APOA-I INDUCED LIPID EFFLUX FROM ADIPOCYTES

By

ALISHA DAWN HOWARD

Bachelor of Science in Chemistry, ACS

Bachelor of Science in Biochemistry

Oklahoma State University

Stillwater, OK

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APOA-I INDUCED LIPID EFFLUX FROM

ADIPOCYTES

Dissertation Approved:

Dr. Jose L Soulages

Dissertation Adviser

Dr. Andrew J Mort

Dr. Ramanjulu Sunkar

Dr. Jack W Dillwith

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Cholesterol (CL) plays a variety of important roles in maintaining cellular functions. CL is required by all animal cells for cell membrane structure and signaling. Cells obtain this CL either through intracellular synthesis or through receptor-mediated endocytosis of extracellular CL (associated with lipoproteins). Despite the ability to readily obtain CL from synthesis and uptake, most cells have only a limited ability to catabolize CL [1]. Homeostasis is maintained through the cell-specific balance of synthesis, uptake, limited catabolism and efflux.

However the means of accumulation, CL homeostasis of cells must be maintained, mainly through the process of CL removal termed reverse cholesterol transport (RCT). RCT involves the generation of high density lipoprotein (HDL) from apolipoprotein (mainly apoA-I) uptake of cellular lipids, including phospholipid (PL) and CL. Because the process of CL delivery to non-liver cells (commonly referred to as peripheral cells when speaking on cholesterol transport) via low density lipoprotein (LDL) was described as 'cholesterol transport', the process of CL removal and transport back to the liver was understandably termed reverse cholesterol transport [2]. Importantly, RCT from macrophages is theorized to be atheroprotective since CL-loaded macrophages are a causative component of atherosclerotic lesions.

Lipoproteins move throughout circulation and are remodeled based on metabolic states. HDL receives CL from cells and delivers it via different routes for eventual transport to the liver where it is processed and excreted as bile salts or recycled in order to maintain CL homeostasis [3].

Despite the fact that the majority of HDL is initially formed from the liver, contribution from other cell types cannot be discounted since HDL is dynamically remodeled (see chapter II) and lipid free apoA-I is constantly released from lipoparticles. As HDL circulates in plasma, several enzymes participate in the remodeling of HDL components

for the purposes of acylation and movement of cholesterol, transferring phospholipids, movement of triglycerides for lipolysis and importantly releasing lipid-poor/lipid-free apoA-I [4]. This release provides cells with substrates needed to form HDL (cellular lipids and apolipoprotein). In light of this, various tissues have the potential to contribute to HDL compositions in circulation regardless of the initial source of the lipoparticle. It is also apparent that characterization of this process from different, contributing cell types is crucial to a complete understanding of HDL regulation.

The initial step in HDL biogenesis involves the binding of lipoproteins (i.e. apoA-I) to the cell surface and the membrane transporter ATP-binding cassette transporter A1 (ABCA1). This is believed to stimulate lipid (mainly CL and PL) efflux. Although the entire RCT pathway is needed for efficient removal of cellular CL, the initial steps are thought to be crucial as evidenced by the lack of this process in Tangier's disease (TD) [5-9]. TD patients have significantly lower HDL levels and high cellular cholesterol ester accumulation [10]. Mutations in ABCA1 have been shown to be the cause of TD [11-13]. The ABCA1 role in HDL biogenesis is seen in the inability of cells from TD patients to efflux lipids to apoA-I [14, 15]. Since the discovery of ABCA1 as a possible lipid transporter, extensive research has been done in macrophage and liver cells identifying transporters involved in lipid efflux to apoA-I or HDL acceptors, however characterization of the process in other cell types is lacking [16-20].

ApoA-I is the major protein component of HDL and is involved in HDL formation and cholesterol removal [18]. ApoA-I-mediated HDL biogenesis is dependent on the levels and activity of the membrane protein ABCA1 [21, 22]. Initiation of lipid efflux (CL and PL) to apoA-I involves binding of apolipoprotein to ABCA1 on the cell membrane [8, 9]. But the mechanism and order of CL/PL lipidation or if this process also involves apolipoprotein internalization for access to intracellular stores of lipids, is unknown.

Adipose tissue is the largest storage site of free cholesterol and functions as a metabolic regulator in the body. For these reasons, the adipocyte role in HDL formation and the mechanism of such must be investigated in order to understand how overall HDL levels and lipid compositions can be influenced by adipose tissue. These studies contribute to this question by characterizing the role and contribution of adipocytes to apoA-I-dependent lipid efflux. Although adipocytes have been shown to efflux cholesterol to apoA-I, characterization of this process is not complete [23, 24].

The mechanism of apoA-I lipidation by adipocytes also needs further investigation. The transporter ABCA1 is suspected to be involved in lipid efflux to apoA-I but direct evidence is lacking. Controversially, apoA-I has also been proposed to enter cells for partial lipidation [25, 26]. Because adipocytes store free CL in their cytosolic lipid droplets (LD), a process involving apoA-I internalization to access these stores is attractive. Previous studies by this laboratory have shown that activation of lipolysis in adipocytes leads to a 22% enhancement

in CL efflux to HDL despite lowered levels of plasma CL in the lipolytic state [27, 28]. This implicates a need for transport from intracellular CL stores. Furthermore, the addition of brefeldin A (BFA), a drug known to interfere with Golgi/ER structure and block vesicular transport as well as ABCA1-mediated CL efflux [29-31], abolished the lipolytic enhancement of CL efflux [27, 28]. This seems to indicate that efflux in adipocytes involves mobilization of intracellular CL stores through BFA-sensitive vesicular transport. These findings have led us to investigate the role of apoA-I internalization in HDL biogenesis from adipocytes.

CHAPTER II

REVIEW OF LITERATURE

i. ***Lipid Transport***

Lipids are transported throughout the body to maintain energy levels and lipid homeostasis. These lipids are solubilized with the help of apolipoproteins that bind to the surface of the lipids and can also participate as co-factors of metabolizing enzymes. The resulting lipoprotein particles are classified based on their densities, which is reflected in their naming. High, low and very low density lipoproteins (HDL, LDL, VLDL) carry lipids to and from cells based on their specializations. Generally, VLDL and LDL transport triglycerides (TG) and CL to peripheral cells and HDL transports CL from peripheral cells back to the liver. VLDL and LDL are known to be atherogenic while HDL is anti-atherogenic [32-34].

HDL can be spherical or disc-shaped depending on its lipid load. Discodial HDL is mainly composed of PL, lipoproteins (mainly apoA-I) and little to no CL. The PL stack as in a truncated bilayer and apolipoproteins wrap around the exposed hydrophobic tails of the PL like a belt [35]. Spherical HDL is composed of a monolayer of PL containing lipoproteins and enzymes, with a neutral core of lipids. As described below, HDL can be further divided into distinct classes based on characteristics such as density, charge and size. Studies have shown these different types of HDL to be different in their functionality and relationship to atherosclerosis risk.

ii. ***Classifications of HDL***

The traditional way to separate and purify different classes of lipoproteins is by ultracentrifugation [33]. The particles separate based on hydrated density as reflected in

their nomenclature [36]. HDL is separated from other serum lipoproteins at d>1.063 g/mL and can be subclassified by further centrifugation into (HDL_2 or HDL_3) based on the amount of lipid (density) in the lipoprotein. HDL_3 is the smaller, denser lipoparticle that can be either disc-shaped or small spherical (1.21>d>1.125 g/mL) containing mainly apoA-I and PL but can also contain small amounts of CL and other lipoproteins (such as apoA-II). HDL_2 is larger, spherical and less dense (1.125>d>1.063 g/mL) and contains lipoproteins (mainly apoA-I), PL, TG, CL and cholesteryl esters.

Other methods of separation have also resulted in different nomenclature of HDL. HDL can be separated by its surface charge using electrophoretic mobility into pre-β-migrating or α-migrating HDL [37]. Pre-β HDL includes both lipid-free/lipid-poor apolipoproteins and discodial HDL. α-Migrating HDL includes spherical lipid loaded HDL as well as some discodial HDL (see table 1).

Table 1. HDL Classifications

Shape of particle	apoA-I Protein component	Lipid component	Electrophoretic Mobility	Subfraction Density (g/mL)	Particle Size (nm)
lipid free/lipid poor apoA-I	apoA-I single molecule	little-no phospholipid	**pre-β**	HDL 3c d~1.171	7.2-7.8
discodial HDL	2-3 apoA-I	mainly phospholipid (small amounts of CL)	**pre-β**	HDL 3b d~1.154	7.8-8.2
				HDL 3a d~1.136	8.2-8.8
spherical HDL	2-4 apoA-I	phospholipid, CL, CE and TG	**αlpha**	HDL 2a d~1.115	8.8-9.7
				HDL 2b d~1.085	8.7-12

iii. ***Sources of HDL and apoA-I***

ApoA-I can be synthesized and secreted from the liver or intestine [38-40]. Some studies have estimated that apoA-I is mainly found in the lipid bound form with only ~3-8% (of total apoA-I) in the lipid-free/lipid-poor form [41]. ApoA-I is stabilized by binding to lipids and lipid-free apoA-I is rapidly removed from circulation by catabolism in the

kidney [42]. ApoA-I collects PL and CL from cells (or possibly from lipoproteins) to form HDL (Fig. 1) [43, 44]. The main sources of nascent HDL are the liver and intestine. In studies, circulating levels of HDL were reduced by ~80% when the transporter ABCA1 was tissue-specifically knocked out of mouse liver cells [42]. Similar experiments with intestinal ABCA1 knockouts revealed an additional ~30% released from the intestine [45]. Although this implies that the liver and intestine have a large influence on HDL compositions and levels, the potential contributions to HDL biogenesis of other tissues cannot be ignored since lipoproteins are dynamically remodeled in circulation and apoA-I constantly released.

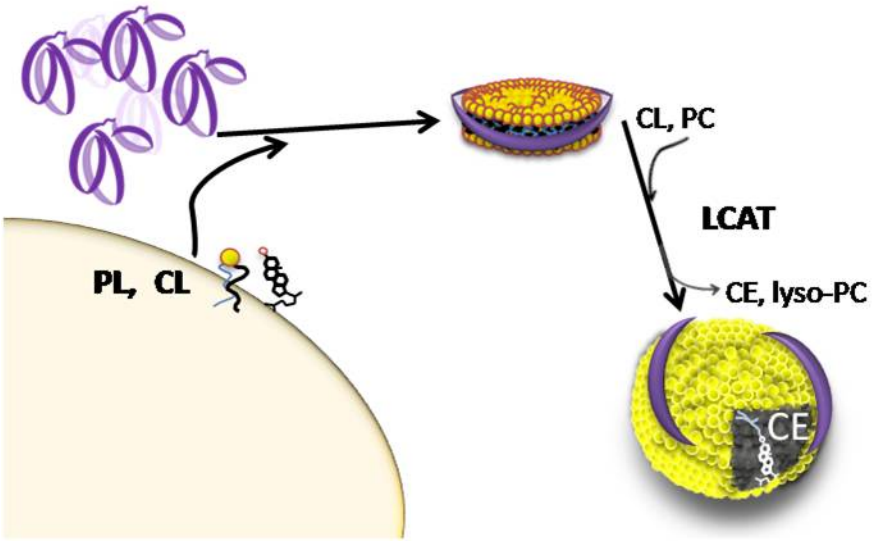

Figure 1. HDL Biogenesis. Apolipoproteins (mainly apoA-I) interact with the cell surface resulting in lipoprotein lipidation by phospholipids (PL) and cholesterol (CL). These nascent lipoparticles form a discodial structure which composes the highest density of HDL fractions. The surface contains the enzyme lecithin:cholesterol acyltransferase (LCAT) which converts CL to cholesterol esters (CE). The CE are more hydrophobic than CL and are relocated internal to the surface lipids-forming spherical HDL.

iv. ***Lipoprotein remodeling and apoA-I release***

HDL is remodeled by plasma factors primarily located on the surface of the lipoprotein particle. Remodeling results in transfer of lipids and release of lipid-free/lipid-poor apoA-I from HDL. CL is deposited into HDL as free cholesterol and converted into cholesteryl esters by lecithin:cholesterol acyltransferase (LCAT) [2]. Cholesteryl esters, which are more hydrophobic than CL, are then relocated into the center of the HDL particle resulting in a conversion from disc-shaped to spherical HDL [46]. HDL becomes larger and less dense as it collects more lipids, either through active flux or diffusion from cell surfaces, and the collected CL is converted to cholesteryl esters by LCAT.

Release of apoA-I occurs in different ways but can be grouped by two general themes. Either enzymes have remodeled HDL into too large of a particle which is unstable and releases apoA-I to downsize and stabilize the structure or enzymes have decreased the size of the lipoparticle and less apoA-I is needed. For instance, as LCAT converts CL to cholesteryl esters, the HDL particle becomes larger and acquires more apoA-I particles [46-48]. This larger HDL, which contains four apoA-I particles is less stable and can release lipid-poor apoA-I to become more stable. Cholesterol ester transfer protein (CETP) is another enzyme that facilitates HDL remodeling by moving cholesteryl esters from HDL to apoB-containing lipoproteins such as VLDL. This activity is accompanied by movement of TG from the other lipoproteins back to HDL [49]. CETP helps generate lipid-poor/lipid-free apoA-I in three different ways. One way is through the TG-enrichment of HDL which becomes the preferred substrate of hepatic lipase (HL) [4, 50]. Hydrolysis of TG by HL results in release of non-esterified fatty acids and remodeling of HDL. The remodeling of HDL into smaller particles results in the release of lipid-poor/lipid-free apoA-I [51, 52]. Secondly, TG-rich HDL is also the preferred substrate of phospholipid transfer protein (PLTP). PLTP activity has been implicated to cause a fusion of lipoparticles which produce a larger, unstable HDL and subsequentially release apoA-I in order to stabilize the particle [53, 54]. Thirdly, CETP promotes the release of apoA-I related to the rate of TG transfer from VLDL. Because this rate is lower than the rate of VLDL catabolism, less net TG may be transferred back to HDL. This results in a lipid-depleted, smaller HDL particle which also releases lipid-poor/lipid-free apoA-I [55].

ApoA-I is also released from chylomicrons during lipoprotein lipase (LPL) hydrolysis of TG [56]. Studies show that radiolabeled chylomicrons release apoA-I for uptake mainly to the HDL fraction after injection into the lymph [57, 58]. This constant remodeling and release of apoA-I provides substrate (apoA-I) for other tissues, besides liver and intestine, to participate in HDL biogenesis (Fig. 2). Despite this, few studies have sought to characterize these processes.

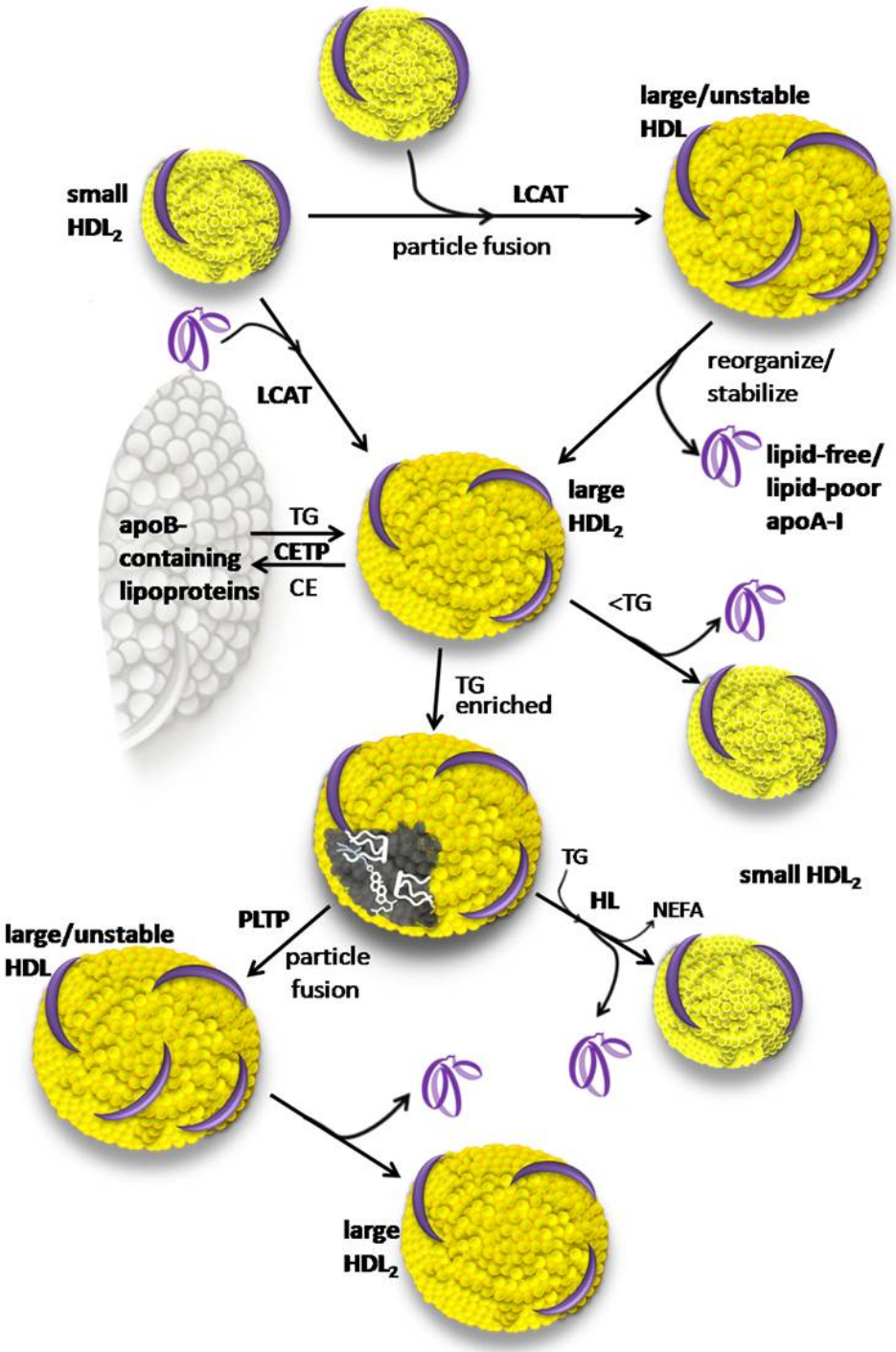

Figure 2. HDL Remodeling and ApoA-I release. HDL is metabolized in circulation resulting in lipid transfers, size changes and lipoprotein release. Diagrammed above are remodeling pathways which may lead to lipid-free/lipid-poor apoA-I release from lipoparticles. Lecithin:cholesterol acyltransferase (LCAT) has been shown to result in lipoparticle fusion which releases apoA-I to help stabilize the resulting large and unstable HDL. Cholesterol ester transfer protein (CETP) activity yields triglyceride (TG)-enriched HDL as well as lipid depleted HDL when TG transfer is incomplete. TG-enriched HDL is a favored substrate for both hepatic lipase (HL) and phospholipid transfer protein (PLTP). HL hydrolyzes the TG and decreases the size of the HDL which releases apoA-I. PLTP has been shown *in vitro* to cause particle fusion resulting in large and small HDL products with release of apoA-I.

*v. **Importance of studies on lipid efflux from adipocytes***

As mentioned above, efflux from the liver and intestine contribute the majority of initial circulating HDL in mice but these studies failed to account for the metabolic potential of adipocytes for influence on these levels [42, 45]. Adipocytes compose the largest reservoir of stored free CL in the body [59, 60]. The mass of the stored CL is proportional to the amount of adipose tissue and TG and in the case of obesity can account for over half of the body's stored CL [61, 62]. Lipolytic mobilization of fat stores has been shown to increase CL efflux to recombinant HDL particles [27]. Studies have also shown correlations in obesity and altered component lipid ratios of circulating HDL [63-65]. Furthermore, accumulation of upper body fat is associated with decreased HDL-CL levels and increased cardiovascular heart disease (CHD) risk [64]. This suggests that overall HDL levels and particle compositions may be greatly affected by lipid efflux from adipocytes. Although apoA-I has been demonstrated to interact with and stimulate lipid efflux from other cell types, the process has not been well characterized in adipocytes. Thus, adipocytes have an unknown potential to influence HDL composition and CL levels.

*vi. **Not Just Concentration: Importance of HDL function***

HDL composition may be an important characteristic affecting functionality. Literature has linked the CL and PL composition of HDL to its downstream efflux potential [66-73]. In light of this, the lipid compositions of circulating HDL may play a more atheroprotective role than the overall HDL levels. Several studies have characterized the PL to CL ratios of apoA-I-dependent lipid efflux from cells such as: macrophages, fibroblasts, smooth muscle cells, erythrocytes and TD fibroblasts [16-20]. The HDL particles formed were shown to vary in composition from CL-rich to CL-poor and for the case of the latter two, no detectable HDL was formed.

Because CL can efflux to HDL via several different pathways, it is reasonable to predict that different HDL subclasses may vary in their success as acceptors. The different mechanisms for efflux from the cell to HDL acceptors involve 1) ABCA1-mediated efflux to apolipoprotein (i.e. apoA-I) acceptors; 2) scavenger receptor BI (SRBI)-mediated flux to HDL; 3) ABCG1-mediated efflux to HDL and 4) passive diffusion to HDL. Studies on the mechanisms, substrate affinities, activities and relative contributions of these pathways have been performed predominately in macrophages.

SRBI is found in many cell types [74-76] and mediates flux of CL to and CE from HDL [76, 77]. The relative contribution of SRBI to CL efflux from macrophages (compared to

the other pathways) seems to be minor compared to ABC A1 and G1 [78-80]. ABCG1 has recently been identified for its ability to efflux CL out of the cell similar to ABCA1 but prefers HDL as an acceptor rather than lipid-free apoA-I [69, 81, 82]. Some studies have predicted that ABCG1 contributes ~20% of overall cellular CL efflux from CL-loaded macrophages [80] but its role in other cell types remains largely uninvestigated. ABCA1 mediates the efflux of both PL and CL to apolipoprotein acceptors [83-85]. Its ability to efflux to HDL as an acceptor is greatly diminished compared to apolipoprotein acceptors [86]. ABCA1 is thought to be essential for the maintenance of HDL levels in circulation as evidenced by extremely low HDL levels in TD patients and ABCA1 knockout mice [10, 42, 45, 87]. Passive diffusion of CL to HDL has been proposed to be significant when the HDL acceptor surface is CL-poor compared to the plasma surface of the cell. This process is believed to be dominant when the cell contains low concentrations of active CL transporters such as ABCA1 and G1 [80, 88].

vii. ***HDL compositions and disease***

Cardiovascular disease is the leading cause of death in developed countries, accounting for ~30% of deaths [89]. Seminal epidemiological studies have shown an inverse relationship between HDL concentration and the risk of CHD [32, 90-93]. Because of this, plasma HDL concentration levels have become a common component of atherosclerotic risk assessment. More recently however, the results from studies seem to suggest that HDL levels alone may not be the determining factor in atherosclerotic risk. Studies have suggested that CHD risk may be inversely related to HDL particle size [63, 94, 95]. In agreement with this, cross-sectional studies have shown HDL_3 to correlate with higher TG levels, apoB (the main protein component of LDL and VLDL), VLDL mass and overall lower HDL concentrations [96]. Also, studies on patients that had recently survived heart attacks found a higher percentage of HDL_3 in these patients compared with controls and found that all patients studied had an inverse relationship of HDL_2 to HDL_3 concentrations [97]. Other studies have found that HDL_2 correlates with lower CHD risk factors [63, 94].

On the other hand, some studies have implicated the protein content of HDL to influence its atheroprotectiveness. Mice transgenic for either human apoA-I or apoA-I/apoA-II showed atherosclerotic lesions to be 15-fold higher in apoA-I/apoA-II mice [98]. This suggests that the protein component of HDL may also influence its ability to promote lipid efflux from macrophage foam cells.

Studies have also shown that HDL function within the RCT pathway directly affects its ability to be atheroprotective. A small study of CHD patients showed that patient serum

efflux capacity correlated to coronary events. This led the authors to speculate that the defective serum was indicative of a defective ABCA1 efflux pathway [99].

There are also studies which break the "high HDL, lowered risk" rule. An example of this are studies showing increased atherosclerosis despite increases in HDL levels, presumably because HDL function in the RCT pathway was impaired [100-102]. In the Honolulu Heart Program Cohort study, a genetic CETP deficiency produced increased CHD in subjects despite increased or unaffected HDL concentrations and increases in HDL size [102]. The authors proposed this unusual correlation to be due to decrease in functionality of the RCT pathway since CETP deficiency has been linked to lower LCAT activity levels. Similarly, clinical trials of the CETP inhibitor torcetrapib showed increased HDL levels as well as increased cardiovascular events and mortality. It is not known if this was due to decreased function of the larger HDL products of CETP inhibition or if it was due to the off-target effects of the drug [101].

Conversely there are examples in which decreased HDL does not lead to an increased CHD risk. For instance, apoA-I_{Milano} is a mutant form of apoA-I found in an Italian family exhibiting lower levels of HDL as well as a decreased risk of CHD [103]. This was later determined to be due to the apoA-I mutation which results in a more efficient apoA-I CL acceptor than wt apoA-I [104]. Similar to this is the apoA-I_{PARIS} mutation which results in dramatically lower HDL without increased evidence of CHD [105, 106]. Other genetic mutations that can cause lowered HDL levels include LCAT deficiency, which also does not result in enhanced CHD (except in compound cases) [107, 108]. Due to these findings it is evident that HDL levels alone cannot be the sole determining risk factor and risk assessment should be extended to include HDL functionality within the RCT pathway.

Thus, since HDL compositions affect functionality and HDL is constantly in flux, then the compositions and classes present in circulation are most likely reflective of tissue energy levels and metabolic needs. This means that although extensive studies in macrophages are warrented due to the direct atheroprotectiveness of CL efflux out of foam cells, studies in other tissues are just as important to determine the type of HDL in circulation that would reach macrophages and other peripheral cells.

viii. ***Mechanisms of nascent HDL formation***

HDL is formed when apoA-I interacts with the cell surface to facilitate removal of cellular lipids. RCT is dependent on HDL to circulate and retrieve CL from overloaded cells. This process is very important as evidenced by the negative correlation of HDL levels and the risk for cardiovascular disease [32]. The most vital component to this entire process seems to be the very beginning steps involving HDL biogenesis. Without

the ability to create HDL and remove CL, cellular CL homeostasis can become imbalanced. Lowered apoA-I/HDL levels and/or functionality translates to a reduced ability to remove cellular CL.

In 1961, patients were identified who exhibited hepatosplenomegaly, severely enlarged lymph nodes and dramatically lower levels of HDL [10]. It was first speculated that this was some type of Niemann-Pick C (NPC) disease which is a lipid storage disorder known to cause lipid droplet accumulations. But unlike NPC disease, these patients showed no eminent signs of central nervous system degeneration. The patients, who were siblings, were residents of an isolated island off the coast of Virginia called Tangier's Island. Thus this lipid storage disorder was labeled as Tangier's disease (TD) (for review see [109]). In 1999, several labs identified the missing essential component of efflux in TD patients to be caused by mutations in the gene coding for the ABCA1 [11-13]. ABCA1 role in HDL biogenesis can be seen in the inability of TD fibroblasts to efflux lipids to apoA-I [14, 15]. Thus ABCA1 emerged as the transporter most likely responsible for lipidation of apolipoproteins and HDL biogenesis [5-9].

Since this discovery ABCA1 has been extensively studied and ABCA1 is known to mediate the efflux of lipids to apolipoprotein acceptors [43]. However, questions related to the mechanism of lipidation remain. Some have proposed that CL efflux occurs simultaneous to PL efflux and others that it is subsequential and independent from the PL loading of apoA-I [5, 7, 110-112]. Also, there are controversies over the apoA-I location of lipid uptake. ApoA-I lipidation has been proposed to occur on the surface of the plasma membrane by the binding of apoA-I to lipid perturbations caused by ABCA1 and solubilization of the lipids into HDL particles [7, 113, 114]. Alternatively, some studies have proposed that lipidation involves the recycling or "retroendocytosis" (i.e. endocytosis followed by exocytosis) of apoA-I as evidenced by colocalization studies with ABCA1 or by monitoring radiolabeled apoA-I release or by cell-surface biotinylation studies [115-120]. Although these studies utilize methods that show apoA-I endocytosis there is disagreement on the level of contribution that this pathway has towards HDL formation. Variation in the proposed functions of apoA-I recycling most likely result from the differences in the methodologies used. Further, many of the methods suffer from an inability to distinguish internalized protein from membrane bound protein and inherent problems due to label exchange and degradation.

A method of labeling developed by this laboratory and employed in these studies, clearly distinguishes internalized and re-secreted protein from cell surface bound protein by using a recombinant apoA-I containing a protein kinase A (PKA) recognition tag (pka-apoA-I) [28, 121]. This construct can be used to ask the question: is apoA-I lipidated with PL and CL from the surface of adipocytes or is the retroendocytosis of apoA-I required for intracellular lipidation or to initiate unknown signaling pathways? Because adipocytes store free CL in their cytosolic lipid droplets (LD), a process involving apoA-I

internalization to access these stores seems plausible. Also, previous studies have implied that efflux in adipocytes involves mobilization of intracellular CL stores through BFA-sensitive vesicular transport [27, 28].

CHAPTER III

MATERIALS AND METHODS

*i. **Materials.***

J774 and 3T3 L-1 cells were purchased from American Type Cell Culture (Manassas, VA). Isoproterenol, dimethyl sulfoxide (DMSO), brefeldin A, glyburide, fatty acid free bovine serum albumin (BSA), isobutyl methyl xanthine (IBMX), dexamethasone, trypsin, insulin, 8-Br-cAMP, GW3965, secondary antibody (anti-rabbit) perioxidase conjugated, streptomycin and penicillin were purchased from Sigma Chemicals Co. (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) was purchased from Cellgro Mediatech, Inc (Herndon, VA). Block lipid transport-4 (BLT4) was received from Chembridge Corp. (San Diego, CA). BLT1 was a gift from Dr. George H. Rothblat. Polyclonal anti-ABCA1 NB400-105 and monoclonal antibodies to the β subunit of ATP synthase clone "3E8" NB600 were purchased from Novus biological (Littleton, CO). Antibody to ATP synthase β clone "3D5" was purchased from Abcam ab14730 (Cambridge, MA). Chemiluminiscence kit and Hybond ECL nitrocellulose membrane for Western blotting was obtained from Amersham Biosciences (Piscataway, NJ). Film for autoradiography and western blotting was Fuji RX Film (Greenwood, SC). Infinity Cholesterol Reagent was purchased from Hitachi (Indianapolis, IN). Phospholipid C kit was purchased from WAKO diagnostics (Richmond, VA). Infinity Triglycerides Reagent was purchased from Thermo (Louisville, CO). Plasmid pET30 EK/LIC was obtained from Novagen (Gibbstown, NJ). [^{32}P]-H_3PO_4 in H_2O was from MP Biomedicals (Irvine, CA). [^{3}H]-choline and [^{3}H]-cholesterol was purchased from PerkinElmer (Waltham, MA). Ni-sepharose resin was purchased from Amersham (Piscataway, NJ). Densitometric analysis was done with AlphaEase Software (Santa Clara, CO). Graphs and statistical calculations were performed using GraphPad (La Jolla, CA).

ii. ***Cell culture***.

J774 macrophages or 3T3 L-1 pre-adipocytes were cultured at 37^0C in 8% CO_2 atmosphere in DMEM supplemented with 10% FBS and 0.01% antibiotics. J774 were allowed to reach 90% confluency prior to experiments. For differentiation of 3T3 L-1 into adipocytes, cells were allowed to grow to confluence then the differentiation into adipocytes was induced by addition of IBMX (111 µg/mL), dexamethasone (0.46 µg/ml), and insulin (1.5 µg/ml) [122]. After 48 h, the cells were incubated in DMEM/10% FBS containing insulin for an additional 48 h. Afterwards the cells were maintained in DMEM/10% FBS. All experiments using adipocytes were conducted 12-14 days after the differentiation period.

iii. ***Analysis of whole well adipocyte or macrophage cell homogenates.***

Cell monolayers from individual 6-well plates containing 14-day old fully differentiated 3T3 L-1 adipocytes or confluent J774 macrophages were scrapped and homogenized.

Total well concentrations of protein were determined by dilution of homogenate in 10% SDS and the absorbance was measured to obtain the second derivative of $A_{nm=281(max)}$ minus the second derivative of $A_{nm=291(min)}$. This allows removal of the disturbance due to DNA abosorbance at $A_{nm=260}$ in the homogenate. The concentration was then calculated using ε"=0.017 to determine the cellular protein.

Next, aliquots of homogenate were used for lipid extraction with chloroform:methanol (2:1 by vol) by the Folch method [123]. Organic phase lipids were then separated by TLC (along with standards) and iodine used to visualize cholesterol (CL) or phospholipids (PL). The spots of both samples and standards were then cut from the plates and lipids extracted from the silica with either 1:1 chloroform:methanol (CL) or 80:20 methanol:chloroform (PL) and dryed. These lipid extracts were then reconstituted as per manufacturer instructions for either a choline-containing phospholipid colorimetric assay (Phospholipid C assay kit, WAKO) or a cholesterol colorimetric assay (Infinity Reagent, Hitachi).

Once well lipid content was determined, the moles of lipids in homogenates were then divided by protein concentration per well in order to standardize each well by protein content. For apoA-I dependent lipid efflux quantification, the sample % efflux value (calculated in *Efflux Assays* below) was multiplied by the total cellular lipid content per mg homogenate protein. This gives the nmol CL or PC / h • mg cellular protein.

iv. ***Cholesterol efflux Assays.***

Fully differentiated 3T3 L-1 adipocytes or J774 macrophages were labeled with [^{3}H]-cholesterol (2 µCi/mL with 44.5Ci/mmol) 22-24 h prior to experiment in the presence of 2 µM GW3965 (or buffer) for adipocytes or 180 µM 8-Br-cAMP (or buffer) for macrophages in 2.5% FBS-DMEM. Previously reports from our laboratory have shown that labeling of adipocytes for 24hrs with [^{3}H]-cholesterol results in a final distribution of labeling to 96% cholesterol and 4% cholesterol esters [44]. Recombinant apoA-I cloned and purified as previously described was used to initiate cellular lipid efflux [121]. At the start of the experiment fresh 0.05% BSA-DMEM was added to the cells with 75 µg/mL apoA-I (or with buffer to determine background levels of efflux). Aliquots of media were taken at indicated time points, briefly centrifuged and [^{3}H]-cholesterol efflux determined by scintillation counting. After allowing efflux to proceed for 5 h the wells were washed and the cholesterol extracted into isopropanol for counting. Whole well [^{3}H]-cholesterol (cpm) was determined by adding the cpm found in the media to the cpm present in the isopropanol extract. The % of cellular CL efflux was calculated at each time point as:
% of CL efflux = cpm media*100/(cpm media + cpm homogenate).

ApoA-I induced lipid efflux was determined as the difference between the mean % of CL efflux in medium containing apoA-I and the average % of lipid efflux found in wells without apoA-I (background efflux). The time course of apoA-I induced CL release (as % total) was fitted by linear regression to estimate the corresponding rate of efflux.

v. ***Phospholipid efflux Assays.***

To measure choline-labeled phospholipid efflux, macrophages or adipocytes were labeled with [^{3}H]-choline [5 µCi/mL] in 2.5% FBS-M199 for 22-24 h. At time zero, 75 µg/mL of recombinant apoA-I in DMEM-0.05% BSA, or buffer as background, was added to each well. Aliquots of media were collected at indicated time points and at the final time point the whole well homogenate and remaining media collected. The organic phase of media (or homogenate) containing radiolabeled lipids was then extracted from the aqueous phase containing the un-incorporated choline by the Folch method [123]. The organic phase lipids were then dryed completely and the [^{3}H]-choline labeled PL were measured by liquid scintillation counting.

The % of cellular PL efflux was calculated from the amounts of [^{3}H]-choline labeled PL determined in media and cell homogenates as:
% of lipid efflux = cpm media*100/(cpm media + cpm homogenate). The mean values and standard deviations of apoA-I induced PL efflux were determined as indicated for CL efflux.

vi. ***Lipid efflux inhibition assays.***

Assays were performed as described above however drugs were pre-incubated with cells 1-2 h prior to experiment by replacing labeling media with DMEM-0.05% BSA experimental media in the presence of BFA (10 µg/mL), GLYB (500 µM), BLT1(10 µM), BLT4 (150 µM) or DMSO as a control. At time zero, the experimental media was re-added with 75 µg/mL of recombinant apoA-I or without apoA-I for a background efflux level. Media and homogenate was collected and protein and lipids analyzed as described above.

To calculate the inhibition of each drug on apoA-I dependent efflux, the efflux slope values of each drug were determined as described above for CL and PL efflux assays. These apoA-I dependent % efflux values were used to calculate inhibition according to: (% inhibition = 100*rate drug / rate control) for each drug replicate. The values were then averaged and expressed as mean with the corresponding standard deviations calculated through propagation of error.

vii. ***Efflux normalization by ABCA1 protein level.***

A semi-quantitative analysis of cellular ABCA1 protein was done by western blotting of aliquots of homogenates. The samples analyzed were obtained from some of the wells not used for lipid efflux studies but contained in the same plate. Protein concentration of homogenates were determined as mentioned section *III.iii*. The volumes of homogenate separated by electrophoresis were adjusted such that equal amounts of protein (70 µg) were loaded for all samples. Proteins were then transferred to nitrocellulose membrane and blotted with rabbit polyclonal anti-ABCA1 (Novus). All samples to be compared (control and GW3965 treated) and macrophages (control and 8-Br-cAMP treated), were loaded within the same gels and analyzed from the same blots. After detection by chemiluminescence the films were scanned and the band intensities determined by densitometry using AlphaEase software (Santa Clara, CA). The band intensities in arbitrary density units (a.u.) were directly compared within the same blots. To compare the results of different blots the band intensities were first normalized using the intensity of adipocytes control as internal reference. CL efflux rate was calculated as described previously and the efflux of each well normalized by mg cellular protein per well.

To normalize the CL efflux rate by ABCA1 protein levels, the a.u. of each sample determined from blots were divided into the apoA-I dependent CL efflux rate determined above. Thus, (nmol CL efflux or % CL Efflux)/h•mg cell protein ÷ (ABCA1 a.u.)/mg cell protein = Efflux Rate/[ABCA1].

viii. ***ApoA-I, apoLp-III and Thrx recombinant proteins****.*

Full-length mature human apoA-I was previously cloned into a vector which incorporates an N-terminal tag containing six-His residues and a five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase A (PKA) [121]. The protein size and identity was confirmed as previously described [121]. The presence of an accessible PKA phosphorylation site on the recombinant apoA-I was confirmed by *in vitro* method previously described [121, 124].

Wild-type *L. migratoria* apolipophorin-III (apoLp-III) cloned into pET32a as previously described [125] was transferred to a pET30 (Novagen, Inc.) expression vector which incorporates an N-terminal six-His residue tag with primers designed to code for the same N-terminal PKA recognition site as pka-apoA-I described above. The final sequence encoded a protein of 217 residues with a mass of 23 kDa. Similarly, thioredoxin (Thrx) was transferred to the same vector incorporating N-terminal His- and PKA recognition site- tags and coding for a protein of 162 residues with a mass of 17.7 kDa. The proteins were expressed separately in *Escherichia coli* and purified by Ni-affinity chromatography using standard procedures. The protein sizes and identities were confirmed by SDS-PAGE and MALDI-TOF peptide mass fingerprinting on a Voyager DE-Pro mass spectrometer in the OSU Core Facility.

ix. ***Cellular Recycling of ApoA-I***

ApoA-I Recycling Assay.

3T3 L-1 adipocytes cultured in six well dishes were radiolabeled by incubation for 5 h at 37^0C with 75 µCi/well of [^{32}P]-orthophosphate (carrier free) in phosphate-free DMEM containing 0.05% BSA. The wells were then washed and fresh media containing isoproterenol (10 µg/ml) with pka-tagged recombinant proteins were added back to each well. After a brief incubation period, the well media was collected and centrifuged at 10,000 rpm for 10 min to pellet any floating cells. Aliquots of medium were used to measure the amount of glycerol (Infinity Triglyceride Reagent) released by cells into the media as an indicator of cell lipolytic rate. The media was then either run onto SDS-PAGE directly or first purified using Ni-affinity chromatography, then separated by gel electrophoresis. Gels were dryed and any pka-tag phosphorylation visualized using autoradiography.

Saturation of apoA-I recycling.

3T3 L-1 adipocytes were radiolabeled as described above. To measure the saturation of apoA-I recycling in adipocytes, fresh media containing pka-apoA-I was added to each well and allowed to incubate for 40 minutes at 37^0C. After 40 minutes, the cell experimental medium was analyzed as described above for pka-apoA-I [^{32}P]-phosphorylation indicative of apoA-I cellular uptake and re-secretion.

Competition of native apoA-I, apoLp-III and Thrx with apoA-I for Recycling

Adipocytes were [^{32}P]-radiolabeled as described above and pka-apoA-I added alone or in combination with human apoA-I, apoLp-III or Thrx to cells to monitor the ability of these proteins to compete with pka-apoA-I for recycling. For competition with human apoA-I, pka-apoA-I (40 µg/mL) was added with increasing concentrations of purified human apoA-I. As a control one well was incubated with 40 µg/mL human apoA-I and 0 µg of pka-apoA-I. For competition with apoLp-III or Thrx, pka-apoA-I (1.5 nmol), pka-apoLp-III (3-6 nmol) or pka-Thrx (3-6 nmol) was added to the media of each well along with 10 µg/mL isoproterenol (to enhance PKA activity). After 40-60 minutes the cell media was collected and analyzed as mentioned previously to monitor for recycling.

Effect of Lipid Efflux Inhibitors on Recycling

Adipocytes were [^{32}P]-radiolabeled in phosphate-free KRBH containing 0.005% BSA. To monitor the effect of lipid efflux inhibition on recycling, inhibiting concentrations of BFA and BLT1 were added to the previously described pka-apoA-I recycling assay. BFA (10 µg/ml) or BLT1 (10 µM) were allowed to pre-incubate 2 h prior to the end of the labeling period and the assay initiated by the addition of 50 µg/mL pka-apoA-I. The cell media was collected after 10, 20 and 40 min and the pka-apoA-I phosphorylation analyzed from purified media as previously described. Glycerol levels were measured (Infinity Triglycerides Reagent, Thermo) by analyzing aliquots of media to ensure that drug incubation did not result in changes in the lipolytic rate.

Effect of antibody to ATP synthase subunit β on pka-apoA-I Recycling

Adipocytes were [^{32}P]-radiolabeled as described previously. 1 h prior to the pka-apoA-I addition, the labeling medium was replaced with fresh medium containing indicated amounts of antibody to ATPase subunit β (either clone 3E8 or 3D5). Pka-apoA-I

(40 µg/mL) was added to the media of each well after the labeling and antibody incubation periods. Aliquots of the media were collected after 60 min and used to analyze both pka-apoA-I and glycerol release into the media as describe previously.

x. ***Relationship of Phospholipid Efflux to ApoA-I Recycling.***

ApoA-I was incubated with radiolabeled and antibody pre-incubated adipocytes as described above. After 60 min cell media was collected and apoA-I associated lipids purified by Ni-affinity chromatography. Protein concentrations of eluants were determined by UV_{280nm} and confirmation of purity and concentration was obtained by SDS-PAGE.

[^{32}P]-labeled phospholipids were extracted from free phosphate and protein fractions by modified Folch [123]. Briefly 20 parts chloroform:methanol [2:1] were added to 1 part protein eluant and mixed. Then choloroform:methanol [2:1] was added such that the organic to eluant ratio became 5:1 and mixed. After separation the upper aqueous phase containing free phosphate and the interface containing proteins were discarded. The lower organic phase containing phospholipids was completely dried then counted using scintillation counting. Counts were then normalized by the amount of apoA-I absorbance at UV_{280nm}. Thin layer chromatography was used to confirm incorporation of [^{32}P] into phospholipids.

xi. ***Relationship of Cholesterol Efflux to ApoA-I Recycling.***

To monitor the relationship of apoA-I recycling and apoA-I dependent cholesterol efflux, recycling and efflux assays were performed in parallel with and without anti-ATPase β. 3T3 L-1 adipocytes were radiolabeled with either [^{3}H]-cholesterol or [^{32}P]-orthophosphate. For CL efflux assays, cells were labeled with [^{3}H]-cholesterol for 19 h in 2.5% FBS-DMEM followed by a 5 h labeling with [^{3}H]-cholesterol in 0.05% BSA-DMEM. For recycling assays adipocyte were [^{32}P]-radiolabeled in 0.05% BSA-DMEM for 4 h. Next all cells were either pre-incubated with or without 60 µg anti-ATPase β clone 3D5 for 1 h prior to pka-apoA-I addition. Recombinant pka-apoA-I was then added to cell media and incubated for either 5 h or 1 h.

For recycling assay analysis, 1 h media was collected and apoA-I purified by Ni-affinity chromatography. Protein concentration was determined by UV_{280nm} and confirmation of purity and concentration was obtained by SDS-PAGE. Gels were dried and subjected to autoradiography and densitometry used to confirm antibody inhibition of apoA-I cell entry and resecretion.

For efflux assay analysis, media was collected at indicated time points, briefly spun and [^{3}H]-cholesterol efflux determined by scintillation counting. After allowing efflux to proceed for 5 h the wells were washed then cholesterol extracted into isopropanol and counted. Whole well [^{3}H]-cholesterol and % of cellular efflux were determined as described above for cholesterol efflux assays.

xii. ***Statistical Analysis***

The statistical significance of differences were determined between means of control and treated sample data using the Student's t-test (Graphpad).

CHAPTER IV

CHARACTERIZATION OF APOA-I DEPENDENT LIPID EFFLUX IN ADIPOCYTES, ROLE OF ABCA1

Abstract

Adipose tissue is a major reservoir of cholesterol and, as such, it may play a significant role in cholesterol homeostasis. The goals of the present studies were to obtain a quantitative characterization of apolipoprotein A-I (apoA-I) dependent lipid efflux from adipocytes and examine the role of ATP-binding cassette transporter A1 (ABCA1) in this process. The rates of apoA-I induced cholesterol and phospholipid efflux were determined. To allow a comparative analysis, parallel experiments were also performed in macrophages. These studies showed that when the rates are normalized by cellular protein or by ABCA1 content, apoA-I induces cholesterol efflux from adipocytes at similar rates as from macrophages. Enhancement of the expression of ABCA1 increased the rates of cholesterol efflux from both adipocytes and macrophages. However, the results also suggested that a non-ABCA1 dependent mechanism could make significant contributions to the basal (basal levels of ABCA1) rate of apoA-I dependent cholesterol efflux. Furthermore, the study of the effect of inhibitors of lipid efflux showed that glyburide and brefeldin A, which affect ABCA1 function, exerted strong and similar inhibitory effects on lipid efflux from both adipocytes and macrophages, whereas BLT1, an SRB-I inhibitor, only exerted a moderate inhibition. Overall these studies suggests that ABCA1 plays a major role in apoA-I dependent lipid efflux from adipocytes and showed high similarities between the abilities of adipocytes and macrophages to release cholesterol in an apoA-I dependent fashion.

Keywords: apoA-I, adipocyte, macrophage, lipid efflux, ABCA1

Abbreviations used: ApoA-I, apolipoprotein A-I; ABCA1, ATP-binding cassette transporter A1; GLYB, glyburide; BFA, Brefeldin A; LXR, Liver X Receptor; BLT, block lipid transport; BSA, bovine serum albumin; FBS, fetal bovine serum; CL, cholesterol; PL, phospholipid; Scavenger Receptor-BI, SRBI

Results

*i. **Lipid efflux from adipocytes**.*

Previous works have shown that incubation of 3T3 L-1 adipocytes with apoA-I results in both CL and PL efflux into the media [23, 24]. Although the previous studies have shown adipocytes capable of apoA-I-induced lipid efflux, no studies have fully characterized the rate, stoichiometry or quantitative scale of this process. In order to characterize apoA-I-dependent efflux from adipocytes, the rate of cholesterol release from [^{3}H]-cholesterol-labeled adipocytes upon apoA-I incubation was determined. Similarly cells radiolabeled with [^{3}H]-choline allowed characterization of the choline-containing PL efflux rate. Parallel experiments were also performed in the murine macrophage cell line J774 to allow the comparison of adipocytes to a well-studied cell-type. Efflux specific to apoA-I induction was calculated by subtracting the background efflux levels from efflux in the presence of apoA-I (Fig. 3A, 3B). The results show that lipid efflux from both adipocytes and macrophages responds to apoA-I induction in a linear fashion. Adipocytes efflux a higher percentage of their cellular PL compared to macrophages but the opposite is true for percent CL efflux in response to apoA-I incubation.

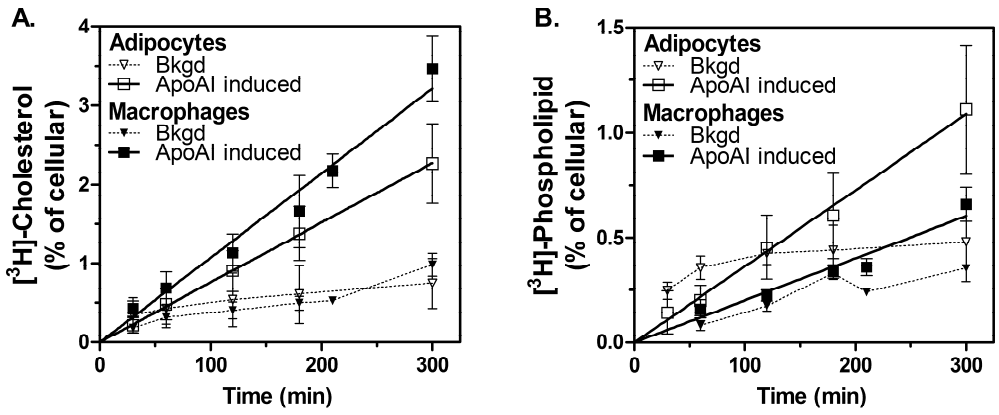

Figure 3. Lipid Efflux from Adipocytes and Macrophages. 3T3 L-1 adipocytes and J774 macrophages were labeled with **A.** [^{3}H]-cholesterol or **B.** [^{3}H]-choline and assayed for efflux into the media upon 75 µg/mL apoA-I incubation (□,■) or with buffer (▽,▼) as described in Methods section *III.iv,v*. The 'apoA-I induced' values for adipocytes (open shapes) and macrophage (filled shapes) data points were calculated by subtracting the background efflux at a particular time point from efflux of wells with apoA-I added at that same time point. Efflux was plotted versus time and error bars represent the mean ± s.d. for two PL and six CL assays in adipocytes as well as two PL and four CL experiments in macrophages with at least triplicates per experiment time point.

ii. ***Drug induction of ABCA1 increases CL efflux***.

Studies have shown that addition of cAMP analogues stimulates lipid efflux through the ABCA1-mediated pathway in macrophages [9, 21, 84, 126, 127]. Therefore the cAMP analogue 8-Br-cAMP (cAMP) was added to cells to enhance the ABCA1-mediated pathway in J774 macrophages (Fig. 4A). As expected this resulted in an increase (2.3 fold, $p < 0.001$) in apoA-I induced CL efflux above untreated macrophages. cAMP is known to increase both ABCA1 mRNA and protein levels in macrophages [9, 84]. Similarly, research has shown that LXR agonists, such as GW3965, increase ABCA1 mRNA levels in adipocytes [128-130]. Therefore the LXR-agonist GW3965 was added to adipocytes for comparison to the cAMP-treated macrophages and apoA-I dependent efflux monitored (Fig. 4B).

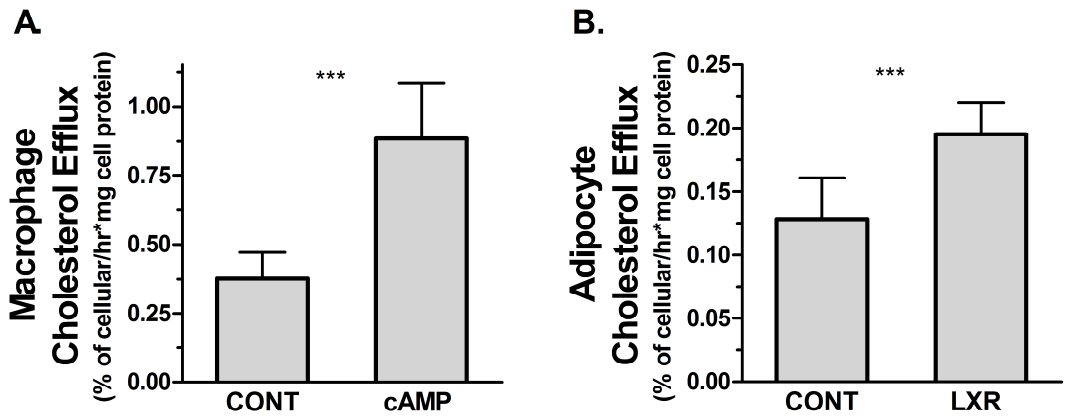

Figure 4. ABCA1 drug induction increases CL efflux from adipocytes. Experiments in **A.** macrophages or **B.** adipocytes were performed as before but cells were treated with either LXR-agonist 180µM 8-Br-cAMP (cAMP) or 2µM GW3965 (LXR) or buffer (CONT) 24 hours prior to assays. ApoA-I induced rates of lipid efflux were determined as mentioned previously using the slopes of the CL efflux assay graphs. The rates were then normalized by mg homogenate protein per well to obtained the average rates of % efflux per hour per mg homogenate protein. The bars represent the mean of four experiments for macrophage (n=15) and three experiments for adipocytes (n=19) for each treatment ± s.d. Significant differences (***, $p < 0.001$) were determined by student's t-test between the means of control and treated samples from both adipocytes and macrophages.

The results showed that the LXR-agonist increased (1.5 fold, $p < 0.001$) the apoA-I dependent CL efflux from adipocytes similar to the cAMP-induction of CL efflux from macrophages. The rates of CL efflux were normalized by the amount of homogenate

protein per sample in order to adjust for differences in protein content per culture well (Fig. 4). This normalization did not affect the fold difference within adipocytes (controls to LXR agonist-treated) or within macrophage (controls to cAMP-treated) efflux but did narrow the difference between adipocytes and macrophages. This is reflective of a variation across the cell types in protein content per culture well and provides one standard for comparison of the assays from adipocytes and macrophages.

iii. ***Molar rates of efflux from adipocytes compared to macrophages***.

Although the macrophages efflux a higher percentage of CL and adipocytes more PL, the percent of cellular (unit) cannot be interpreted to mean that macrophages efflux more CL and adipocytes more PL. To be able to compare in such a way, the total lipid contents of the different cell-types first need to be determined. Because adipocytes are expected to store larger amounts of cholesterol, conversion of lipid efflux as a percentage into molar lipid efflux was necessary in order to compare the efflux levels across cell types. The first step, in converting the units of efflux from percentage to moles, involved determining the homogenate lipid levels of both adipocytes and macrophages. The total lipid content of cells was determined by extraction of homogenate lipids, TLC separation, re-extraction, and finally quantified by assay as described in Methods section *III.iii*. All PL species were extracted from one TLC spot but the phospholipase D colorimetric assay measures mainly the phosphatidylcholine (PC) and sphingomyelin (SM) present. The resulting values for total CL and PC/SM (representative of overall PL efflux) in the homogenates were summarized in table 2. The difference in control and treated (either with cAMP or GW3965) homogenate lipid compositions were found to show no significant difference in lipid compositions and therefore all homogenate values within cell-types were averaged together. As expected, measured homogenate cholesterol levels showed that adipocytes contain more cholesterol per mg protein with a CL/PL ratio near one compared to macrophages with a CL/PL ratio closer to 0.75. The quantification for macrophages was consistent with previous reports of J774 homogenate lipid compositions [131]. The amount of homogenate lipid was then used to calculate the moles of CL and PL released into the media (table 2). The conversion of apoA-I dependent lipid efflux from a percentage to a molar efflux rate resulted in further equalization of efflux levels from both control adipocytes and macrophages although the treatment of macrophages with cAMP was still higher (1.3 fold) than the rate of CL efflux from LXR-treated adipocytes. A similar analysis of apoA-I dependent PL efflux from both adipocytes and macrophages was performed as described previously and is also summarized in table 2.

Table 2.
Homogenate Lipid Compositions and apoA-I dependent efflux of 3T3 L-1 adipocytes and J774 macrophages.

	Adipocytes	LXR-treated Adipocytes	Macrophages	cAMP-treated Macrophages
[a] Cellular Phospholipid **(nmol PC/SM per mg protein)**	45.7 ± 8.3 (25)	—[†]	33.9 ± 8.4 (34)	—
[a] Cellular Cholesterol **(nmol CL per mg protein)**	46.2 ± 11.2 (29)	—	25.7 ± 7.6 (32)	—
[b] Phospholipid Efflux **(nmol PC/SM per h • mg protein)**	0.100 ± 0.018 (33)	n.d.	0.0409 ± 0.010 (45)	0.159 ± 0.042 (44)
[b] Cholesterol Efflux **(nmol CL per h • mg protein)**	0.210 ± 0.051 (138)	0.309 ± 0.077 (46)	0.165 ± 0.050 (68)	0.387 ± 0.12 (70)
CL/PL Efflux ratio	2.1 ± 0.64	n.d.	4.0 ± 1.6	2.4 ± 1.0

[a] Cellular contents of choline-containing phospholipids (PC and SM) and cholesterol (CL) were determined as indicated in Methods section *iii*. Data are expressed as mean values ± s.d. The number of independent data points, different wells, is included in parenthesis.

[b] The rates of lipid efflux (mean ± s.d.) were obtained by linear regression. Data from 5 experiments (J774 cells) and 8 experiments using 3T3 L-1 cells with 3 experiments for GW3965 (LXR)-treated 3T3 L-1 cells are included. The number of data points used in the calculation of the rates is included in parenthesis. The efflux stoichiometry in moles of CL per mol of PL was calculated from the average rates of efflux. The standard deviation of the CL/PL ratio was estimated from the standard deviations of the rates of CL and PL efflux and the propagation of error equation.

n.d., not determined.

[†]the homogenate lipid compositions for treated cells were insignificantly different than values for control cells and were therefore represented as mean ± s.d. in the control cell columns.

*iv. **Comparison of adipocyte and macrophage lipid efflux ratios (CL:PL).***

The ability of adipocytes to efflux both CL and PL is an important establishment because it highlights the ability of adipocytes to contribute to nascent HDL levels. Furthermore, apoA-I-dependent efflux ratios (CL:PL) from adipocytes evaluated against macrophages allows a comparative analysis of each cell type's efflux pathway. The majority of apoA-I-dependent efflux from macrophages occurs via an ABCA1-mediated pathway thus the CL to PL efflux should have a characteristic ratio [18-20, 131]. To analyze the newly formed lipoparticle CL to PL composition, the quantitative lipid efflux assays mentioned previously were used to calculate the molar CL to PL ratios for adipocytes or macrophages (table 2). Since just the CL and PC/SM TLC spots were analyzed by colorimetric assay, the resulting ratio of efflux is actually CL to PC/SM. However, PC

has been shown to be the major phospholipid induced to efflux to apoA-I, thus these ratios are expected to be representative of the overall CL:PL efflux ratios [19, 132]. Although homogenates contained CL:PL ratios closer to 0.8-1.0 in both macrophages and adipocytes, ratios of CL:PL efflux changed significantly. Consistent with previous reports, control macrophages demonstrated an efflux ratio close to ~4 but in a cAMP-treated state the efflux ratio approached 2 [18-20, 131]. Similar to cAMP-macrophages the adipocyte CL:PL ratio in table 2 is approximately 2. Furthermore the ratio of CL:PL for cAMP-treated macrophages in the absence of control levels of efflux is also very similar to the ratio for adipocytes (1.85 ± 1.3). Thus, apoA-I elicits CL and PL efflux from adipocytes at ratios similar to the lipid efflux ratios of the ABCA1-mediated apoA-I dependent efflux pathway of cAMP-macrophages.

v. ***Normalization of lipid efflux by ABCA1 protein levels in adipocytes and macrophages.***

Adipocyte 3T3 L-1 cells have previously been shown to express ABCA1 and efflux CL upon apoA-I incubation [23, 24, 27]. However, although the mRNA expression levels have been studied in relation to efflux levels, the protein expression levels have not. The amount of ABCA1 mRNA relative to protein appears to differ by tissue type and protein levels are regulated on both the transcriptional level and by protein degradation [17, 133, 134]. Thus it has been concluded that analysis of ABCA1 mRNA levels cannot be solely used to predict efflux ability of cells [21]. In agreement with this, research has shown disparity between the level of ABCA1 mRNA levels and efflux levels in adipocytes [24].

The stoichiometric similarity of adipocyte lipid efflux compared to macrophages suggests the involvement of ABCA1 in apoA-I dependent lipid efflux from adipocytes. To further investigate this, lipid efflux assays were performed simultaneously with western blot analysis of ABCA1 from adipocyte and macrophage homogenates. Aliquots of homogenates were used for ABCA1 blotting by combining adipocytes and macrophage samples into different lanes within the same blots such that the adipocyte ABCA1 concentration could be determined relative to macrophage concentrations. The representative blot in figure 5A shows that ABCA1 is present in both adipocytes and macrophages as expected. As summarized by the graph of anti-ABCA1 relative densities from combined experiments, it is also apparent that the level of expression is increased in LXR-agonist treated adipocytes and cAMP-treated macrophages compared to controls (Fig. 5B). Also adipocytes seem to express ABCA1 at slightly higher levels than macrophages in the control states, but cAMP seems to result in a much stronger induction of ABCA1 protein expression in macrophages than LXR-treatment of adipocytes.

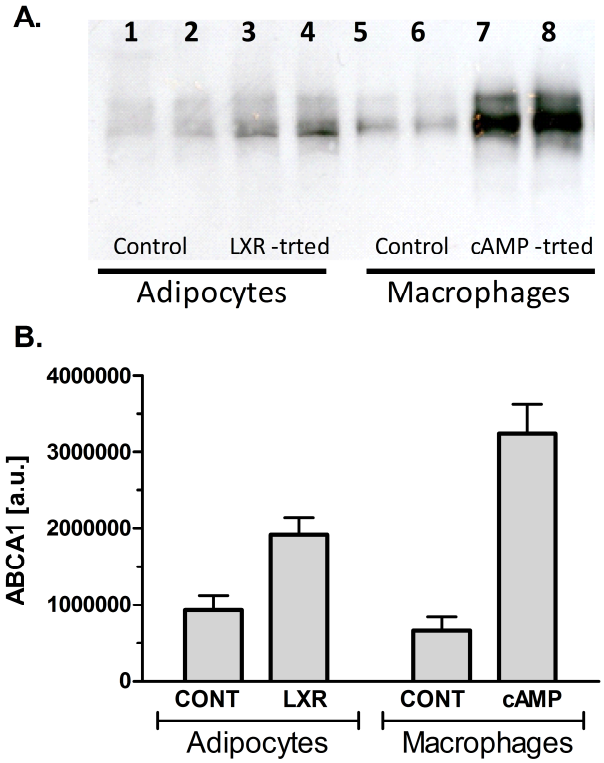

Figure 5. Adipocyte and Macrophage ABCA1 Protein Expression Levels. A. Comparison of ABCA1 protein levels from adipocytes to macrophages. 3T3 L-1 adipocyte and J774 macrophage homogenate samples were loaded onto SDS-PAGE and transferred to nitrocellulose for western blotting with anti-ABCA1. Amounts loaded per lane were normalized by the protein concentrations of homogenates. Samples were loaded such that each lane contained approximately 70µg of homogenate protein. Western blot is demonstrative of samples from 3 separate experiments with n=7 for each treatment state. Samples to be compared to each other were run within the same blot and normalized by homogenate protein per lane. Blot is representative of 4-6 technical replicate blots per sample per independent experiment with 3 experiments per treatment and cell type. **B.** Western blot densities of anti-ABCA1. Blots were normalized to each other by internal sample densities and arbitrary units combined into replicate samples. Replicates samples were combined by treatment and cell type and bars represent means ± s.d. for three independent experiments (n=7). Significant differences ($p < 0.001$) were determined by student's t-test between the means of control and treated samples from both adipocytes and macrophages.

In order to evaluate the efflux rate per ABCA1, the densitometric arbitrary units (a.u.) representing ABCA1 protein levels were then used to normalize the CL and PL efflux rates obtained from assays (Fig. 6A, 6B). The ABCA1 normalized CL efflux rate shows that adipocytes efflux at an identical rate as macrophages per ABCA1. The results also show an interesting pattern for CL efflux per ABCA1 protein (Fig. 6B). Induction of ABCA1 protein expression by either the LXR agonist or cAMP resulted in a decrease in efficiency of efflux per ABCA1. This large difference seems to implicate either an apoA-I induced but ABCA1-independent mechanism in control cells or that induction of

ABCA1 protein expression is not the only determinant in stimulation of cholesterol efflux to apoA-I. It is important to remember that this ratio does not represent overall efflux but instead similar levels of apoA-I induced efflux normalized by low levels of ABCA1 expression (Fig. 5B, 6A).

Despite these differences, the rate of cholesterol efflux normalized by ABCA1 protein was similar in the LXR-agonist treated adipocytes compared to cAMP-treated macrophages (Fig. 6B). Because cAMP-treated macrophages are believed to efflux lipids to apoA-I mainly through ABCA1, this is further evidence that adipocytes also efflux lipids to apoA-I mainly through an ABCA1-mediated pathway.

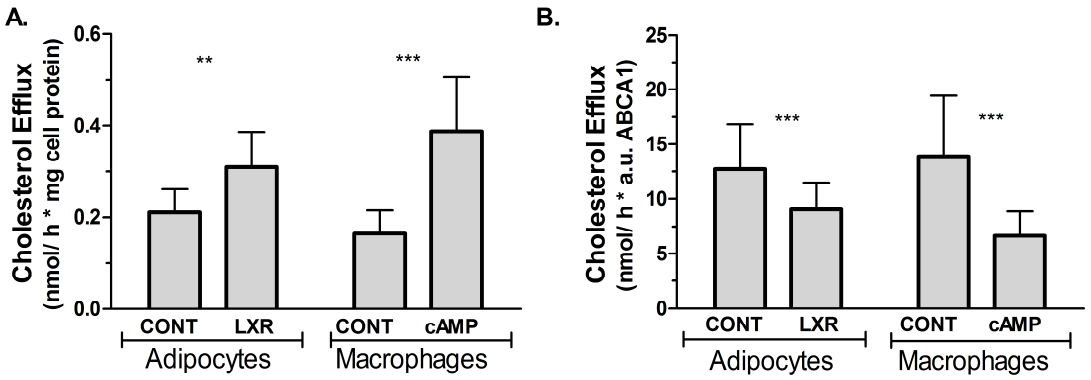

Figure 6. Effect of ABCA1 expression levels on apoA-I dependent CL Efflux from Adipocytes or Macrophages. A. Rates of CL efflux normalized by total cellular protein content. The experiments were performed as indicated in methods. Adipocytes and macrophages were treated with either the LXR-agonist, 2μM GW3965 (LXR), or 180μM 8-Br-cAMP (cAMP), respectively, or with buffer (CONT) for 24h prior to assays. The rates of apoA-I induced CL efflux normalized by protein content were determined from the slopes of the efflux assay time courses (% of cellular CL released into medium vs time). The average rates were then converted into nmoles of CL/h·mg cell protein using the cholesterol contents of the cells. The mean values ± s.d. for adipocytes were obtained from three independent experiments and include 19 data points. The data for macrophages were obtained from four experiments (n=15). Significant differences (***, $p < 0.001$; **, $p < 0.005$) were determined by student's t-test between the means of control and treated samples for both adipocytes and macrophages. **B.** Rates of CL efflux normalized by ABCA1 protein levels. The average rates of apoA-I induced CL efflux shown in figure 6A were converted into nmoles of CL/h·ABCA1 protein using the average relative levels of ABCA1 expression shown in the figure 5B. Each bar represents the mean ± s.d. which was calculated using the propagation of errors equation and the standard deviations of ABCA1 levels and rates of efflux. The differences between the means of control and treated cells were significant (***, $p < 0.001$) for both adipocytes and macrophages.

vi. ***Inhibition of apoA-I dependent lipid efflux from adipocytes by ABCA1-targeting drugs.***

To further investigate the role of ABCA1 in apoA-I dependent efflux from adipocytes, efflux assays were performed in the presence of several drugs known to reduce apoA-I dependent lipid efflux via inhibition of the ABCA1 transporter.

BFA is a drug known to block transport vesicle formation [29, 30, 135-137]. While BFA does not inhibit the endocytic transport of all proteins [136, 138, 139], it has been shown to inhibit the recycling of ABCA1 [31, 140]. Use of BFA allows manipulation of adipocyte ABCA1 availability to participate in efflux pathways. Although BFA has been shown to inhibit ABCA1, its broad disruption of cellular structures may cause disruption of lipid efflux from adipocytes via other unknown mechanisms. Therefore other ABCA1-targeting inhibitors were also used. The block lipid transport (BLT) family of inhibitors was first developed by Nieland *et. al.* while scanning for inhibitors that block the SRBI-mediated lipid transfer in cells [141]. SRBI mediates cholesterol ester uptake from, or CL efflux to HDL [76, 77]. However, while it was found that BLT1-3 and BLT5 remained highly specific to SRBI-mediated CL efflux, BLT4 cross inhibited ABCA1-mediated CL efflux [142]. Therefore in these adipocyte lipid efflux assays, BLT4 is used as a unique inhibitor of both the ABCA1 and SRBI pathways of efflux. Also BLT1 was used as a specific inhibitor of the non-ABCA1 SRBI-mediated CL efflux (to HDL) pathway. Finally, the sulfonylurea glyburide (GLYB) which is known to block several ABC transporters including ABCA1 was used [5, 85, 143-148]. GLYB is a powerful inhibitor for use in the apoA-I dependent lipid efflux assays to elucidate if apoA-I-induced efflux from adipocytes involves ABCA1.

Experiments to analyze the effects of these drugs on apoA-I dependent CL and PL efflux involved similar efflux assays as described previously but were performed in the presence of inhibiting BFA, GLYB, BLT4 or BLT1 concentrations. The drugs were allowed to preincubate with the cells prior to the addition of apoA-I. The resulting efflux versus time graphs were used to obtain the rate of efflux for each drug and control. To calculate the inhibition that each drug imposed on apoA-I dependent lipid efflux, the efflux rates of each drug were divided by the rates of the controls. The resulting inhibitions were expressed as a percent of the control efflux for CL or PL across both adipocyte and macrophages (Fig. 7).

Figure 7 shows strong inhibition by GLYB and BFA in both macrophages and adipocytes of the PL and CL efflux induced by apoA-I. BFA falls within the expected (~58-85%) inhibition compared to what has been studied previously for fibroblasts but is higher than previous work in macrophages [140, 149]. This could be because previous works in macrophages were limited to 2 hours of inhibition and highlight the time-sensitivity of BFA's effect. GLYB also strongly inhibited CL and PL efflux of both macrophages and

adipocytes. In addition to GLYB and BFA, adipocytes were also incubated with BLT1 and BLT4. Similar to GLYB and BFA, BLT4 also inhibited CL efflux but was only half as effective in inhibition of PL efflux. BLT4 had been reported to strongly inhibit CL release to apoA-I however its effects on PL efflux had not been examined until now [142]. Similarly BLT1 slightly blocked CL efflux but its inhibition of PL efflux from adipocytes was much less pronounced. It is interesting to note that while the BLT drugs inhibited cholesterol efflux from adipocytes more than they inhibited phospholipid efflux, the opposite is true of GLYB and BFA.

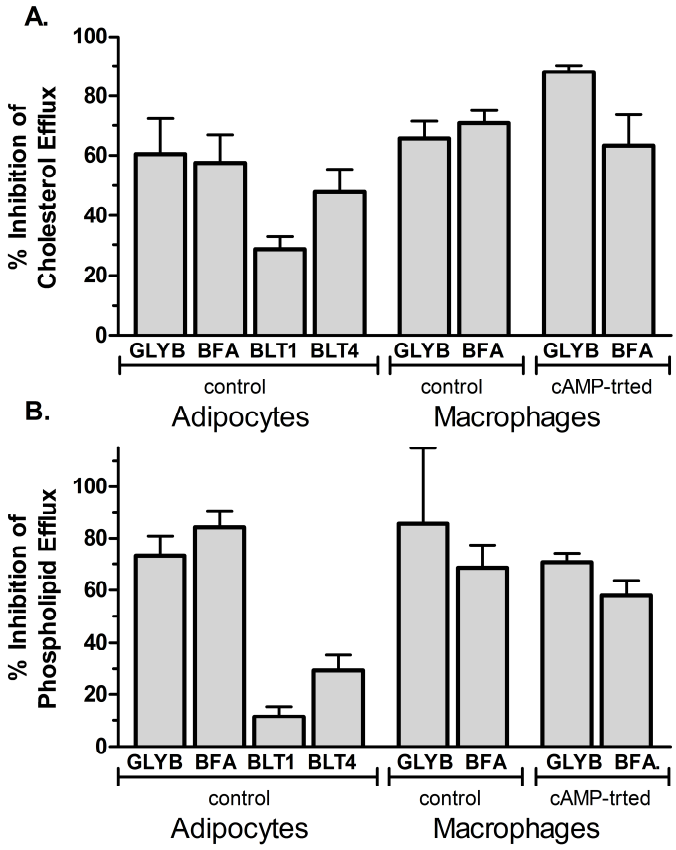

Figure 7. Effect of ABCA1-inhibiting drugs on apoA-I dependent efflux from adipocytes compared to macrophages. The effect of the drugs GLYB (500µM), BFA (36µM), BLT1 (10µM) and BLT4 (150µM) on apoA-I-dependent cholesterol efflux from macrophages and adipocytes was determined as described in Methods section *III.vi*. **A.** Inhibition of apoA-I induced cholesterol efflux from adipocytes and macrophages. The bars represent the mean ± the s.d. of 2-4 independent experiments for adipocytes and 1 experiment for macrophages with n=6-14 for adipocytes and n=3 for macrophages. **B.** Effects of the same drugs on inhibition of apoA-I dependent phospholipid efflux from adipocytes and macrophages. The bars represent the mean ± s.d. of one experiment with n=3-4.

It has been reported that, in the case of glyburide inhibition of PL efflux, the inhibition seen is due largely to increases in background (i.e., lipid efflux to apoA-I free media) rather than large decreases in apoA-I induced lipid efflux [111, 142]. However for most of the efflux assay experiments, the majority of each drug's inhibition resulted from decreased efflux in the presence of apoA-I rather than in than increases in background levels of efflux (data not shown). In the few cases where increases in background contributed to the majority of the inhibition observed, the overall % inhibition values showed no significant difference from other experiments. Thus the inhibitions by these drugs most likely reflect a real inhibition of ABCA1-mediated apoA-I dependent lipid efflux in which any experimental variability in background efflux levels are appropriately controlled for, thus giving an accurate reflection of the inhibition ability of each drug.

CHAPTER V

THE β-SUBUNIT OF ATP SYNTHASE CONTROLS CELLULAR UPTAKE AND RESECRETION OF APOA-I IN ADIPOCYTES BUT DOES NOT CONTROL APOA-I INDUCED LIPID EFFLUX

Abstract

The initial steps in the formation of nascent HDL involve lipid loading of apoA-I. Cellular uptake of apoA-I may be important for the assembly of HDL and may also regulate the circulating levels of the apoA-I and/or HDL. ApoA-I is shown here to be internalized and re-secreted (apoA-I recycling) by adipocytes in a saturatable and specific process. ABCA1 is involved in cellular binding, uptake and lipidation of apoA-I. The following studies however, suggest apoA-I recycling is a receptor-mediated process independent of ABCA1 function [121]. Additional results show that a monoclonal antibody against the β-subunit of ATP synthase blocks apoA-I recycling but has no effect on the rate of apoA-I induced cholesterol or phospholipid efflux. It is concluded that a) ATP synthase β, which had been previously identified as an apoA-I receptor [150], is directly involved in cellular recycling of apoA-I and, b) cellular recycling of apoA-I is not required for apoA-I induced lipid efflux demonstrating that apolipoprotein lipidation and recycling are independent processes.

Keywords: apoA-I, adipocyte, lipid efflux, ABCA1, recycling, ATP synthase β

Abbreviations used: ApoA-I, apolipoprotein A-I; ABCA1, ATP-binding cassette transporter A1; BFA, Brefeldin A; BSA, bovine serum albumin; FBS, fetal bovine serum; apoLp-III, apolipophorin III; Thrx, thioredoxin; ATPase β, ATP synthase F_1 subunit β;

Results

i. ***Pka-ApoA-I enters the cell by a saturatable and specific pathway.***

In order to study the uptake and re-secretion of apoA-I and its role in the process of efflux a recombinant protein was developed containing full length mature human apoA-I and two tags including a His-tag for protein purification and a protein kinase (PKA) consensus sequence containing tag [27, 28]. The pka-tag allows monitoring of protein cell entry. The concept of the assay is based on the idea that cell entry of the tagged protein allows for its phosphorylation by intracellular PKA. Also, if the protein is re-secreted, the level of the tag-phosphorylation analyzed from the media is related to the level of protein recycling. By radiolabeling the ATP pool with [^{32}P]-orthophosphate, the phosphorylated pka-apoA-I collected from cell media could then be visualized by autoradiography allowing for direct evidence of apoA-I recycling.

To examine the levels to which apoA-I might be taken up by the cell and re-secreted into the cellular medium, adipocytes were [^{32}P]-radiolabeled and increasing amounts of pka-apoA-I were added to the experimental media. Following a 30 minute incubation time, media was collected and phosphorylation of pka-apoA-I analyzed. The coomassie gel (Fig. 8A) shows the increasing levels of protein added to the cell media while the autoradiogram (Fig. 8B) shows increasing levels of phosphorylation. These results definitively show that apoA-I is recycled in adipocytes. Importantly, comparison of the last two lanes of both the gel and autoradiogram reveal that the increase in phosphorylation becomes saturated. To better visualize this saturation, the densitometric phosphorylation a.u. of pka-apoA-I were normalized by the protein amount and graphed to examine the level of apoA-I uptake and re-secretion (Fig. 8C). From the graph, apoA-I appears to be readily entering and exiting the cell until the process starts saturating near 45 µg/mL. The two major possibilities for uptake of apoA-I involve either receptor-mediated endocytosis and/or bulk phase endocytosis through membrane recycling. Bulk phase endocytosis would involve binding of apoA-I to the plasma membrane and slow internalization as the membrane turns over. Instead the saturation of uptake seen from increasing apoA-I concentration in the experimental media seems to implicate a saturatable receptor mediated pathway (K_D ~1µM).

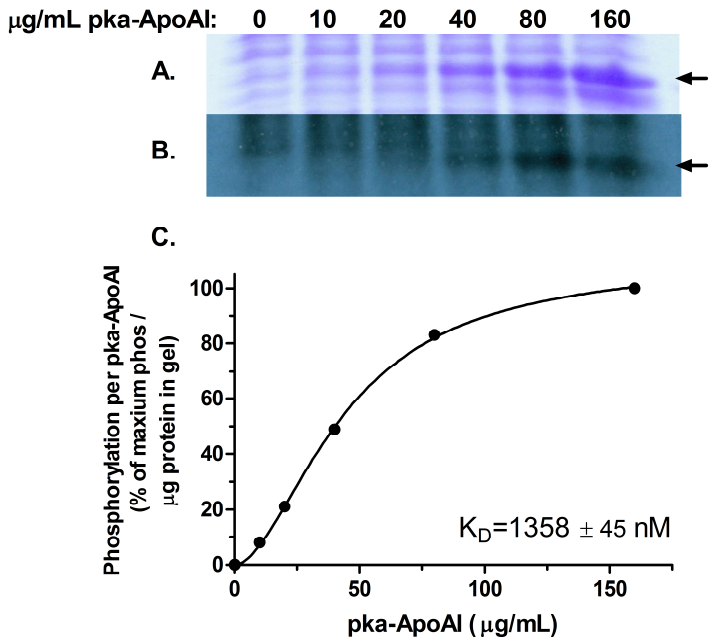

Figure 8. Saturation of apoA-I entry and exit from the cell. Recycling assays of pka-apoAI were performed in [^{32}P]-prelabeled adipocytes as described in Methods section *III.ix*. **A.** Coomassie stained gel representing 10% of each well's media per lane. Arrow indicates the 31kDa pka-apoA-I. **B.** Autoradiogram of the gel in A showing increased levels of phosphorylation per protein. **C.** Graph of the level of pka-apoA-I phosphorylation (from part B) normalized by the amount of pka-apoA-I protein (from part A) versus the increasing amounts of apoA-I added. The equilibrium binding constant (K_d) of recombinant pka-apoA-I was calculated from the saturation curve (from part C) using the graphing program GraphPad.

To evaluate the recycling pathway further, increasing levels of native human apoA-I were added to compete with pka-apoA-I for cell entry. Since human apoA-I contains no PKA-tag, decreases in phosphorylation indicate that pka-apoA-I is specifically being blocked from cell entry. Cells were [^{32}P]-radiolabeled and a constant concentration of pka-apoA-I was maintained with varying amounts of competing human apoA-I added. Media was collected after the incubation time and separated by gel (Fig. 9B). Pka-apoA-I travels as a 31 kDa protein while un-tagged human apoA-I travels at its native size (28kDa) below it. Results show that pka-apoA-I loses phosphorylation as the human apoA-I concentration is increased (Fig. 9A). A control (Fig. 9A, 9B, lane1) was added to show that incubation of human apoA-I does not induce secretion of any unknown phospho-proteins at the same size as pka-apoA-I. Phosphorylation of pka-apoA-I was then plotted versus the competing human apoA-I concentrations to assess the level of competition between human and recombinant apoA-I (Fig. 9C). Analysis of the inhibitor-response curve reveals that human apoA-I behaves similar to recombinant pka-apoA-I with

K_i ~1µM. This implies that recombinant and human apoA-I are most likely competing for cell entry from the same receptor.

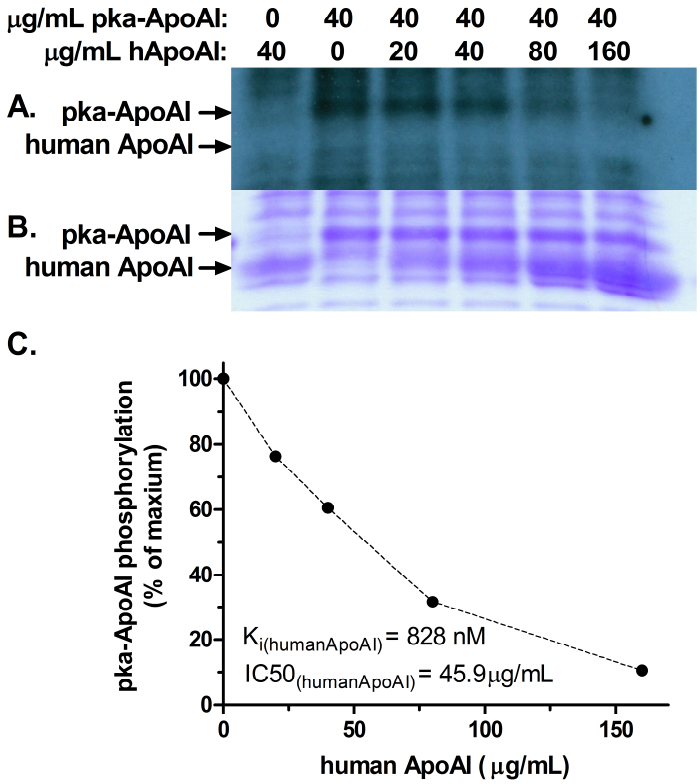

Figure 9. Human apoA-I competes with recombinant pka-apoA-I for entry into the cell. Recycling assays of pka-apoA-I were performed as described previously with increasing amounts of human apoA-I added. **A.** Autoradiogram showing that human apoA-I competes with pka-apoA-I for entry and exit from the cell. **B.** Coomassie gel with 10% of the well media loaded. Shows pka-apoA-I concentration constant and human apoA-I is increasing. **C.** Graph of autoradiogram versus the human apoA-I concentrations in part B. GraphPad software was used to plot the log dose-response curve and calculate the K_d of inhibition (K_i) and IC50 of human apoA-I. The K_i of 828nM (23 µg/mL) has 95% CI of 422 to 1625nM and the IC50 of 45.9 µg/mL has 95% CI of 23.4 to 90.2 µg/mL.

ii. ***Effect of ApoLp-III and Thrx on recycling of pka-ApoA-I.***

Since human apoA-I competed with pka-apoA-I for cell entry, experiments were designed to further test the specificity of the recycling receptor. This was investigated by experiments designed to perform the apoA-I recycling assays in the presence of other types of proteins. ApoLp-III was chosen to test whether the apoA-I receptor may have

affinity for other apolipoproteins. Similar to apoA-I, it is mainly alpha helical and binds to the lipids of lipoparticles [151]. Thrx was also chosen as a generally unrelated protein to monitor the level of specificity needed to compete for recycling. The coding regions of these proteins were cloned into plasmids such that the recombinant proteins contained a HIS-tag for purification as well as the PKA-recognition site tag similar to the pka-apoA-I construct (Fig. 10).

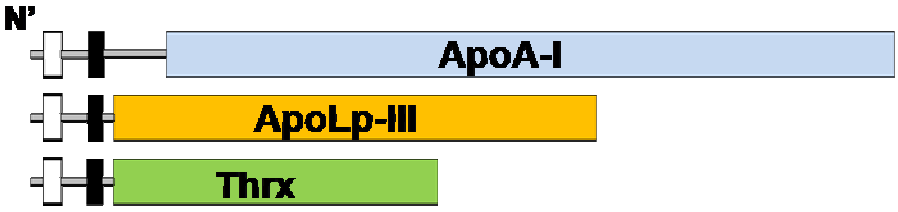

Figure 10. ApoA-I, ApoLp-III and Thrx pka-tag recombinant protein constructs. Sequence coding for mature apoLp-III and Thrx were cloned into plasmids such that the recombinant proteins would contain a N-terminal HIS-tag to facilitate purification and PKA-recognition site tag to enable use of the construct in recycling assays as described in Methods. The recombinant proteins are diagrammed above to show the different coding regions. The open boxes represent the 6 histidine residue tag and the closed boxes represent the PKA recognition sequence RRASV. Intervening amino acid sequence between tags and protein sequence is represented by the thin gray bar and sequence coding for either ApoA-I, ApoLp-III or Thrx is represented by blue, orange or green boxes, respectively. The dimensions of each region are proportional to number of amino acids such that relative (primary) sizes are visualized.

Because all the recombinant proteins used for this study contain a pka-recognition site phosphorylation tag, the internalization of apoA-I as well as the other two proteins can be monitored (Fig. 10). For the experiments, increasing amounts of either apoLp-III or Thrx were added simultaneously with apoA-I to [^{32}P]-radiolabeled adipocytes. Pka-apoA-I, which was shown previously to be internalized and re-secreted from adipocytes, can be added to culture media with or without the potential competitors, and the recycling monitored by analysis of the medium following the incubation time.

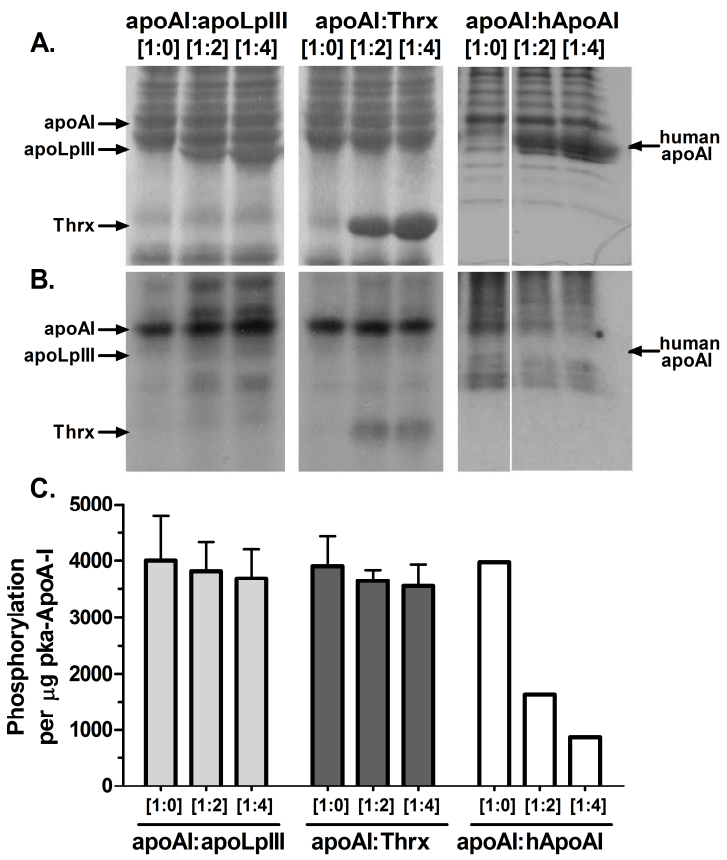

Figure 11. The effect of apoLp-III and Thrx on apoA-I recycling. Competition recycling assays of pka-apoA-I were performed as described previously (Methods section *III.x*) with increasing amounts of apoLp-III (3 or 6 nmol) or Thrx (3 or 6 nmol) or human apoA-I (as described previously) added to culture wells along with pka-apoA-I (1.5nmol). **A.** Representative Coomassie-stained gel of media samples with arrows indicating location of pka-apoA-I, apoLp-III, Thrx or human apoA-I. **B.** Autoradiogram of gel (from A) showing [^{32}P]-phosphorylation of pka-recombinant proteins. **C.** Average phosphorylation of pka-recombinant proteins as determined by autoradiography of media gels (arbitrary units of densitometry). Bars represent the mean ± s.d. of two independent experiments for apoA-I:apoLp-III (n=5) and one experiment for apoA-I:Thrx (n=3) and human apoA-I. The differences between pka-apoA-I densities of phosphorylation were found to be not significant by student's t-test ($p>0.05$) for both apoLp-III and Thrx competition assays.

As can be seen in figure 11, pka-apoA-I is recycled through adipocytes as expected (lanes 1, 6, 9) and although added with apoA-I to cells, the proteins apoLp-III and Thrx do not compete with apoA-I for cell entry (Fig. 11C). In contrast, addition of human apoA-I resulted in competition with pka-apoA-I for recycling as determined previously (Fig. 9, 11).

Interestingly, although apoLp-III and Thrx don't reduce the pka-apoA-I internalization, they themselves are internalized and re-secreted but to what seems to be a much lower

degree than apoA-I (apoLp-III ~30 fold less and Thrx ~7.5 fold less relative to pka-apoA-I; Fig. 12C). These results show that receptor-mediated apoA-I recycling has a higher specificity for apoA-I than other proteins (including some apolipoproteins).

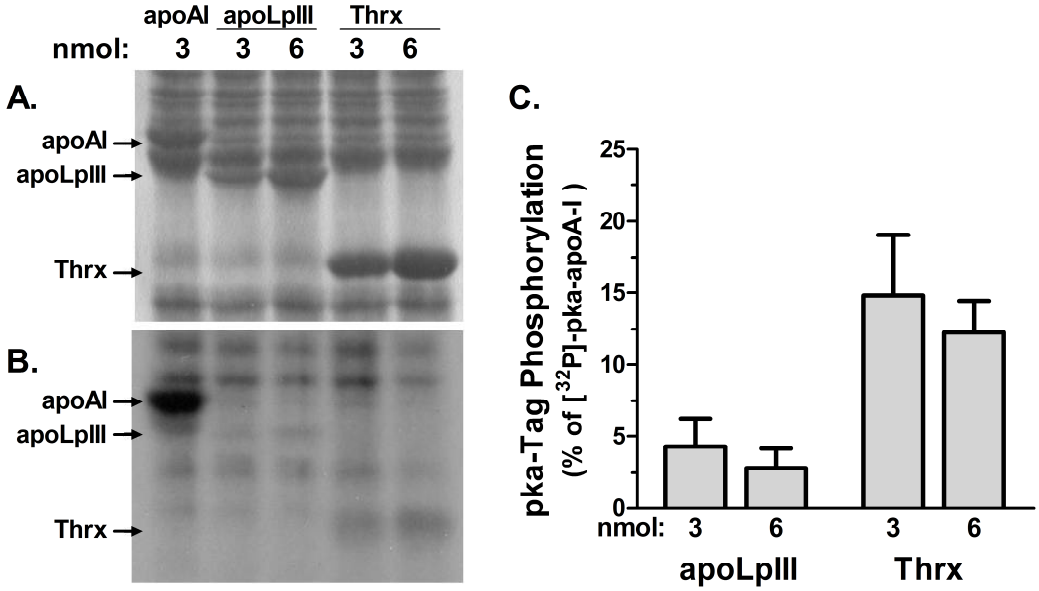

Figure 12. Recycling of apoLp-III and Thrx compared to apoA-I in adipocytes. Recycling assays of apoLp-III and Thrx were performed as described previously with increasing amounts of pka-apoLp-III (3 or 6 nmol) or pka-Thrx (3 or 6 nmol) or pka-apoA-I (3 nmol) added alone to culture wells. **A.** Representative Coomassie-stained gel of media samples (15% volume loaded) showing location of pka-apoA-I, pka-apoLp-III and pka-Thrx. **B.** Autoradiogram of gel (from A) showing [^{32}P]-phosphorylation of pka-recombinant proteins. **C.** Average phosphorylation of pka-recombinant proteins as determined by autoradiography of media gels (arbitrary units of densitometry). Bars represent the mean ± s.d. of two independent experiments for apoLp-III (n=5) and one experiment for Thrx (n=3). The differences between pka-tag phosphorylation of 3 and 6 nmol incubations of either apoLp-III or Thrx were found to be not significant by student's t-test ($p>0.05$).

iii. ***Inhibitors of ABCA1 (BFA) and SRBI (BLT1) do not inhibit apoA-I uptake and resecretion.***

Next the role that the receptors ABCA1 or SRBI may play in the lipidation of apoA-I was investigated through use of the inhibitors BFA and BLT1. To examine the possibility that ABCA1 may be mediating both apoA-I cellular uptake and resecretion along with lipid efflux, BFA was used to block ABCA1 recycling and cell surface availability [31]. As shown previously, BFA is also a strong inhibitor of ABCA1-mediated efflux to apoA-I (Chapter IV section v). Additionally BLT1 was used to investigate SRBI as

another possible apoA-I recycling receptor. BLT1 is known as a strong inhibitor of SRBI-mediated CL efflux to HDL [141], and previous work has also shown it to be capable of weak inhibition of ABCA1-mediated efflux in adipocytes (Chapter IV section v).

For experiments, cells were [^{32}P]-radiolabeled and the drugs BFA and BLT1 were allowed to pre-equilibrate before addition of pka-apoA-I. Cell media was collected after the specified incubation times and pka-apoA-I purified using Ni-chromatography. Purified pka-apoA-I was run onto SDS gels (Fig. 13A) and exposed to film to visualize pka-apoA-I phosphorylation (Fig. 13B). Because the lipolytic activity level of the cell regulates the PKA activity level and glycerol release into the media, medium glycerol levels are indicative of PKA activity levels. Thus, to control for changes in PKA activity possibly induced by the drugs, an aliquot of media was used to measure glycerol levels.

As can be seen in figure 13B, the phosphorylation is stronger in the samples with a 40 minute medium incubation time. Phosphorylation of these samples was analyzed by densitometry as mentioned previously and normalized by medium glycerol levels. The results represent the levels of apoA-I recycling in the presence treatment with either BFA or BLT1 (Fig. 13C).

Although the results show that apoA-I is retroendocytosed, these drugs, which were previously shown to inhibit apoA-I lipidation, have no inhibition on the uptake and re-secretion of apoA-I. This implies that apoA-I recycling involves a receptor besides those shown to be inhibited by BFA (ABCA1) and BLT1 (SRBI) and leads to the possibility that apoA-I recycling through adipocytes may be independent from its lipidation. Similarly, other studies have concluded that apoA-I internalization in macrophages may not significantly contribute to overall efflux levels and that only a low level of internalized apoA-I co-localizes with ABCA1 [119, 152].

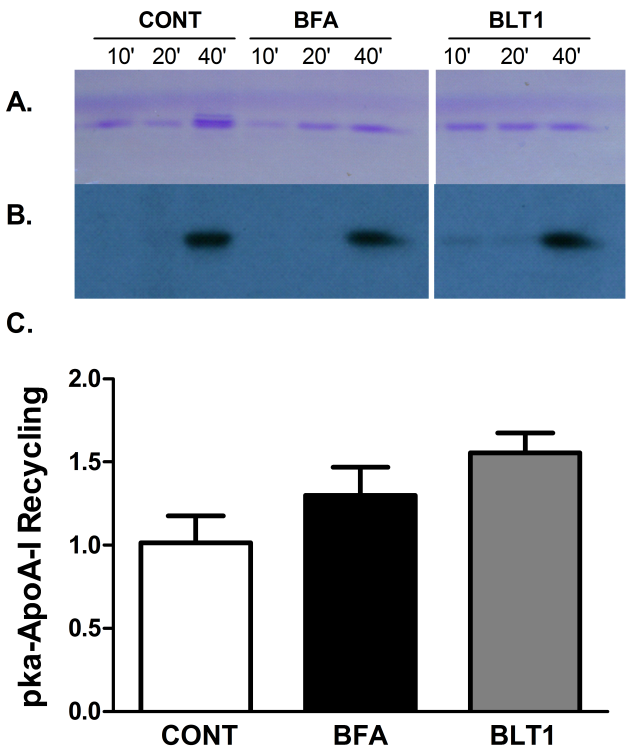

Figure 13. Efflux drugs do not inhibit pka-apoA-I uptake and resecretion. [^{32}P]-prelabeled adipocytes were pre-equilibrated with BFA (10 µg/ml) or BLT1(10 µM) for 2h prior to incubation with 50 µg/mL pka-apoA-I. The media was collected and analyzed for tag-phosphorylation and rate of glycerol release as described previously. **A.** Coomassie gel showing pka-apoA-I purified per time point. **B.** Autoradiogram of the dried gel (from part A) showing phosphorylation of purified apoA-I. **C.** Graph of autoradiogram normalized by protein amount (from part A, 40' samples) and medium glycerol rates. Error bars represent the mean ± s.d., n=2. Sample's were not significantly different as determined using the students t-test ($p>0.05$).

*iv. **Antibody to ATP synthase beta subunit blocks cell entry and exit of apoA-I.***

The above studies have shown that apoA-I recycling involves a pathway separate from those involved in ABCA1-mediated efflux. Therefore, claims that cell surface ATP synthase acts as an apoA-I receptor were investigated.

Ectopically expressed ATP synthase F_1 subunit β (ATPase β) was first discovered to bind apoA-I when Martinez *et al.* used immobilized apoA-I to probe solubilized membrane proteins [150]. Subunits of the traditionally mitochondrial protein have been shown to be present on the cell surface of hepatocytes, lymphocytes, endothelial cells and adipocytes but not erythrocytes nor Chinese hamster ovary cells [150, 153-155]. Although studies are lacking for all these cell types, the complete ATPase complex with both the

membrane-embedded F_0 and the soluble F_1 (containing α,β,γ,δ,ε subunits) subdomains has been shown to be present in isolated rat hepatocytes [156]. Ectopically expressed ATPase has been shown to exhibit ATP synthesis and hydrolysis activities in both hepatocytes and endothelial cells [156-158]. Furthermore, antibodies to α and β subunits of the F_1 subdomain have been shown to inhibit the synthase/hydrolase activities of ATPase [156-158].

Because cell surface expressed ATPase β had been shown to bind apoA-I [150], the effect that β subunit antibodies had on apoA-I uptake and re-secretion from adipocytes was investigated. For this experiment, [^{32}P]-prelabeled adipocytes were pre-incubated with increasing amounts of anti-ATPase β prior to the experiment. Two monoclonal antibodies to the beta subunit of ATP synthase (clones 3E8 and 3D5) were chosen to study the effect on apoA-I uptake. After antibody pre-incubation, apoA-I was added to the experimental medium and the media removed after the incubation period. Cell entry of apoA-I and exit back into the cell media was monitored by the phosphorylation of the pka-tag. ApoA-I was purified from the media using Ni-affinity chromatography then analyzed by SDS-PAGE and autoradiography as shown in figure 14A and 14B respectively.

The results show that apoA-I uptake and re-secretion is accompanied by phosphorylation of the construct's pka-tag (lane 1, Fig. 14B) and that this phosphorylation is blocked by increasing amounts of anti-ATPase β clone 3D5 (lane 2-7, Fig. 14B). Although anti-ATPase β clone 3D5 successfully blocked apoA-I recycling, the other clone (3E8) investigated did not have a significant effect on apoA-I phosphorylation levels (Fig. 14C, $p>0.05$). To further classify the inhibition by clone 3D5, phosphorylation of pka-apoA-I was plotted versus antibody dose (Fig. 14D). The results indicate that the level of recycled apoA-I rapidly falls upon incubation with antibody until reaching a low at approximately 35% of maximum phosphorylation. This is comparable to the inhibition of endothelial cell-surface ATP synthase activities observed in a previous study by binding of a monoclonal antibody (Mab3D5) to the domains near the active site of the beta subunit [158]. The log dose-response of the inhibition was plotted and the curve was used to calculate an IC50 for the antibody clone 3D5 at ~10.6 μg/mL (95% CI of 4.722 to 23.88 μg/mL). PKA activity levels were also checked through measurement of glycerol release into the media (Fig. 14E). The glycerol levels showed no significant change, demonstrating that changes in pka-apoA-I phosphorylation were not due to changes in intracellular PKA activity (Fig. 14E, $p>0.05$).

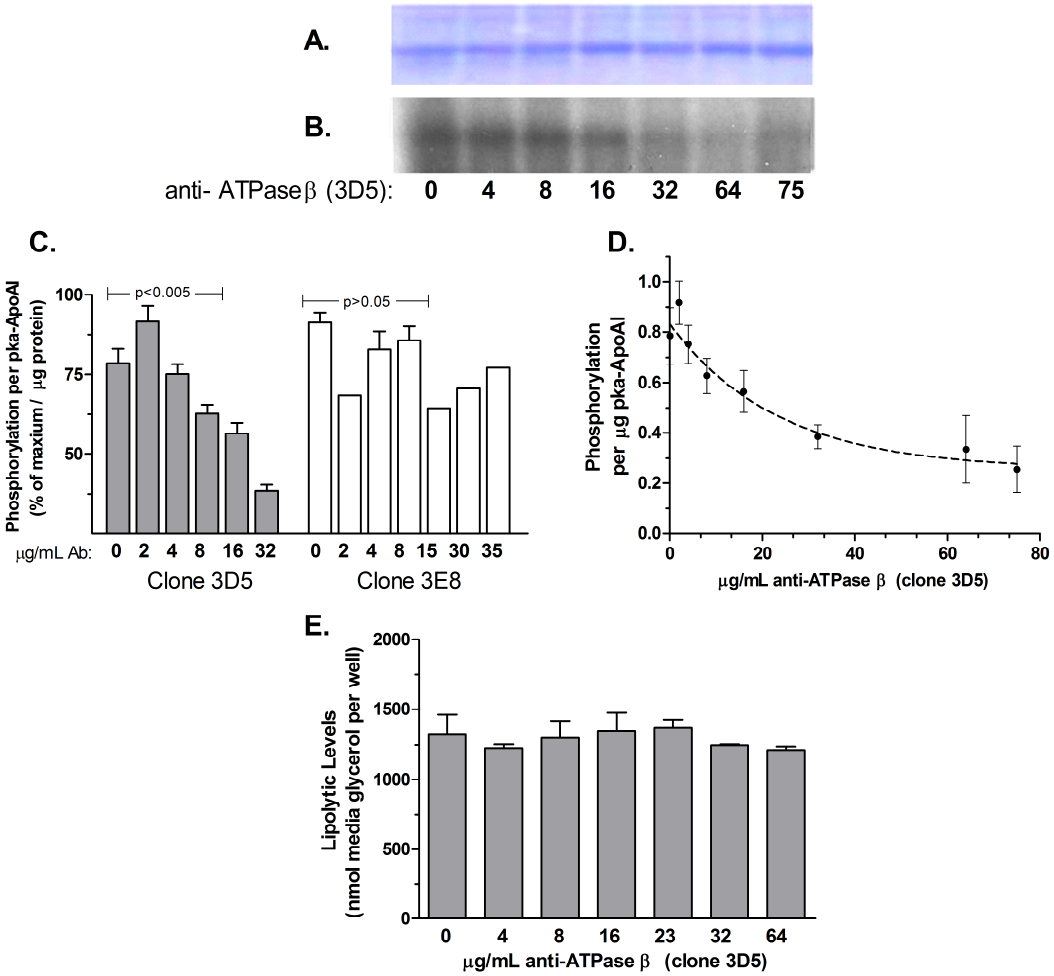

Figure 14. The effect of antibody to ATP synthase (β subunit) on cellular uptake and release of pka-apoA-I.

Recycling assays of pka-apoA-I were performed as described previously with increasing amounts of antibody to ATPase β (either monoclonal 3D5 or 3E8) allowed to pre-incubate 1 h prior to addition of pka-apoA-I (40 µg/mL). The media was collected and analyzed to detect tag pka site phosphorylation as described previously. **A.** Representative Coomassie blue stained gel and **B.** autoradiogram displaying phosphorylation of purified pka-apoA-I protein. **C.** Protein and phosphorylation amounts (such as from A and B) were quantified and plotted by the amount of antibody added. Error bars represent standard error of the mean from two independent experiments per clone with at least duplicates per sample. Significance of differences was determined from 0 to 8 µg/mL of antibody with student's t-test and using one-way ANOVA with post tests for linear trend (3D5, $p<0.0001$ and 3E8, $p>0.05$) using GraphPad software. **D.** Phosphorylation of pka-apoA-I released into media (as determined by densitometry of gel and autoradiogram) was plotted versus antibody dose. **E.** Glycerol released into media was measured to monitor lipolytic and PKA activity levels. Error bars in D and E represent mean ± s.d. with n=2-6 from 2 independent experiments.

v. ***Antibody to ATP synthase beta subunit does not affect apoA-I dependent cholesterol efflux levels.***

Because previous studies were able to pharmacologically uncouple apoA-I recycling from apoA-I lipidation using BFA, cholesterol efflux was measured to confirm that antibody blocking of pka-apoA-I cellular entry has no effect on cholesterol efflux to apoA-I. Cholesterol efflux experiments involve [^{3}H]-cholesterol pre-labeling for 24 hours in media containing a decreased amount of serum compared to the previous [^{32}P]-labeling experiments. Previous literature has shown that cell medium serum levels influence ATPase ectopic cell surface expression in HepG2 hepatocytes and the human T cell line CEM [159, 160]. Under serum starvation conditions ATP synthase surface expression is induced [159, 160]. Because surface levels of ATPase are influenced by medium serum concentrations, a control [^{32}P]-labeling recycling assay was performed in the same manor and media conditions as [^{3}H]-cholesterol efflux experiments to ensure the significant antibody blocking of pka-apoA-I cell entry.

As can be seen in figure 15, the cholesterol efflux rate to apoA-I is unchanged even when co-incubated with anti-ATPase β, which is shown to block pka-apoA-I cell entry under similar experimental conditions (Fig. 15A, 15B). It should be noted that, although 60 µg of antibody significantly blocks the cellular uptake of pka-apoA-I, the percent inhibition is only approximately 40%, whereas under previous experimental conditions recycling inhibition was at approximately 60%. This difference is most likely due to changes in the labeling-medium conditions of the different experiments which may affect cell surface expression of ATPase β. Indeed, studies have shown that medium levels of cholesterol and serum concentrations dramatically affect the cell surface expression of ATP synthase and thus may have varying affects on our pka-apoA-I recycling assays [159, 161]. This highlights the importance of monitoring cell entry in the same experiment and manor as the cellular cholesterol efflux measurements.

However, despite this 40% inhibition, cholesterol efflux remains unaffected. These results are in line with other works showing that ATPase activity when blocked by inhibition factor 1 (IF_1) shows no effect on efflux to apoA-I, and moreover shows that the apoA-I cell entry and release is unrelated to the overall lipidation of apoA-I [150].

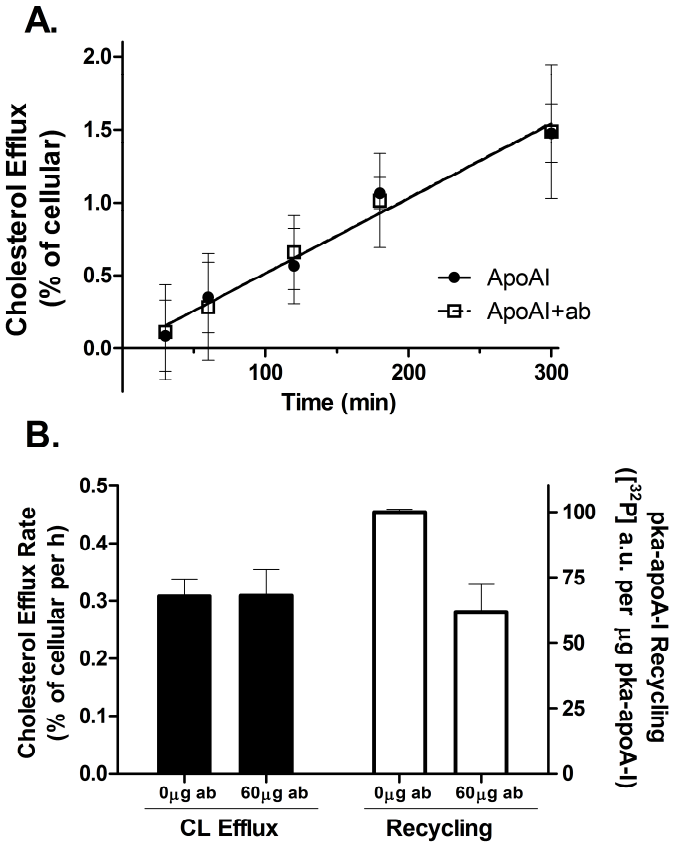

Figure 15. Effect of anti-ATPase β on ApoA-I-dependent cholesterol efflux. 3T3 L-1 adipocytes were radiolabeled with [^{3}H]-cholesterol as mentioned in methods and pre-equilibrated 1 h prior to pka-apoA-I addition with or without 60 µg anti-ATPase β clone 3D5. **A.** Efflux assay media was collected at indicated time points and cholesterol efflux rate calculated from the time courses of the CL efflux and represented in B. **B.** For 'Recycling' data, 1 h media was collected and apoA-I purified. Protein concentration was determined as mentioned in methods (*III.xv*). The media was collected and analyzed for tag-phosphorylation as described previously. Bars in part A represent mean ± s.d. of n=4 and solid bars in part B (for efflux rate) represent the standard error of the slope from part A and the open bars (for recycling assays) represent the mean ± s.d. of n=4.

vi. ***Antibody to ATP synthase beta subunit does not affect apoA-I-dependent phospholipid efflux levels.***

The mechanism of apoA-I lipidation involves both CL and PL loading of apoA-I, but it is unclear if this occurs simultaneously or in steps. Although our results have shown CL efflux to be independent from apoA-I recycling, PL efflux was also examined to clearly define the relationship of apoA-I recycling and HDL formation.

The effect on the level of PL efflux was investigated by adding increasing amounts of anti-ATPase β to cells. Cells were prepared by the radiolabeling and antibody addition as described previously. After apoA-I incubation, experimental media was collected and Ni-affinity purified to obtain effluxed lipids associated to apoA-I. The lipids were extracted by modified Folch to remove the free phosphate and protein fractions from the organic phase containing radiolabeled phospholipids. Scintillation counting of the phospholipids were normalized by apoA-I UV absorbance levels at 280nm and plotted by the amounts of antibody added to the media (Fig. 16). The results show that phospholipid efflux levels associated to apoA-I are not significantly affected by antibody levels (Fig. 16, student's t-test $p>0.05$).

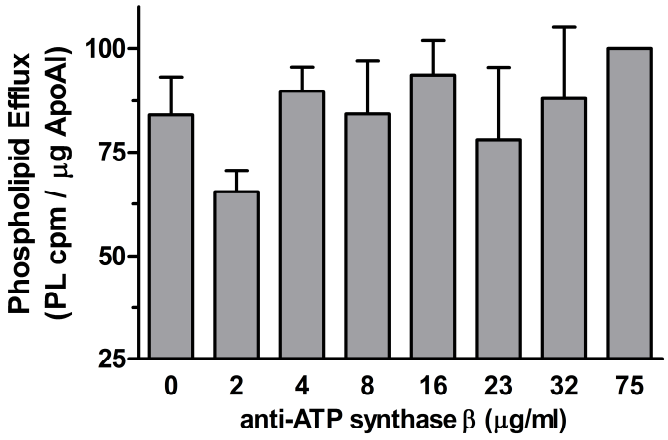

Figure 16. Effect of anti-ATPase β on ApoA-I-dependent phospholipid efflux. [^{32}P]-prelabeled adipocytes were pre-equilibrated with anti-ATPase β clone 3D5 at the indicated concentrations for 1 h prior to pka-apoA-I (40 μg/mL) addition. ApoA-I was then purified from the experimental medium and protein concentration was determined by UV_{280nm}. Phospholipids were then extracted (as described in Methods *III.xiv*) and [^{32}P]-labeled phospholipids measured by scintillation counting and normalized by protein UV absorbance levels. Error bars represent mean ± s.d. of n=2-6 from two independent experiments.

CHAPTER VI

DISCUSSION

i. ***Characterization of apoA-I dependent lipid efflux from adipocytes***.

As the largest storage site of free cholesterol in the body, adipocytes have the potential to influence circulating HDL [59, 60]. Studies have shown correlations in obesity and altered component lipid ratios of circulating HDL [63-65]. Upper body fat accumulation is associated with lower HDL levels and increased CHD risk [64]. This suggests that lipid efflux from adipocytes has the potential to significantly alter overall HDL levels and compositions. ApoA-I has been demonstrated to utilize ABCA1 for cholesterol and phospholipid efflux from other cell types but the process had not been well characterized in adipocytes until now. These studies which have furthered the characterization of both CL and PL apoA-I dependent efflux from adipocytes highlight the ability of adipocytes to contribute to the HDL population.

Quantification of lipid efflux

The studies presented above have compared the molar rates of lipid efflux to apoA-I from both adipocytes and macrophages. The radiolabel pulse-chase efflux assay is a common assay to assess the efflux potential of cells in culture, acceptors and transporters. The efflux data of these assays are commonly expressed as the percent of label (in experimental media) out of the total cellular label. Although this is adequate for comparison of treatments or differences in assay conditions, it is inadequate for comparisons across cell types due to variance in lipid content. In order to evaluate a cell's potential contribution to HDL, the molar content of the lipids effluxed must be established. Our results showed that adipocytes efflux cholesterol at similar molar rates as macrophages but at lower rates than cAMP-treated macrophages. Conversely, the rate

of phospholipid efflux from adipocytes was higher compared to macrophages. This resulted in a higher ratio of CL:PL efflux from macrophages (~4) compared to adipocytes (~2) but in a similar ratio for cAMP-treated macrophages compared to adipocytes. This means that adipocytes release HDL particles that are CL-poor compared to control macrophages, are the same as cAMP-treated macrophages and are CL-rich compared to smooth muscle cells and some fibroblasts [16, 162]. Because cAMP is believed to hyperactivate lipid efflux to apoA-I via the ABCA1-mediated efflux pathway and the stoichiometry of efflux from adipocytes matches that of cAMP-treated macrophages, these results support a role for ABCA1 in the apoA-I dependent lipid efflux from adipocytes.

Homogenate lipid compositions

The CL and PL lipid content determination of homogenates confirmed that adipocytes contain higher levels of CL as reflected in the higher CL per PL compared to macrophage homogenates. These lipid measurements were similar to previous literature measuring adipocyte tissue lipid compositions as well as previous literature on J774 macrophages [59, 131]. Other works have also identified changes in homogenate lipid compositions after treatment with cAMP or LXR-agonists [163, 164]. Interestingly, 8-Bromo-cAMP treatments in those studies resulted in CL accumulation in epithelial cells, while use of a LXR-agonist resulted in lower CL accumulation in HepG2 cells. Contrary to these reports, our studies of treatment with either cAMP or GW3965 resulted in a slight decrease in both CL and PL homogenate lipid levels which was determined to be insignificant by student's t-test (data not shown, $p>0.05$). This difference is most likely due to differences in assay conditions including medium compositions and longer incubation periods of cells of those previous studies with the drugs.

Role of ABCA1

Results of the efflux assay studies in combination with western blotting have provided evidence that ABCA1 protein levels are correlated to apoA-I dependent lipid efflux. These assays showed first, that ABCA1 is expressed in adipocytes at similar levels as the expression level in macrophages. And secondly, they show that the LXR-agonist GW3965 increased both ABCA1 protein levels (2 fold) and efflux (1.5 fold) from adipocytes. ABCA1 induction in macrophages by cAMP resulted in a 4.7 fold increase in ABCA1 protein levels with a 2.3 fold increase in CL efflux.

Additionally, it was shown that CL efflux to apoA-I from adipocytes occurs at the same rate as from macrophages *when* the rates are normalized by the amount of ABCA1

protein expressed in cells. These results implicate ABCA1 to be in control of apoA-I dependent lipid efflux from both adipocytes and macrophages.

Surprisingly, the rate of efflux per ABCA1 protein, or efficiency of efflux, was higher for untreated cells compared to the ABCA1-induced cells. This could be explained in two different ways. One possibility is that there is some factor responsible for a low level of apoA-I-inducible cholesterol efflux that is downstream or independent of ABCA1-mediated efflux. This would result in higher efficiency of efflux per ABCA1 in control cells if this other factor is not increased in the treated cells. Therefore this efficiency would not be seen in treated cells because it is overcast by the increased levels of ABCA1-mediated efflux. This factor could be another protein that may facilitate a higher efficiency of efflux through or supplemental to the ABCA1 pathway. The concentration or activity of this other factor is probably regulated similar to the basal regulation of ABCA1, since there is a similar level of efficiency between adipocytes and macrophages per expression of ABCA1. However, because this higher efficiency of efflux is not seen in the experimentally treated states, it could not be a protein upregulated by cAMP or LXR. This means that while treatment of cells with either cAMP or LXR-agonists increases ABCA1 levels, it does not increase another unknown component of the efflux pathway which would allow for the more efficient efflux per ABCA1 level seen in the control cells. Potential candidates include SRBI and ABCG1, which are both known to efflux CL to ABCA1-formed HDL [67, 81]. Through studies in various cell types, LXR is known to upregulate ABCG1 but not SRBI and conversely, cAMP is known to upregulate SRBI but not ABCG1 [165-169]. Because studies in adipocytes are lacking, it is unknown how these drugs may affect these non-ABCA1 transporters of adipocytes. But if similarly regulated, it is possible that the LXR- or cAMP-treatment would result in less SRBI or ABCG1 per ABCA1 compared to the uninduced state. Thus, ABCG1, SRBI or some other unknown protein factor may influence the efficiency of the ABCA1 efflux pathway.

Another possibility is that the level of efflux efficiency could be due to the levels of functionally active ABCA1 compared to overall homogenate levels of ABCA1. Studies show that the cell surface expression levels of ABCA1 are linked to its ability to mediate apoA-I-dependent lipid efflux [43]. In control cells where ABCA1 levels are lower, higher cell-surface localization would help maintain a higher efficiency efflux level. However, in induced cells where ABCA1 levels are upregulated, ABCA1 may be located not only on the cell surface but also in processing or *en route*. This would result in division of the efflux by a higher ABCA1 concentration than that which is actually responsible for it and would seemingly result in a lower efficiency. Although this is possible, this is less likely since the non-active ABCA1 levels would have to be the same for both the LXR-treatments in adipocytes and cAMP-treatments in macrophages since the efficiency is similarly repressed.

Despite the different possibilities, the ratios of CL efflux per ABCA1 protein levels remained constant when comparing the control cell-types to each other or the treated cell-types to each other. This again suggests that ABCA1 is the major transporter involved in lipid efflux to apoA-I from adipocytes in both basal and LXR activated states.

To further investigate the role of ABCA1 in apoA-I dependent lipid efflux from adipocytes, the inhibition effects of the ABCA1-targeting drugs GLYB and BFA on lipid efflux from both adipocytes and macrophages were compared. The results showed that GLYB and BFA inhibit apoA-I dependent efflux from adipocytes at similar levels as those seen in macrophages. Additionally, this work represents the first to study the effect of the drugs BLT1 and BLT4 not only on cholesterol efflux inhibition but also phospholipid efflux inhibition. BLT1 only slightly inhibited PL efflux to apoA-I, which is expected since BLT1 targets SRBI-mediated lipid efflux to HDL and not ABCA1-mediated apoA-I dependent efflux. BLT1 also partially inhibited CL efflux, which can be explained by reasoning that BLT1 may be blocking SRBI-mediated efflux to lipoparticles formed after apoA-I addition to the cells within the time frame of the assays. However, in the case of GLYB/BFA, PL efflux inhibition seems to be the same or slightly higher (ns GLYB CL-PL; ** p=0.0012 BFA CL-PL) than the inhibition of CL efflux. If real, this difference could also be explained by downstream CL-enrichment of nascent HDL particles, such as SRBI/ABCG1-mediated efflux or diffusion. Different from both these cases however, is inhibition by BLT4. BLT4 is unique in that it inhibits not only ABCA1-mediated efflux of lipids to apoA-I but also SRBI-mediated efflux to HDL. Contrary to previous reports in ABCA1-overexpressed HEK293 cells, our assays in adipocytes show BLT4 does not inhibit CL efflux to the same degree as GLYB or BFA but instead is slightly lower [142]. Since BLT4 has been shown to block ABCA1, it was also surprising that BLT4 did not inhibit PL efflux as strongly as it did CL efflux. However, this difference can be explained by BLT4's ability to also inhibit the possible downstream HDL CL-enrichment by SRBI. This unique study on the effects of BLT1 and BLT4 on both CL and PL efflux to apoA-I and comparison to GLYB/BFA provides support for the idea that nascent HDL particles may be further CL-enriched. The degree to which this CL-enrichment occurs may vary by cell-type and requires further studies to understand the effect on circulating HDL compositions and functionality.

ii. ***ApoA-I recycling in adipocytes***.

Despite many recent studies, the relationship of apoA-I recycling to efflux and the localization of apoA-I lipidation is still highly controversial [25, 26]. The works presented here show that apoA-I uptake and resecretion is a specific receptor-mediated process that can be blocked by an antibody to the β subunit of ATPase. Definitively,

blocking of apoA-I recycling was shown to have no significant effect on phospholipid or cholesterol efflux to apoA-I acceptors.

Cell surface ATPase subunit β as an apoA-I receptor

ATPase is expressed ectopically on the cell surface in several cell lines [150, 153-155]. It is also functionally active on the cell surface although separate studies have shown differences in either hydrolase or synthesis activities [150, 156-158, 170]. Consistent with apoA-I binding to cell surface ATPase, ATPase subunit β has been shown to bind to alpha helical proteins such as vasoconstrictor coupling factor 6, the inhibitory protein (IF_1) and apoE [171-173] as well as to the proteolytic fragment angiostatin [154]. Because ATPase β has been shown to bind these proteins, it seems possible that it may bind other apolipoproteins which share the apoA-I alpha helical nature. But, these studies have shown that the apoA-I recycling is not inhibited by addition of competing amounts of apoLp-III. This implies that the specific interaction of apoA-I with cell surface ATPase governs apoA-I recycling in adipocytes. Whether apoE, which was shown to bind to solubilized ATPase, binds to cell surface ATPase requires further study.

Role of apoA-I-ATPase β interaction

Studies investigating the function of the apoA-I interaction with ATPase have indicated that the interaction leads to a decrease in cytosolic lipid droplet (LD) accumulation in adipocytes [174]. Interestingly, those studies also showed that antibodies to the β subunit mimic the apoA-I interaction which caused the decreases in cytosolic LD accumulation. In the present studies, apoA-I was blocked from cell entry and resecretion by anti-ATPase β, but lipid efflux to apoA-I receptors remained un-modified. This led to the conclusion that apoA-I uptake and re-secretion is not involved in lipid efflux. However, in light of the previous activity studies where the apoA-I binding response is mimicked and not blocked by antibody, apoA-I binding to ATP synthase may result in signaling which is not blocked by the antibody in our recycling assays. This signaling would have to be independent of recycling since our assays show that apoA-I cell entry and exit is blocked by anti-ATPase β, but other possible downstream signals elicited by apoA-I binding would not be ruled out.

One the other hand, apoA-I has been shown to stimulate hydrolase activity in hepatocytes [150] while antibodies have been shown to inhibit both ATP synthesis and hydrolysis in several cell types [150, 156-158, 170, 174]. ATPase hydrolase activity has been linked to HDL endocytosis via a non-SRBI receptor in hepatocytes [150]. Those studies have proposed that ATPase-mediated ADP production leads to the activation of the G-protein

coupled receptor $P2Y_{13}$ on the hepatocyte surface which modulates HDL endocytosis [175, 176]. ApoA-I stimulation of this pathway, through ADP production, was inhibited by antibodies to the ATPase subunit β [150]. Research in adipocytes is limited but some studies have proposed that HDL endocytosis in adipocytes may be SRBI-related instead of ATPase mediated [155, 177]. Contrarily, the above works show that the SRBI inhibitor BLT-1 has no significant affect on pka-apoA-I uptake and re-secretion. The above studies on this novel pathway are preliminary and need further work to establish the role of the apoA-I-ATPase interaction in adipocytes. Furthermore results show only ~65% inhibition of pka-apoA-I phosphorylation indicating that other pathways, not involving cell surface ATPase, may also be involved in apoA-I recycling. As such, blocking of apoA-I-ATPase mediated recycling would not affect those other possible pathways. This would account for studies linking apoA-I recycling to the ABCA1 endocytic pathway [117, 120, 178].

Overall these studies have shown that the apoA-I-ATPase interaction functions in adipocyte apoA-I recycling and further that this apoA-I uptake does not play a major role in lipid efflux to apoA-I.

iii. ***Physiological Relevance***

The adipocyte contribution to overall HDL levels was found to be at the same level of contribution as J774 macrophages in cell culture. Because this is a cell by cell comparison, we would expect the influence of adipocytes on HDL in circulation to be much larger than the contribution of macrophages since the adipocytes far outnumber macrophages. Also, the characterization of HDL lipid compositions revealed that the lipoparticles formed from the apoA-I dependent lipid efflux from adipocytes were CL-poor compared to control macrophages. This means that this CL-poor (relative to macrophages) HDL particle will be a more efficient acceptor of CL efflux from cells. Therefore, because HDL is dynamically remodeled and apoA-I available to induce lipid efflux from adipocytes, adipose tissue is expected to be a significant regulator of the HDL compositions in circulation. Also, since the ability of HDL to accept CL from CL-loaded foam cells is believed to be responsible for the atheroprotective nature HDL, the HDL biogenesis from adipocytes can only be characterized to aid this process.

It has also been shown by works in this laboratory that CL efflux increases from adipocytes to HDL acceptors in the lipolytic state [27]. The cellular source of this CL efflux was not the plasma membrane and the lipolytic mobilization involved BFA-sensitive vesicular transport. This study showed that adipocytes do have the metabolic potential to affect circulating cholesterol levels.

Although those works had revealed the importance metabolic state on adipocyte CL efflux to HDL, the influence of adipocytes on HDL biogenesis had not been characterized until now. Due to the prevalence and convergence of obesity and heart disease the continued study of adipocyte influence on these processes are an important public health priority. The studies presented above have laid the groundwork for such studies by characterizing the HDL compositions, contributions and mechanisms of apoA-I dependent lipid efflux from adipocytes. As such, these studies are crucial cornerstones for future studies on the influence of metabolic states on lipid efflux from adipocytes.

iv. ***Concluding Remarks***

The studies presented here have sought to characterize the apoA-I dependent lipid efflux of adipocytes. The rates of CL and PL efflux from adipocytes upon apoA-I incubation were determined and show that lipid efflux from adipocytes is induced by apoA-I at a similar rate as from macrophages. The CL to PL stoichiometric efflux ratio of adipocytes was also similar to the lipid efflux ratio seen in macrophages, implicating by comparison the ABCA1-mediated pathway in adipocytes.

To investigate the role of ABCA1, the apoA-I dependent lipid efflux levels were normalized by cellular ABCA1 protein content. This 'efficiency' of lipid efflux was analyzed in both adipocytes and macrophages and the comparison showed that apoA-I induces lipid efflux at the same rate per ABCA1 protein from adipocytes as from macrophages. To further test the role of ABCA1, the drugs GLYB, BFA and BLT4, which target the ABCA1 efflux pathway, were used to analyze apoA-I-dependent efflux from adipocytes. The results show patterns of inhibition expected for apoA-I induction of efflux via an ABCA1-mediated pathway. Collectively, the studies provide strong support for ABCA1 as the main transporter involved in lipid efflux from adipocytes to apoA-I.

Investigation of apoA-I internalization through use of pka-tagged apoA-I revealed that apoA-I is recycled in adipocytes [121]. pka-ApoA-I recycling was found to be a specific process that human apoA-I could compete with, but not apoLp-III or Thrx. The recycling was also saturatable and this lead to the proposition of receptor involvement in apoA-I cellular uptake and re-secretion. First, ABCA1 was tested as the possible recycling receptor but the ABCA1-inhibitor BFA failed to block apoA-I recycling. Similarly the drug BLT1, which blocks SRBI-mediated CL flux to HDL, also had no effect on apoA-I recycling. The next receptor to be tested was the ectopically expressed cell surface ATP synthase subunit β. Surprisingly, the phosphorylation of pka-apoA-I was blocked by use of a monoclonal antibody to ATPase β.

Finally, the ability of anti-ATPase β to block apoA-I internalization was used as a tool to directly analyze the relationship of apoA-I recycling to lipidation. CL and PL efflux assays carried out in the presence of anti-ATPase β showed that lipid efflux to apoA-I remained unaffected by antibody blocking of apoA-I recycling. This is the first direct study to show that apoA-I recycling is unrelated to apoA-I lipidation.

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VITA

Alisha Dawn Howard

Candidate for the Degree of

Doctor of Philosophy

Thesis: APOA-I INDUCED LIPID EFFLUX FROM ADIPOCYTES

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data:

Born in Oklahoma, US. Daughter of Dennis and Tanya. Sister of David and Daniel. Wife of Eric Howard.

Education:

Bsc. in Chemistry, A.C.S. certified, Oklahoma State University. 2001-2005
Bsc. in Biochemistry, Oklahoma State University. 2002-2005

Completed the requirements for the Doctor of Philosophy in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in May, 2010.

Experience:

Graduate Student Research Assistant, Department of Biochemistry, Oklahoma State University. Mentor: Dr. Jose L. Soulages, Ph.D. Research work: "ApoA-I dependent lipid efflux from adipocytes." 2006-2010.

Undergraduate Student Lab Assistant, Department of Biochemistry, Oklahoma State University. Mentor: Dr. Estela L. Arrese, Ph.D. Research work: Lipolysis in *Manduca sexta*, focus on proteins triglyceride-lipase and lipid storage droplet protein-1. 2004-2006.

Teaching Assistant, Department of Chemistry, Oklahoma State University. Instructor: Dr. Mark Rockley, Ph.D. Class: General Chemistry I for majors laboratory section. 2003.

Honors, Awards and Other Professional Activities:

2010-present	American Heart Association, Student Member
2009-present	Oklahoma Academy of Sciences, Student Member
2009	OSU Microarray Workshop and Certification
2008-present	Gamma Sigma Delta, Graduate Honor Society
2008	OSU Mass Spectroscopy Workshop and Certification
2006-present	CASNR Fellowship, Oklahoma State University
2006-present	Biochemistry and Mol. Biology Graduate Student Association
2003-present	Phi Lambda Upsilon, Graduate Chemistry Honor Society

Name: Alisha Dawn Howard Date of Degree: May, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: APOA-I INDUCED LIPID EFFLUX FROM ADIPOCYTES

Pages in Study: 72 Candidate for the Degree of Doctor of Philosophy

Major Field: Biochemistry and Molecular Biology

Findings and Conclusions:
These studies have sought to characterize the apoA-I dependent lipid efflux of adipocytes. The rates of CL and PL efflux from adipocytes upon apoA-I incubation were determined and show that lipid efflux from adipocytes is induced by apoA-I at a similar rate as from macrophages. The CL to PL stoichiometric efflux ratio of adipocytes was also similar to the lipid efflux ratio seen in cAMP-treated macrophages, implicating by comparison the ABCA1-mediated pathway in adipocytes.
The 'efficiency' of apoA-I dependent lipid efflux, that is, lipid efflux normalized by ABCA1 cellular protein content, was found to be the same in both adipocytes and macrophages. To further test the role of ABCA1, the drugs GLYB, BFA and BLT4, which target the ABCA1 efflux pathway, were used to analyze apoA-I-dependent efflux from adipocytes. The results show patterns of inhibition expected for apoA-I induction of efflux via an ABCA1-mediated pathway. Collectively, the studies provide strong support for ABCA1 as the main transporter involved in lipid efflux from adipocytes to apoA-I.
Investigation of apoA-I internalization through use of pka-tagged apoA-I revealed that apoA-I is recycled in adipocytes. PKA-ApoA-I recycling was found to be a specific process that human apoA-I could compete with, but not apoLp-III or Thrx. The recycling was also saturatable and this lead to the proposition of receptor involvement in apoA-I cellular uptake and re-secretion. To investigate this, ABCA1 was tested as a potential recycling receptor but the ABCA1-inhibitor BFA failed to block apoA-I recycling. Similarly the drug BLT1, which blocks SRBI-mediated CL flux to HDL, also had no effect on apoA-I recycling. The next receptor to be tested was the ectopically expressed cell surface ATP synthase subunit β. Surprisingly, the phosphorylation of pka-apoA-I was blocked by use of a monoclonal antibody to ATPase β.
Finally, the ability of anti-ATPase β to block apoA-I internalization was used as a tool to directly analyze the relationship of apoA-I recycling to lipidation. CL and PL efflux assays carried out in the presence of anti-ATPase β showed that lipid efflux to apoA-I remained unaffected by antibody blocking of apoA-I recycling. This is the first direct study to show that apoA-I recycling is unrelated to apoA-I lipidation.

ADVISER'S APPROVAL: Dr. Jose Soulages