CHOLESTEROL EFFLUX IN ADIPOSE TISSUE

By

PHILIP BENJAMIN VERGHESE

Bachelor of Science in Chemistry
University of Pune
Pune, India
1995

Master of Science in Biochemistry
University of Pune
Pune, India
1997

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Dissertation Approved:

Dr. Jose L. Soulages

Dissertation Advisor

Dr. Chang-An Yu

Dr. Robert L. Matts

Dr. Jack W. Dillwith

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER I

Introduction

Cholesterol is a lipid molecule indispensable for the normal functioning of mammalian cells. It plays structural and functional roles in the cell membrane and functions as precursor for bile acids and steroid hormones. Other than this classic concept of cholesterol functions, it has been proposed that cholesterol plays an important role in protein and lipid trafficking, signal transduction, and generating cell surface polarity [1-4]. For the normal execution of the above functions, the total cellular cholesterol level and distribution among the organelles and compartments of membrane is critical. Mammalian cells synthesize their own cholesterol endogenously and receive cholesterol exogenously through diet. The biosynthesis of the cholesterol molecule involves multiple steps and requires several enzymes. The exogenous influx of cholesterol is through lipoproteins, mainly low-density lipoproteins (LDL) via LDL receptor pathway [5, 6]. Cells also constantly efflux out excess cholesterol from organs. The synthesis, influx, efflux and distribution of cholesterol are tightly and precisely regulated. Several sterol response elements and transcriptional regulators such as liver-X-receptors (LXRs), retinoid- X-receptor (RXR), and peroxisome proliferators-activated receptor α,β, and γ (PPAR α,β,γ) take part to maintain the sterol balance in mammalian system. Numerous pathways exist to acquire cholesterol when it is required and efflux it when it is in abundance. Defects in the cholesterol metabolic and transport pathways result in numerous fatal disease conditions in humans and animals.

Excess of free cholesterol (FC) is toxic to cells [7]. The potential consequences of the toxicity induced by free cholesterol are loss of membrane fluidity, disruption of membrane domains, interruption of signal transduction, initiation of cell death, intracellular cholesterol crystallization, and formation of toxic sterols [7]. Cells prevent FC toxicity by FC efflux and acetyl-Coenzyme A acetyltransferase I (ACATI) mediated FC esterification [7]. Cholesterol efflux is indispensable to the cellular cholesterol homeostasis. Cholesterol ester hydrolase (CEH) reverses the FC esterification process. The synthesis and hydrolysis of cholesterol ester (CE) take place constantly in the cells and thus contribute significantly to FC efflux process [8].

Reverse cholesterol transport (RCT) is the process by which excess unesterified cholesterol is transported from peripheral tissues to the liver for excretion from the body [9, 10]. The FC efflux is one of the decisive early steps of the RCT and high-density lipoprotein (HDL) serves as the main acceptor for the cellular cholesterol released from the peripheral cells [11]. Apolipoprotein A-I (apoA-I) is the major protein component of HDL and plays important structural and functional roles. Human apoA-I is a 243-residue protein consisting of ten amphipathic helices synthesized primarily in the liver and the intestine [12]. Deficiency and naturally occurring mutations in apoA-I fail to produce normal HDL levels and particles [13]. The plasma levels of HDL and apoA-I are inversely linked with the risk of cardiovascular diseases. Hence it is suggested that RCT is an important physiological process that protects against cardiovascular diseases [14].

The rate of cellular cholesterol removal to apoA-I and HDL acceptors depends on multiple factors such as cholesterol abundance and organization in the plasma membrane [12-14], the content and nature of ATP-binding cassette transporter A1 (ABCAI) [14-17]

and scavenger receptor class B Type1 (SR-BI) receptors [12, 18, 19] and the nature and concentration of external sterol acceptors [20].

Cholesterol Efflux Pathways

There are at least five major pathways through which cholesterol is transported out of peripheral cells:**(i)** ABCAI mediates cholesterol and phospholipid transfer to lipid poor external acceptors like apoA-I, which is an early step in HDL formation [21] (Fig. 1-1). This is a unidirectional energy dependent pathway [22]. ATP-binding cassette (ABC) proteins consist of a large family of membrane receptors that transports a wide variety of substances including lipids, sterols, ions, peptides, proteins and drugs across both intracellular and extracellular membranes [23, 24]. ABCAI conforms to the general architecture of the ABC transporters, consists of two sets of six trans-membrane domains and has two paired cytoplasmic Walker and an ABC signature motif [24]. Defects in ABCAI receptor cause Tangier disease, a condition described by increase in cardiovascular diseases due to accumulation of cholesterol in tissue macrophages and decreased HDL associated cholesterol in blood plasma [25]. ApoA-I is the major component of HDL in the circulation and evidence from several studies suggest that deficiency in ABCAI receptor or external acceptor apoA-I could seriously decrease the plasma HDL concentrations, which could increase artherogenic risk. Even though several studies attempted to elucidate the mechanism of ABCAI mediated cholesterol efflux, it is still unclear and under intense debate.

(ii) The HDL formed through ABCAI or other processes and other external acceptors can promote cholesterol efflux by acquiring cholesterol from the membranes

[12, 13] (Fig. 1-2). This is a concentration dependent and passive process. One such passive process is the aqueous diffusion of cholesterol from cell surface onto external acceptors. Another passive process, micorsolubilization, refers to the capability of apolipoproteins to acquire cholesterol and phospholipids independently of lipid transporters [26, 27].

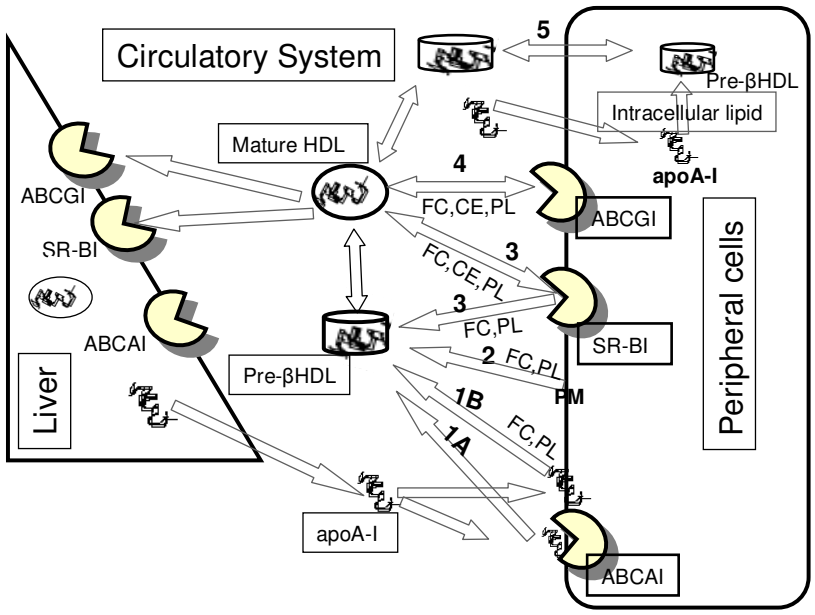


Fig. 1. Cholesterol Efflux Pathways: 1. ABCA-I and apoA-I mediated FC and PL efflux. **2**. Passive diffusion mediated FC and PL efflux. **3**. SR-BI mediated lipid efflux. **4**. ABCGI mediated lipid efflux. **5**. Retroendocytosis mediated lipid efflux.

(iii) The HDL can interact with SR-BI and efflux free cholesterol out of the cell and influx cholesterol ester in to the cells [18, 19] (Fig. 1-3). This process is energy

independent. SR-BI is a member of CD-36 family proteins, which contains two transmembrane domains and one large extracellular loop. Liver and steroidgenic cells are the major sites of SR-BI synthesis [18]. The ligands of SR-BI include, HDL, LDL, very low density lipoproteins (VLDL), reconstituted discoidal HDL (rHDL), other lipoproteins and large cholesterol ester rich spherical lipoproteins. The direction of lipid transfer is bidirectional. Defects in SR-BI receptor reduced the clearance of HDL cholesterol from plasma in human and mice and this could lead to development of atherosclerosis [18].

(iv) ATP-binding cassette transporter GI (ABCGI) mediates cholesterol and phospholipids transfer to mature HDL molecules but not to lipid free apoA-I [28, 29] (Fig. 1-4). ABCGI is a half-transporter (one ATP binding site), which forms homodimers and the ABCGI mRNA is expressed ubiquitously among human tissues [30]. ABCGI gene silencing by RNA interference results in reduced efflux of cholesterol and phospholipids to mature HDL [31]. Overexpression of ABCGI protects liver, macrophages and several other tissues from massive lipid accumulation during high fat diet in murine models [31]. ABCGI also mediates the transport of cholesterol to LDL [32] . The data from several studies propose that ABCGI reorganizes and increases the availability of cholesterol enriched membrane domains for HDL molecules but not to apoA-I [33]. A direct HDL and ABCGI interaction is still under debate. Like the ABCAI lipid efflux, the mechanism of ABCGI lipid efflux is also under intense debate.

(v) All of the above mechanisms suggest a direct and/or indirect interaction of lipoprotein to membrane receptors and or membrane lipids for cholesterol efflux. The cell surface event of direct and/or indirect interactions leads to transport of cholesterol and

phospholipids from internal locations to the plasma membrane (PM) and transferred to PM bound HDL or lipid free apolipoproteins. Several lines of evidence suggest an alternative model of cholesterol efflux mechanism where HDL and lipid free apolipoproteins are transported inside the cell via receptor-mediated endocytosis. The endocytosed lipoproteins remove cholesterol from intracellular locations, probably from lipid droplets and are resecreted without degradation (retroendocytosis) (Fig. 1-5). In 1985, the first study in macrophages with gold labeled HDL and (^{125}I) HDL suggested that HDLs bind to cell surface receptors and are internalized and transported to non-lysososmal compartments. Finally, the HDL is secreted from the cells to tissue culture media without degradation [34]. Later the Schmitz et al in a different study suggested that the retroendocytosis process is damaged in tangier disease fibroblast, expressing a defective ABCAI receptor [35]. Since then several other studies suggested endocytosis and vesicular transport of HDL in different physiologically relevant cell lines [27, 36-39]. At present the HDL retroendocytosis is controversial. Shortly after the first paper on retroendocytosis of HDL, another study observed absence of internalization of (^{125}I) HDL in fibroblast and macrophages [40]. Further more the authors suggested that HDL stimulate the transport of lipids from intercellular locations to cell surface, rather than undergoing retroendocytosis [40, 41].

The retroendocytosis process was further tested in lipid free apoA-I. With a fluorescent labeled apoA-I and ABCAI-green fluorescent protein (GFP) fusion protein, the mechanism of ABCAI mediated efflux to apoA-I was studied in HeLa cells [42]. This ABCAI overexpression study suggested that ABCAI and apoA-I on the cell surface are internalized into early endosomes and can either recycle back to the plasma membrane or

traffic to late endocytic compartments. The apoA-I can collect lipids from late endocytic vesicles and traffic back to the PM and be released as the nascent HDL particle [42]. The apoA-I trafficking is an ABCAI dependent process [42]. Cyclosporin A is accepted as an inhibitor of ABCAI turnover in PM, but increases overall total expression of ABCAI in cells. This inhibitor considerably reduces the (^{125}I) apoA-I uptake and re-secretion in macrophage cells [39]. Also several studies suggested that many classical endocytic proteins directly or indirectly linked with ABCAI. One such protein is RhoGTPase Cdc42 which interacts with ABCAI and modulates lipid efflux [27]. None of these studies suggested the mechanism of the receptor mediated retroendocytosis. The five pathways mentioned are separate and may function through different mechanisms.

Mechanism of apoA-I and ABCA-1 mediated lipid efflux

The in vitro and in vivo data from several studies suggest that ABCAI and lipid free apoA-I are critical for the biogenesis of pre β- HDL and lipid efflux from peripheral cells. As indicated previously, the mechanism of apoA-I and ABCAI mediated efflux is still not well understood, though extensively studied. At present three models have been proposed to explain the apoA-I and ABCAI mediated lipid efflux.

The first model suggests that there is no direct interaction between apoA-I and ABCAI, but have spatial proximity between the protein and receptor ([43] Fig. 1-1B). ABCAI rearranges the lipid organization in plasma membrane, which increases the phosphatidylserine (PS) and enables apoA-I binding to phosphatidylserine in the exofacial side of the plasma membrane (PM). This study used a fluorescent labeled Cy5-apoA-I and ABCAI-green fluorescent protein (GFP) to determine apoA-I binding curves

and translational diffusion coefficients (D_T) in membranes. The study concluded that saturation of surface binding of apoA-I to membrane does not correlate with the increase in ABCAI receptor (over expression) in the membrane and the translational diffusion coefficient (D_T) of fluorescent Cy5-apoA-I correlates with molecular interaction with lipids rather than with a protein receptor. Later this report was challenged by Smith et al [44] by showing that ABCAI induction was coupled with a small increase in PM exofacial PS and the apoA-I is not competed by annexin V, a PS binding protein. Further more, with a photo-bleaching assay, the authors showed that Cy5- apoA-I does not rapidly diffuse in cell surface and speculated that apoA-I is directly or indirectly bound to an integral protein, probably with ABCAI [44].

In a different study, a variation of the above model was presented where ABCAI redistributes cholesterol to cell-surface sites, and it becomes available for removal by apolipoproteins. This study suggested a microsolubilization model of lipid efflux whereby apolipoproteins concurrently remove phospholipids and cholesterol from cell-surface domains formed by ABCAI [45].

The second model suggests a direct physical interaction between apoA-I and ABCAI at the cell surface and which is probably required for the lipid efflux facilitated by these two proteins [46] (Fig. 1-1). Several chemical cross-linking and immunoprecipitation studies concluded that apoA-I directly interact with ABCAI. The major drawbacks of these studies are failure in obtaining consistent stoichometery and binding affinity values for apoA-I and ABCAI interaction in increasing (up regulation) and decreasing (down regulation) receptor conditions and inability to give evidences to

suggest binding of apoA-I to ABCAI is a necessary step for lipid efflux. It is clear from the cross-linking studies that direct interaction between apoA-I and ABCAI is not sufficient for the movement of cholesterol out of the cell.

The recent third model suggests a hybrid of first two models and consists of three steps. In the first step, a small regulatory pool of apoA-I directly interacts with ABCAI (low affinity binding site) thereby increasing the transfer of phospholipids to exofacial surface of the plasma membrane. In the second step, the reorganized phospholipid in the outside surface creates membrane tension and is relieved by formation of exovesiculated lipid domains. The exovesiculated lipid domains formed promote high affinity binding of apoA-I (high affinity binding site) to these structures. In the third step, apoA-I spontaneously solubilizes the exovesiculated lipid domains to produce discoidal nascent HDL particle [47, 48]. The data from the above studies and several other studies were put together to create this more coherent hybrid model. But the authors failed to address the requirement and specificity of the very low amount of apoA-I binding directly to ABCAI. Also, the model suggested a spontaneous solubilization of lipid domains to produce discoidal nascent HDL particle, which should have produced an exponential lipid efflux kinetics at initial hours, which is not observed in several studies.

Current Perspectives on Cholesterol efflux

In the last decade considerable progress has been made in understanding the components of cholesterol efflux and HDL biogenesis. But the function and interactions of identified components of cholesterol efflux and mechanisms of the pathways are not well understood. There is no unique specificity for apoA-I in lipid efflux in different

cultured peripheral cells. The lipoproteins, apoA-I1, apo-III (insect lipoprotein), apoA-IV apoE, apoC and even an amphipathic peptide have efflux properties the similar to apoA-I in in vitro experiments. But the apoE-/- mutant and apoA-I -/- apoE-/- double mutant studies in mice suggest that apoA-I is the key apolipoprotein that reduces the accumulation of cholesterol from atherosclerotic lesions and skin, even in the presence of other lipoproteins in equal amounts [49-52]. The specific in vivo properties of apoA-I and the role of apoA-I in mediating cholesterol efflux in different peripheral tissues is not understood.

ABCAI is expressed widely through out the mouse and human organs but the role and contribution of each organ in HDL biogenesis is not known. In the classical RCT model, the peripheral cells are the primary location for the prebeta-HDL biogenesis and ultimately transferred as mature HDL to the liver [9, 10]. Also, it has been suggested from ABCA-/- mutant mice studies that RCT is determined by different metabolic states in the peripheral organs and not by the concentration of plasma HDL and apoA-I [53]. But a recent study in chow fed ABCAI-/- liver mutant mice challenges the classical RCT model and proposed that the liver is the single most significant source of plasma HDL (82%), where pre- β HDL is synthesized [54]. Another study with ABCA-/- intestine mutant suggests that intestine also plays a significant role in the biogenesis of HDL (30%) through ABCAI receptor [55]. These studies were conducted by specifically mutating the liver and intestine ABCAI. But the studies on the total peripheral cholesterol efflux contribution per day per kg of body weight in mice suggest an opposing view to the above 2 studies [56]. There is significant contribution of cholesterol efflux from peripheral cells. Considering the complexities and cross talk involved between pathways

and tissue secretory components, these specific mutation studies are not sufficient to conclude the contribution of peripheral cells in the HDL biogenesis and total RCT. Even though muscle, adipose tissue, skin and other peripheral tissues express comparable or elevated amounts of cholesterol and ABCAI as of liver and intestine, their significance in the contribution of lipid efflux by HDL biogenesis and maturation is undermined by the above studies. The tissue that contributes the greatest mass of cholesterol to the RCT process is till unknown

As explained earlier there are several cholesterol efflux pathways. The relative contribution of lipid efflux by different pathways and components is not known. Recent study in cultured macrophages in the serum environment with ABCAI and SR-BI receptor inhibitors, it has been suggested that the contribution of these receptors in mediating cholesterol efflux is low (<30%) compared to the background efflux (>70%) [57]. A large percentage of efflux is not mediated by these receptors in the presence of serum lipoproteins. It is possible that there are other uncharacterized receptors/proteins and pathways involved in the cholesterol/lipid efflux process.

Adipose Tissue

Adipose tissue is a complex organ involved in synchronizing a variety of biological processes including energy metabolism, neuroendocrine , and immune function [58-61]. Adipose tissue is a major secretory organ and the secretory products communicate locally and with other organs like liver, brain and skeletal muscles [62]. The cross- talk between the secretory products and other organs are very important for the normal energy homeostasis of the body and a disturbance in the balance of the

secreted products will result in several disease conditions like insulin resistance leading to type 2 diabetes, heart diseases, and stroke (metabolic syndrome) [62]. Leptin, Adiponectin, resistin, lipoprotein lipase, acylation stimulating protein, fast-induced adipose factor, visfatin and vaspin are the few studied among hundreds of secretory adipokines of adipose tissue which are important for the normal energy metabolism. Human adipose tissue consists of various types of cells that include mature adipocyes (50-70%), preadipocytes (20-40%), endothelial cells (1-10%), macrophages (10-30%) and other cells (unknown percentage) [63]. The percentage cell composition of adipose tissue fluctuates according to different fat depot locations, metabolic state and disease conditions [63]. It has been demonstrated that the macrophages get attracted more during increasing fat accumulation in body and a significant amount of protein expressed in adipose tissue has macrophage characteristics [63]. Obese animals express high levels of inflammatory proteins like TNF-alpha, IL-6, IL-8, IL-18, MIF, and IL-1Beta , which are the key initiation factors for the development of metabolic syndrome. The increased secretion of these inflammatory proteins by adipose tissue are possibly due to the increasing macrophage accumulation in adipose tissue [63]. Metabolic syndrome is now suggested as a state of impaired function of the secretory products of adipose tissue and associated cells. These findings and speculations underline the prerequisite of further extensive study of adipose tissue metabolism.

Whole body energy metabolism is regulated by adipose tissue through its stored lipids; modulated by intrinsic and extrinsic signals. The stored lipids in adipose tissue are in a specialized organelle called lipid droplets (LD), which are formed by a monolayer of phospholipids and sterols surrounding a hydrophobic core of neutral lipids, mainly

consisting of triacylglycerol (TAG), and sterol esters. Adipose tissue constitutes one of the largest reservoirs of cholesterol in vertebrates [61]. The size of the cholesterol pool in adipose tissue is dependent on the mass of adipose tissue and, thus, proportional to the cellular triglyceride (TG) content [64, 65]. As in several other cell types, most of the cellular cholesterol of adipocytes resides in the plasma membrane and the lipid droplets. The lipid droplets found in tissues such as liver, adrenal glands, and enterocytes contain cholesterol, mostly, in the form of cholesterol esters. However, in adipocytes most of the lipid-droplet cholesterol is found in the non-esterified form [65, 66].

The cholesterol content of peripheral cells such as adipose tissue is controlled through the balance between uptake, release, synthesis, and catabolism of cholesterol [67]. Because the capacity of cholesterol synthesis and catabolism of adipocytes is very low [64, 66], the cholesterol content of adipose tissue is dependent on the balance between efflux and influx of cholesterol. Therefore, the rate of efflux to extracellular cholesterol acceptors could affect the cholesterol content of adipose tissue to a higher extent than in other tissues, such as liver, which have an active cholesterol metabolism. Maintenance of the cholesterol balance of adipose tissue is likely to be important to the proper metabolic functioning of the tissue [68, 69]. Moreover, since adipose tissue contains one of the largest pools of free cholesterol, the output of free cholesterol from this peripheral tissue might affect the homeostasis of cholesterol metabolism, in general.

Cholesterol Efflux in Adipose Tissue

The dynamic role of adipose tissue in energy balance through its ability to accumulate (Lipogenesis; deposition of lipids) or release (Lipolysis; hydrolysis of

triacylglycerol) fatty acids, and the fact that there is a correlation between the TG and cholesterol contents of adipocytes suggests that the metabolism of these two lipids could be coupled. Because both TG and cholesterol are mostly confined to the lipid droplet, the changes in TG content that normally occur in adipocytes, for instance after fasting or intense physical activity [64, 67, 68], could be accompanied by changes in the rate of cholesterol efflux to external acceptors. Although there is scarce specific information about the possible role of the metabolic state of adipocytes on the rate of cellular cholesterol efflux, studies showing that differentiation of pre-adipocytes [69] and development of obesity[70] are associated to changes in the cholesterol content of adipocytes membranes suggest that changes in the metabolism of TG could affect the transport of cholesterol.

Previous studies have shown that adipocytes release cellular cholesterol to HDL [71, 72] and apoA-I [71, 73-76]. The effect of break down of TG on the rate of cellular cholesterol efflux from adipocytes to apoA-I or HDL and the efflux mechanism involved are not well studied in adipose tissue. However, as is the case for most tissues, the pathways and mechanisms involved in cholesterol efflux in adipose tissue, and their relative contribution to the overall efflux process and RCT, have not been fully established.

CHAPTER II

Stimulation of lipolysis enhances the rate of cholesterol efflux to HDL in adipocytes

Introduction

Adipose tissue constitutes one of the largest reservoirs of cholesterol in vertebrates [61].The size of the cholesterol pool in adipose tissue is dependent on the mass of adipose tissue and, thus, proportional to the cellular triglyceride (TG) content [64, 65]. As in several other cell types, most of the cellular cholesterol of adipocytes resides in the plasma membrane and lipid droplets. The lipid droplets found in tissues such as liver, adrenal glands, and enterocytes contain cholesterol, mostly, in the form of cholesterol esters. However, in adipocytes most of the lipid droplet cholesterol is found in the non-esterified form [65, 66]. The cholesterol content of peripheral cells is controlled through the balance between uptake, release, synthesis, and catabolism of cholesterol [77]. Because the capacity of cholesterol synthesis and catabolism of adipocytes is very low [64, 66], the cholesterol content of adipose tissue is dependent on the balance between efflux and influx of cholesterol. Therefore, the rate of efflux to extracellular cholesterol acceptors could affect the cholesterol content of adipose tissue to a higher extent than in other tissues, such as liver, which have an active cholesterol metabolism. Maintenance of the cholesterol balance of adipose tissue is likely to be important to the proper metabolic functioning of the tissue [73, 78]. Moreover, since adipose tissue

contains one of the largest pools of free cholesterol, the output of free cholesterol from this peripheral tissue might affect the homeostasis of cholesterol metabolism, in general. High density lipoprotein (HDL) plays a major role in the removal of cholesterol from peripheral tissues [11]. The rate of cellular cholesterol removal to HDL depends on multiple factors, such as the cholesterol abundance in plasma membrane [12, 13] the function of ABCAI [79, 80] and SR-BI receptors [12, 18-20] , and the nature and concentration of external sterol acceptors [20]. As is the case for other tissues, the mechanisms involved in cholesterol efflux in adipose tissue, and their relative contribution to the overall efflux process, have not been fully established.

The dynamic role of adipose tissue in energy balance through its ability to accumulate or release fatty acids, and the fact that there is a correlation between the TG and cholesterol contents of adipocytes suggests that the metabolism of these two lipids could be coupled. Because both TG and cholesterol are mostly confined to the lipid droplet, the changes in TG content that normally occur in adipocytes could be accompanied by changes in the rate of cholesterol efflux to external acceptors. For instance, an increase in plasma cholesterol levels has been observed after fasting or intense physical activity, [67-69]. Although there is scarce specific information about the possible role of the metabolic state of adipocytes on the rate of cellular cholesterol efflux, studies showing that differentiation of pre-adipocytes [69] and development of obesity [70] are associated to changes in the cholesterol content of adipocyte membranes suggest that changes in the metabolism of TG could affect the transport of cholesterol. Previous studies have shown that adipocytes release cellular cholesterol to HDL [71, 72].

The purpose of the current study was to examine the effect of lipolysis on the rate of cellular cholesterol efflux from 3T3 L1 adipocytes to discoidal reconstituted HDL.

Experimental Procedures

Materials. 3T3 L-1 cells were purchased from American Type Cell Culture (Manassas, VA). Polyclonal anti-ABCAI antibody was obtained from Novus-Biologicals (Littleton, CO). Monoclonal antibody for plasma membrane Na^+K^+-ATPase was purchased from the

Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Horseradish peroxidase (HRP) tagged antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Fatty acid free bovine serum albumin, protease inhibitors (phenyl-methyl sulfonyl fluoride, leupeptin, aprotinin, antipain), isobutyl methyl xanthine (IBMX), dexamethasone, trypsin, biotin, sodium pyruvate, insulin, 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). [^{3}H]-Cholesterol (60 Ci/mmoL) was from Perkin-Elmer (Waltham, Massachusetts). Chemiluminiscence kit for Western blots was obtained from Amersham Biosciences (Piscataway, NJ).

Cell Culture. 3T3 L-1 pre-adipocytes were cultured at 37^0C in 8% CO_2 atmosphere in high glucose DMEM supplemented with 10% FBS and 0.01% streptomycin and penicillin antibiotics. One day after confluence, the differentiation into

adipocytes was induced by addition of IBMX (111 µg/mL), dexamethasone (0.46 µg /ml), and insulin (1.5 µg /ml) in the medium that was also supplemented with biotin (4 µg/ml) and sodium pyruvate (100 µg /ml) [81]. After 48 h, the cells were incubated in DMEM/10 % FBS containing insulin, biotin, and sodium pyruvate for additional 48 h. Afterwards, the cells were maintained in DMEM/10% FBS. All experiments were conducted 12 days after completion of the differentiation period.

Preparation of Reconstituted Discoidal HDL Particles. Liposomes were obtained by dispersing a thin film of dimyristoyl-phosphatidylcholine (DMPC) in PBS buffer, pH 7.5 [82]. ApoA-I was purified from human plasma and kept in 2 M guanidine hydrochloride at -20^{0}C [83]. A dialyzed aliquot of apoA-I in PBS buffer pH 7.5 was added to the DMPC vesicles at a lipid:protein ratio of 1:1 (w/w) and incubated at 24^0 C for 12 h. Samples of the reconstituted HDL particles (rHDL) were analyzed by gel filtration-FPLC using a Superose 6 column (Pharmacia, Piscataway, NJ) and nondenaturing gradient gel electrophoresis as described [82].

Cholesterol Efflux. Adipocytes cultured in twelve well dishes were radiolabeled by incubation for 24 h with [^{3}H]-cholesterol (2 µCi/ml) in DMEM containing 2.5% FBS. The media was removed and the cells were incubated for 3 hours in serum-free DMEM containing 0.2% BSA (DMEM/BSA) and, before the beginning of the experiment, rinsed twice with DMEM/BSA. The experiments were started by addition of fresh DMEM/BSA (basal lipolysis) or DMEM/BSA supplemented with 1µg/ml of isoproterenol (high lipolysis). Cholesterol efflux to rHDL was determined at 37^0 C in the corresponding,

basal or high lipolysis media containing HDL at a concentration of 50 µg of apoA-I/ml. Background cholesterol efflux was determined in the corresponding basal and high lipolysis media containing no acceptors. Microliter aliquots of the medium were collected at specific time intervals to estimate the fraction of [^{3}H]-cholesterol released into the medium by scintillation counting. At the end of the incubation, the cells were washed with phosphate buffered saline (PBS) twice and the lipids extracted with 2-propanol [84]. The lipid extract was used to determine the fraction of radiolabeled cholesterol remaining in the cells. Cholesterol efflux was expressed as the percentage of radiolabeled cholesterol released into the media as: 100x [^{3}H]-Cholesterol in media /([^{3}H]-Cholesterol in media + [^{3}H]-Cholesterol in cells). The effect of BFA on cholesterol efflux was studied by addition of BFA to the DMEM/BSA medium at a final concentration of 10 µg /ml.

Ultracentrifugation. Samples of medium were fractionated by ultracentrifugation in a KBr density gradient as previously reported [82]. Aliquots of the fractions were used to determine [^{3}H]-cholesterol radioactivity. The density of the fractions was determined by refractometry. The subcellular distribution of [^{3}H]-cholesterol is determined by discontinuous sucrose gradient centrifugation of homogenates of 3T3L-1 cells. After the corresponding treatment, the cells were washed with PBS and harvested by scrapping in cold 10 mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, and protease inhibitors. A cell homogenate was prepared with a tight fitting Dounce homogenizer. The homogenate was adjusted to 50% sucrose, then 5.0mL each of 40%, 30%, 20%, 10% and homogenization buffer without sucrose was layered on top of the gradient. After centrifugation in SW28 rotor at 23,000 RPM for 18 h the gradient was

fractionated in equal volumes and an aliquot of the fractions were scintillation counted to determine the radioactivity.

Lipolysis. The extent of lipolysis was estimated from the fatty acids released into the cell medium. Fatty acids were determined spectrophotometrically using the enzyme coupled assay NEFA B,Wako (Richmond, VA) .

Isolation of Plasma Membrane (PM). After the corresponding treatment, the cells were washed with PBS and harvested by scrapping in cold 10 mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, and protease inhibitors. A cell homogenate was prepared with a tightfitting Dounce homogenizer. Plasma membranes were isolated from the homogenate using the fractionation procedure previously described [85]. The homogenate was centrifuged at 19,000g for 20 min. The pellet obtained from this centrifugation was resuspended in 0.25 M sucrose/10 mM Tris-HCl buffer, layered onto a discontinuous sucrose gradient, 0.75 and 1.12 M sucrose, and centrifuged at 100,000g in a SW40 rotor for 75 min. The band at the interphase of the 0.75 M and 1.12 M sucrose layers (plasma membrane) was collected, diluted with 50 mM Tris-HCl buffer pH 7.4, and centrifuged at 40,000g for 30 min.

Western Blot Analysis. Aliquots of the membrane preparations containing 15 μg of protein were mixed with reducing SDS-sample buffer and subjected to SDS-PAGE in 8% acrylamide: bisacrylamide gels. The proteins were transferred to a nitrocellulose membrane and probed with a rabbit anti-mouse/human ABCAI (1:750 dilution), and anti-

SR-BI (1:1000). Parallel Western blots were run to determine the abundance of Na^+/K^+ ATPase using a monoclonal anti-Na^+/K^+ ATPase antibody (1:10 dilution). All secondary antibodies were used at a 2500-fold dilution. The bands were visualized by chemiluminescence and quantified by densitometry.

Lipid Analysis. Cholesterol contents of plasma membrane fractions were determined spectrophotometrically using the enzyme coupled assay Infinity™ Cholesterol (Thermo Electron Corporation). The cholesterol data were normalized for protein content. Protein contents were determined using the bicinchoninic acid method (Micro BCA Protein Assay Reagent Kit, Pierce Biotechnology).

Fluorescence Anisotropy. Fluorescence anisotropy measurements were performed at 24 ^{0}C in a K2 spectrofluorometer (ISS, Urbana, IL) as previously described [86]. Plasma membrane samples, 1 ml containing 50 μg/ml of protein, were incubated with 0.75 nmoles of DPH (1, 6- diphenyl-1, 3, 5-hexatriene) for 30 min. Sample temperature was controlled with a circulating water bath. Emission intensities were measured at 431 nm with excitation at 355 nm. Steadystate fluorescence anisotropy, *r*, was calculated as: $r = (I_{vv} - G\, I_{vh}) / (I_{vv} + 2GI_{vh})$

I_{VV} and I_{VH} are the parallel and perpendicular polarized fluorescence intensities measured with vertically polarized excitation light. The intensities were corrected for scattering using unlabeled samples. Instrumental polarization selectivity was corrected by means of the G factor, which is calculated from the ratio of parallel and perpendicular intensities determined with horizontally polarized excitation light.

Statistical Analysis. The statistical significance of the data was evaluated with the paired Student's t-test using the program Instat (Graphpad).

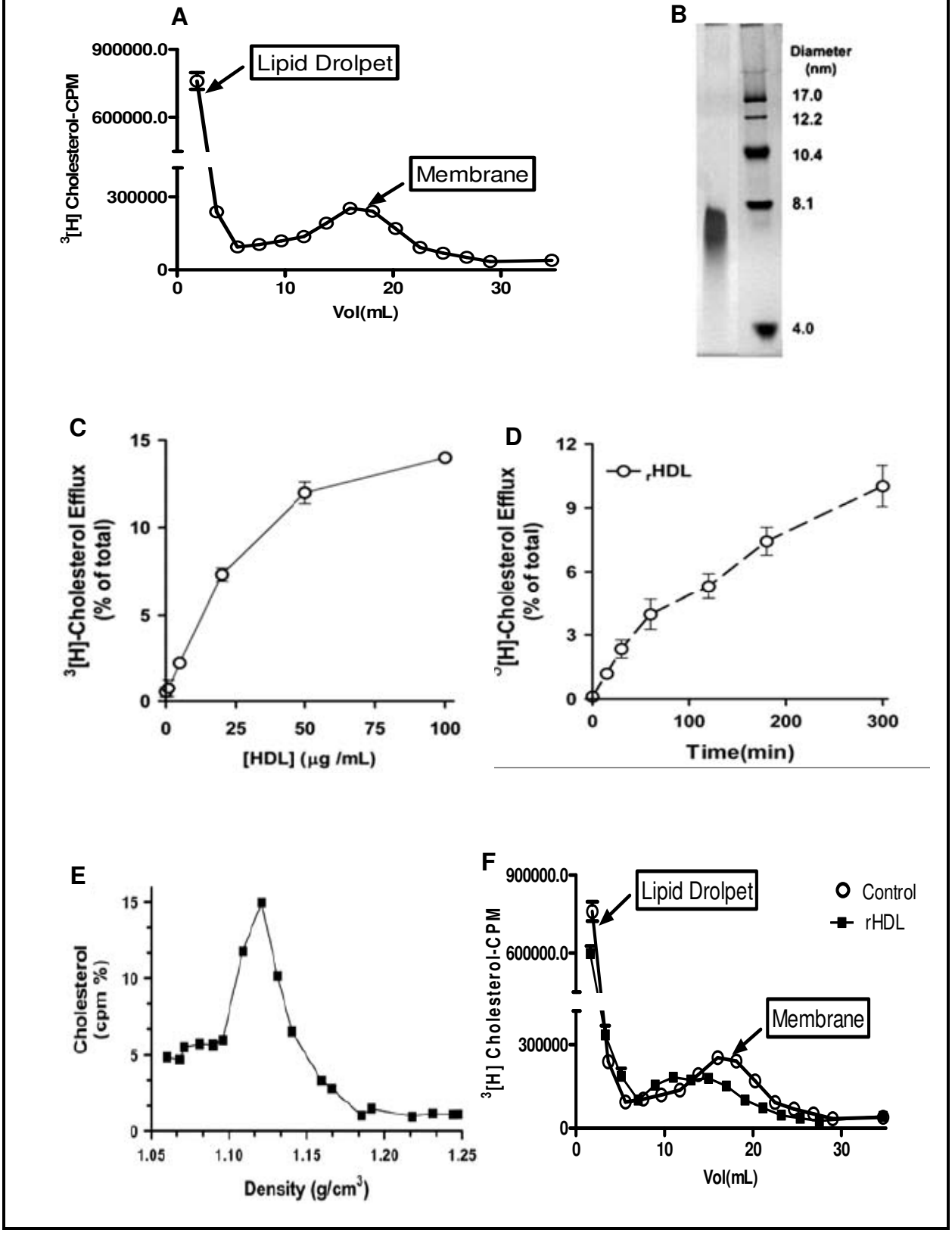
Results

Cellular cholesterol efflux to rHDL. The rate of cholesterol efflux to rHDL was studied in adipocytes that were prelabeled with [^{3}H]-cholesterol for 24 h. Under these conditions 96% of the radiolabeled cholesterol is found as such, and the remaining 4% is found as cholesterol esters (data not shown). This distribution of radiolabeled cholesterol is nearly identical to the relative mass contents of cholesterol and cholesterol ester previously reported for adipocytes [65, 66, 73] suggesting that in 24 h the incorporated radiolabeled cholesterol reaches a physiological distribution. The subcellular distribution of [^{3}H]- cholesterol was determined by sucrose gradient centrifugation of homogenates of 3T3L-1 cells (Fig. 2A). The [^{3}H]- cholesterol distribution profile shows that the lipid droplet is the major location of cellular cholesterol (approximately 60%).

To study cholesterol efflux HDL particles (rHDL) were reconstituted using human apoAI and phosphatidylcholine at a 1:40 molar ratio. Using this molar ratio the lipoproteins obtained are highly homogeneous. Analysis of the lipoproteins obtained by nondenaturing gradient gel electrophoresis shows a single major band with an apparent diameter of ~7.8 nm (Figure 2B). Figure 2C shows that HDL promotes [^{3}H]-cholesterol efflux from adipocytes in a dose dependent manner**.** At the maximum concentration of lipoprotein tested (100 μg apoA-I/ml), and after five hours of incubation, HDL promotes

the release of approximately 14% of the total cellular cholesterol. A representative time course of cholesterol efflux from adipocytes to HDL (50 µg/ml) is shown in Figure 2D.

Figure 2E shows the density distribution of the radiolabeled cholesterol found in the cell medium after incubation of [^{3}H]-cholesterol pre-labeled adipocytes in DMEM containing HDL. The profile of cholesterol distribution was obtained by ultracentrifugation of the medium in a density gradient of KBr. The position of the maximum of radioactivity in the density gradient (density~ 1.11 g/cm3) is consistent with the association of the cholesterol released by the cells with HDL. Figure 2F shows the change in the subcellular distribution of [^{3}H]- cholesterol in the presence of the external acceptor, HDL in 3T3L-1 cells. The distribution profile of the subcellular [^{3}H]-cholesterol was obtained by ultracentrifugation of cell homogenate on a discontinuous sucrose gradient for 18 h. The profile of [^{3}H]-cholesterol suggests that HDL receives cholesterol from lipid droplets and membranes.


A
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Lipid Drolpet
Membrane
^{3}[H] Cholesterol-CPM
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20
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Vol(mL)
B
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12.2
10.4
8.1
4.0
C
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10
5
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^{3}[H]-Cholesterol Efflux
(% of total)
0
25
50
75
100
[HDL] (μg /mL)
D
12
9
6
3
0
^{3}[H]-Cholesterol Efflux
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0
100
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Time(min)
E
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rHDL
0
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20
30
Vol(mL)

Fig. 2. Cellular Cholesterol Efflux from Adipocytes to Discoidal HDL. A) Distribution of the [^{3}H]-cholesterol in fractions from the sucrose gradient centrifugation of an homogenate of 3T3L-1 cells **B)** HDL-dose response: [^{3}H]- cholesterol pre-labeled adipocytes were incubated for 5 h at 37^{0}C in DMEM containing different concentrations of rHDL; **C)** Non denaturing gel electrophoresis of the reconstituted HDL particles used in the studies. The lipoprotein (lane 1) and size markers (lane 2) were stained with Coomassie Blue; **D)** Time course of cholesterol efflux: adipocytes were incubated in DMEM medium containing HDL (50 μg apoA-I/ml). Samples from three wells were taken at different time points. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity. The cholesterol efflux data were corrected for the radiolabeled cholesterol released in the absence of HDL. Data represent the percent of cellular cholesterol released in the presence of HDL (average values +/- S.E). Data are from six wells for the HDL dose response and three wells for the time course experiment; **E)** DMEM-medium containing HDL was collected after incubation for 5 h with [^{3}H]-cholesterol pre-labeled adipocytes. The medium was subjected to ultracentrifugation in KBr density gradient. Aliquots of the density gradient were used for radioactivity and density determination. **F)** Distribution of the [^{3}H]-cholesterol in fractions from the gradient centrifugation of an homogenate of 3T3L-1 cells incubated in the presence and absence of external acceptor rHDL.

Effect of lipolysis on the rate of cholesterol efflux to rHDL.

To examine the effect of lipolysis on cholesterol efflux, [^{3}H]-cholesterol prelabeled cells were incubated in DMEM/BSA in the absence or in the presence of the non-specific β-adrenergic agonist, isoproterenol. In the presence of isoproterenol, the rate of lipolysis, as estimated from the fatty acid (FA) released into the incubation media, increases ~7-8 fold, from ~3.2 to ~27 nmol FA/mg of cellular protein.h. The effect of

lipolysis on the efflux of cholesterol from adipocytes to discoidal HDL is shown in Figure 3. After 180 min incubation with rHDL, the average extent of cholesterol efflux in adipocytes with basal lipolytic activity was 8.06 +/- 0.38% and increased to 9.99 +/- 0.32%, when lipolysis was activated. A similar conclusion was obtained comparing the efflux data obtained at 300 min. At both time points, activation of lipolysis promotes a significant increase in the extent of cholesterol efflux to discoidal HDL, 23.8% increase at 180 min, P<0.001, and 22.3% at 300 min, P<0.0001.

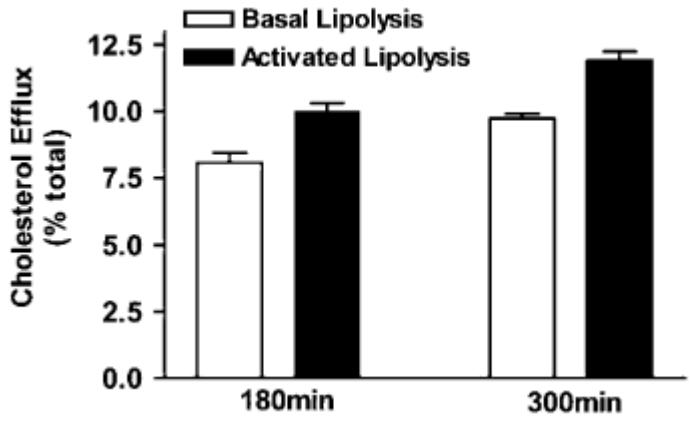

Fig. 3. Effect of Lipolysis on the of Rate Cellular Cholesterol Efflux to HDL. Adipocytes were labeled with [^{3}H]-cholesterol as indicated in experimental procedures and incubated in DMEM containing rHDL (50 μg apoA-I/ml) for 180 min or 300min, at 37^0 C. "High lipolysis" and "Basal Lipolysis" refer to cells incubated in the medium with or without isoproterenol, respectively. Cholesterol efflux is represented as percentage of the total cellular cholesterol as indicated in methods. The data plotted were corrected for the radiolabeled cholesterol released in the absence of HDL and represents the average values +/- the S.E. of 9 experiments for 180 min and 10 experiments for 300 min. Student's t-test analysis indicated significant differences between the mean values of cholesterol efflux (high vs basal lipolysis) at both 180 min (P<0.001) and 300min (P < 0.0001).

Activation of lipolysis promotes a decrease in the cholesterol content of plasma membranes.

To study a possible role of the cholesterol content of the plasma membranes in the enhancement of cholesterol efflux induced by activation of lipolysis, plasma membranes of [^{3}H]-cholesterol labeled adipocytes were isolated from cells incubated in DMEM medium in the absence of HDL after a five hour period of incubation in the absence or in the presence of isoproterenol. The isolated membranes were analyzed for protein and radiolabeled cholesterol content (cpm). As shown in the Figure 4A, activation of lipolysis promotes a decrease in the content of radiolabeled cholesterol of the plasma membrane. Nearly identical changes are observed when membranes are isolated from adipocytes incubated for periods of 1 or 3 hours (data not shown). The decrease in cholesterol content of the plasma membrane is accompanied by an increase in the cholesterol content of the lipid droplets (data not show). Given the fact that changes in cholesterol content usually affect the mobility and/or packing of the lipid molecules in the bilayer [87], possible changes in the physical properties of the plasma membranes were investigated by determining the fluorescence anisotropy of DPH. Figure 4B shows that activation of lipolysis promotes a significant decrease in the fluorescence anisotropy of DPH from 0.154 +/- 0.005 to 0.141 +/- 0.004. The lower fluorescence anisotropy of DPH in membranes isolated from cells with high lipolytic activity is consistent with the observed decrease in cholesterol content of the membranes.

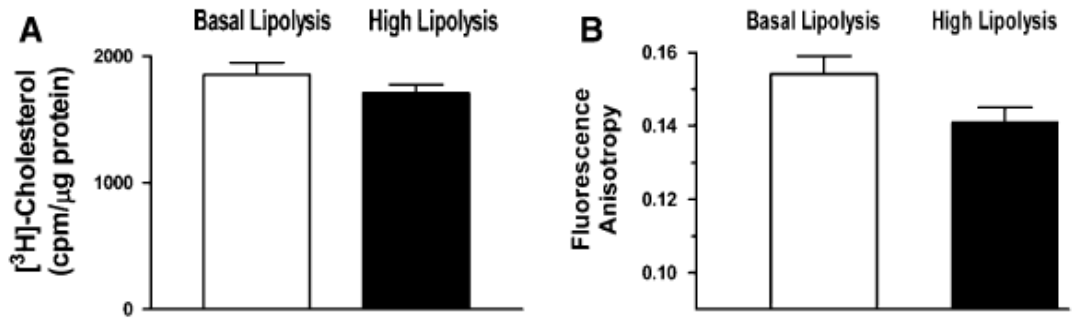

Fig. 4. Effect of Lipolysis on the Cholesterol Content of Plasma Membrane of Adipocytes. [^{3}H]-Cholesterol pre-labeled cells were incubated in 75 cm^2 tissue culture flasks for 5 h in DMEM media. The media of samples labeled "High Lipolysis" contained isoproterenol. After incubation, the cells were homogenized, and plasma membranes isolated as described in methods**. Panel A** shows the ratios of radiolabeled cholesterol to protein content observed in the membranes. Bars represent the mean values of two membrane preparations +/- the difference. **Panel B** shows the data of anisotropy of fluorescence of DPH obtained in membrane preparations isolated from cells with basal and high lipolytic activity. Triplicate samples containing 50 μg/ml of protein were incubated with DPH at a molar ratio of 100:1 (phospholipid to DPH) for 30 min. Fluorescence anisotropy (mean +/- SD) was determined at 24^{0}C.

Effect of lipolysis on ABCAI and SR-BI levels in plasma membrane.

A possible role of the cholesterol transporters ABCAI and SR-BI in the enhancement of cholesterol efflux was evaluated by studying the protein levels of the transporters in plasma membrane isolated form adipocytes under basal and activated lipolytic conditions. Plasma membranes were isolated from the cell monolayer by ultracentrifugation in a sucrose gradient. The isolated fraction floated at the interface between the 0.75 M and 1.12 M sucrose and, compared to the cell homogenate, was highly enriched in Na^+/K^+ ATPase. Western blotting of plasma membrane proteins with a polyclonal ABCAI antibody showed no changes in the level of ABCAI protein after a

sustained five hour activation of the lipolysis (Fig. 5A). In contrast some experiments, we observed a slight decrease in the level of ABCAI protein in plasma membrane. Furthermore, no changes in the membrane levels of ABCAI were observed when the cells were incubated in the presence of apoA-I or rHDL. Western blotting of plasma membrane proteins isolated from control, or isoproterenol treated, cells with a polyclonal SR-BI antibody did not reveal a lipolysis related variation in SRB1 content, either (Fig. 5A). Overall, the results suggest that the observed increase in cholesterol efflux during lipolytic activation is not related to increases in the plasma membrane levels of ABCAI or SR-BI.

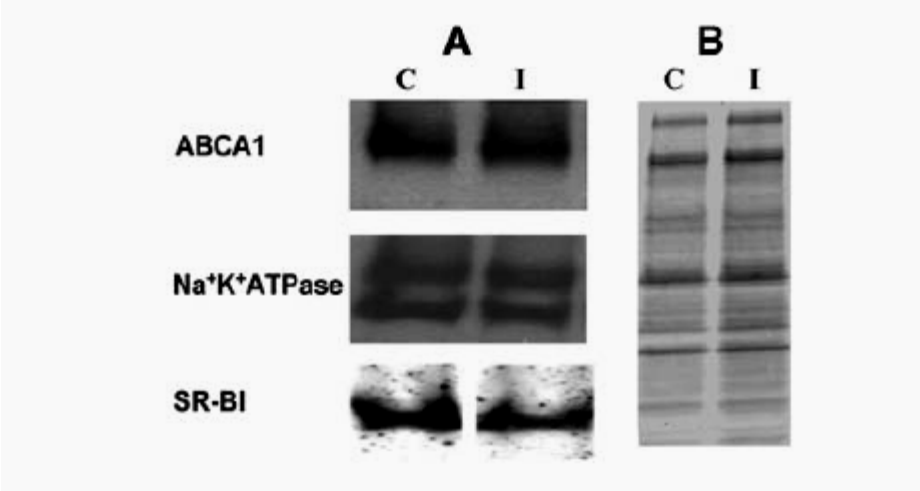

Fig. 5. Effect of Lipolysis on ABCAI and SR-BI Protein Contents. A) Western blotting of ABCAI, Na^+/K^+ ATPase and SR-BI receptor was performed in plasma membrane samples (15 µg of potein/sample) after SDS-PAGE, as described in experimental procedures. **B)** Coomassie blue stained SDS-PAGE of plasma membrane proteins (15 µg). Membrane samples were isolated from adipocytes incubated 300 min in DMEM (samples labeled "C") or in DMEM containing isoproterenol (samples labeled "I").

Effect of Brefeldin A on the enhancement of cholesterol efflux to rHDL.

The lack of correlation between the cholesterol content of plasma membrane and the rate of cholesterol efflux suggested that changes in the dynamics of intracellular transport of lipids could be involved in the induction of cholesterol efflux associated to activation of the lipolysis. To investigate this possibility the efflux of cellular cholesterol was studied in the presence and in the absence of Brefeldin A, which is known to alter vesicular lipid and protein transport by promoting fusion of Golgi and endoplasmic reticulum compartments [88, 89]. Under basal lipolytic conditions Brefeldin A promoted a minor, not significant, decrease in cholesterol efflux to HDL (Fig. 6), 6% ($P<0.1$). On the other hand, when lipolysis is activated, BFA reduces the enhancement of cholesterol efflux to HDL to the level observed in cells with basal lipolytic activity (Fig. 6). Thus, when lipolysis is activated, Brefeldin A promotes a decrease in the extent of cholesterol efflux, from 11.52 +/-0.55 to 9.69 +/- 0.22 % ($P<0.01$).

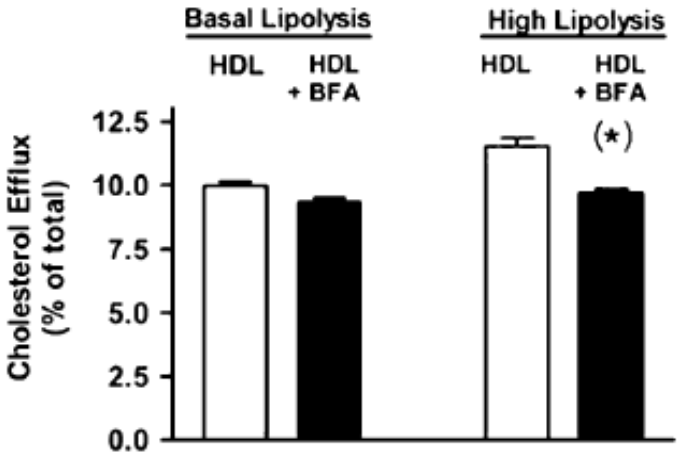

Fig. 6. Effect of Brefeldin A on the Rate of Cholesterol Efflux to rHDL. 3T3L-1 adipocytes were incubated for 5 h in DMEM with, or without, Brefeldin A at 10 µg/ml. HDL was used at a concentration of 50 µg of protein/ml. Lipolysis was activated by incubating the cells in media containing isoproterenol. Each bar represents the mean values (%) +/- the standard error of cellular cholesterol efflux of three experiments. Comparison of the means through the t-test rendered P-values of 0.057 for the effect of BFA under basal lipolysis, and 0.0062 for the effect of BFA under high lipolysis.

DISCUSSION

The investigation of the effect of the lipolytic state on adipocytes on the rate of cellular cholesterol efflux is of interest because, due to its large content of unesterified cholesterol, adipose tissue could play a major role in the homeostasis of cholesterol metabolism and transport. The relative cholesterol content of adipose tissue varies with the degree of obesity of the individual. However, adipose tissue always represents a major reservoir of cholesterol and in cases of major obesity it may contain over 50% of the total body cholesterol [90]. The present study shows that activation of the lipolysis is associated to an increase in adipocyte cholesterol efflux to HDL. These findings suggest

the existence of a metabolic link between cellular transport of cholesterol and the lipolytic activity of adipose tissue. This metabolic link argues against the view of adipose tissue as a passive cholesterol buffering sink. The increase in efflux associated to lipolysis could be partially responsible of the increase in plasma cholesterol that is observed after long periods of fasting or intense physical activity [64, 67, 68].

An enhancement of cholesterol efflux to HDL may occur when the cholesterol content of the plasma membrane increases. In this case, mechanisms of cholesterol efflux, such as passive simple diffusion or transporter facilitated diffusion would lead to an increase in the rate of cholesterol efflux to apoA-I or HDL [12]. However, our study indicates that activation of lipolysis triggers a decrease in the cholesterol content of the plasma membrane. Therefore, the enhancement in the rate of cholesterol efflux to HDL cannot be attributed to the cholesterol content of the membrane. If this were the case we should expect a decrease in cholesterol efflux to HDL when lipolysis is activated. The biochemical mechanisms that affect the cholesterol content of the plasma membrane in adipocytes are not known. However, it seems that multiple stimuli can trigger changes in cholesterol content of the plasma membrane of adipocytes. Previous studies have shown that “obese adipocytes” contain a lower concentration of plasma membrane cholesterol than lean adipocytes [70]. Moreover, changes in the plasma membrane cholesterol were observed during the differentiation of adipocytes [69, 73].

The cholesterol transporters ABCAI and SR-BI play a significant role in the transfer of cholesterol between cells and extracellular acceptors [57, 91]. It has been shown that overexpression of the scavenger receptor BI leads to an increase in the rates of cholesterol efflux to HDL in a variety of cells [92-94]. A mechanism of enhancement

of efflux to HDL involving an increase in SR-BI expression could be involved in adipocytes. However, SR-BI is poorly expressed in adipose tissue [18] and our study showed no significant changes in the expression of SR-BI protein upon activation of lipolysis. For these reasons, a role of SR-BI expression on cholesterol efflux, or in the enhancement of cholesterol efflux associated to lipolysis, is unlikely. The role of ABCAI in mediating cholesterol efflux to HDL is not clear. It has been shown that overexpression of ABCAI in transfected macrophages does not increase the efflux of cellular cholesterol to HDL [46]. On the other hand, fibroblasts isolated from subjects with Tangier disease have a reduced efflux of cholesterol to HDL [79, 80] suggesting that ABCAI is somehow involved in the cellular transfer of cholesterol to HDL. Our study showed no significant changes in ABCAI levels and therefore the increase in efflux cannot be directly related to the expression of ABCAI. However, as discussed below, ABCAI may play an indirect role in the enhancement of cholesterol efflux that takes place when the lipolysis is activated. Given the unlikely direct roles of the levels of cholesterol and cholesterol transporters of plasma membrane in the enhancement of cholesterol efflux, a possible role of vesicular transport was investigated using BFA, which is known to alter vesicular lipid and protein transport by promoting fusion of Golgi and endoplasmic reticulum compartments [88, 89]. BFA was shown to inhibit cholesterol efflux to lipid free apoA-I while mimicking several of the cellular effects observed in cells isolated from patients affected by Tangier disease [42, 95, 96]. This study showed that cholesterol uptake by HDL is mostly unaffected by Brefeldin A under basal lipolytic conditions suggesting that BFA sensitive vesicular transport is not required for the basal output of cholesterol to HDL. On the other hand, BFA abolished the lipolysis-induced

enhancement of cholesterol efflux to HDL, suggesting that BFA-sensitive vesicular transport is necessary to allow the enhancement of cholesterol efflux that accompanies β-adrenergic activation of the lipolysis. Since BFA has been shown to prevent the recycling of ABCAI from intracellular vesicles to the plasma membrane [42, 95], an indirect role of ABCAI in the enhancement of cholesterol efflux is possible. A mechanism of cholesterol efflux mediated by activation of vesicular transport could explain the apparent contradiction given by the fact that the release of cellular cholesterol increases when the cholesterol content of the plasma membrane is reduced.

CHAPTER III

Cholesterol efflux and apoA-I recycling in adipose tissue

Introduction

Reverse cholesterol transport (RCT) is the process by which excess unesterified cholesterol (UC) is transported from peripheral tissues to the liver for excretion from the body [9, 10]. RCT is relevant because the peripheral cells acquire cholesterol through uptake of lipoproteins and de novo synthesis, but are unable to catabolize it. The free cholesterol (FC) efflux is one of the decisive early steps of the RCT and high-density lipoprotein (HDL) serves as the main transport vehicle for the cellular cholesterol released from the peripheral cells to liver [11]. Apolipoprotein A-I (apoA-I) is the major protein component of HDL that promotes the efficient transfer of excess peripheral cell cholesterol to make them an efficient acceptor and transporter. Deficiency and naturally occurring mutations in apoA-I fail to produce normal HDL levels and particles [13]. The plasma levels of HDL and apoA-I are inversely linked with the risk of cardiovascular diseases. Hence it is suggested that RCT is an important physiological process that protects against cardiovascular diseases [14].

Several studies suggest that ABCAI is required for the initial and normal lipidation of apoA-I to from pre-beta HDL; an efficient acceptor molecule, intermediate to mature HDL (HDL-C). In the absence of ABCAI, the apoA-I is rapidly catabolized [97]. ABCAI is expressed widely through out the mice and human organs but the role and

contribution of each organ in HDL biogenesis is not known. In the classical RCT model, the peripheral cells are the primary location for the pre-beta HDL biogenesis and these molecules are ultimately transferred as mature HDL to the liver [9, 10]. But a recent study in liver-specific ABCAI-knockout (KO) mice challenges the classical RCT model and proposed that the liver is the single most significant source of plasma HDL-C [54]. Another study with intestine specific ABCA-KO mice suggests that intestine also plays a significant role in the biogenesis of HDL through ABCAI receptor [55]. These studies suggest that the 2 organs that synthesize apoA-I are also primarily responsible for lipidating apoA-I through ABCAI. But the studies on the total peripheral cholesterol efflux contribution per day per kg of body weight in mice suggest an opposing view to the above 2 studies [56]. Even though muscle, adipose tissue, skin and other peripheral tissues express comparable or elevated amounts of cholesterol and ABCAI than liver and intestine, their significance in the contribution of lipid efflux by HDL biogenesis has not been studied. The tissue that contributes the greatest mass of cholesterol to the RCT process is also unknown.

Adipose tissue contains more cholesterol than most other organs when expressed per protein or organ mass [56]. Thus, adipose tissue may contribute significantly to the biogenesis of HDL and RCT process. Because the capacity of cholesterol synthesis and catabolism of adipocytes is very low [64, 66], the cholesterol content of adipose tissue is dependent on the balance between efflux and influx of cholesterol.

Our studies as well as others have shown that adipose tissue has the ability to efflux cholesterol to HDL and apoA-I [72, 73, 83]. The rate of cellular cholesterol

removal by apoA-I depends on the plasma membrane levels of cholesterol [13, 79] and ATP binding cassette transporter A1, ABCAI [80] in several cell lines. However, as is the case for most tissues, the mechanisms involved in cholesterol efflux in adipose tissue, and their relative contribution to the overall efflux process have not been fully established. This study was directed to gain insights into; a) new pathways of cholesterol efflux in adipocytes. b) the effect of lipolysis on the rate of cellular cholesterol efflux to lipid free apoA-I.

Experimental Procedures

Materials: 3T3 L-1 cells were purchased from American Type Cell Culture (Manassas, VA). Polyclonal anti-ABCAI antibody was obtained from Novus-Biologicals (Littleton, CO). Monoclonal antibody for plasma membrane Na^+K^+-ATPase was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Horseradish peroxidase (HRP) tagged antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Fatty acid free bovine serum albumin, protease inhibitors (phenyl-methyl sulfonyl fluoride, leupeptin, aprotinin, antipain), isobutyl methyl xanthine (IBMX), dexamethasone, trypsin, biotin, sodium pyruvate, insulin, 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). [^{3}H]-Cholesterol (60 Ci/mmoL) was from Perkin-Elmer (Waltham, Massachusetts). [^{32}P] orthophosphate (carrier free) was purchased from MP Biochemicals. Chemiluminiscence kit for Western blots was obtained from Amersham Biosciences (Piscataway, NJ).

***Cell Culture and Lipoproteins*:** 3T3 L-1 pre-adipocytes were cultured at 37 ^{0}C in 8% CO_2 atmosphere in high glucose DMEM supplemented with 10% FBS and 0.01% streptomycin and penicillin antibiotics. One day after confluence, the differentiation into adipocytes was induced by addition of IBMX (111 μg/mL), dexamethasone (0.46 μg/ml), and insulin (1.5 μg/ml) in the medium that was also supplemented with biotin (4 μg/ml) and sodium pyruvate (100 μg/ml) [15]. After 48 h, the cells were incubated in DMEM/10

% FBS containing insulin, biotin, and sodium pyruvate for additional 48 h. Afterwards the cells were maintained in DMEM/10% FBS. All experiments were conducted 12 days after completion of the differentiation period.

Human lipid-free apoA-I was isolated, stored and prepared for cell culture studies as described previously [98]. Recombinant apoA-I was prepared by cloning full-length human apoA-I into a pET33b vector (Novagen), which incorporates an N-terminal tag encoding for a six-His tag and a five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA)

Cholesterol Efflux: Adipocytes cultured in six or twelve well dishes were radiolabeled by incubation for 24 h with [^{3}H]-cholesterol (2 μCi/ml) in DMEM containing 2.5% FBS. The media was removed and the cells were incubated for 3 hours in serum-free DMEM containing 0.2% BSA (DMEM/BSA) and, before the beginning of the experiment, rinsed twice with DMEM/BSA. The experiment started by addition of fresh DMEM/BSA (basal lipolysis) or DMEM/BSA supplemented with 10 μg/ml of isoproterenol (high lipolysis). Cholesterol efflux to apoA-I was determined at 37 ^{0}C in the corresponding, basal or high lipolysis, media containing the acceptors at a concentration of 50 μg of apoA1/ml. Background cholesterol efflux was determined in the corresponding basal and high lipolysis media containing no acceptors. Microliter aliquots of the medium were collected at specific time intervals to estimate the fraction of [^{3}H]-cholesterol released into the medium by scintillation counting. At the end of the incubation, the cells were washed with phosphate buffered saline (PBS) twice and the lipids extracted with 2-propanol. The lipid extract was used to determine the fraction of

radiolabeled cholesterol remaining in the cells. Cholesterol efflux was expressed as the percentage of radiolabeled cholesterol released into the media as:

100x [^{3}H]-Cholesterol in media /([^{3}H]-Cholesterol in media + [^{3}H]-Cholesterol in cells).

The effect of inhibitors on cholesterol efflux was studied by pre-incubating the pharmacological agents, BFA (10 µg/mL), Glyburide (500 µM), BLT-1(10 µM), and BLT-4 (150 µM) with [^{3}H]-cholesterol labeled cell media for 2 h. After the preincubation, the cells were incubated for an additional 5 h at 37°C under the same concentrations of inhibitors in the presence or absence of 50 µg/ml lipid-free apoA-I to measure cholesterol efflux. Background cholesterol efflux was determined by adding corresponding drug solvents in the media containing no acceptors. Microliter aliquots of the medium were collected at specific time intervals to estimate the fraction of [^{3}H]-cholesterol released into the medium by scintillation counting. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity as described above.

Isolation of Lipid Droplets. Lipid droplets were obtained as previously described [99].The post-nuclear supernatant containing 0.25 M sucrose was overlaid with 5.0 ml of 50 mM Tris-HCl buffer containing, 1 mM EDTA and protease inhibitors, and centrifuged in a SW40 rotor for 3 h at 200,000 g and 4 °C. The lipid droplets were collected from the top layer.

Lipolysis. The extent of lipolysis was estimated from the fatty acids released into the cell media. Non-esterified fatty acids were determined with the NEFA kit (Wako Chemicals USA, Inc).

***Western Blots*:** Aliquots of the cell homogenate of 3T3L-1 (basal lipolysis), 3T3L-1(high lipolysis), HEK 293, HepG2, J774.1 (un-stimulated), J774.1 (stimulated with 8-Br-cAMP (300 mM) for 16 hours) and PM and LD preparations of 3T3L-1 cells containing 20 µg of protein were mixed with reducing SDS-sample buffer and subjected to SDS-PAGE in 8% acrylamide gels. The proteins were transferred to a nitrocellulose membrane and probed with a rabbit anti mouse/human ABCAI (1:750 dilution), and anti-SR-BI (1:1000). Parallel Western blots were run to determine the abundance of Na^+/K^+ ATPase using a monoclonal anti- Na^+/K^+ ATPase antibody (1:10 dilution). All secondary antibodies were used at a 2500 fold dilution. The bands were visualized by chemiluminescence and quantified by densitometry.

ApoA-I cellular Uptake and Re-secretion. Full-length recombinant human apoA-I was cloned into a pET33b vector (Novagen), which incorporates an N-terminal tag encoding for a six-His tag and a five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA). The protein was expressed in *E. coli* and purified by Ni-affinity chromatography. The activity and specificity of PKA against recombinant apoA-I was tested by *in vitro* phosphorylation with bovine PKA and γ-[^{32}P]-ATP. Human apoA-I isolated from serum was not phosphorylated by PKA whereas the recombinant protein was highly phosphorylated by PKA.

In vivo Phosphorylation of apoA-I and PKA specificity: 3T3-L1 fibroblasts derived adipocytes cultured in six well dishes were double radiolabeled by incubation for 4 h with 80 µCi/well of [^{32}P] orthophosphate (carrier free) and 2 µCi/ml [1,2^{3}H]-cholesterol for 24 h at 37^{0}C in phosphate-free DMEM containing 0.12% BSA. At the end of the labeling period, 10 ug/mL isoproterenol (high lypolysis), DMSO (basal lipolysis)

and PKA inhibitor H-89 (40 μM) were added to the appropriate wells. Recombinant apoA-I (50 μg) was also added immediately and cell media collected at 60 and 120 minutes. The collected cell media was loaded into a Ni-affinity columns, the columns were extensively washed with 20 mM imidazole and eluted the recombinant apoA-I with 1M imidazole. The eluted proteins were separated by SDS-PAGE on 4-20% gels and subjected to autoradiography to visualize the phosphorylation.

Kinetics of apoA-I phosphorylation: The kinetic of apoA-I phosphorylation was monitored by incubating the [^{32}P] orthophosphate (carrier free) pre-labeled cells with 10, 20, 40 80 and 100 micrograms of recombinant apoA-I per milliliter of cell media. After the incubation of apoA-I for 40 min, the proteins in the cell media were separated by SDS-PAGE on 4-20% gels. The gels were subjected to autoradiography to visualize the phosphorylation.

Competition Assay: The specificity of apoA-I phosphorylation was monitored by incubating the [^{32}P] orthophosphate (carrier free) pre-labeled cells with a 40 microgram of recombinant apoA-I per milliliter of cell media and 10, 20, 40, 80 and 160 microgram of human apoA-I per milliliter of cell media. After incubation for 40 min, the proteins in the cell media were separated by SDS-PAGE on 4-20% gels. The gels were subjected to autoradiography to visualize the phosphorylation.

Inhibitor study on uptake and re-secretion: The effect of inhibitors on retroendocytosis was studied by pre-incubating the pharmacological agents, BFA (10 μg/mL), Glyburide (500 Na^+/K^+ M), and BLT-1(10 μM), with [3H]- labeled and [^{32}P] orthophosphate (carrier free) cell media for 2 h. After the preincubation, the cells were incubated for an additional 40 min at 37°C with the same concentrations of inhibitors in

the presence or absence of 50 microgram of apoA-I per milliliter of cell media. After incubation for 40 min, the proteins in the cell media were separated by SDS-PAGE on 4-20% gels. The gels were subjected to autoradiography to visualize the phosphorylation.

Lipid Extraction*:* To determine the phospholipid and cholesterol acquired by apoA1 during the efflux process, the lipids in the eluted protein were extracted with 2:1 chloroform/ methanol mixture. The organic phase was collected and dried. The lipids were separated by thin layer chromatography (TLC) on Silica Gel K6 plates using chloroform/methanol/water (50:25:4) as the developing solvent. The phospholipids spots were visualized by autoradiography and the spots corresponding to cholesterol were scraped and their radioactivity determined by liquid scintillation counting.

Results

Cellular cholesterol efflux to apoA-I

The rate of cholesterol efflux to human plasma apoA-I was studied in adipocytes that were pre-labeled with [^{3}H]-cholesterol for 24 h. Under these conditions 96% of the radiolabeled cholesterol in adipose tissue is found as such, and the remaining 4% is found as cholesterol esters. This distribution of radiolabeled cholesterol is nearly identical to the relative mass contents of cholesterol and cholesterol ester previously reported for adipocytes [65, 66, 73] suggesting that in 24 h the incorporated radiolabeled cholesterol reaches a physiological distribution.

A representative time course of cholesterol efflux to apoA-I (50 µg/mL) is shown in figure 7. The amounts of [^{3}H] cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H] cholesterol released into the medium during the 5 h incubation. At the concentration of lipoprotein tested (50 µg apoA-I/ml), and after 5 h of incubation, the total cellular cholesterol efflux is approximately 2.8% and lipid free apoA-I induced cholesterol efflux is approximately 1.8% of the total cellular cholesterol.

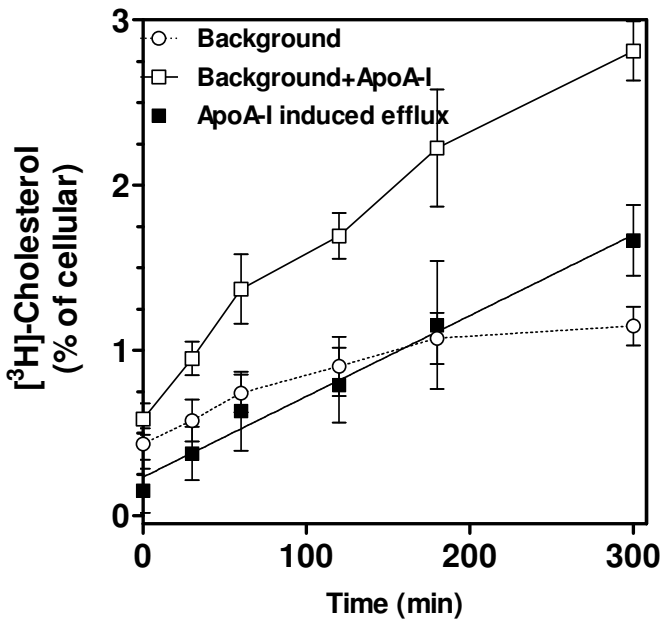

Fig. 7. Cellular Cholesterol Efflux from Adipocytes to Lipid Free apoA-I. Time course of cholesterol efflux: Adipocytes grown in six-well plates were labeled with [^{3}H]-cholesterol and incubated for 5 h at 37^{0}C in DMEM containing lipid-free apoA-I (50 μg/ml). Samples from three wells were taken at different time points. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity. The mean fraction % of radiolabeled cholesterol detected in the media is shown in the graphs. The apoA-I induced cholesterol efflux values were obtained by subtracting the total cholestrol efflux values (apoA-I (50 ug/mL) from background efflux values (without apoA-I incubation). The data plotted is average value of two independent experiments with three internal replicates ± SD)

Effect of lipolysis on the rate of cholesterol efflux from adipocytes to apoA-I.

To examine the effect of lipolysis on cholesterol efflux to lipid free apoA-I, [^{3}H]-cholesterol prelabeled cells were incubated in DMEM/BSA in the absence or in the presence of the non-specific β-adrenergic agonist, isoproterenol. In the presence of isoproterenol, the rate of lipolysis, as estimated from the fatty acids released into the

incubation media, increases ~7-8 fold, from ~3.2 to ~27 nmol FA/mg of cellular protein/h.

Figure 8 shows the effect of lipolysis on the rate of cholesterol efflux to the lipid free human and recombinant apoA-I acceptor. The figure shows the average values of rate of cellular cholesterol efflux per minute obtained when adipocytes were incubated in DMEM/BSA containing apoA-I (50 μg/ml). The amounts of [^{3}H] cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H] cholesterol released into the medium/min during the 5 h incubation. The data shows that activation of lipolysis does not induce a significant increase in the rate of cholesterol efflux to lipid free human apoA-I; 0.0043%/min, ±0.0006 and 0.0050%/min, ±0.0007 ($P>0.05$) and recombinant apoA-I; 0.0041%/min, ±0.0008 and 0.0050%/min, ±0.0013 ($P>0.05$).

The data in figure 8 also demonstrate that the human lipid free apoA-I and recombinant apoA-I have the same cholesterol efflux kinetic characteristics. Since the efflux properties are the same for human lipid free apoA-I, a recombinant apoA-I containing an N- terminal histidine tag and protein kinase A (PKA) site was used for further experiments.

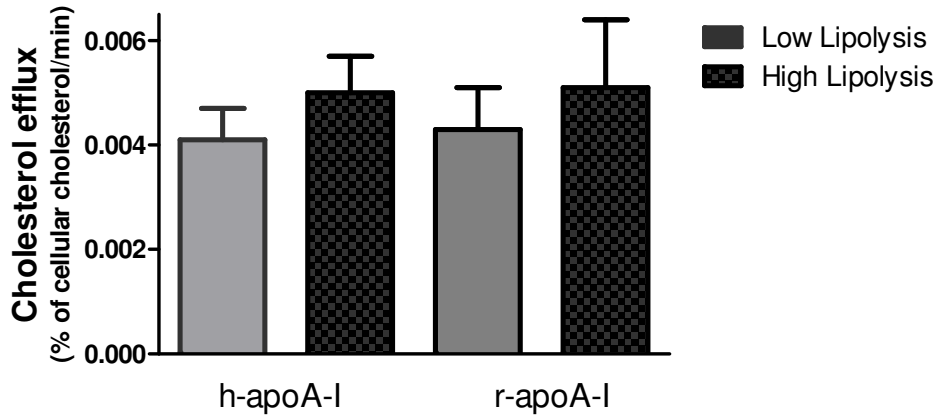

Fig 8. Effect of Lipolysis on the Rate of Cholesterol Efflux to apoA-I. Adipocytes grown in six-well plates were labeled with [^{3}H]-cholesterol and incubated for 5 h at 37^{0}C in DMEM containing lipid-free human apoA-I (h-apoA-I) and recombinant apoA-I (r-apoA-I) (50 μg/ml). "High lipolysis" and "Basal Lipolysis" refer to cells incubated in the corresponding media with or without isoproterenol (10 μg/ml), respectively. Samples of the media were collected at 5 h and analyzed for radioactivity. The mean fraction % of radiolabeled cholesterol detected in the media is shown in the graphs. The data plotted (average value of four independent experiments ± SE) were corrected for radiolabeled cholesterol released in the absence of acceptor.

Effects of inhibitors on ABCAI and SR-BI-mediated cholesterol efflux to apoA-I

ABCAI and SR-BI are abundantly expressed in liver, macrophages, intestine, brain, kidney, steroidogenic and endothelial cells. ABCAI is up-regulated with cyclic AMP analog in macrophages and several other cell types. The cyclic AMP induced up-regulation was exploited to study the ABCAI function in these cell types.

Figure 9 A shows that adipose cells abundantly express ABCAI in comparison with cells that are known to have high expression, like HepG2 (human liver cells) and cAMP stimulated J774.1(macrophage cells). HEK293 (kidney cells) is known to express low levels of ABCAI.

The cyclic AMP analog and apoA-I does not up-regulate the ABCAI expression in adipose cells (Fig. 9 B). The SR-BI is adequately expressed in adipose cells and does not up-regulate the expression with cyclic AMP treatment (Fig. 9 C).

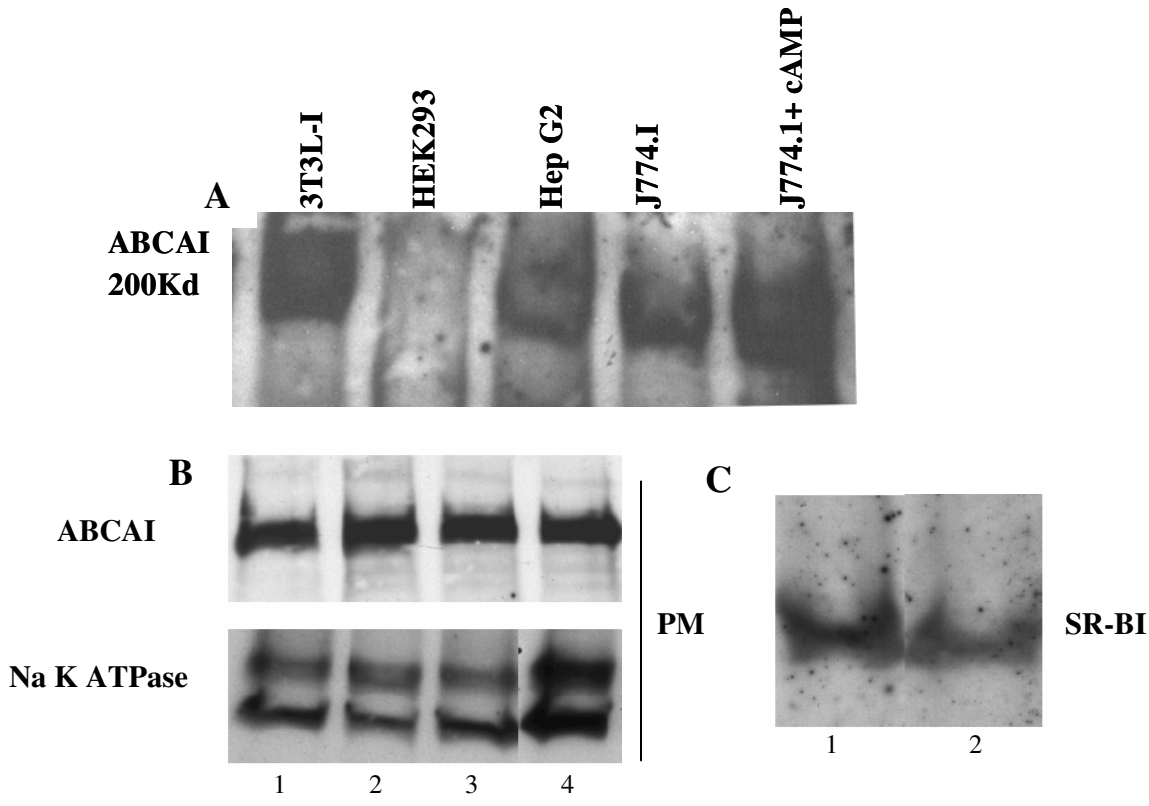

Fig. 9 ABCAI Protein Content In Adipose Tissue And Other Cell Lines A) Western blot showing ABCAI protein levels in homogenates of different cell lines. Western blot was performed after separating 20 µg of protein/sample by SDS-PAGE, as described in experimental procedures. **B)** Western blotting of Na^+/K^+ ATPase and ABCAI of plasma membrane of 3T3L-1 adipocytes was performed after separating 15 µg of protein/sample by SDS-PAGE, as described in experimental procedures. The samples were obtained after incubation of the cells for 5 h in the absence or in the presence of isoproterenol (without acceptors); lane 1&2 respectively and cholesterol acceptor apoA-I (50 µg of protein/ml); lane 3 and cholesterol acceptor apoA-I and isoproterenol; lane 4. **C)** Western blot of SR-BI receptor from plasma membranes of adipocytes incubated for 5 h in DMEM; lane 1, or in DMEM containing isoproterenol, lane 2 .

To gain quantitative data on the contribution of cholesterol efflux pathways in relation to lipid free apoA-I in adipocytes, we used 4 inhibitors which are previously shown to inhibit receptor mediated ((Glyburide, Block Lipid Transfer-1&4 (BLT-1 & BLT-4)) and non-receptor mediated pathways (BFA) in other cell lines. BFA, the most extensive studied inhibitor, blocks the vesicular lipid and protein transport by promoting fusion of Golgi and endoplasmic reticulum compartments in the cells [89]. It is reported that Glyburide and BLT-4 blocks the ABCAI and apoA-I dependent pathway and BLT-1 blocks HDL and SR-BI mediated pathway in cultured cell lines. The above mentioned inhibitors, glyburide, BLT-1 and BLT-4 have not been studied in adipose tissue in relation to cholesterol efflux.

Cholesterol efflux was measured from cells labeled with [^{3}H]-cholesterol for 24 h, followed by a 2 h preincubation with inhibitors. This was followed by a 5 h incubation with inhibitors in the presence of 50 µg of protein per milliliter of lipid-free apoA-I. The amounts of [^{3}H] cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H] cholesterol released into the medium during the 5 h incubation.

Figure 10A shows that the rates of cholesterol efflux to lipid free apoA-I in the presence of inhibitors. The non-receptor mediated BFA (10 g/mL) had a 63% inhibition in cholesterol efflux to apoA-I (Fig. 10 B). The ABCAI and apoA-I mediated inhibitors, glyburide (500 µM) and BLT-4 (150 µM) had 99% and 50% inhibition respectively and the SR-BI and HDL mediated inhibitor, BLT-1(10 µM) had 40 % inhibition in cholesterol efflux to apoA-I (Fig. 10 B). The inhibition studies suggested that i) lipid free apoA-I mediated cholesterol efflux may involve both receptor mediated and non-receptor

mediated vesicular transport. ii) ABCAI may play an important role in the lipid free apoA-I mediated cholesterol efflux, since glyburide and BLT-4 significantly inhibited the cholesterol efflux ii) in addition of vesicular transport and ABCAI mediated pathways several other pathways may exist.

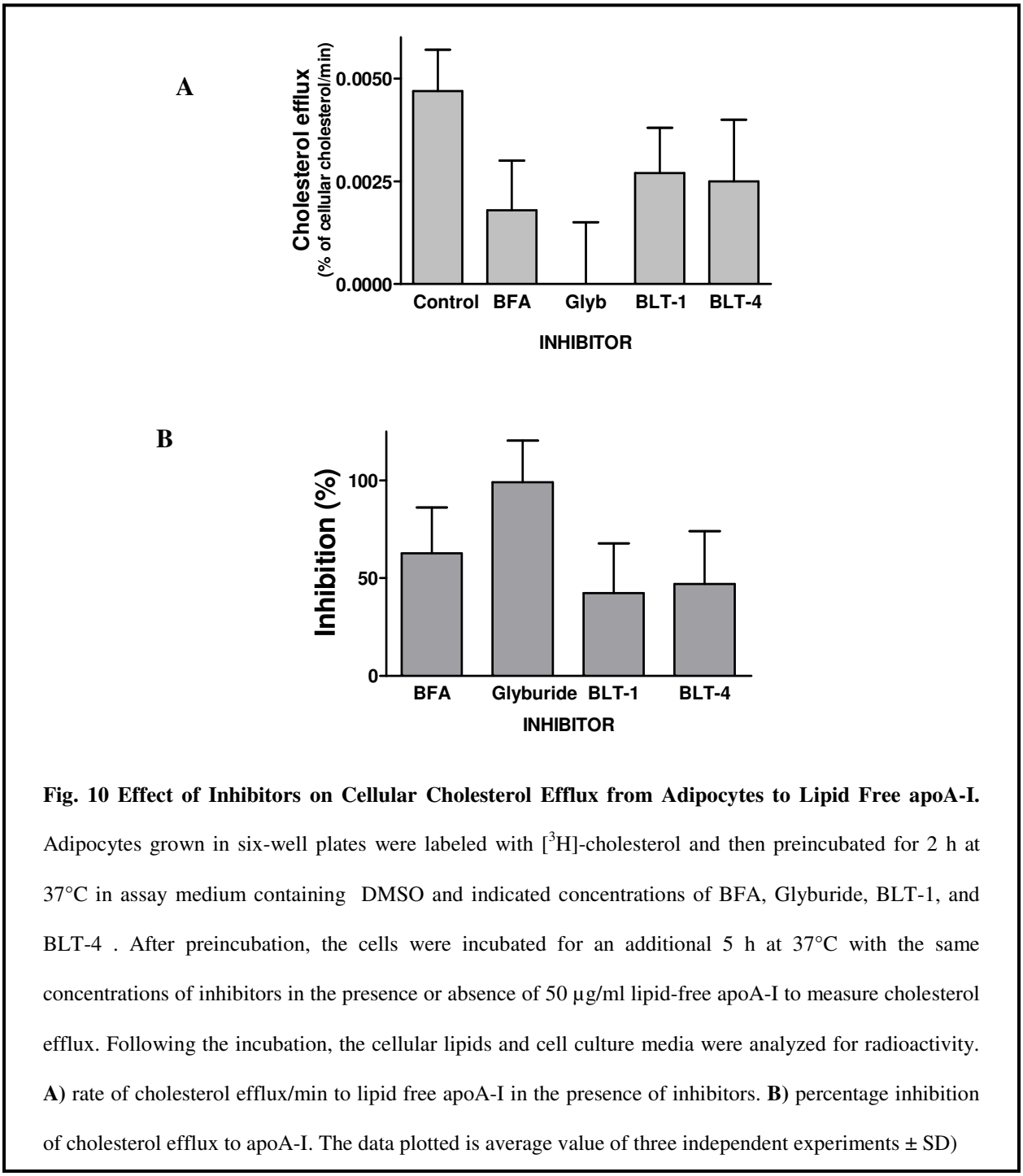

Fig. 10 Effect of Inhibitors on Cellular Cholesterol Efflux from Adipocytes to Lipid Free apoA-I. Adipocytes grown in six-well plates were labeled with [^{3}H]-cholesterol and then preincubated for 2 h at 37°C in assay medium containing DMSO and indicated concentrations of BFA, Glyburide, BLT-1, and BLT-4 . After preincubation, the cells were incubated for an additional 5 h at 37°C with the same concentrations of inhibitors in the presence or absence of 50 µg/ml lipid-free apoA-I to measure cholesterol efflux. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity. **A)** rate of cholesterol efflux/min to lipid free apoA-I in the presence of inhibitors. **B)** percentage inhibition of cholesterol efflux to apoA-I. The data plotted is average value of three independent experiments ± SD)

Uptake and re-secretion of apoA-I by 3T3L-1 adipocytes

The stored lipids in adipose tissue are in a specialized organelle called lipid droplets. Adipose tissue constitutes one of the largest reservoirs of cholesterol in vertebrates [61]. Cellular cholesterol of adipocytes resides more in lipid droplets than PM and most of the lipid-droplet cholesterol is found in the non-esterified form. The high amount of intracellular cholesterol depot, receptor sensitive inhibitor studies and the presence of ABCAI in the lipid droplet (Fig. 11) prompted to us to gain further insights into the possible pathways involved in apoA-I mediated cholesterol efflux. Hence we investigated the ability of adipocytes to internalize and re-secrete apoA-I into the cell medium (retroendocytosis).

This question was investigated using a novel approach in which a full-length recombinant human apoA-I with an N terminal five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA) was constructed. The protein was expressed in *E. coli* and purified by Ni-affinity chromatography. The activity and specificity of PKA against recombinant apoA-I was tested by in vitro phosphorylation with bovine PKA and γ-[^{32}P]-ATP. Human apoA-I isolated from serum was not phosphorylated by PKA whereas the recombinant protein was highly phosphorylated by PKA (Fig. 12).

This apoA-I variant allowed studying uptake and re-secretion of apoA-I through the appearance of phosphorylated apolipoprotein in the cell culture medium. Because protein phosphorylation can only occur intracellularly, the presence of phosphorylated apoA-I in the cell medium clearly indicates that the protein was internalized and subsequently secreted by the cell. 3T3L-1 fibroblasts derived adipocytes cultured in six

well dishes were double radiolabeled by incubation for 4 h with 80 μCi/well of [^{32}P] orthophosphate (carrier free) and 2 μCi/ml [^{3}H]-cholesterol for 24 h at 37^{0}C in phosphate-free DMEM containing 0.12% BSA. At the end of the incubation period cell media was collected at different time points and loaded into a Ni-affinity column to purify the recombinant apoA-I. The eluted proteins were separated by SDS-PAGE on 4-20% and subjected to autoradiography to determine the phosphorylation.

The data in figure 13 A demonstrates the presence of phosphorylated apoA-I in the medium of control cells; (low lipolysis), or in the medium of cells incubated with isoproterenol; (high lipolysis). Also the phosphorylation of apoA-I observed in low and high lipolysis is sensitive to the PKA inhibitor, H-89 (Fig.13 A). The intensity of phosphorylation of apoA-I in the medium from control cells was lower than that observed in the medium of cells with high rate of lipolysis (Fig. 13 B). Most of this difference is probably due to the fact that PKA is activated by isoproterenol, which is a β-adrenergic agonist. However, the novel approach used evidently showed retroendocytosis of apoA-I in adipose cells.

To show that the recycling apoA-I is lipid loaded, the phospholipid and cholesterol acquired during the recycling process is extracted with chloroform/ methanol mixture. The figure 14 shows a representative autoradiogram of the phosphatidylcholoine spots separated in a TLC plate. The separated cholesterol spots were scraped and scintillation counted to determine the specific cholesterol efflux to apoA-I, which is similar to the results discussed earlier (data not shown).

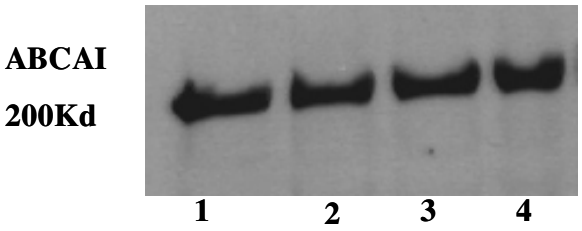

Fig. 11. ABCAI in Lipid Droplet of 3T3L-1: Western blotting of lipid droplet of 3T3L-1 adipocytes was performed after separating 15 μg of protein/sample by SDS-PAGE, as described in experimental procedures. The samples were obtained after incubation of the cells for 5 hours in the absence or presence of the cholesterol acceptor apoA-I . Lane 1&3: shows control (low lipolysis) cells incubated with and without apoA-I respectively. Lane 2&4: shows cells incubated with isoproterenol (high lipolysis) with and without apoA-I respectively.

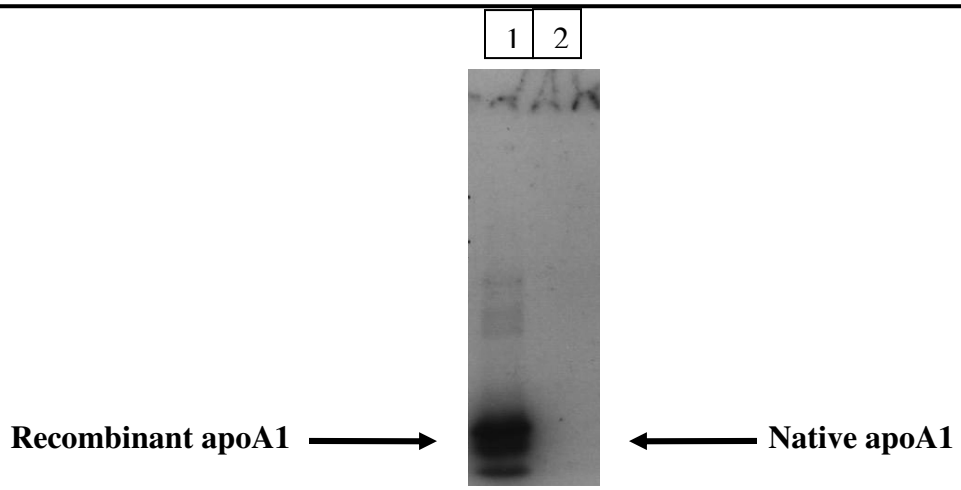

Fig. 12. In vitro Phosphorylation of Recombinant Lipid Free apoA-I: The specificity of PKA against recombinant apoA-I was tested with commercially purchased bovine PKA and γ-[^{32}P]-ATP as described in experimental procedures. Lane 1: recombinant apoA-I with PKA site. Lane2: B human apoA-I isolated from serum.

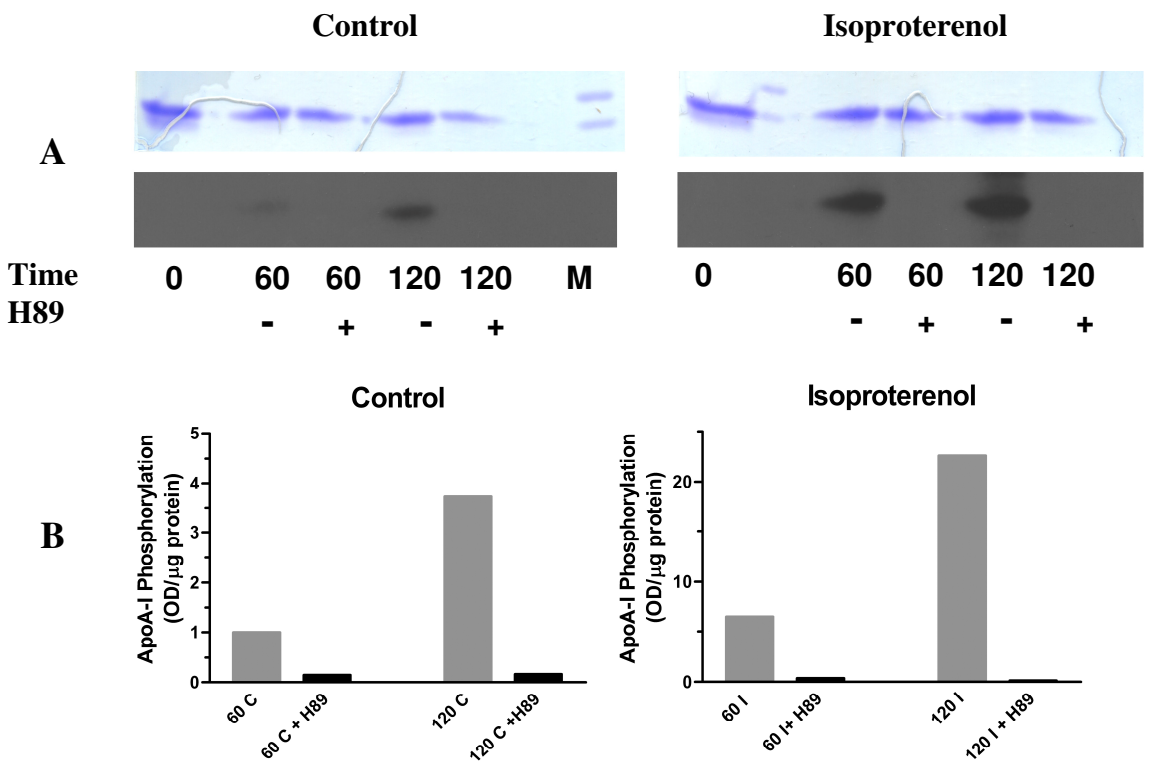

Fig. 13. Uptake and Re-secretion of apoA-I in Adipose Cells: 3T3L-1 adipocytes were radiolabeled by incubation with 2 µCi/ml [^{3}H]-cholesterol for 24 h and for 4 h at 37^{0}C with 80 µCi/ml of [^{32}P]-phosphate in phosphate-free DMEM containing 0.12% BSA. Cellular uptake and subsequent re-secretion of apoA-I was monitored by incubating the pre-labeled cells with recombinant apoA-I (50µg/ml) containing a phosphorylation site for PKA. After incubation for 60 and 120min, apoA-I was purified from the cell culture media by Ni-affinity chromatography. Proteins were separated by SDS-PAGE on 4-20% gels. **Panel A** shows the apoA-I region of the Coomassie Blue-stained gel (upper) and the corresponding autoradiogram (lower). The cells were incubated in the same experimental condition with H-89 PKA inhibitor as indicted in the figure. **Panel B** shows the corresponding normalized phosphorylation of panel A **Left panel** purified apoA-I from the medium of cells with low lipolysis); **Right panel**: purified apoA-I from the medium of cells incubated with apoA-I with high lipolysis.

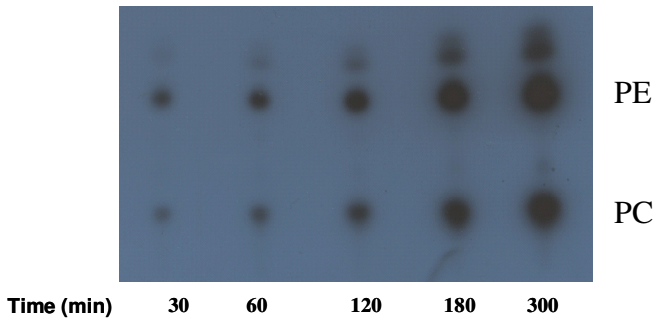

Fig. 14. Lipidatated apoA-I of Retroendocytosis. 3T3L-1 adipocytes were radiolabeled by incubation with 2 μCi/ml [^{3}H]-cholesterol for 24 h and for 4 h at 37^{0}C with 80 μCi/ml of [^{32}P]-phosphate in phosphate-free DMEM containing 0.12% BSA. Cellular uptake and subsequent re-secretion of apoA1 was monitored by incubating the pre-labeled cells with recombinant apoA-I (50μg/ml) containing a phosphorylation site for PKA. apoA-I from the corresponding time points were purified from the cell culture media by Ni-affinity chromatography. Aliquot of purified protein was used to extract lipids by chloroform/ methanol mixture and separated by TLC. The autoradiograph of the TLC spots; Phosphatidylcholine and Phosphatidylethanolamine is shown.

Figure 15 shows that the apoA-I phosphorylation in adipocytes increases with increasing concentration of apoA-I up to 100 μg of protein per milliliter. The apoA-I phosphorylation is also sensitive to the human apoA-I competition (Fig. 16). This suggests that recombinant apoA-I recycling pathway is specific and occurs through the same pathway as of human apoA-I.

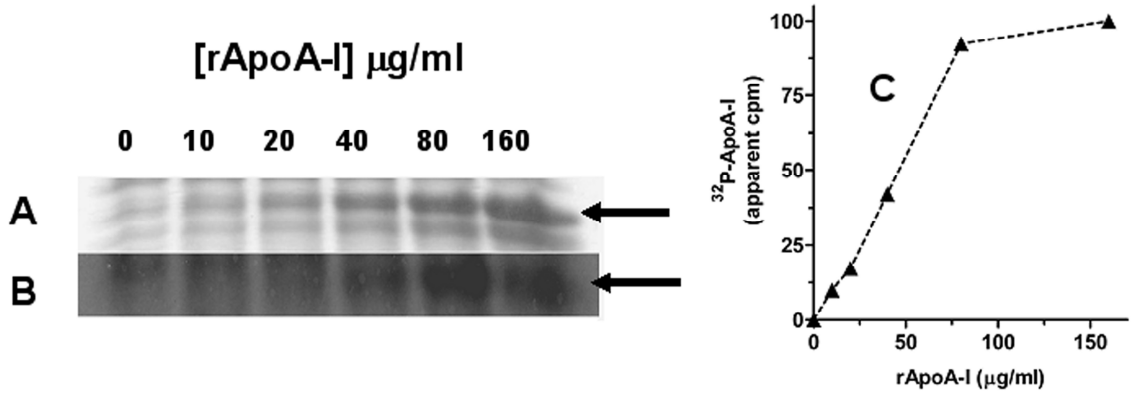

Fig. 15. Uptake and Re-secretion Increases with Increasing apoA-I Amount in Media. apoA-I phosphorylation increase with 3T3L-1 adipocytes were radiolabeled by incubation with 80 μCi/ml of [^{32}P]-phosphate in phosphate-free DMEM containing 0.12% BSA for 4 h. Cellular uptake and subsequent re-secretion of apoA-I was monitored by incubating the pre-labeled cells with recombinant apoA-I with various amounts. After incubation for 1 h, the proteins in the cell media were separated by SDS-PAGE on 4-20% gels. **Panel A** shows the apoA-I region of the Coomassie Blue-stained gel. **Panel B shows** the corresponding autoradiogram of the Coomassie Blue-stained gel. **Panel C** shows the corresponding normalized phosphorylation of panel A & B

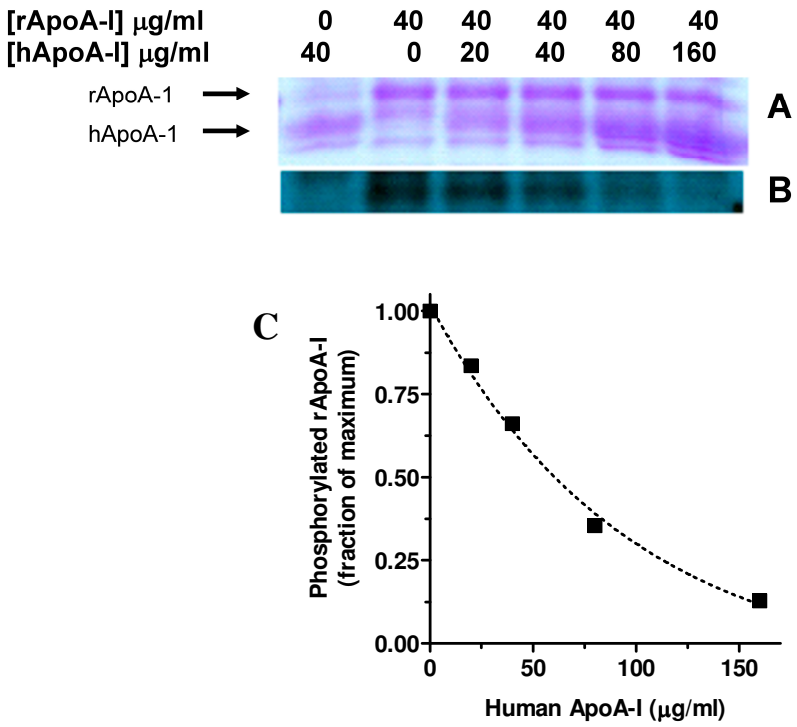

Fig. 16. The Retroendocytosis of apoA-I is Specific: 3T3L-1 adipocytes were radiolabeled by incubation with 80 μCi/ml of [^{32}P]-phosphate in phosphate-free DMEM containing 0.12% BSA for 4 h. The specificity of uptake and re-secretion was monitored by incubating the pre-labeled cells with a constant amount of recombinant apoA-I and a varying amount of human apoA-I isolated from serum. After incubation for 1 h, the proteins in the cell media was collected and were separated by SDS-PAGE on 4-20% gels. **Panel A & B** shows the apoA-I regions of the Coomassie Blue-stained gel (upper) and the corresponding autoradiogram (lower). **Panel C** shows the corresponding normalized phosphorylation of apoA-I.

To further investigate the mechanism of retroendocytosis of apoA1 in adipocytes, the phosphorylation was measured from 3T3L-1 cells double labeled with [^{3}H] cholesterol and [^{32}P] orthophosphate (carrier free) for 24 and 4 h respectively, followed

by a 2 h preincubation with inhibitors, which was followed by a 40 min incubation with inhibitors in the presence and absence of 50 μg of protein per milliliter of lipid-free apoA-I. At the end of the incubation period cell media was collected at different time points and loaded into a Ni-affinity column to purify the recombinant apoA-I. The eluted proteins were separated by SDS-PAGE on 4-20% gel and subjected to autoradiography to determine the phosphorylation.

Figure 17, Panel A&B shows the phosphoylation of apoA-I in the presence and absence of inhibitors. Figure 17 C shows the rate of phosphorylation of apoA-I normalized for the protein amount and Panel D shows the phosphorylation of apoA-I normalized for protein amount and rate of lipolysis (rate of lipolysis relates to the level of PKA activity in cells) in the presence and absence inhibitors. The results suggest that the rate of apoA-I uptake and re-secretion is independent of receptor and non-receptor sensitive drugs used in this study.

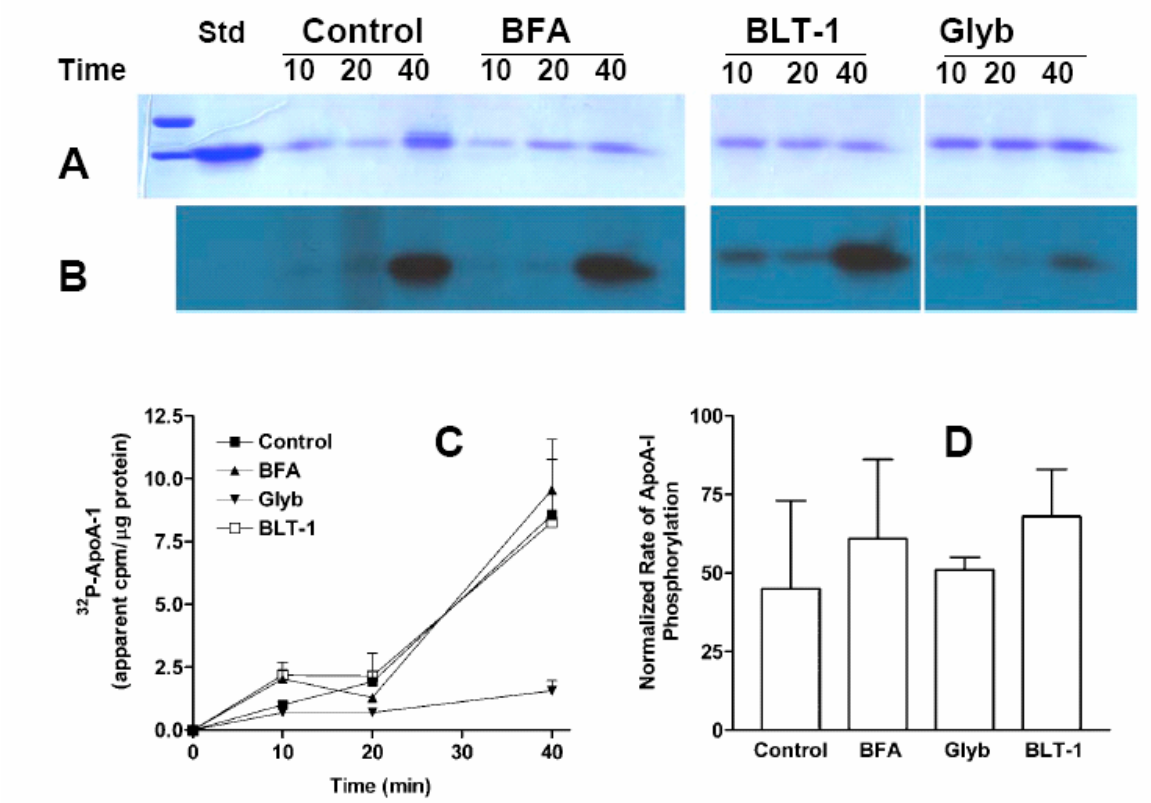

Fig. 17. Retroendocytosis of apoA-I is not blocked by inhibitors in adipose cells: 3T3L-I adipocytes were radiolabeled by incubation with 2 μCi/ml [^{3}H]-cholesterol for 24 h and for 4 h at 37^{0}C with 80 μCi/ml of [^{32}P]-phosphate in phosphate-free DMEM containing 0.12% BSA. Cellular uptake and subsequent re-secretion of apoA-I was monitored by incubating the pre-labeled cells with recombinant apoA-I (50 μg/ml) containing a phosphorylation site for PKA in the presence or absence of inhibitors; BFA, Glyburide, and BLT-1 as described in experimental procedures. After incubation for 10, 20 and 40 min, apoA-I was purified from the cell culture media by Ni-affinity chromatography. Proteins were separated by SDS-PAGE on 4-20% gels. **Panel A &B** shows the apoA-I region of the Coomassie Blue-stained gel and (upper) and the corresponding autoradiogram (lower). **Panel C** shows the corresponding phopshorylation of apoA-I normalized with protein amount. **Panel D** shows the corresponding phopshorylation of apoA-I normalized with protein amount and rate of lipolysis.

Discussion

Our previous study established a metabolic link between lipolysis and rHDL cholesterol efflux in adipocyte. The investigation of the effect of the lipolytic state and rate of cellular cholesterol efflux is of interest in adipocytes because of its large content of stored unesterified cholesterol and role in energy homeostasis. Due to the large amount of free cholesterol stored in adipose tissue compared to most other organs, including liver and muscle, it could play a major role in the homeostasis of cholesterol metabolism. In continuation with our previous study, the current study investigated the effect of lipolysis on apoA-I cholesterol efflux, the major apolipoprotein of HDL in circulation. Data from this study shows that the activation of lipolysis does not increase adipocyte cholesterol efflux to apoA-I. This observation does not undermine the link existing between lipolysis and cholesterol efflux. But the data suggests that lipid free apoA-I is not an efficient acceptor like pre β –HDL and may interact with different lipid domains in PM or intracellular location for the cholesterol absorption. This confines the ability of apoA-I to access the modulated cholesterol domains of membranes in the cells during the activation of lipolysis. It has been shown that apoA-I interacts with non-lipid raft domains in the PM and rHDL has the ability to interact with both non-lipid raft and lipid raft domains in macrophages and other cell lines [100-104].

It is very important to understand the apoA-I cholesterol efflux pathways, because of its prime role in the biogenesis of HDL. ABCAI and SR-BI are the two known cell receptor proteins that mediate apoA-I and HDL cholesterol efflux. The lipoproteins and receptors play an important role in cholesterol metabolism in mammalian system and abnormality in these components are correlated with far-reaching pathologies [31, 105].

This study shows that apoA-I induced cholesterol efflux in adipocytes at 5 h period is 1.8 % of the total cellular cholesterol (fig. 7) and the efflux is dose dependent (data not shown). Several pharmacological drugs have been demonstrated to inhibit ABCAI and SR-BI mediated free cholesterol efflux in several cell lines. Glyburide inhibits ABCAI mediated lipid efflux in macrophage cells, muscle cells, fibroblasts HEK293 cells, by reducing apoA-I binding to cell surface [15, 38, 106]. BLT-1 inhibits SR-BI receptor mediated efflux to lipoproteins while BLT-4 blocks both ABCAI and SR-BI in HEK293 cells [32]. BFA blocks vesicular transport in cells. The efficacy to specifically inhibit cholesterol efflux pathways and mechanism of inhibition of these pharmacological agents will provide new insights in the understanding of cholesterol efflux process in adipose cells. In this study, the tested cholesterol efflux inhibitors, BFA, glyburide, BLT-1 and BLT-4 inhibited apoA-I induced cholesterol efflux significantly. The inhibition of ABCAI and SR-BI receptors and vesicular transport by the drugs suggested that apoA-I induce cholesterol efflux through multiple pathways in adipose cells. These cholesterol efflux pathways could be activated through vesicular transport (with and without the involvement of receptors), ABCAI, SR-BI, ABCGI and other unidentified pathways and receptors.

The stored lipids in adipose tissue are in a specialized organelle called LD. The adipose tissue constitutes one of the largest reservoirs of free cholesterol in vertebrates [61]. Cellular cholesterol of adipocytes resides more in LD than PM and most of the lipid-droplet cholesterol is found in the non-esterified form (Fig. 2 A). Studies in macrophages and endothelial cells suggest that HDL and apoA-I bind to cell surface receptors, internalize, transport to non-lysososmal compartments and remove cholesterol

from intracellular locations. Finally, the lipoproteins are secreted from the cells to tissue culture media without degradation [34, 39, 42]. But a different study did not find evidence for lipoprotein internalization in macrophages cells [40]. At present the HDL and apoA-I retroendocytosis is under debate.

The higher amount of intracellular cholesterol present in LD than PM, the decrease of cholesterol content in the PM during the activation of lipolysis [83], receptor sensitive inhibitor studies and the presence of ABCAI in the lipid droplet led us to the hypothesize that adipocytes have the ability to internalize and re-secrete apoA-I into the cell medium and play a potential role in the cholesterol homeostasis. The retroendocytosis studies are still under dispute mainly because of the ambiguous techniques employed in studying this process. ^{125}I labeled apoA-I, apoA-I-green fluorescent protein (GFP) fusion protein and electron microscopy techniques are commonly used to study retroendocytosis. But the biochemical evidence for this process is mainly obtained from data of ^{25}I experiments. This technique has serious limitations in identifying the membrane bound, internalized and re-secreted lipoproteins because the externally residing lipoproteins and re-secreted lipoprotein cannot be distinguished.

To investigate the possibility of up-take and re-secretion of apoA-I in adipose cells, we used a novel approach in which a full-length recombinant human apoA-I with an N terminal five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA) was constructed. Because the protein phosphorylation can only occur intracellularly, the presence of phosphorylated apoA-I in the cell medium clearly indicates that the protein was internalized and subsequently

secreted by the cell. The constructed recombinant protein was further tested for their phosphorylation ability and PKA specificity in vitro and in vivo. Our studies unambiguously showed that adipose cells uptake and re-secrete lipidated apoA-I in time specific manner. The uptake and re-secretion of recombinant apoA-I is challenged by native human apoA-I. This suggests that both recombinant and native apoA-I share a common pathway for internalization and secretion. We have used BFA (vesicular sensitive), Glyburide (ABCAI inhibition), and BLT-1 (SR-BI inhibition) to further understand the pathway of recycling of apoA-I in adipose tissue. At the concentration used, all three drugs had no effect on the uptake and recycling of apoA-I in adipose tissue. The autoradiograph of the gluburide inhibitor study shows decreased phosphorylation of re-secreted apoA-I. This decreased phosphorylation observed is due to the inhibition of cellular lipolysis by glyburide. Once the phophorylation was normalized for protein amount and rate of lipolysis, the glyburide treatment showed no significant inhibition effect compared to the control experiments. This also shows that glyburide interferes with cellular process other than its known actions. In conclusion, we clearly show that adipose tissue uptakes and re-secretes lipidated apoA-I molecules. The observed retroendocytosis may not occur through the known receptors or components of cholesterol efflux. Several cell lines express low levels of ABCAI, SR-BI and ABCGI but have normal cholesterol efflux and function through poorly identified or unidentified pathways [107]. The contribution of cholesterol efflux by retroendocytosis and the physiological relevance of this process is unknown.

In brief our studies show that activation of lipolysis does not induce a significant increase in the rate of cholesterol efflux to lipid free apoA-I. The known vesicular and non-vesicular cholesterol efflux inhibitors inhibit global cholesterol efflux significantly. We have evidently presented that lipid free apoA-I undergo retroendocytosis in adipose tissue and the cholesterol efflux drugs had no effect on the uptake and recycling of apoA-I.

CHAPTER IV

Cholesterol efflux and apoA-I recycling in macrophages

Introduction

Reverse Cholesterol Transport (RCT) is relevant because peripheral cells acquire cholesterol through uptake of lipoproteins and de novo synthesis, but are unable to catabolize it. The free cholesterol (FC) efflux is one of the decisive early steps of the RCT and high-density lipoprotein (HDL) serves as the main transport vehicle for the cellular cholesterol released from the peripheral cells to liver [11]. Apolipoprotein A-I (apoA-I) is the major protein component of HDL that promotes the efficient transfer of excess peripheral cell cholesterol to make them an efficient acceptor and transporter. Deficiency and naturally occurring mutations in apoA-I fail to produce normal HDL levels and particles [13]. The plasma levels of HDL and apoA-I are inversely linked with the risk of cardiovascular diseases. Hence it is suggested that RCT is an important physiological process that protects against cardiovascular diseases [14].

Monocyte derived macrophage cells accumulate cholesterol ester (CE) rich lipoproteins like LDL and apolipoprotein B (apoB) containing particles from plasma and vascular compartments via specific receptors [24, 108]. The CE and lipid rich macrophages morphologically appear as foam cells. These foam cells are the hallmark of early and intermediate atherosclerotic lesions [109]. HDL and apoA-1 stimulate cholesterol efflux from macrophage foam cells and reduce cholesterol accumulation and atherogenesis in animals [110-112]. The overall contribution of macrophages in the

cholesterol homeostasis and RCT process is unknown but in relation to atherosclerosis, macrophage-specific RCT is extremely crucial in preventing the disease.

Macrophage cholesterol efflux has been extensively studied. Several of these studies suggested that the reteroendocytosis of lipoproteins occur in macrophages for cholesterol efflux [27, 36-39]. But the retroendocytosis of lipoproteins in macrophages or other cell lines are under intense debate because of the ambiguous techniques employed to elucidate this process. In this study we aim to determine the uptake and re-secretion of apoA-I in macrophages with an unambiguous technique.

Experimental Procedures

Materials: J774.1 macrophage cells were purchased from American Type Cell Culture (Manassas, VA). Polyclonal anti-ABCAI antibody was obtained from Novus-Biologicals (Littleton, CO). Horseradish peroxidase (HRP) tagged antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Fatty acid free bovine serum albumin, protease inhibitors (phenyl-methyl sulfonyl fluoride, leupeptin, aprotinin, antipain), 8-Br-cAMP, 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). [^{3}H]-Cholesterol (60 Ci/mmoL) was from Perkin-Elmer. [^{32}P] orthophosphate (carrier free) was purchased from MP Biochemicals. Chemiluminiscence kit for Western blots was obtained from Amersham Biosciences (Piscataway, NJ).

Cell Culture and Lipoproteins: J774.1 cells were cultured at 37°C in 8% CO_2 atmosphere in high glucose DMEM supplemented with 10% FBS and 0.01% streptomycin and penicillin antibiotics. All experiments were conducted when cells were at 85%-90% confluence.

Recombinant apoA-I was prepared by cloning full-length human apoA-I into a pET33b vector (Novagen), which incorporates an N-terminal tag encoding for a six-His tag and a five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA)

Cholesterol Efflux: J774.1 macrophage cells in 12 well dishes were stimulated with 8-Br-cAMP and radiolabeled with 2 μCi/ml [^{3}H]-cholesterol for 24 h. The media

was removed and the cells were incubated for 3 hours in serum-free DMEM containing 0.2% BSA (DMEM/BSA) and, before the beginning of the experiment, rinsed twice with DMEM/BSA. The experiment was started by addition of fresh DMEM/BSA with 8-Br-cAMP. Cholesterol efflux to apoA-I (50 µg/mL) was determined at 37 ^{0}C in the media of cells that were stimulated with 8-Br-cAMP for 24 h and control cells with 8-Br-cAMP added just before the experiments. Background cholesterol efflux was determined in the media containing no acceptors. Microliter aliquots of the medium were collected at specific time intervals to estimate the fraction of [^{3}H]-cholesterol released into the medium by scintillation counting. At the end of the incubation, the cells were washed with phosphate buffered saline (PBS) twice and the lipids extracted with 2-propanol . The lipid extract was used to determine the fraction of radiolabeled cholesterol remaining in the cells. Cholesterol efflux was expressed as the percentage of radiolabeled cholesterol released into the media as:

100x [^{3}H]-Cholesterol in media /([^{3}H]-Cholesterol in media + [^{3}H]-Cholesterol in cells).

The effect of inhibitors on cholesterol efflux was studied by pre-incubating the pharmacological agents, BFA (10 µg/mL), Glyburide (500 µM), and BLT-4(150 µM) with [^{3}H]-cholesterol labeled cell media for 2 h. After the preincubation, the cells were incubated for an additional 5 h at 37°C with the same concentrations of inhibitors in the presence or absence of 50 µg/ml lipid-free apoA-I to measure cholesterol efflux. Background cholesterol efflux was determined by adding corresponding drug solvents in the media containing no acceptors. Microliter aliquots of the medium were collected at specific time intervals to estimate the fraction of [^{3}H]-cholesterol released into the

medium by scintillation counting. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity as described above.

***Western Blots*:** Aliquots of the cell homogenate of J774.1 (un-stimulated), J774.1 (stimulated with 8Br-cAMP (300 mM) for 24 h) cells containing 20 µg of protein were mixed with reducing SDS-sample buffer and subjected to SDS-PAGE in 8% acrylamide gels. The proteins were transferred to a nitrocellulose membrane and probed with a rabbit anti mouse/human ABCAI (1:750 dilution). All secondary antibodies were used at a 2500 fold dilution. The bands were visualized by chemiluminescence.

ApoA-I cellular Uptake and Re-secretion. Full-length recombinant human apoA-I was cloned into a pET33b vector (Novagen), which incorporates an N-terminal tag encoding for a six-His tag and a five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA). The protein was expressed in *E. coli* and purified by Ni-affinity chromatography. The activity and specificity of PKA against recombinant apoA-I was tested by *in vitro* phosphorylation with bovine PKA and γ-[^{32}P]-ATP. Human apoA-I isolated from serum was not phosphorylated by PKA whereas the recombinant protein was highly phosphorylated by PKA.

In vivo Phosphorylation of apoA-I: J774.1 cells in 12 well dishes were stimulated with 8-Br-cAMP and 2 µCi/ml [^{3}H]-cholesterol for 24 h. The cells were then radiolabeled by incubation for 4 h with 80 µCi/well of [^{32}P] orthophosphate (carrier free) in phosphate-free DMEM media containing 0.12% BSA. At the end of the labeling period, 300 µM of 8-Br-cAMP was added to the stimulated cells. Recombinant apoA-I (50 µg) was also added immediately and cell media was collected at 15, 30, and 60 minutes. The collected cell media was loaded into a Ni-affinity column, was washed the column

extensively with 20mM imidazole and eluted the recombinant apoA-I with 1M imidazole. The eluted proteins were separated by SDS-PAGE on 4-20% gels and subjected to autoradiography to visualize the phosphorylation.

Results

Cellular cholesterol efflux to apoA-I

The rate of cholesterol efflux to apoA-I was studied in J774.1 that were pre-labeled with [^{3}H]-cholesterol for 24 h. The distribution of radiolabeled cholesterol is nearly identical to the relative mass contents of cholesterol suggesting that in 24 h the incorporated radiolabeled cholesterol reaches a physiological distribution. It has been shown that ABCAI is up-regulated with cyclic AMP analogs in macrophages [113]. In this study, we used 300 µM of 8-Br-cAMP to up-regulate ABCAI in J774.1 cells (Fig.18 A).

A representative time course of cholesterol efflux to apoA-I (50 µg/mL) is shown in figure 18B. The amounts of [^{3}H] cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H] cholesterol released into the medium during the 5 h incubation. At the concentration of lipoprotein tested (50 µg apoA-I/ml), and after 5 h of incubation with un-stimulated and stimulated cells, the total cellular cholesterol efflux is approximately 7% and 15% respectively. We observed approximately 30 fold up-regulation of the total ABCAI protein content in J774.1 cells.

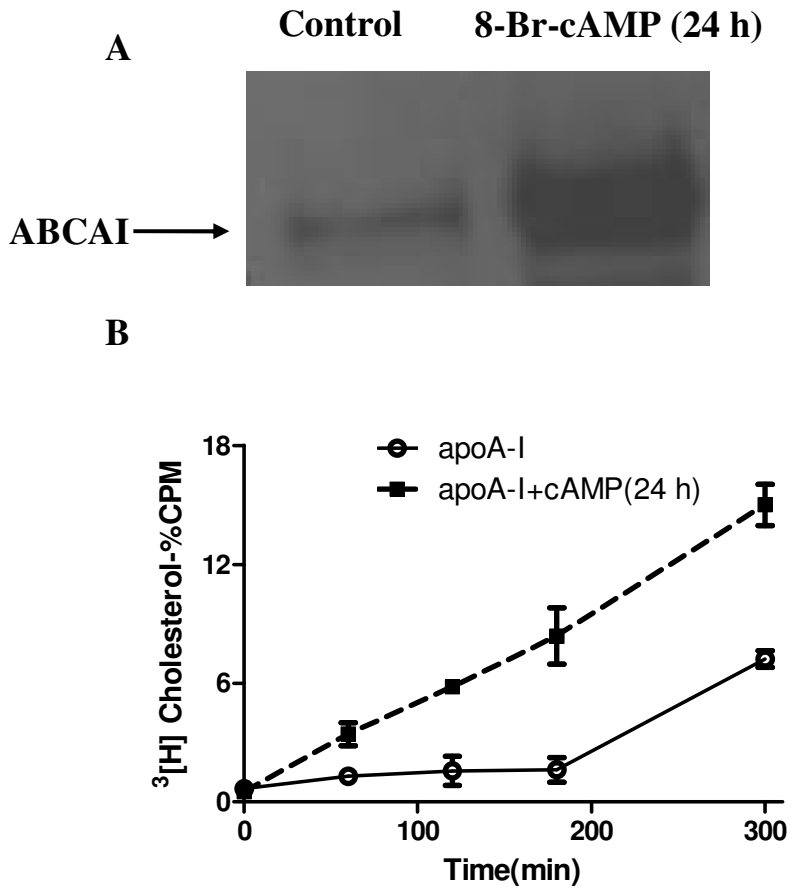

Fig. 18. Cellular Cholesterol Efflux from J774.1 to Lipid Free apoA-I. A. Western blot showing up-regulation of ABCAI protein content in J774.1 cells after incubation with 300 µM of8-Br-cAMP for 24 h. **B.** Time course of cholesterol efflux. J774.1 cells were radiolabeled by incubation for 24 h with [^{3}H]-cholesterol (2 µCi/ml) and 8-Br-cAMP in DMEM containing 2.5% FBS. Cholesterol efflux to apoA-I (50µg/mL) was determined at 37 ^{0}C in the media of cells that was stimulated with 8-Br-cAMP for 24 h and control cells with 8-Br-cAMP added just before the experiment. Samples from three wells were taken at different time points. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity. The mean fraction % of radiolabeled cholesterol detected in the media is shown in the graphs. The data plotted is average value of two independent experiments with three replicates ± SD.

Effects of inhibitors on ABCAI and SR-BI-mediated cholesterol efflux to apoA-I

ABCAI and SR-BI are abundantly expressed in macrophages cells. To gain quantitative data on the contribution of cholesterol efflux pathways in relation to lipid free apoA-I in macrophages, we used 3 inhibitors which are previously shown to inhibit receptor mediated (Glyburide, Block Lipid Transfer-4 (BLT-4)) and non-receptor mediated pathways (BFA). BFA, the most extensive studied inhibitor blocks the vesicular lipid and protein transport by promoting fusion of Golgi and endoplasmic reticulum compartments in the cells [89]. It is reported that Glyburide and BLT-4 blocks the ABCAI and apoA-I dependent pathway in cultured cell lines.

Cholesterol efflux was measured from stimulated (300 µM 8-Br-cAMP) cells labeled with [^{3}H] cholesterol for 24 h, followed by a 2 h preincubation with inhibitors. This was followed by a 5 h incubation with inhibitors in the presence of 50 µg of protein per milliliter of lipid-free apoA-I. The amounts of [^{3}H] cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H] cholesterol released into the medium during the 5 h incubation.

Figure 19 shows that the percentages of cholesterol efflux to lipid free apoA-I in the presence of inhibitors. The non-receptor mediated BFA (10 g/mL) had a 59% inhibition in cholesterol efflux to apoA-I. The ABCAI and SR-BI mediated inhibitors, glyburide (500 µM) and BLT-4 (150 µM) had 92% and 73% inhibition respectively(Fig. 19). In agreement with earlier studies, our inhibition studies also suggested that lipid free apoA-I mediated cholesterol efflux may involve both receptor mediated and non-receptor

mediated vesicular transport and ABCAI may play an important role in the lipid free apoA-I mediated cholesterol efflux, since glyburide significantly inhibited the pathway.

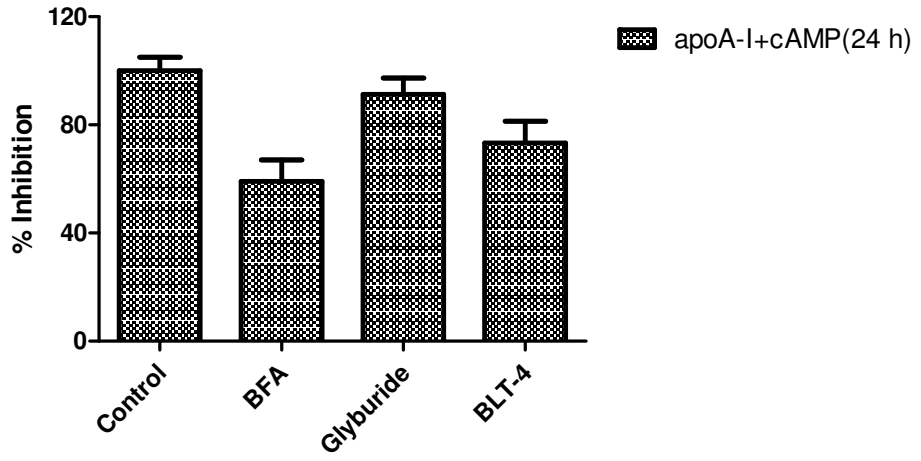

Fig. 19. Effect of Inhibitors on Cellular Cholesterol Efflux from J774.1 to Lipid Free apoA-I. J774.1 were labeled with [^{3}H]-cholesterol and then preincubated for 2 h at 37°C in assay medium containing DMSO and indicated concentrations of BFA, Glyburide, and BLT-4 . After the preincubation, the cells were incubated for an additional 5 h at 37°C with the same concentrations of inhibitors in the presence or absence of 50 µg/ml lipid-free apoA-I to measure cholesterol efflux. Following the incubation, the cellular lipids and cell culture media were analyzed for radioactivity. The figure represents the percentage inhibition of cholesterol efflux to apoA-I. The data plotted is average value of two independent experiments with three replicates ± SD)

Uptake and re-secretion of apoA-I by J774.1 macrophage cells

Several studies reported that the reteroendocytosis of lipoproteins occur in macrophages for cholesterol efflux [27, 36-39]. But the retroendocytosis of lipoproteins in macrophages or other cell lines are under intense debate because of the ambiguous

techniques employed to elucidate this process. In this study we used a novel approach to determine the uptake and re-secretion of apoA-I in macrophages.

A full-length recombinant human apoA-I with an N terminal five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA) was constructed. The protein was expressed in *E. coli* and purified by Ni-affinity chromatography. The activity and specificity of PKA against recombinant apoA-I was tested by in vitro phosphorylation with bovine PKA and γ-[^{32}P]-ATP. Human apoA-I isolated from serum was not phosphorylated by PKA whereas the recombinant protein was highly phosphorylated by PKA (Fig. 12).

This apoA-I variant allowed studying uptake and re-secretion of apoA-I through the appearance of phosphorylated apolipoprotein in the cell culture medium. Because protein phosphorylation can only occur intracellularly, the presence of phosphorylated apoA-I in the cell medium clearly indicates that the protein was internalized and subsequently secreted by the cell. J774.1 stimulated cells (300μM 8-Br-cAMP) cultured in 12 well dishes were double radiolabeled by incubation for 4 h with 80 μCi/well of [^{32}P] orthophosphate (carrier free) and 2 μCi/ml [^{3}H]-cholesterol for 24 h at 37^{0}C in phosphate-free DMEM containing 0.12% BSA. At the end of the incubation period cell media was collected at different time points and loaded into a Ni-affinity column to purify the recombinant apoA-I. The eluted proteins were separated by SDS-PAGE on 4-20% and subjected to autoradiography to determine the phosphorylation.

Figure 20 A demonstrates that the rapoA-I is not phosphorylated in the medium of J774.1 stimulated cells. We have simultaneously tested stimulated J774.1 cells with another cAMP analogue, isoproterenol to confirm our results. This also demonstrated absence of

phosphorylated apoA-I in the media (data not shown). This suggests that apoA-I does not undergo retroendocytossis in macrophages under tested conditions. To further extend out studies to other cell lines and evaluate the uptake and re-secretion experiments, we have tested retroendocytosis of apoA-I in human liver cells (HepG2 cells). The figure 20B shows that the apoA-1 is phoshporylated in a time dependent manner.

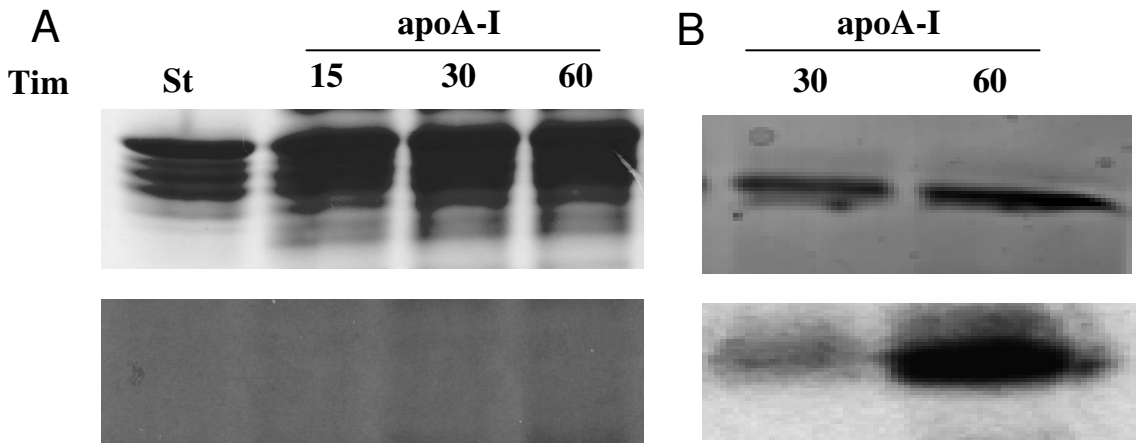

Fig. 20. Uptake and Re-re-secretion of apoA-I in J774.1 and HepG2 Cells: J774.1 (stimulated) and HepG2 cells were radiolabeled by incubation with 4 h at 37^0C with 80 μCi/ml of [^{32}P]-phosphate in phosphate-free DMEM containing 0.12% BSA. Cellular uptake and subsequent re-secretion of apoA-I was monitored by incubating the pre-labeled cells with recombinant apoA-I (50μg/ml) containing a phosphorylation site for PKA. After incubation for 15, 30 and 60 min with J774.1 cells and 30 and 60 min with HepG2 cells, the cell apoA-I was purified from the cell culture media by Ni-affinity chromatography. Proteins were separated by SDS-PAGE on 4-20% gels. **A)** The apoA-I region of the Coomassie Blue-stained gel (upper) and the corresponding autoradiogram (lower) of J774.4 cells. **B**). The apoA-I region of the Coomassie Blue-stained gel (upper) and the corresponding autoradiogram (lower) of HepG2 cells.

Discussion

HDL and apoA-1 stimulate cholesterol efflux from macrophage foam cells and reduce cholesterol accumulation and atherogenesis in mouse models [110-112]. It is very important to understand the apoA-I cholesterol efflux pathways, because of its prime role in the biogenesis of HDL. ABCAI and SR-BI are the two known cell receptor proteins that mediate apoA-I and HDL cholesterol efflux. The lipoproteins and receptors play an important role in cholesterol metabolism in mammalian systems and abnormality in these components are correlated with far-reaching pathologies [31, 105].

As shown previously, our studies also confirmed that apoA-I efficiently efflux cholesterol from macrophages. The apoA-I induced cholesterol efflux is approximately 15% of the total cellular cholesterol. Our previous study has shown that adipose tissue cholesterol efflux to apoA-1 is approximately 1.8% of the total cellular cholesterol. This result illustrates the difference in the characteristic properties of peripheral cells in relation to apolipoprotein induced cholesterol efflux. Considering the total cellular cholesterol present in the adipose tissue, the 1.8% cholesterol efflux is significant. But the difference in the percentage efflux of the total cellular cholesterol is the individual characteristic property of peripheral cells. Also it is confirmed that the pharmacological drugs like BFA, glyburide and BLT-4 have significant effect on the global cholesterol efflux pathways. The inhibition of ABCAI and SR-BI receptors and vesicular transport by the drugs suggested that apoA-I induce cholesterol efflux through multiple pathways in J774.1 cells. These cholesterol efflux pathways could be activated through vesicular transport (with and without the involvement of receptors), ABCAI, SR-BI, ABCGI and other unidentified pathways and receptors.

Several studies reported that the reteroendocytosis of lipoproteins occur in macrophages for cholesterol efflux [27, 36-39]. But the retroendocytosis of lipoproteins in macrophages or other cell lines are under intense debate because of the ambiguous techniques employed to elucidate this process.

To investigate the possibility of up-take and re-secretion of apoA-I in macrophage cells, we used a novel approach in which a full-length recombinant human apoA-I with an N terminal five amino acid recognition sequence (RRASV) for the catalytic subunit of cAMP-dependent protein kinase (PKA) was constructed. Because the protein phosphorylation can only occur intracellularly, the presence of phosphorylated apoA-I in the cell medium clearly indicates that the protein was internalized and subsequently secreted by the cell. The constructed recombinant protein was further tested for their phosphorylation in vitro abilty.

Our studies unambiguously showed that adipose and HepG2 cells uptake and re-secrete lipidated apoA-I in time specific manner. But with the same approach and with two different cAMP stimulating drugs, apoA-I did not undergo retroendocytosis in J774.1 cells. This result is in opposite to the proposed hypothesis of apoA-I retroendocytosis in macrophages published from several labs. The result suggests that cells do not prefer some cholesterol efflux pathways and have specific routes of intracellular lipid traffic to PM for the availability for the external acceptors. It is important to note here that shortly after the first paper on retroendocytosis of HDL, another study observed absence of internalization of (^{125}I) HDL in fibroblast and macrophages[40]. Further the authors suggested that in macrophages, HDL stimulate the transport of lipids from intercellular locations to cell surface, rather than undergoing retroendocytosis [40, 41].

CHAPTER V

Summary

The current studies are important to understand the reverse cholesterol transport (RCT) in vertebrates. Reverse cholesterol transport is the process by which excess unesterified cholesterol is transported from peripheral tissues to the liver for excretion from the body. Several lines of evidence suggest that defects in RCT contribute to the development of atherosclerosis. Adipose tissue constitutes a major site of cholesterol storage and, as such, it may play a role in the regulation of circulating cholesterol levels. The understanding of metabolic link between the lipolytic state (hydrolysis of triacyglycerol) of adipocytes and the mechanism of release of cellular cholesterol to external cholesterol acceptors like apolipoproteinA-I (apoA-I) and high density lipoprotein (HDL) is necessary to elucidate the role of adipose tissue in whole body cholesterol homeostasis.

Our study shows that β-adrenergic activation of the lipolysis significantly increases the extent of cholesterol efflux to reconstituted discoidal HDL particles. The enhancement of cholesterol efflux is not due to the enrichment of plasma membrane cholesterol, or to the levels of the cholesterol transporters ABCAI and SR-BI. The activation of lipolysis is accompanied by an increase in BFA-sensitive vesicular transport that in turn enhances cholesterol efflux to HDL. The study supports a metabolic link between the lipolytic activity of adipocytes and the rate of cellular cholesterol efflux to HDL.

The activation of lipolysis does not induce a significant increase in the rate of cholesterol efflux to lipid free apoA-I. The known vesicular and non-vesicular cholesterol efflux inhibitors inhibit global cholesterol efflux significantly. We have evidently presented that lipid free apoA-I undergo retroendocytosis in mouse adipose tissue, human liver cells but not in human macrophages. The known cholesterol efflux inhibitory drugs had no effect on the uptake and recycling of apoA-I.

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APPENDIX

ABCAI and SR-BI siRNA adenovirus construction and testing

Introduction

Reverse cholesterol transport (RCT) is the process by which excess unesterified cholesterol is transported from peripheral tissues to the liver for excretion from the body [9, 10]. The FC efflux is one of the decisive early steps of the RCT and high-density lipoprotein (HDL) and apoA-I serve as the main acceptors for the cellular cholesterol released from the peripheral cells [11]. The current data indicate that apoA-I interact with ABCAI receptor for the initial lipidation and further through several intermediate steps form the discoidal and mature HDL molecules. The discoidal and mature HDL molecules interact with SR-BI receptor of peripheral cells to acquire excess lipids and further deliver to liver. ABCAI and SR-BI are considered as important receptors in the biogenesis of HDL and whole body cholesterol homeostasis. It is important to study the role of these receptors in different peripheral tissues. In order to quantitatively determine the efflux capacities of ABCAI and SR-BI receptors, we decided to prepare adenoviral siRNA for these receptors. Our objective was to prepare adenovirus containing interfering RNA to silence the ABCAI and SR-BI receptors.

ABCAI and SR-BI siRNA adenovirus construction and testing

Small interference (si) RNA synthesis and transfection: For ABCAI RNA inhibition, siRNA 5'AACAGGTTTGGAGATGGTTAT 3' was designed from the full-length mouse ABCAI sequence corresponding to position 6717-6737 of NM_01345 (Genbank accession number). For SR-BI RNA inhibition, siRNA 5'AAGCAGCAGGTGCTCAAGAAT 3' was designed from the full-length mouse SR-BI sequence corresponding to position 301- 321of NM_016741 (Genbank accession number) respectively. The specificity to the targeted genes were confirmed by searching the targeting segments with NCBI blast.

The sense and antisense DNA oligonucleotides that encode the hairpin siRNA template (55-mer oligonucleotide) were synthesized commercially by Integrated DNA Technologies (Coralville, IA). The oligos were annealed to form the siRNA template were inserted and ligated into the Shuttle Vector 1.0 CMV (pSilencer adeno 1.0 CMV system, Ambion, Catalog # 5790). The Negative Control Shuttle Vector supplied with the kit that contains a siRNA template sequence that lacks significant homology to the mouse, human, and rat genome databases can serve as a negative control.

The linearized Shuttle Vector containing the siRNA template and the linearized Adenoviral LacZ Backbone plasmid were mixed and transfected in HEK-293 (kidney) cells by calcium phosphate method according to the manufacturer's protocol (Ambion, Catalog # 5790). Recombination of the two plasmids within the HEK-293 cells resulted in the production of recombinant adenovirus.

A titration experiment was done to optimize the amount of recombinant adenovirus for the best expression of the hairpin siRNA. HEK 293 cells were infected

with dilutions of the viral stock and after 48 hours, the cells were fixed and stained with the antibody specific for the adenovirus hexon protein. Signal was detected with a secondary antibody conjugated with horseradish peroxidase (HRP) specific to the antihexon antibody. Subsequent treatment of the cells with DAB substrate turned the infected cells to dark brown,which were counted to determine the virus titer. (Adeno-X Rapid Titer Kit, Clontech). Figure 21 shows different dilutions of the HEK 293 cells infected with ABCAI adenovirus.

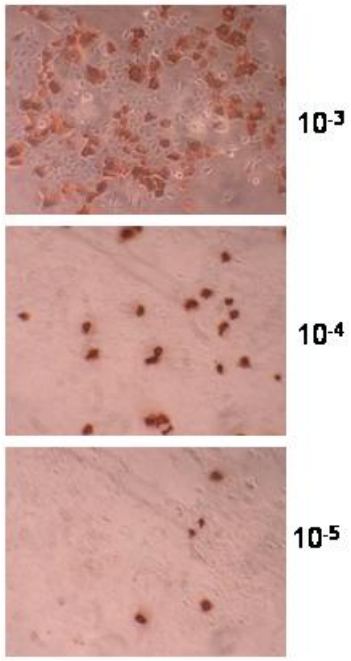

Fig. 21. Dilutions of the HEK 293 cells infected with ABCAI adenovirus as indicated. The photos were taken using a 10X objective.

3T3L-1 and J774.1 cells in 12 well plates were incubated with 10^{-2} dilution of adenovirus containing ABCAI siRNA for 24 h, 48 h and 72 h. After the incubation, cells

were homogenized and aliquots of the homogenate containing 20 µg of protein were mixed with reducing SDS-sample buffer and subjected to SDS-PAGE on 8% acrylamide gels. The proteins were transferred to a nitrocellulose membrane and probed with a rabbit anti-mouse/human ABCAI (1:750 dilutions). The bands were visualized by chemiluminescence. Fig. 22 shows the representative western blot result of J774.1 cell homogenate. Results showed no changes in the level of ABCAI protein under the conditions tested. Further tests are required to optimize the conditions and titer of the virus.

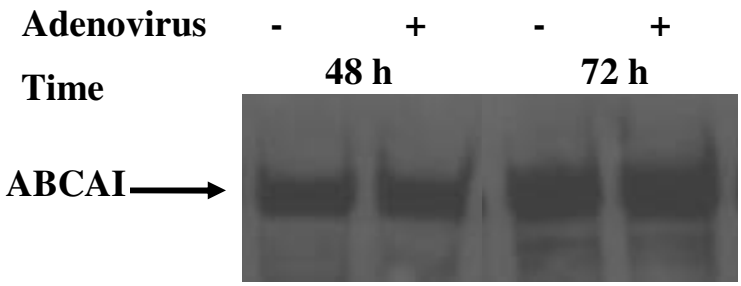

Fig. 22. ABCAI Protein Levels after the Treatment of ABCAI siRNA Containing Adenovirus in J774.1 cells. A) Western blotting of ABCAI performed in homogenate samples (20 µg of protein/sample) after SDS-PAGE.

VITA

Philip Benjamin Verghese

Candidate for the Degree of

Doctor of Philosophy

Thesis: CHOLESTEROL EFFLUX IN ADIPOSE TISSUE

Major Field: Biochemistry & Molecular Biology

Education:
BSc. in Chemistry, University of Pune, Pune, India. 1992-1995

MSc. in Biochemistry, University of Pune, Pune, India. 1995-1997

Graduate Diploma in Business Studies (GDBS), Dublin Business School, Dublin, Ireland. 2000-2001

Completed the requirements for the Doctor of Philosophy in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in December, 2007.

Experience:
Graduate Research Associate, Department of Biochemistry & Molecular Biology, Oklahoma State University, Stillwater, OK. 2001-Present

Research Fellow, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Kerala, India.1997-1998

Professional Memberships:

American Association for the Advancement of Science

Name: Philip Benjamin Verghese Date of Degree: December, 2007

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CHOLESTEROL EFFLUX IN ADIPOSE TISSUE

Pages in Study: 93 Candidate for the Degree of Doctor of Philosophy

Major Field: Biochemistry & Molecular Biology

Scope and Method of Study:
The current studies are important to understand the reverse cholesterol transport (RCT) in vertebrates. Reverse cholesterol transport is the process by which excess unesterified cholesterol is transported from peripheral tissues to the liver for excretion from the body. Several lines of evidence suggest that defects in RCT contribute to the development of atherosclerosis. Adipose tissue constitutes a major site of cholesterol storage and it may play a role in the regulation of circulating cholesterol levels. The understanding of metabolic link between the lipolytic state (hydrolysis of triacyglycerol) of adipocytes and the mechanism of release of cellular cholesterol to external cholesterol acceptors like apolipoproteinA-I (apoA-I) and high density lipoprotein (HDL) is necessary to elucidate the role of adipose tissue in whole body cholesterol homeostasis.

Findings and Conclusions:
Our study shows that β-adrenergic activation of the lipolysis significantly increases the extent of cholesterol efflux to reconstituted discoidal HDL particles. The enhancement of cholesterol efflux is not due to the enrichment of plasma membrane cholesterol, or to the levels of the cholesterol transporters ABCA1 and SR-BI. The activation of lipolysis is accompanied by an increase in BFA-sensitive vesicular transport that in turn enhances cholesterol efflux to HDL. The study supports a metabolic link between the lipolytic activity of adipocytes and the rate of cellular cholesterol efflux to HDL. The activation of lipolysis does not induce a significant increase in the rate of cholesterol efflux to lipid free apoA-I. The known vesicular and non-vesicular cholesterol efflux inhibitors inhibit global cholesterol efflux significantly, but not specific cholesterol efflux to apoA-I. We have evidently presented that lipid free apoA-I undergo retroendocytosis in mouse adipose cells, human liver cells but not in human macrophage cells. The known cholesterol efflux inhibitory drugs had no effect on the uptake and recycling of apoA-I.

ADVISER'S APPROVAL: Jose L. Soulages