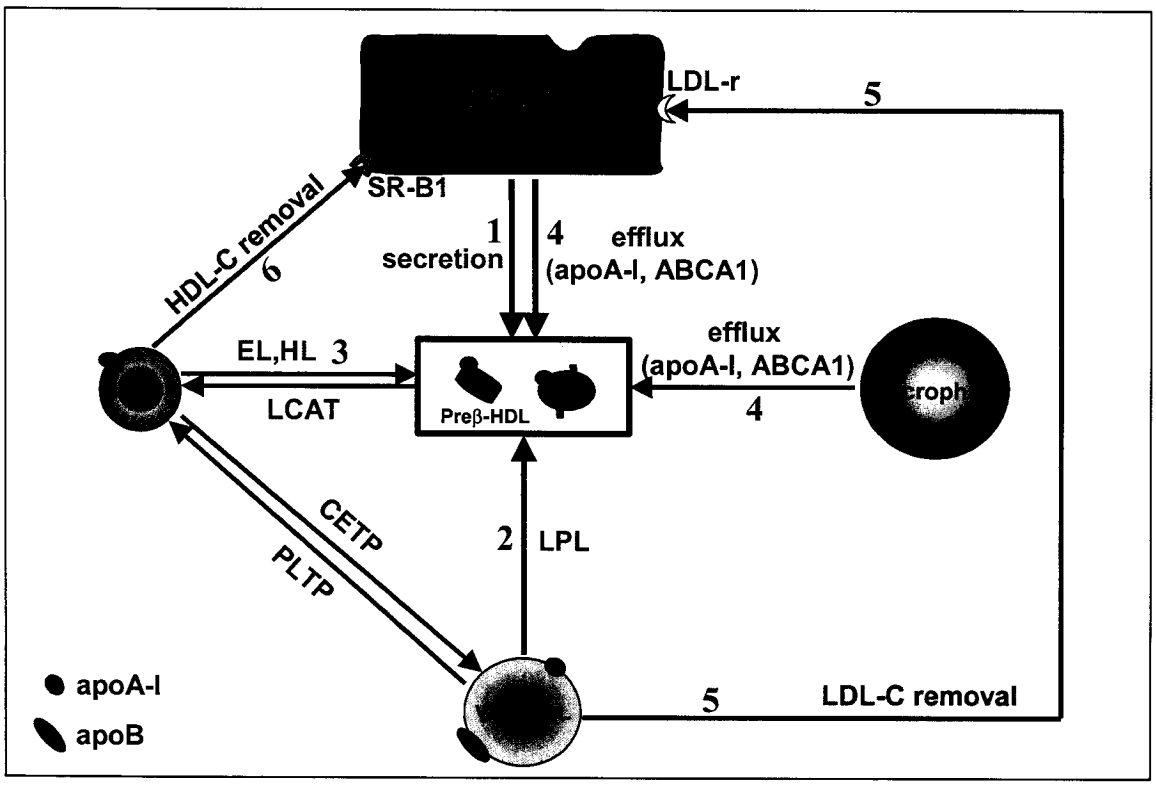

Figure 4-2: Contribution of Hepatocytes to the HDL Metabolism

Preβ-HDL or LpA-I particles are generated: 1) from the hepatocyte as nascent HDL particles; 2) through the LPL-mediated catabolism of apoB-containing particles; 3) through the remodeling or regeneration of preβ-HDL particles through the activities of EL, HL, CETP, and PLTP; and 4) by the interaction of apoA-I and ABCA1 mediated cellular cholesterol efflux at the both hepatocyte and macrophage cell surface. 5) HDL-cholesterol is removed by either direct SR-B1 pathway or indirect LDL-receptor pathway.

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