EFFECT OF INTRACELLULAR CHOLESTEROL TRAFFICKING ON ITS EFFLUX TO APOA-I

WEN CHI

NATIONAL UNIVERSITY OF SINGAPORE
2005

EFFECT OF INTRACELLULAR CHOLESTEROL TRAFFICKING ON ITS EFFLUX TO APOA-I

BY

WEN CHI
(*B.SC., Wuhan University*)

A THESIS SUBMITTED
FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY
NATIONAL UNIVERSITY OF SINGAPORE
2005

ACKNOWLEDGEMENTS

I would like to express my heartfelt thanks and appreciation to my supervisor, Associate Professor, Li Qiutian, Department of Biochemistry, National University of Singapore, for his keen supervision, valuable suggestion and discussion, patient guidance and encouragement during my study.

I would like to give my special thanks to Ms. Tan Boon Kheng for her wonderful assistance and unfailing help. I would also like to express my appreciation to my friends, Shaoke, Zhili, Miaolv, Qingsong, Weishi, Qiping, Yiliang, Bojun, Minglei, Yushan and Qukun for their help and most of all, their valuable friendship. Some of them gave me the generous support and understanding when I was in the hardest time. They have really made my postgraduate life meaningful and unforgettable. I am also grateful to National University of Singapore for awarding me a research scholarship.

Last but no least, I would like to express my deepest appreciation to my beloved parents, sister and girl friend for their dedicated love, confidence, support, understanding and patience to stand by me throughout all the time we spent together. This thesis is dedicated to them with my deepest love.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS USED IN TEXT

ABC	ATP-binding cassette
ABCA1	ATP-binding cassette transporter A1
ACAT	acyl coenzyme A:cholesterol acyltransferase
Acrylamide	N,N'-mthylenbisacrylamid eectrophoresis prity reagent
ApoA-I	apolipoprotein A-I
APS	ammonium persulfate
BHK	baby hamster kidney
CD	cyclodextrin
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CO_2	carbon dioxide
Cyto. D	cytochalasin D
DMSO	dimethyl sulfoxide
EMEM	Minimum Essential Medium Eagles
ER	endoplasmic reticulum
ERGIC	ER/Golgi intermediate compartment
FC	free cholesterol
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IFN	interferon
Jasp.	jasplakinolide

LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LPDS	lipoprotein deficient serum
LXR	liver X receptor
MβCD	methyl-β-cyclodextrin
MT	microtubule
MTOC	microtubule organizing center
NPC1	Niemann-Pick C1
PBS	phosphate-buffered Saline
PFA	paraformaldehyde
PL	phospholipids
PPAR	peroxisome proliferator-activated receptor
PVDF	polyvinglidene fluoride
RCT	reverse cholesterol transport
RXR	retinoic acid receptor
SDS	sodium dodecyl sulfate
SR-BI	scavenger receptor class-B type I
SREBP	sterol regulatory element-binding protein
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TLC	thin layer chromatography
U18666A	3β-[2-(diethylamino) ethoxy] androst-5-en-17-one

SUMMARY

High density lipoprotein (HDL) or its apolipoproteins remove excess free cholesterol from cells to maintain cellular cholesterol homeostasis. This process, which is defined as reverse cholesterol transport (RCT), prevents the excessive cholesterol accumulating on the vessel wall and the development of atherosclerosis which causes significant morbidity and mortality in the developed societies.

In this study, apolipoprotein A-I (apoA-I) and methyl-β-cyclodextrin (MβCD) were used as cholesterol acceptors to investigate the mechanism of intracellular cholesterol trafficking and its effect on cholesterol efflux in human fibroblast. ApoA-I is the main protein of HDL that plays a key role in cholesterol efflux in vivo. It has been pointed out that apoA-I could bind directly to the exofacial face of the caveolae to facilitate FC desorption (Saito et al., 1997) and stimulate the translocation of intracellular cholesterol to the plasma membrane (Oram and Yokoyama, 1996) and subsequent enhancement of the efflux of intracellular cholesterol (Sviridov and Fidge, 1995). On the other hand, MβCD is a non-specific acceptor for cholesterol. It gets cholesterol from both caveolae and non-caveolae membrane domains. The results obtained in this study confirm that the kinetics of cholesterol effluxes to apoA-I or MβCD were clearly different in most cases, probably due to the fact that apoA-I and MβCD take cholesterol from different cholesterol pools. Here, the cholesterol was from two different cholesterol pools: plasma membrane derived cholesterol, which was directly labeled with ^{3}H-cholesterol, and de novo synthesized cholesterol labeled by using ^{3}H-acetate as the precursor.

Before cholesterol efflux, the cells were treated with different drugs which would affect the microtubules, the actin filaments, the Golgi apparatus and the ER, respectively, to examine if these subcellular organelles are involved in cholesterol trafficking and efflux. From the results of this study, it is known that caveolae is the key regulator of intracellular cholesterol trafficking and efflux. Disassembly of caveolae by cholesterol depletion markedly increased the cholesterol efflux to apoA-I. Disruption of actin microfilaments which are necessary for caveolae integrity also significantly enhanced cholesterol efflux. This result is a further support for the conclusion that caveolae are very important for cholesterol trafficking and efflux. One of the other conclusions can be drown through this study is that Golgi apparatus appears to play a minor role in the movement of nascent cholesterol from ER to plasma membrane. It seems that microtubules, U18666A-inhibited cholesterol intracellular trafficking and ER-plasma membrane contacts did not affect cholesterol efflux at any significant level. However, more investigations are needed to verify these observations.

CHAPTER 1. INTRODUCTION

1.1 Cellular cholesterol homeostasis and atherosclerosis

1.1.1. Cellular cholesterol homeostasis

Cholesterol is an essential component of cell-surface membranes. It functions to maintain the fluidity of cell membranes which separate the cell from its extracellular environment. It also provides the material for synthesis of bile acids and steroid hormones. At the cellular level, cholesterol homeostasis is maintained by regulated cholesterol uptake, de novo synthesis, intracellular transport and efflux.

1.1.1.1. Cholesterol uptake

Extrahepatic cells obtain cholesterol by endogenous synthesis and from circulating low density lipoprotein (LDL) particles, which are taken up via specific cell-surface receptors. Brown and Goldstein first demonstrated the presence of high-affinity LDL-binding sites on the surface of normal cells (Brown and Goldstein, 1986). LDL binds to LDL-receptors that cluster in clathrin-coated pits, specialized invaginations in the cell-surface, followed by formation of clathrin-coated vesicles, which subsequently become uncoated. Thereafter, a complex vesicular pathway selectively sorts both proteins and lipids that enter the lysosomes for subsequent metabolism, releasing unesterified cholesterol to other

intracellular sites and plasma membrane (Fielding and Fielding, 1997). The number of LDL-receptors expressed on the cell-surface is controlled by negative-feedback regulation involving the cells' demand for cholesterol and membrane-bound transcription factors termed as sterol regulatory element-binding proteins (SREBPs) (Horton et al., 2002). When the concentration of cholesterol in the cell rises or demands for cholesterol are low, transcription of the LDL-receptor is suppressed (Brown and Goldstein, 1986; Horton et al., 2002); this slows down plasma LDL clearance and, consequently, the accumulating particles tend to undergo oxidative damage by free radicals. In contrast, when cellular cholesterol levels fall or demands for cholesterol are high, gene transcription is induced to enhance expression of LDL receptors and LDL clearance. These regulatory mechanisms serve to maintain a constant level of unesterified cholesterol, despite fluctuations in cellular requirements and exogenous supplies.

The cell surface scavenger receptor class-B type I (SR-BI) functions as a HDL receptor that mediates nonendocytic, selective uptake of cholesterol. Unlike the classical LDL receptor pathway, in which the entire lipoprotein is internalized in clathrin-coated pits and degraded (Brown and Goldstein, 1986), HDL binds to SR-BI and the core cholesteryl ester (CE) is delivered to cross the plasma membrane without endocytosis and degradation of the entire HDL particle (Pittman et al., 1987). This two-step process is termed as 'selective-uptake pathway', with the first step being lipoprotein binding to the extracellular domain of SR-BI clustered in caveolae and the second step the transfer of lipids from HDL particle to cross the plasma membrane (Krieger, 1999).

1.1.1.2. Cholesterol synthesis

Cholesterol synthesis is a complex biosynthetic process which begins with acetyl-CoA and involves more than 27 enzymes, many of which are located in the endoplasmic reticulum (ER) (Urbani and Simoni, 1990). The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase catalyzes the rate-limiting reaction in cholesterol biosynthesis pathway (Brown and Goldstein, 1980). LDL-derived cholesterol suppresses intracellular cholesterol synthesis by depressing the activity of HMG-CoA reductase.

1.1.1.3. Intracellular cholesterol distribution

The correct cholesterol intracellular distribution is essential for many biological functions of mammalian cells. In the biosynthetic secretory pathway, cholesterol concentration is lowest in the ER. It increases through the Golgi apparatus, with the highest concentration in the plasma membrane (Liscum and Munn, 1999).

Although cholesterol is synthesized in ER, cholesterol concentration in the ER is very low, comprising only 0.5-1% of total cellular cholesterol (Lange et al., 1999). However, cholesterol concentration in the ER membrane is crucial for cellular cholesterol homeostasis because many aspects of cholesterol regulation are under tight feedback control and are sensitive to the cholesterol concentration in the ER.

The cholesterol content of the Golgi apparatus is intermediate between those of the ER and the plasma membrane (Mukherjee et al., 1998). It has been proposed that rafts rich in

cholesterol form in the Golgi apparatus and are selectively transported to the periphery from the *trans*-Golgi (Simons and Ikonen, 1997; Ikonen, 2001).

In mammalian cells, the plasma membrane normally contains majority of cellular free cholesterol (FC) (Liscum and Munn, 1999). In several extrahepatic cell lines, including fibroblasts, only small part of plasma membrane cholesterol localized in exofacial leaflet of the membrane (Fielding and Fielding, 1997). FC first transported to caveolae is subsequently distributed to other plasma membrane domains, or released to extracellular acceptors (Fielding and Fielding, 1996; Smart et al., 1996; Uittenbogaard et al., 1998). FC in excess may be esterified by acyl coenzyme A:cholesterol acyltransferase (ACAT) and stored as CE droplets within the cytoplasm (Chang et al., 1997).

Intracellular cholesterol trafficking is responsible to maintain the proper cellular cholesterol distribution and cholesterol efflux.

1.1.1.4. Cholesterol efflux

The removal of excess FC from cells by HDL or its apolipoproteins is important for maintaining cellular cholesterol homeostasis. Multiple mechanisms for cellular cholesterol efflux exist. Efflux of FC via aqueous diffusion occurs within all cell types but is inefficient (Phillips et al., 1987). Efflux of cholesterol is enhanced when SR-BI is present in the cell plasma membrane (Jig et al., 1997). Both diffusion-mediated and SR-BI–mediated effluxes occur to phospholipid-containing acceptors (ie, HDL and lipidated

apolipoproteins); in both cases, the flux of cholesterol is bidirectional, with the direction of net flux depending on the cholesterol gradient. The ATP-binding cassette transporter A1 (ABCA1) mediates efflux of both cellular cholesterol and phospholipid. In contrast to SR-BI–mediated flux, efflux via ABCA1 is unidirectional, occurring to lipid-poor apolipoproteins. The details of these three mechanisms will be discussed further in section 1.3.

1.1.2. Atherosclerosis

Atherosclerosis causes significant morbidity and mortality in the developed societies. The magnitude of this problem is profound, as atherosclerosis claims more lives than all types of cancer combined. Although currently it is a problem of the developed world, the World Health Organization predicts that global economic development could lead to an epidemic of atherosclerosis in the developing countries that acquire Western life style. Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in large- and medium-size arteries. Macrophages internalize modified lipoproteins and cell debris and this process is not regulated by cholesterol. In contrast to other cells, macrophages accumulate lipid in cell bodies. Released from lysosomes, FC is transferred to the ER either directly or indirectly via the plasma membrane. In the ER, cholesterol is esterified by ACAT to protect the cell from the cytotoxicity of excess unesterified cholesterol (Chang, 1997). CEs have lower water solubility than cholesterol and appear as cytosolic lipid droplets. The lipid laden macrophages have foamy shape and are termed as foam cells which gradually impinge on the vessel lumen and hinder

blood flow. This process can last for decades until an atherosclerotic lesion, leading to thrombosis and compromised oxygen supply to target organs such as the heart and brain. The loss of heart and brain function as a result of reduced blood flow is termed as heart attack and stroke, respectively (Stocker and Keaney, 2004).

1.2. Reverse cholesterol transport (RCT): Function of HDL

RCT originally discovered by Glomset (Glomset, 1968) is a pathway transporting excess cholesterol from extrahepatic cells and tissues to the liver for the synthesis of bile acids and subsequent excretion from the body. By reducing the accumulation of cholesterol in the wall of arteries, RCT may prevent development of atherosclerosis. Approximately 10 mg cholesterol per kilogram body weight is synthesized by extrahepatic tissues every day and must be transferred to the liver for effective catabolism (Dietschy et al., 1993). HDL particles are thought to play the key role in this protective system against atherosclerosis.

The process of RCT is complex. Lipid-poor apoA-I mediates cholesterol efflux from cells. Cholesterol is converted to CE by enzyme lecithin-cholesterol acyltransferase (LCAT) within HDL. CEs are transferred from HDL to apolipoprotein B–containing lipoproteins by cholesteryl ester transfer protein (CETP). Then HDL CEs are used for bile acids biosynthesis in the liver (Daniel, 2003). Preβ_1-HDL, a discoid lipid-poor particle, is the initial acceptor of cellular cholesterol (Sviridov and Nestel, 2002). It may be originated mainly from the surface components of hydrolyzed triglyceride-rich lipoproteins in the

blood stream. Accumulation of cholesterol into the particles transforms the pre β_1-HDL into larger spherical lipoprotein particles, namely, pre β_2-HDL, which is the substrate for LCAT. FC on the surface of pre β_2-HDL is converted to more lipophilic CEs by LCAT, leading to an expansion of particles into spherical shape. These particles acquire more cholesterol from preβ-HDL (Sasahara et al., 1998) and are transformed into larger α_2-HDL and α_1-HDL. The HDL particles finally transfer cholesterol to the liver by at least two distinct processes: (i) HDL particles dock to the SR-BI receptors which express on the cell membrane of the hepatocyte; (ii) HDL particles exchange CEs for triglyceride from remnant particles and LDL, a process driven by the action of CETP. Finally, triglyceride and phospholipids are hydrolyzed by hepatic lipase. Particles are remodeled into smaller α_3-HDL particles and lipid-free apoA-I, which in turn are rapidly re-lipidated by cellular cholesterol and phospholipids to form new preβ_1-HDL. As discussed above, HDL cholesterol is finally delivered to the liver. Hepatic cholesterol can then be excreted from the body either as FC directly or after conversion to bile acids. The liver is the only organ which can substantially influence net excretion of cholesterol from the body and cholesterol delivered by lipoproteins is a primary source of substrates for biliary lipid secretion as bile acids and cholesterol (Angelin, 1995).

An inverse relationship between HDL levels and the incidence of atherosclerotic coronary artery disease has been supported by numerous epidemiological studies. It seems that HDL cholesterol is associated with protection against coronary artery disease because HDL levels indicate the efficiency of RCT. Many of the factors that increase HDL level are antiatherogenic, due to the complex interrelationships of HDL and

triglycerides-rich lipoprotein metabolism and other metabolic pathways. Each interposition influencing HDL will have to be prospectively evaluated (Tall, 1990).

1.3. Pathways mediating cellular cholesterol efflux

Cholesterol efflux, in which excess cellular FC is released from cells to HDL particles, is the first step of RCT. It is a complex process and multiple mechanisms exist, depending on the particular cell type and its metabolic state, different membrane cholesterol pools and the nature of the acceptor particles. . There are 3 known mechanisms of FC efflux: (1) aqueous diffusion, (2) SR-BI-mediated FC efflux, and (3) ABCA1-mediated efflux. Each mechanism and its relative importance will be discussed briefly here (Yancey et al., 2003).

1.3.1. Aqueous diffusion

The simplest and most basic mechanism for cellular cholesterol efflux is aqueous diffusion in which individual cholesterol molecules desorb from the plasma membrane, diffuse through the aqueous phase and are captured by phospholipids containing acceptor particles. This process is passive and driven by the cholesterol concentration gradient (Johnson et al., 1991).

The FC transfer rate from cells to medium is influenced by both cell and acceptor properties. At low acceptor concentrations, the FC transfer rate is dependent on the

frequency of diffusion mediated collisions between cholesterol molecules and acceptor particles. At high acceptor particle concentrations, the desorption of cholesterol molecules from the surface of cells becomes the rate-limiting step; there is a high activation energy acquired for this step, and cholesterol transfer rates are strongly temperature-dependent (Phillips et al., 1987).

The rate of FC efflux via aqueous diffusion is highly dependent on the structure of the acceptor particle. The size of the acceptor particle is important because it affects the diffusion-mediated collisions with cholesterol molecules on the cell surface. Large particles are inefficient acceptors due to the limited access to the cell surface (Rothblat et al., 1999). If the distance between the FC donor and acceptor is very small, the time required for diffusion between them will decrease obviously (Fielding and Fielding, 2001). In contrast, two other factors will slow the simple diffusion of FC from cell surface. Majority of FC in the plasma membrane is in the cytofacial leaflet of the bilayer (Fielding and Fielding, 1997). The rate of cholesterol exchange between the cell membrane leaflets is relatively slow compared to FC efflux (Raggers et al., 2000). As a result, in the absence of other factors, only the exofacial pool of plasma membrane FC (typically 3-5% of total FC) will contribute directly to simple diffusion. Secondly, the cell surface is bounded by an ‘unstirred water layer’ which forms a significant diffusion barrier. The extent of ‘unstirred water layer’ is inversely proportional to the solute diffusion coefficient (Pohl et al., 1998).

1.3.2. SR-BI mediated cholesterol efflux

Early studies showed that different kinds of cells exhibit significantly different cholesterol efflux rates to phospholipids containing acceptors (Rothblat and Phillips, 1982) due to several possible reasons, such as variability in the fluidity or cholesterol content of the plasma membrane, in the thickness or composition of the extracellular matrix, which might affect the access of cholesterol acceptors to the plasma membrane, or in different expression levels of lipoprotein receptor in the plasma membranes of different cell types (Rothblat et al., 1999). Subsequent studies have shown that these differences are attributable to different expression levels of SR-BI (Ji et al., 1997; Jian et al., 1998). This conclusion is supported by the experiment that efflux is accelerated from COS-7 cells transiently transfected with SR-BI compared with efflux from control COS-7 cells (de la Llera-Moya et al., 1999).

Besides stimulating the efflux of FC, expression of SR-BI also drives the influx of FC. Thus, movement of FC via SR-BI is bidirectional and the net movement of FC via SR-BI depends on a preexisting cholesterol concentration gradient (Kellner-Weibel et al., 2000; de la Llera-Moya et al., 2001).

The detailed mechanism by which SR-BI facilitates the bidirectional flux of FC is not very clear. Although not proven, it is often assumed that SR-BI facilitates the diffusion mechanism of FC flux. Binding of the acceptor particles in close apposition to SR-BI could possibly enhance the aqueous diffusion by concentrating the acceptor particles at the cell surface. However, it has been proven that high-affinity binding to cell surface receptors alone is not sufficient to stimulate the efflux of FC, because the expression of

CD36 in COS-7 cells markedly enhances the high-affinity binding of HDL but does not increase efflux. The data indicate that SR-BI changes the plasma membrane cholesterol organization and then enhances the bi-directional cholesterol flux between cells and extracellular acceptors (de la Llera-Moya et al., 1999). The presence of SR-BI on the plasma membrane creates an environment whereby the rate of exchange of FC molecules is increased. In such a situation the net movement of FC between cell surface and acceptor articles is not influenced by SR-BI. Instead, the net movement of FC is decided by the FC gradient that exists between the acceptors and the cell surface. It is likely that the SR-BI-induced changes in plasma membrane organization involve caveolae and/or lipid rafts (Rothblat et al., 1999) because in some cell types, SR-BI is localized to caveolae and lipid rafts, areas of the membrane that are rich in both cholesterol and sphingolipids (Babitt et al., 1997; Graf et al., 1999).

1.3.3. ABCA1 mediated cholesterol efflux

1.3.3.1. Discovery of ABCA1

The discovery of ABCA1 came from the study of the patients with Tangier disease, a rare recessive disorder. These patients present with very low levels of lipid-free apoA-I and HDL, accumulation of CE in macrophage-rich tissues and large orange tonsils (Fredrickson, 1964). In the cholesterol enriched fibroblasts and macrophages from patients with Tangier disease, efflux of cholesterol and phospholipid to lipid-free apoA-I is markedly reduced but efflux to HDL as acceptors is normal (Francis et al., 1995). Since

apoA-I is unable to sequester cholesterol and phospholipid to generate discoidal pre-β-HDL, the apoA-I protein is rapidly degraded (Knight, 2004).

In 1999, several groups using different strategies identified ABCA1 as the defective gene in Tangier disease patients, and proposed that the protein controls the transfer of both cholesterol and phospholipid to apoA-I, the initial step in HDL synthesis (Brooks-Wilson et al., 1999; Bodzioch et al., 1999; Rust et al., 1999).

ABCA1 belongs to the ATP-binding cassette (ABC) family of genes encoding transmembrane proteins which have common structural characters. Each member of the ABC family is believed to transport a specific set of molecules (e.g. ions, amino acids, proteins, sugars, phospholipids and a range of drugs) across the plasma membrane, as well as intracellular membranes of the ER and mitochondria using ATP (Dean et al., 2001). Each transporter contains either one or two copies of structural elements: a hydrophobic region of six transmembrane domains and a hydrophilic cytosolic ATP-binding cassette, comprising two conserved peptide motifs (Walker A and Walker B motifs) and a unique amino acid signature sequence between each Walker motif (Walker et al., 1982). ABCA1 contains two of these units, covalently linked by a highly hydrophobic segment, which is assumably an essential element for the translocation of lipids.

1.3.3.2. Mechanism of ABCA1 meditaed cholesterol efflux: ApoA-I – ABCA1 interactions

In contrast to aqueous diffusion and SR-BI–mediated FC flux, the cholesterol efflux mediated by ABCA1 is unidirectional and net efflux of cellular FC would always occur via this mechanism independent of the cholesterol gradient. The preferred cholesterol acceptor for ABCA1 is lipid-poor apolipoproteins especially apoA-I. There has been considerable controversy over the mechanism of action of ABCA1, particularly in relation to the binding of acceptor molecules.

ABCA1-mediated lipid (cholesterol and phospholipid) efflux requires an acceptor apolipoprotein containing an amphipathic helix, such as apoA-I, apoA-II or apoE. It is also known that ABCA1 activity induces the formation of novel structures that protrude from the plasma membrane and bind apolipoproteins (Lin and Oram, 2000). Probably the phosphatidylserine exofacial flopping generates a biophysical microenvironment required for the docking of apoA-I at the cell surface. This has led to the hypothesis that apoA-I binds to a region of the membrane modified by ABCA1, a conclusion supported by the different lateral mobilities of ABCA1 and apoA-I on the cell surface (Chambenoit et al., 2001). Subsequent study pointed out that a cytotoxic pool of FC, which is located in the plasma membrane, is readily available for efflux to apoA-I, and ABCA1 may be involved in the removal of cytotoxic cholesterol (Kellner-Weibel et al., 2003). On the other hand, chemical cross-linking and immunoprecipitation analysis showed that apoA-I binds directly to ABCA1 (Wang et al., 2000), and natural mutations in the extracellular loops of ABCA1 extinguish cholesterol efflux and direct interaction of ABCA1 and apoA-I. Furthermore, a specific mutation in the first loop of ABCA1 (W590S) reduced cholesterol efflux but not cross-linking activity, indicating that acceptor binding is

necessary, but not sufficient, for cholesterol efflux (Fitzgerald et al., 2002). Recently, a novel, highly conserved motif (VFVNFA) of the ABCA1 C terminus was identified. This conserved motif was required for lipid efflux and alteration of this motif eliminated its binding of apoA-I (Michael et al., 2004).

Since HDL cholesterol and phospholipid levels are very low in plasma from Tangier disease patients and ABCA1 is identified as the defective gene in those patients, it was initially proposed that ABCA1 transport both these lipids across the plasma membrane directly. Upon ATP-binding and hydrolysis, cholesterol and phospholipids were rapidly flipped from the inner to the outer leaflet of the membrane bilayer, to be sequestered by lipid-poor apoA-I.

However, later study suggests that cholesterol is not the substrate for ABCA1 and is effluxed from cells via a two-step mechanism. When FC efflux from cells expressing high levels of ABCA1 was inhibited, phospholipid efflux to apoA-I still occurred. Moreover, when this conditioned media containing phospholipids-apoA-I complexes was transferred to ABCA1-deficient cells, it stimulated efflux of FC, but not phospholipids (Fielding et al., 2000). Other researchers showed that ABCA1 did not bind cholesterol directly and apoA-I binding to ABCA1 was closely associated with phospholipid translocation, but not FC efflux (Wang et al., 2001). These experiments indicate a two-step mechanism for FC efflux. Firstly, ABCA1 actively transfers phospholipids (PLs) across the membrane to lipid-poor apoA-I; this generates discoidal phospholipid-apoA-I complexes, which can acquire FC subsequently. So ABCA1 is believed to function

indirectly as a cholesterol efflux regulatory protein to promote preβ1-HDL formation (Owen and Mulcahy, 2002). In recent study, the author pointed out that apoA-I binds to ABCA1 which induces the formation of the perturbed PL bilayer by its PL transport activity in the first step. The hydrophobic α-helices in the C-terminal domain of apoA-I insert into the region of the perturbed PL bilayer and induce the second step of lipidation of apoA-I and formation of nascent HDL particles (Vedhachalam et al., 2004).

On the other hand, another hypothesis has been proposed to explain the mechanism through which ABCA1 plays a role in cholesterol efflux. In the mechanism named membrane solubilization phospholipids and cholesterol are mobilized simultaneously in whatever proportions they are present by lipid free apoA-I as discrete units (Gillotte et al., 1998; 1999). But the precise mechanism has not been elucidated.

1.3.3.3. Regulation of ABCA1 expression

Transcription of the ABCA1 gene and cell-surface expression of ABCA1 protein are tightly regulated by many metabolites, including sterols, cAMP, *cis*-retinoic acid, peroxisome proliferator-activated receptor (PPAR) agonists, and interferon γ (IFN- γ).

Cholesterol loading is known to increase ABCA1 mRNA and protein level. This increase is reversed when cellular cholesterol is removed by incubation with HDL (Langmann et al., 1999). This effect is thought to be mediated by the stimulation of liver X receptor (LXR) nuclear hormone receptor that is activated by oxysterol ligands. Physiological LXR ligand 22-(*R*)-hydroxycholesterol and LXR selective agonist TO-901317 increase

ABCA1 mRNA level by more than 3-fold. This enhancement is absent in peritoneal macrophages isolated from LXRα and LXRβ knockout mice (Repa et al., 2000).

Previous study illustrated that RAW264 macrophages treated with 8-bromo-cAMP showed parallel increases in ABCA1 mRNA and protein levels, incorporation of ABCA1 into the plasma membrane, binding of apoA-I to cell surface ABCA1 and apoA-I-mediated cholesterol efflux (Oram et al., 2000). However, the regulatory motif in the human promoter that binds cAMP and activates the ABCA1 gene has yet to be identified. In normal macrophages, PPARα and PPARγ agonists increase ABCA1 mRNA expression and apoA-I mediated cholesterol efflux, whereas no effects are observed in macrophages from patients with Tangier disease (Chinetti et al., 2001). LXRα mRNA was induced also by these agonists. Furthermore, the addition of both PPAR and LXRα activators had an additive effect on induction of ABCA1 expression. However, no functional PPAR response element has been identified in the ABCA1 promoter. It appears that PPAR agonists may indirectly modulate ABCA1 gene expression by activation of the LXRα pathway and illustrate a complex interaction between PPARα, PPARγ and LXRα in the cellular regulation of ABCA1 gene expression.

In contrast, IFNγ reduces ABCA1 expression, thereby reducing apoA-I-mediated cholesterol and phospholipid efflux in mouse peritoneal macrophages and foam cells (Panousis and Zuckerman, 2000). This suggests that by decreasing cellular cholesterol efflux through pathways that include up-regulation of ACAT and down-regulation of

ABCA1, IFNγ may facilitate the conversion of macrophages to foam cells, promoting the progression of atherosclerosis.

1.4. Intracellular cholesterol trafficking and cholesterol efflux

1.4.1. From lysosome to plasma membrane and the cell interior

An important cholesterol source is LDL which is internalized and delivered to lysosomes. CEs are carried largely in the core compartment of LDL particles, after its hydrolysis, FC is rapidly released from lysosomes and appears in the plasma membrane (Brasaemle and Attie 1990; Johnson et al., 1990). LDL-cholesterol transport from lysosomes to plasma membrane is inhibited by U18666A but not affected by agents that disrupt the cytoskeleton (Liscum, 1990). The Niemann-Pick C1 (NPC1) protein is the key regulator responsible for exit of LDL cholesterol from lysosomes. Cells with defective NPC1 accumulate unesterified cholesterol in lamellar bodies derived from lysosomes and exhibit markedly impaired rates of esterification of LDL cholesterol (Pentchev et al., 1994). NPC cells are defective in the delivery of lysosomal cholesterol to the plasma membrane (Neufeld et al., 1996). Consistent with the findings, NPC1 overexpression increases the rate of delivery of endosomal cholesterol to the plasma membrane, providing further support for the role of NPC1 in this trafficking pathway (Millard et al., 2000). However, the details about function of NPC1 have not yet been pinpointed. The

NPC1 protein may function in cholesterol modulated late endocytic vesicular transport (Blanchette-Mackie, 2000).

LDL-cholesterol is not only mobilized to the plasma membrane but is also transported to the ER, where cholesterol may become esterified by ACAT (Liscum and Munn, 1999). This process is inhibited by hydrophobic amines, such as U18666A (Liscum and Faust, 1989), imipramine (Rodriguez-Lafrasse et al., 1990) and progesterone (Butler et al., 1992). This latter pathway is energy dependent (Skiba et al., 1996) but plasma membrane independent (Underwood et al., 1998).

.

1.4.2. From ER to plasma membrane

The nascent cholesterol synthesized in ER is transferred to plasma membrane rapidly (half time of ~10 min) (DeGrella and Simoni, 1982; Kaplan and Simoni, 1985). The transport is ATP-dependent and inhibited at 15°C. Treatment with brefeldin A, which disrupts Golgi apparatus, does not affect nascent cholesterol transport from ER to plasma membrane (Urbani and Simoni, 1990). This raises the possibility that nascent cholesterol is transported to plasma membrane via a pathway bypass Golgi apparatus. When cholesterol arrives in the plasma membrane, it is found first in the caveolae. Most of the cholesterol is transported out of the membrane if native plasma is present (Fielding and Fielding, 1995). In the absence of extracelluar lipoproteins, cholesterol migrates to the surrounding non-caveolae membrane (Smart et al., 1996).

1.4.3. From plasma membrane to cell interior

Plasma membrane cholesterol is constitutively transported into the cell interior and returned to the cell surface rapidly (Lange et al., 1993). This movement of plasma membrane cholesterol to cell interior is inhibited by different reagents. Some of these, such as hydrophobic amines U18666A (Härmälä et al., 1994), sphingosine (Härmälä et al., 1993) and progesterone (Lange, 1994), inhibit several intracellular cholesterol transport pathways. Some drugs which disrupt cellular cytoskeleton also inhibit cholesterol from plasma membrane to ER. This indicates that intact intermediate filament is important to this pathway (Evans, 1994). These results induce the speculation that the movement of plasma membrane cholesterol to cell interior is mediated by vesicular transport. However, such intermediate has not been fully identified (Liscum and Munn, 1999).

1.4.4. Function of `caveolae

Caveolae are FC-rich, clathrin-free plasma membrane invaginations with a characteristic diameter of 50 to 100 nm (Smart et al., 1999). The core membrane structure of caveolae is enriched in cholesterol, gangliosides, ceramide, diacylglycerol (Liu and Anderson, 1995), phosphatidylinositol diphosphate (Pike and Casey, 1996) and the integral membrane protein caveolin which is a 22-kDa cholesterol binding protein (Murata et al., 1995). The involvement of caveolin in cholesterol transport is consistent with several previous observations. (i) The nascent cholesterol synthesized in ER first appears in the

caveolae domain of the plasma membrane (Smart et al., 1996). (ii) Progesterone, which blocks cholesterol transport, causes caveolin accumulation in the lumen of the ER (Smart et al., 1996). (iii) Oxidase treatment of caveolae cholesterol causes caveolin to dissociate from plasma membrane and redistribute to intracellular vesicles that co-localize with Golgi apparatus markers (Smart et al., 1994). (iv) The caveolin mRNA levels and caveolin expression are very sensitive to the FC content of the cell. An increase in LDL–FC internalization was associated with proportional cellular FC and upregulation of caveolin (Fielding et al., 1997). On the other hand, cholesterol efflux from HepG2/cav cells, which are transfected with human caveolin-1 and then express caveolin-1 mRNA, a high abundance of caveolin-1 protein, and the formation of caveolae on the plasma membrane, is 45% higher than that from parent HepG2 cells when human apoA-I was used as acceptor (Fu et al., 2004).

Depletion of caveolar FC with cyclodextrin (CD) led to down-regulation of caveolin mRNA and cell surface protein levels (Hailstones et al., 1998). There is evidence suggesting that caveolae efflux cellular cholesterol to HDLs, the principal acceptor of cellular cholesterol in the RCT pathway (Fielding and Fielding**,** 1995). More recently, HDL and caveolin-1 were proved co-localized in caveolae by immunoelectron microscopy in endothelial cells loaded with cholesterol (Chao et al., 2003). However, the direct evidence about this process is absent and the data available remain controversial, partly due to the different cell types used and the different analytical methods adopted.

1.4.5. Function of Golgi apparatus

It is now recognized that Golgi apparatus plays a key role in cholesterol sorting and trafficking. The first noticeable evidence for Golgi involvement in cholesterol movement was shown by freeze-fracture electron microscopy using filipin as a probe for FC (Coxey et al., 1993). After addition of LDL to cultured fibroblasts for 24 h, cholesterol is enriched within specific compartments of the Golgi. This cholesterol enrichment in Golgi apparatus was seen even in NPC fibroblasts. It was suggested that the Golgi was involved in LDL cholesterol transport from lysosomes to the plasma membrane. Treatment of cells with brefeldin A which can disrupt Golgi apparatus resulted in enhanced cholesterol delivery to ACAT. This could be due to LDL-cholesterol destined for the plasma membrane being redistributed to ER by blocking the Golgi dependent pathway (Neufeld et al., 1996). Subsequent studies confirmed the finding that an intact Golgi apparatus is not necessary for the flow of LDL-cholesterol to the ER. The LDL-cholesterol movement to ACAT is normal even when the Golgi apparatus is severely disrupted by brefeldin A (Underwood et al., 1998). It is still unclear whether the Golgi apparatus is involved in the plasma membrane-independent route of LDL-cholesterol transport to the ER.

There were results suggesting that newly synthesized cholesterol was transported from ER to plasma membrane via a vesicular system (Kaplan and Simoni, 1985). However, an efficient alternate pathway for nascent cholesterol movement was proposed when severe disruption of Golgi apparatus did not alter the kinetics of cholesterol arrival at the plasma membrane (Urbani and Simoni, 1990).

1.5. Aims of this project

Cholesterol is an essential component of cellular membranes. Cholesterol homeostasis is maintained by regulating cholesterol uptake and de novo synthesis, intracellular transport and efflux. Many diseases are related to defective cholesterol metabolism such as coronary heart disease, Tangier disease and Alzheimer's disease. The mechanisms involved in maintaining cholesterol homeostasis are very complicated and not clear yet. The aim of this study is to investigate the effect of intracellular cholesterol trafficking on cholesterol efflux in human fibroblast. I labeled cellular cholesterol through two different ways, directly incubating cells with ^{3}H-cholesterol or incubating cells with ^{3}H-acetate which will be converted to ^{3}H-cholesterol via do novo synthesis route. The labeled cells were treated with different drugs which would affect microtubule, actin network, Golgi apparatus or ER to test if these factors contribute to cholesterol trafficking and efflux. Cholesterol efflux was performed using lipid free apolipoprotein A-I or methyl-β-cyclodextrin (MβCD) as acceptors. Apolipoprotein A-I is the major protein component of HDL, while MβCD is one of the simplest and commonly used extracellular cholesterol acceptors. It contains cyclic oligosaccharides that are believed to be able to dissolve lipids in their hydrophobic core. The significance of ABCA1 expression and intracellular cholesterol trafficking in cholesterol efflux is then discussed.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The following reagent was purchased from Ajax Chemicals Pty Limited (9 short street, Auburn, N.S.W. 2144, Australia)

Anhydrous Diethyl Ether

The following reagent was purchased from Amersham (Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England)

BCS Scintillation Cocktail

The following reagents were purchased from BioRad (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California 94547)

Rabbit Anti-Goat IgG (H+L)-HRP Conjugated, Goat Anti-Mouse IgG (H+L)-HRP Conjugated, Prestained Broad Range Precision Protein^{TM} Standards, Ammonium Persulfate, Acrylamide (N,N'-Methylenbisacrylamid Electrophoresis Purity reagent)/Bis Solution, Electrophoresis Purity Reagent Bromophenol Blue, RC DC Protein Assay Kit (including DC Protein Assay Reagent A, DC Protein Assay Reagent B, DC Protein Assay Reagent S, RC Protein Assay Reagent I, RC Protein Assay Reagent II)

The following reagent was purchased from Calbiochem (EMD Biosciences, Inc., 10394 Pacific Center Court, San Diego, CA 92121)

Fluor save™ Reagent

The following reagent was purchased from Cambrex (Cambrex Corporation, One Meadowlands Plaza, East Rutherford, New Jersey 07073)

EMEM (Minimum Essential Medium Eagles)

The following reagent was purchased from Chemicon International (28820 Single Oak Drive, Temecula, CA 92590)

Re-Blot Plus Strong Solution

The following reagent was purchased from Duchefa Biochemie (A. Hofmanweg 71, 2031 BH Haarlem, the Nethelands)

polysorbate 20 (TWEEN 20)

The following reagent was purchased from Epicentre (726 Post Road, Madison, WI 53713 USA)

Brefeldin A

The following reagent was purchased from Hayman Limited (Eastways Park, Witham, Essex, CM8 3YE England)

Ethyl Alcohol A.R. Quality

The following reagent was purchased from HyClone (Hyclone, 925 West 1800 South, Logan, UT 84321)

Fetal bovine serum

The following reagents were purchased from Invitrogen (**Faraday Avenue, Carlsbad, California U.S.A.)**

Glutamine, Penicillin-streptomycin, Goat Serum

The following reagent was purchased from Intracel (Intracel, 93 Monocacy Boulevard, Unit A8, Frederick, MD 21701)

Fetal Bovine Lipoprotein Deficient Serum

The following reagents were purchased from J. T. Baker (Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg NJ 08865 U.S.A.)

Tris (Base), Chloroform

The following reagent was purchased from Mallinckrodt Chemicals (Mallinckrodt Laboratory Chemicals, A Division of Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865)

Anhydrous Methyl Alcohol

The following reagents were purchased from Merck (Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany)

Triton® X-100, Glycin, Sodium Hydroxide Pellets, Acetic Acid, n-Hexan, Isopropanol, 25 TLC aluminium sheets 20×20cm Silica Gel 60 F254, 3β-[2-(diethylamino) ethoxy] androst-5-en-17-one (U18666A)

The following reagents were purchased from Molecular Probes (Eugene, OR 97402-0469, 29851 Willow Creek Road, Eugene, OR 97402, United States)

Alexa Fluor® 488 Goat anti-mouse IgG, Jasplakinolide,

The following item was purchased from Pall Corporation (2200 Northern Boulevard, East Hills, NY 11548)

Bio Trace™ PVDF(polyvinglidene fluoride) Transfer Membrane

The following reagents were purchased from Perkin Elmer (Perkin Elmer® Life and Analytical, Science, Boston, MA, USA)

[1,2-^{3}H(N)]-Cholesterol, [^{3}H]-Acetic Acid Sodium Salt

The following reagents were purchased from Pierce (Pierce Biotechnology, Inc., Rockford, IL)

Super Signal West Femto Maximum Sensitivity Substrate, Super Signal West Pico Chemiluminescent Substrate, Bond-Breaker™ TCEP solution, CL-X Posure™ Film, 8×10 inches, Clear Blue X-Ray Film

The following reagent was purchased from Roche Diagnostics (F. Hoffmann-La Roche Ltd, Diagnostics Division, Grenzacherstrasse 124, CH-4070 Basel, Switzerland)

Complete EDTA-free Protease inhibitor cocktail tablets,

The following reagents were purchased from Senta Cruz Biotechnology (2145 Delaware Avenue, Santa Cruz, Califomia 95060)

Anti-ABCA1, goat polyclonal IgG

The following reagents were purchased from Sigma (Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103)

Trypsin-EDTA solution, Apolipoprotein A-I, DMSO (Methyl sulfoxide), Monoclonal anti-β-Actin clone AC-74, TO-901317, Phosphatidylinositol-specific Bacillus cereus Phospholipase C, Deep Blue Dyed Latex Beads, Methyl-β-cyclodextrin (MβCD), TEMED (N,N,N',N'-Tetramethyl-Ethylenediamine), Heptane, Nocodazole, Cytochalasin D, Lauryl sulfate (SDS), Paraformaldehyde (PFA)

The following reagent was purchased from Spectrum Chemical Mfg. Cotp. (S. San pedro Street, Gaedena, California)

Hydrochloric Acid

The following reagent was purchased from Singapore Oxygen Air Liquid Pte Ltd (Soxal, Singapore)

Carbon dioxide (CO_2), Pure Liquid Nitrogen

2.1.2. Media and buffers

2.1.2.1. Reagents for cell culture

Complete EMEM medium

Minimum Essential Medium Eagles supplemented with 15% (v/v) uninactivated fetal bovine serum and a mixture of L-glutamine (2 mM, final concentration, the same below), penicillin (100 unit/ml) and streptomycin (100 µg/ml)

Delipidated EMEM medium

Minimum Essential Medium Eagles supplemented with 7.5% (v/v) uninactivated lipoprotein deficient serum (LPDS) and a mixture of L-glutamine (2 mM, final concentration, the same below), penicillin (100 unit/ml) and streptomycin (100 µg/ml)

Frozen medium

Minimum Essential Medium Eagles supplemented with 40% (v/v) uninactivated fetal bovine serum with 10% DMSO and 2mM L-glutamine

Lysis buffer

50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100,

Serum-free EMEM medium

Minimum Essential Medium Eagles supplemented with L-glutamine (2 mM, final concentration, the same below), penicillin (100 unit/ml) and streptomycin (100 µg/ml)

PBS

1.76 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 9 mM KCl, pH 7.4

Trypsin-EDTA

0.05% trypsin and 0.02% EDTA

2.1.2.2. Reagents for Western Blotting

4×Resolving gel buffer

1.5 M Tris-HCl, pH 8.8

4×Stacking gel buffer

0.5 M Tris-HCl, pH 6.8

5×Loading buffer (for 10 ml stock)

1 g SDS, 5 g sucrose, 0.01 g Electrophoresis purity reagent bromophenol blue, 3.125 ml 1 M Tris-HCl (pH6.8), 1 ml TCEP, 3 ml distilled water, mixed well by stirring overnight

10×Transfer buffer stock

30.3 g Tris and 144.13 g glycine in 1 liter distilled water.

Blocking buffer

5% Anlene skimmed milk in TBS

5×Running buffer

15.15 g Tris-HCl, 72 g glycine and 5 g SDS in 1 liter distilled water

TBS

6.075 g Tris and 8.766 g NaCl dissolved in 1 liter distilled water, pH adjusted to 7.5

TBST

0.1% TWEEN 20 in TBS buffer

Transfer buffer

100 ml 10×transfer buffer topped up to 800 ml with distilled water, mixed well, then 200 ml methanol added

2.1.3. Instruments and other general consumables

The instruments and general consumables used in this work include BECKMAN LS6000 Scintillation Counter (Beckman Coulter, Inc., 4300 N. Harbor Boulevard, Fullerton, CA), Biological Safety Cabinet Class II (Gelman Science Inc., Washtenaw County Circuit Court), CO_2 Incubator (Heraeus Kulzer Australia Pty Ltd., Unit 3, 4 Gibbes Street, Chatswood, Australia), Dry-Bath (Medical Research Council, 20 Park Crescent, London W1B 1Al, UK), Eppendorf Centrifuge 5810R (B. BRAUN, Postfach 1120, 34209 Melsungen, Germany), pH meter (Beckman Coulter), OLYMPUS IX71 Fluorescence Microscope(Olympus Technologies Singapore Pte Ltd, 41 Science Park Road, #04-17/18 The Gemini, Singapore Science Park II, Singapore), Orbital Shaker 100 (ARMALAB, ARMALAB, LLC, Bethesda, MD), Oven, Water Bath (MEMMERT**,** Memmert GmbH+Co.KG, Schwabach, Germany), PowerPac Basic Power Supply 100 (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California 94547), Vortex machine (VWR Scientific, 1310 Goshen Parkway, West Chester, PA), Ultrasonic Water Bath (ITS Science and Medical, 219 Henderson Road, Henderson Industrial Park, Singapore), Blue MAXTM 50 ml/15 ml Polypropylene Conical Tube, 1ml/2ml/5ml/10ml/50ml nonpyrogenic serological pipet (Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ, USA), Fast Turn Cap Mini Poly-Q^{TM} Vial (Beckman

Coulter, Inc., 4300 N. Harbor Boulevard, Fullerton, CA), MULTIDISH 12 wells, 48 wells and Easy Flask 75 V/C, 25 V/C (NUNC, Apogent Company, NUNC A/S Kamstrupvj.90, Roskilde, Denmark).

2.2. Cell culture

The GM00730A cells were obtained from NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research and maintained in complete EMEM medium at 37°C in a humidified atmosphere containing 5% CO_2.

A new flask was initiated according to following procedure:

1) The complete EMEM medium was warmed up to 37°C before use. The pipettes and culture flasks were irradiated with ultraviolet rays for 30 min in the culture hood.

2) The cells were removed from liquid nitrogen tank and placed in 37°C water bath as soon as possible. After being thawed out, the cells were rapidly transferred into a 25-cm^2 flask. Another 5 ml warm complete EMEM medium was added into the flask. The flask was placed in HERA cell CO_2 incubator at 37°C in a humidified atmosphere containing 5% CO_2 overnight.

3) The medium in the flask was replaced by fresh complete EMEM medium on the next day.

4) The cells were passaged after 3-4 days when the cells were about 80%-90% confluence.

Cells were passaged according to following procedure:

1) The cells were washed with warm PBS buffer once.
2) Two ml trypsin-EDTA was added to a 75-cm^2 flask for 3 min, and the flask was beaten gently to dislodge the cells.
3) The action of trypsin was stopped with 6 ml complete EMEM medium. The cells were resuspended and an appropriate volume was transferred to a new flask, which was subsequently added with 15 ml of complete EMEM medium per flask.

Cells were frozen in liquid nitrogen for further usage according to following procedure:
The subconfluent cells were trypsinised and spun down at 300 ×g for 5 min at room temperature. The cell pellet was resuspended in 2 ml frozen medium and stored in NUNC Cryo Tube™ vials. The tubes were kept at 4°C for half hour, -20°C for 2 hours, and -80°C overnight and transferred into liquid nitrogen tank on the following day.

2.3. ABCA1 overexpression

2.3.1. ABCA1 overexpression in complete EMEM medium

The cells were seeded in plates in complete EMEM medium. When cells were about 80%-90% confluence, complete EMEM medium was replaced by complete EMEM medium containing 5 µM TO-901317 and further incubated for 24 hours.

2.3.2. ABCA1 overexpression in delipidated medium

The cells were seeded in plate in complete EMEM medium. When cells were about 80%-90% confluence, cells were washed with PBS once and delipidated EMEM medium containing 5 µM TO-901317 was added. The cells were incubated for 48 hours in the presence of TO-901317.

2.4. Protein determination

Samples for protein determination were prepared according to following procedure:

1) Culture plate was put on ice. Culture medium was removed from culture wells using micropipette.
2) Two ml ice-cold PBS buffer was used to wash the cells.
3) Appropriate volume of ice-cold lysis buffer was added to the culture wells. The cells were scraped off using cell scraper.
4) Cell lysate was collected into eppendorf tubes and it was put on ice for 30min. Then cell lysate was spun at 13,000 ×g for 10 min at 4°C to remove whole cells and nucleus. The post-nuclear supernatant was stored at -20°C for further use.

Protein determination was performed with the Bio Rad RC DC Protein Assay Kit II.

1) A standard curve (0-1.5 mg BSA/ml) was prepared each time the assay was performed. BSA stock solution concentration was 1.5 mg/ml.

2) A total of 25 µl of standard BSA solution and the sample were added into clean, dry eppendorf tubes.
3) RC reagent I (125 µl) was added into each tube. The tubes were vortexed and incubated for 1 min at room temperature.
4) RC reagent II (125 µl) was added into each tube. The tubes were vortexed and centrifuged at 13,000 ×g for 10 min at room temperature.
5) The supernatant was discarded by inverting the tubes on clean, absorbent tissue paper. Liquid should be dried completely from the tubes.
6) DC Reagent S (5 µl) was added to each 250 µl of DC Reagent A. This solution was then referred to as Reagent A' and 127 µl Reagent A' would be required for each standard or sample assay (Regent A' should be prepared freshly. It is stable for one week. If precipitate forms, solution should be warmed and vortexed.).
7) Regent A' (127 µl) was added to each tube. The tubes were vortexed and incubated at room temperature for 5 min or until precipitate is completely dissolved. The tubes should be vortexed before proceeding to the next step.
8) DC Regent B (1 ml) was added to each tube. The tubes were vortexed immediately and incubated at room temperature for 15 min.
9) The liquid was transferred into cuvettes. Absorbance was read at 750 nm. The absorbance would be stable for at least 1 hour.

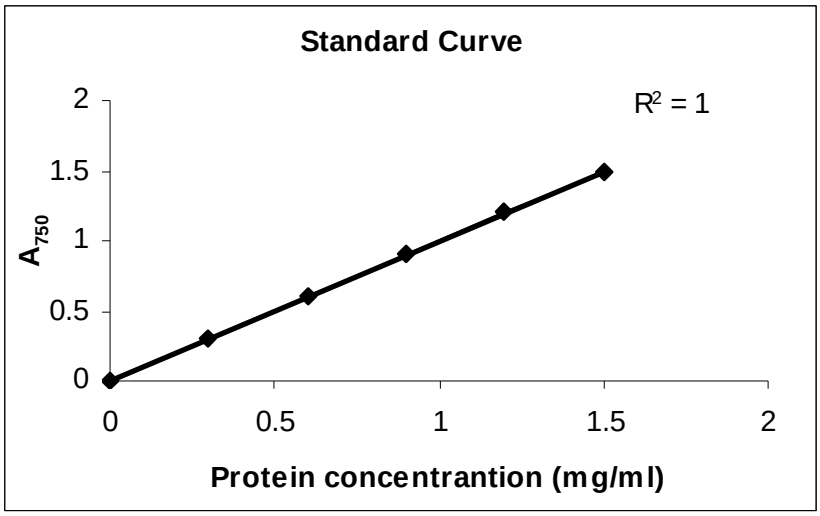


Fig. 2.1 A typical protein assay standard curve

2.5. Western Blotting

Samples for Western Blotting were prepared according to following procedure:

1) One volume 5×loading buffer was added into 4 volume post-nuclear supernatant and vortexed thoroughly.
2) The samples were boiled at 100°C for 5 min and spun at 13,000×g for 2 min. Then the samples were ready for western blotting loading.

Western Blotting was performed according to the well established protocol. Briefly, the resolving gel solution (8%) was mixed well and allowed to degas before ammonium persulfate (APS) and TEMED were added. All reagents were quickly mixed and poured into a mini-gel casting chamber. A depth of 2.0 cm from the top was left empty. The

resolving gel was overlaid with water immediately to separate the resolving gel buffer from air and was allowed to stay for 30 min. When the resolving gel had solidified, water was removed; the stacking gel solution (4%) was prepared and poured on top of the resolving gel. A comb was inserted into the stacking gel immediately. The stacking gel solution was allowed to stay for 30 min to solidify. After the stacking gel solidified, the comb was removed.

Samples (30 µg protein) or 5 µl of precision protein standard were loaded into each well. The gel was then electrophoresised at 40 volt when the dye advanced in the stacking gel. The voltage was adjusted to 100 volt when the dye entered the resolving gel. The electrophoresis was terminated till the dye front ran near the bottom of the resolving gel. The resolving gel was isolated from gel casting chamber and soaked in the pre-cooled transfer buffer together with the PVDF membrane, sponge and filter paper of the same size as the gel for about 10 min. The gel sandwich was stacked in the order of sponge, filter paper, gel, membrane, filter paper and sponge. The proteins were transferred from gel onto PVDF membrane at 100 volt for 90 min at 4°C. The membrane was then blocked in blocking buffer overnight at 4°C or for 1 hour at room temperature with shaking. The membrane was then incubated in 10ml of blocking buffer containing 1:500 diluted anti-ABCA1 antibody or 1:1000 diluted anti-actin antibody overnight at 4°C or for 1 hour at room temperature with shaking. The membrane was then washed with TBST buffer four times to remove the excess first antibody and then incubated in blocking buffer containing 1:2000 diluted rabbit anti-goat antibody for detection of ABCA1 or 1:5000 diluted anti-mouse antibody for detection of actin for 1 hour. The

membrane was then thoroughly washed with TBST buffer four times to remove the excess secondary antibody. Finally the membrane was submerged in a mixture of equal volume of super signal west pico chemiluminescent substrate and enhancer. The membrane was removed from the mixture and placed against the film in the cassette. The film was developed after appropriate exposure by following the manufacturer's instructions.

2.6. Cholesterol efflux

2.6.1. Efflux of plasma membrane derived cholesterol to apoA-I

1) Cells were seeded in 48 well culture plates in complete EMEM medium.
2) When about 80%-90% confluence, complete EMEM medium was replaced by 200 µl complete EMEM medium containing 5 µM TO-901317 to induce ABCA1 overexpression. The cells were incubated in the above medium for 24 hours.
3) The complete EMEM medium containing 5 µM TO-901317 was then replaced by 150 µl serum-free EMEM medium containing 1 µCi ^{3}H-cholesterol and cultured for 24 h.
4) The cells were washed with 37°C PBS containing 0.25% BSA twice and 37°C serum-free EMEM medium once.
5) The cells were incubated in 150 µl serum-free EMEM medium containing 10 µg/ml apoA-I for indicated time.

6) Incubation medium was collected at indicated time point and centrifuged at 13,000 ×g for 5 min to remove floating cells.
7) Medium was aliquoted (100 µl) to measure the CPM by liquid scintillation counting.
8) The cells in plates were dissolved in solution containing 0.1 N NaOH and 0.5% SDS. The cell lysate was used to measure the CPM of the cells by liquid scintillation counting.
9) The level of cholesterol efflux was calculated as following:

   Total medium CPM/(total medium CPM + total cellular CPM)×100%

2.6.2. Efflux of plasma membrane derived cholesterol to MβCD

1) Cells were seeded in 48 well culture plates in complete EMEM medium.
2) When about 80%-90% confluence, complete EMEM medium was replaced by 150 µl serum-free EMEM medium containing 1 µCi ^{3}H-cholesterol. The cells were labeled for 24 h.
3) The cells were washed with 37°C PBS containing 0.25% BSA twice and 37°C serum-free EMEM medium once.
4) The cells were incubated in 150 µl serum-free EMEM medium containing 0.1% MβCD for indicated time.
5) Culture medium was collected at indicated time points and centrifuged at 13,000 ×g for 5 min to remove floating cells.

6) Medium was aliquoted (100 µl) to measure the CPM in medium by liquid scintillation counting.
7) The cells in plates were lysed in solution containing 0.1 N NaOH and 0.5% SDS. The cell lysate was used to measure the CPM in cells by liquid scintillation counting.
8) The level of cholesterol efflux was calculated as following:

   Total medium CPM/(total medium CPM + total cellular CPM)×100%

2.6.3. Efflux of de novo synthesized cholesterol to apoA-I

1) Cells were seeded in 12 well culture plates in complete EMEM medium.
2) When about 80%-90% confluence, the cells were washed with PBS once. Delipidated EMEM medium (1 ml) containing 5 µM TO-901317 was added in each well to induce ABCA1 overexpression. The cells were incubated in the medium for 48 hours.
3) The delipidated EMEM medium containing 5 µM TO-901317 was then replaced by 500 µl delipidated EMEM medium containing 10 µCi/ml ^{3}H-acetate. The cells were cultured for 2 h.
4) Cells were washed with 37°C PBS containing 0.25% BSA twice and 37°C serum-free EMEM medium once.
5) The cells were incubated in 500 µl serum-free EMEM medium containing 10 µg/ml apoA-I for indicated time.

6) Culture medium was collected at indicated time point and centrifuged at 13,000 ×g for 5 min to remove the floating cells. Supernatant (400 µl) was aliquoted.
7) The cells in plate were lysed in 500 µl solution containing 0.1 N NaOH and 0.5% SDS for each well.
8) The aliquoted medium and the solution containing cell lyses were extracted with 400 µl hexane:isopropanol (3:2, v/v) for 3 times respectively. The combined organic phase was used to measure CPM in medium and cells by liquid scintillation counting.
9) The level of cholesterol efflux was calculated as following:

   Total medium CPM/(total medium CPM + total cellular CPM)×100%

2.6.4. Efflux of de novo synthesized cholesterol to MβCD

1) Cells were seeded in 12 well culture plates in complete EMEM medium.
2) When about 80%-90% confluence, the cells were washed with PBS once. Delipidated EMEM medium (1 ml) was added into each well. The cells were incubated in the medium for 48 hours.
3) The delipidated EMEM medium was then replaced by 500 µl delipidated EMEM medium containing 10 µCi/ml ^{3}H-acetate. The cells were incubated for 2h.
4) The cells were washed with 37°C PBS containing 0.25% BSA twice and 37°C serum-free EMEM medium once.

5) The cells were incubated in 500 µl 0.1% MβCD in serum-free EMEM medium for indicated time.
6) Incubation medium was collected at indicated time point and centrifuged at 13,000 ×g for 5 min to remove the floating cells. The supernatant (400 µl) was aliquoted.
7) The cells in plates were lysed in 500 µl solution containing 0.1 N NaOH and 0.5% SDS for each well.
8) The aliquoted medium and the solution containing cell lyses were extracted with 400 µl hexane:isopropanol (3:2, v/v) for 3 times respectively. The combined organic phase was used to measure CPM in medium and cells by liquid scintillation counting.
9) The level of cholesterol efflux was calculated as following:
   Total medium CPM/(total medium CPM + total cellular CPM)×100%

2.7. Thin layer Chromatography (TLC)

1) Chamber preparation. Developing solvent was prepared by mixing the hexane/ethyl ether/acetic acid (70:30:2, v/v) thoroughly by shaking or vortex. Appropriate volume of developing solvent was added into the chamber so that it was 0.5 cm deep in the bottom of the chamber. The chamber was sealed and left for at least 1 hour so that the atmosphere in the chamber became saturated with the developing solvent.

2) TLC plate preparation. The TLC plate was cut into appropriate size. A pencil was used to draw a line across the plate 1 cm above the bottom of the plate. Under the line, light marks were used to indicate the names of the samples. Enough space between the samples should be left so that they would not run together, about 4 samples on a 5 cm wide plate was advised. The plates should be handled carefully so that the coating of adsorbent would not be disturbed. The plate was activated at 100°C for 1 hour before use.

3) Sample application. Lipid samples were extracted with hexane/isopropanol (3:2, v/v) for 3 times and the organic phases were combined together and dried under nitrogen flow at room temperature or in freezing dryer. The lipid pellet was dissolved in appropriate volume of chloroform/methanol (2:1, v/v). The solution was then applied to the TLC plate with a 1 µL microcap. This was done by taking a microcap and dipping it into the solution of the sample to be spotted. Then, the end of the microcap was gently touched to the adsorbent on the origin in the place where marked for the sample. All of the contents of the microcap were let to run onto the plate and the spot dried in the air. Care was taken not to disturb the coating of the adsorbent.

4) TLC development. The prepared TLC plate was placed in the developing chamber, which was then sealed, and left undisturbed. TLC development was stopped when the solvent was about half a centimeter below the top of the plate.

5) Visualization of the spots. The TLC plate was taken out of the developing chamber and dried in the air. The plate was sprayed with visualizing solvent and then heated at 100°C for 5 min immediately after spraying.

6) Cholesterol analysis. The spot representing cholesterol was scraped off. The ^{3}H radioactivity of the spot representing cholesterol and the rest of the lane was measured separately. The percentage of ^{3}H-acetate converted into ^{3}H-cholesgerol was estimated by: ^{3}H radioactivity in cholesterol / (^{3}H radioactivity in cholesterol + ^{3}H radioactivity in rest part) ×100%. It has been found that more than 90% of the radioactivity was found in ^{3}H-cholesterol.

2.8. Fluorescence Immune Staining

1) Cells were seeded in 24 well culture plates in complete EMEM medium. There was a clean cover slip placed in each well.

2) When cells were about 40%-50% confluence, the complete EMEM medium was replaced by serum-free EMEM medium containing 100 nM jasplakinolide or 1μg/ml cytochalasin D for 24 hours.

3) The medium was sucked out. The cells were washed with cold PBS for 3 times. Then 4% PFA in PBS (0.5 ml) was added into each well for 20 min at room temperature to fix the cells.

4) PFA was sucked out. The cells were washed with PBS for 3 times. PBS (400 µl) containing 0.2% (v/v) Triton-X-100 was added into each well and incubated with the cells for 30 min at room temperature.

5) The PBS containing Triton-X-100 was replaced by 250 µl PBS containing 10 % goat serum. The cells were incubated with goat serum for 1 hour.

6) PBS (200 µl) containing 10% goat serum and 1:250 mouse monoclonal anti-Actin was added into each well and incubated with cells over night at 4°C.

7) The cells were washed with PBS for 3 times, each time for 5 min.

8) PBS (200 µl) containing 10% goat serum and 1:1000 Alexa Fluor® 488 Goat anti-mouse IgG was added into each well and incubated with cells for 2 hours at room temperature. Plate was wrapped in foil to avoid light.

9) Cells were washed with PBS 3 times, 5 min for each time. Then the cover slips were taken out from each well and mounted with Fluor save™ Reagent to protect the fluorescence in the cells.

10) The cover slips were observed with the fluorescence microscope.

CHAPTER 3. RESULTS

3.1. Effects of TO-901317 on ABCA1 expression and cholesterol efflux

3.1.1. Effect of TO-901317 on ABCA1 expression

TO-901317 is a nonsteroidal ligand of LXR which is known to regulate ABCA1 expression (Repa et al., 2000). ABCA1 (220 kDa) and β-actin (42 kDa) expression levels were determined by Western Blotting. Relatively levels of ABCA1 expression were estimated by densitometric analysis and the ratio was calculated. Fig. 3.1 and Fig. 3.2 show that the density ratios of ABCA1/actin in human fibroblasts treated with 5 µM TO-901317 were about 70% higher than the rations in control human fibroblasts. The result verifies that 5 µM TO-901317 treatment increase ABCA1 protein level about 70% whether the cells were cultured in complete EMEM medium or in delipidated EMEM medium.

	Control	TO-901317 treated
Density ratio (ABCA1/actin)	1.01	1.78

1.78/1.01=1.76

Fig. 3.1 Effect of TO-901317 on ABCA1 expression in fibroblasts cultured in complete EMEM medium. Cells were cultured in complete EMEM medium with or without 5 µM TO-901317 for 24 hours and then harvested and ABCA1 protein level was determined by Western Blotting.

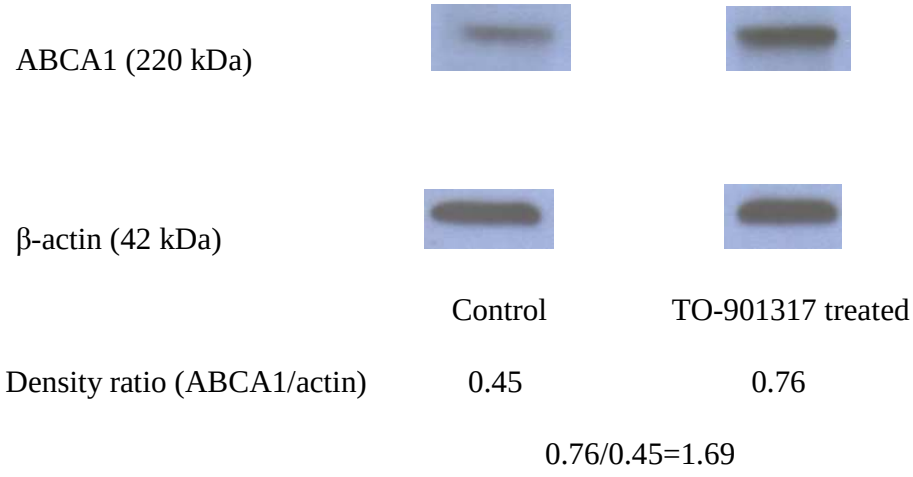


Fig. 3.2 Effect of TO-901317 on ABCA1 expression in fibroblasts cultured in delipidated EMEM medium. Cells were cultured in delipidated EMEM medium with or without 5 μM TO-901317 for 48 hours and then harvested for ABCA1 protein determination by Western Blotting.

3.1.2. Effect of TO-901317 on cholesterol efflux to apoA -I

Fig. 3.3 and Fig. 3.4 show that cells treated with 5 μM TO-901317 exhibited higher level of cholesterol efflux (~ 2.5 fold) when apoA-I was used as the acceptor, whether ^{3}H-cholesterol was used to label the cells or it was newly synthesized by the cells. The increased cholesterol efflux is assumably due to the higher ABCA1 protein level expressed in TO-901317 treated cells. These results confirm that ABCA1 plays a very important role in cholesterol efflux to apoA-I.

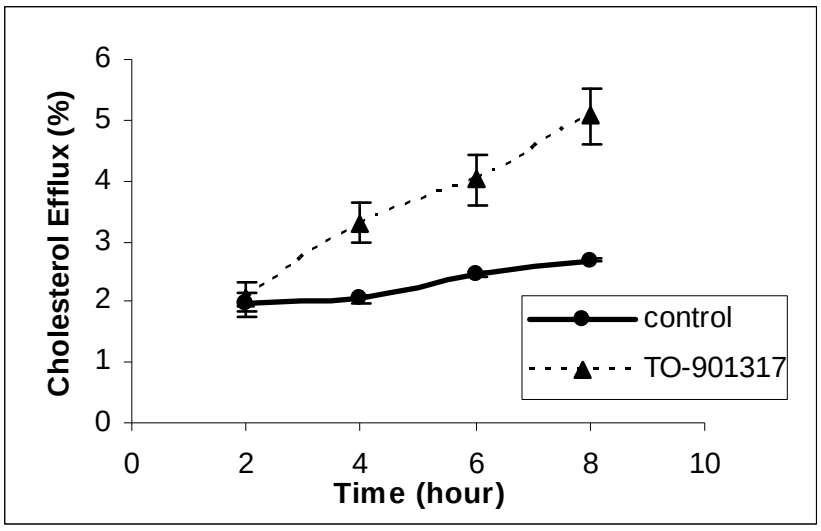


Fig. 3.3 Effect of TO-901317 on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with or without 5 μM TO-901317 for 24 hours and then labeled with 1 μCi/ml ^{3}H-cholesterol for 24 hours. After washing, 10 μg/ml apoA-I in serum-free EMEM medium was added to start cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

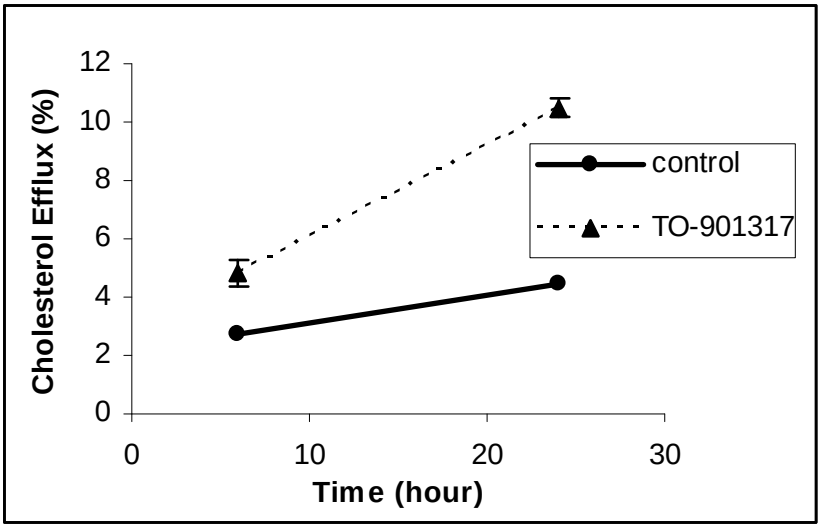


Fig. 3.4 Effect of TO-901317 on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with or without 5 µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate the cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

The level of cholesterol efflux in control cells is too low and shows no significant change over time up to 24 h. In the following experiments, hence, all experiments on cholesterol efflux to apoA-I will be performed in cells treated with 5 µM TO-901317, unless mentioned otherwise.

3.1.3. Effect of TO-901317 on cholesterol efflux to cyclodextrin

In contrast to apoA-I, when CD was used as the acceptor for cholesterol efflux from the fibroblasts, more cholesterol had effluxed from the cells within shorter period of time. However, this process appeared independent of ABCA1 as there was no difference in the level of cholesterol efflux in the presence and absence of TO-901317 (Fig. 3.5).

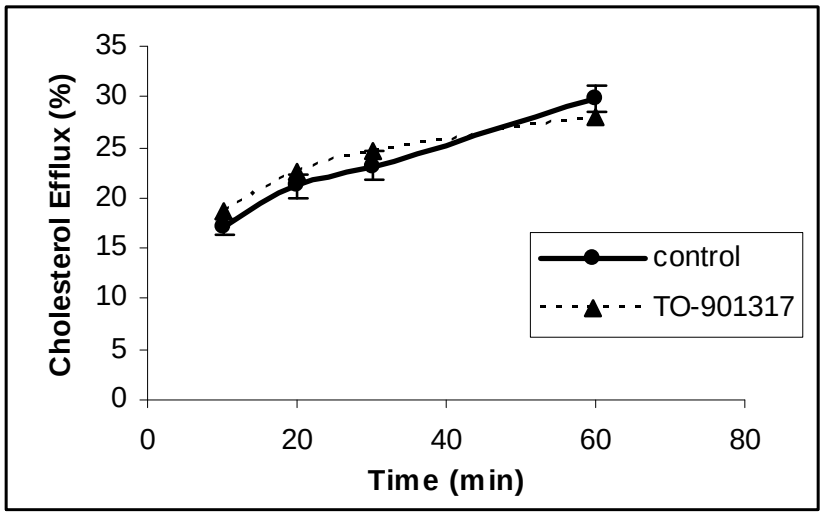


Fig. 3.5 Effect of TO-901317 on efflux of plasma membrane derived cholesterol to MβCD. Cells were cultured in complete EMEM medium with or without 5 μM TO-901317 for 24 hours and then labeled with 1 μCi/ml ^{3}H-cholesterol for 24 hours. After washing, 0.1% MβCD in serum-free EMEM medium was added to start the efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.2. Plasma membrane in cholesterol efflux: role of caveolae

After MβCD treatment, the cellular cholesterol content was reduced by 75% compared with that in control cells (Fig. 3.6). Also, such reduction in cellular cholesterol was a rapid process and it completed within half hour of MβCD treatment. It is known that MβCD may selectively remove cholesterol from membranes and disrupt the structure of caveolae. It would be interesting to see if caveolae is involved in cholesterol efflux to apoA-I. Fig. 3.7 shows that the level of cholesterol efflux to apoA-I increased up to 4 fold when the cells were pre-treated with MβCD. This confirms that caveolae do play a role in cellular cholesterol efflux to apoA-I.

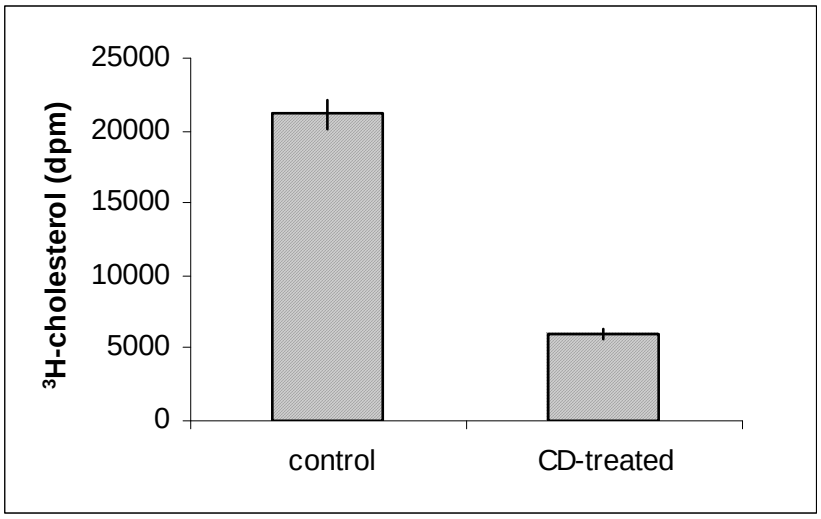


Fig. 3.6 Effect of MβCD treatment on cellular cholesterol content. Cells were labeled with 1 μCi/ml ^{3}H-cholesterol for 24 hours. After washing with PBS, cells were incubated in serum-free EMEM medium with or without 10 mM MβCD for 30 min. Thereafter, the medium was removed and the cellular ^{3}H-cholesterol was quantitated with the liquid scintillation counter. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

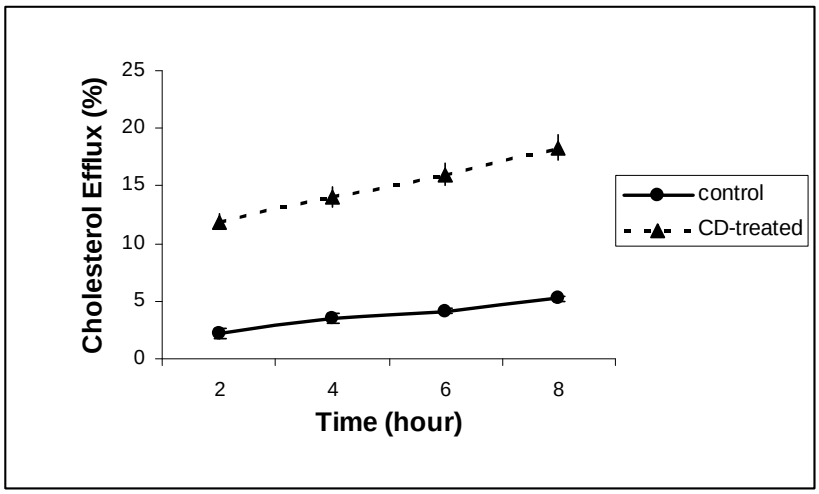


Fig. 3.7 Effect of MβCD extraction on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 μM TO-901317 for 24 hours and then labeled with 1 μCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 10 mM MβCD for 30 min. Thereafter, 10 μg/ml apoA-I in serum-free EMEM medium was added to start efflux. The medium was collected at indicated time points and the ^{3}H-cholesterol in the medium and in the cells was quantitated by liquid scintillation counting. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.3. Effects of drugs on cholesterol efflux

3.3.1. Brefeldin A

Incubation of BFA with the fibroblasts did not affect the efflux of plasma membrane derived cholesterol to apoA-I (Fig. 3.8). However, the efflux of newly synthesized cholesterol to apoA-I was suppressed (Fig. 3.9). These results suggest that cholesterol efflux is regulated somehow by its intracellular trafficking. Because BFA is known to disrupt the structure and function of Golgi apparatus, the above difference between the efflux of plasma membrane cholesterol and the do novo synthesized cholesterol indicates

that Golgi apparatus may play a role in cholesterol trafficking from ER to plasma membrane. On the other hand, when MβCD was used as the acceptor for cholesterol efflux, pre-treatment of the cells with BFA did not affect the efflux whether the cholesterol is pre-existing in the cell plasma membrane or newly synthesized (Fig. 3.10 and Fig. 3.11). These results imply that the de novo synthesized cholesterol may transfer from ER to plasma membrane via two separate pathways, the Golgi-dependent pathway and the Golgi-independent pathway.

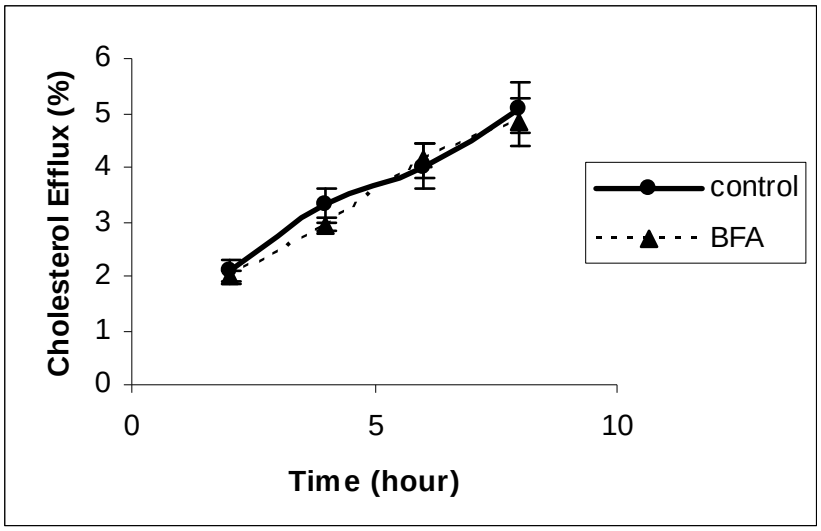


Fig. 3.8 Effect of BFA on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 µM TO-901317 for 24 hours and then labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 10 µg/ml BFA for 2 hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to start cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

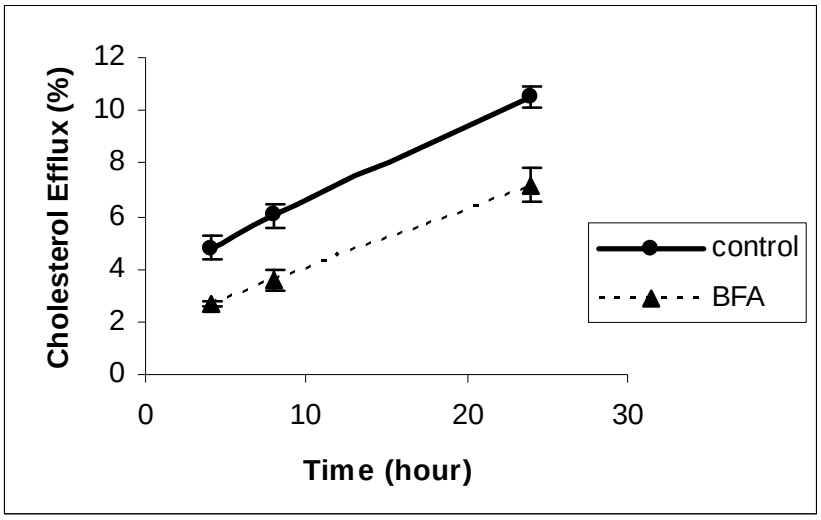


Fig. 3.9 Effect of BFA on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with 5 µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 10 µg/ml BFA for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

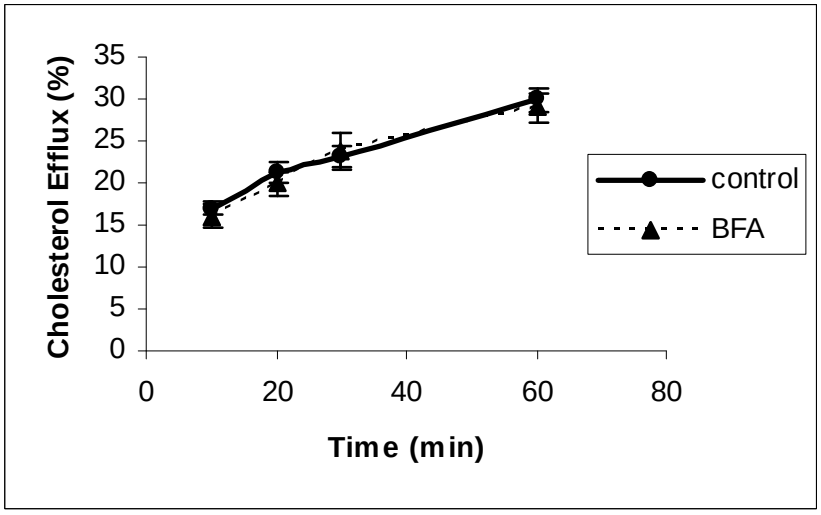


Fig. 3.10 Effect of BFA on efflux of plasma membrane derived cholesterol to MβCD. Cells were labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 10 µg/ml BFA for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

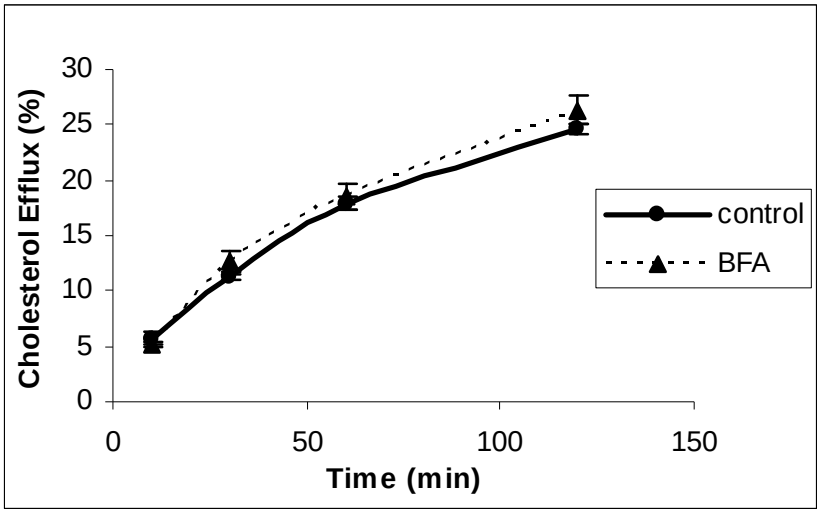


Fig. 3.11 Effect of BFA on efflux of de novo synthesized cholesterol to MβCD. Cells were cultured in delipidated EMEM medium for 48 hours and then labeled with 10 μCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 10 μg/ml BFA for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.3.2. Nocodazole

The efflux of plasma membrane derived cholesterol was not affected by nocodazole treatment whether the efflux was to apoA-I (Fig. 3.12) or to MβCD (Fig. 3.14). On the other hand, the efflux of newly synthesized cholesterol to MβCD was inhibited as much as 25% by nocodazole treatment after the efflux was continued for 2h (Fig. 3.15.), while the efflux of newly synthesized cholesterol to apoA-I was not inhibited but enhanced slightly by nocodazole treatment (Fig. 3.13). As MβCD and apoA-I acquire cholesterol from cell surface via different mechanisms and even potentially from different membrane

domains, the difference in efflux of newly synthesized cholesterol caused by different acceptors implies the existence of independent nascent cholesterol transport pathways, with which cholesterol was delivered to different plasma membrane microdomains and acquired consequently by different acceptors. From the results obtained, it appears that microtubules are involved in the transport of nascent cholesterol for efflux.

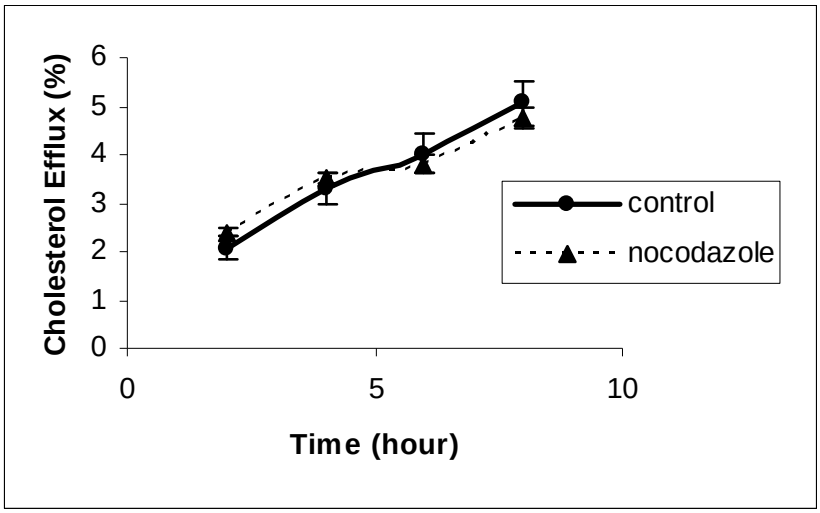


Fig. 3.12 Effect of nocodazole on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 µM TO-901317 for 24 hours and then labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 20 µM nocodazole for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

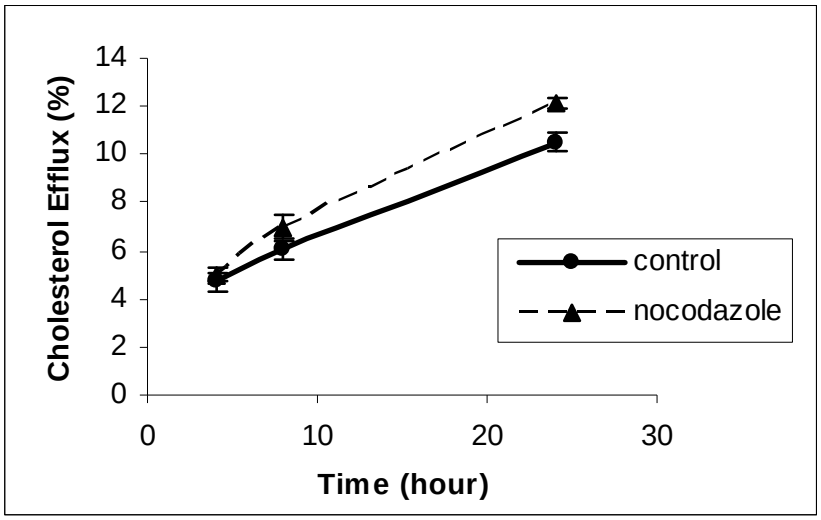


Fig. 3.13 Effect of nocodazole on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with 5 µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 20 µM nocodazole for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

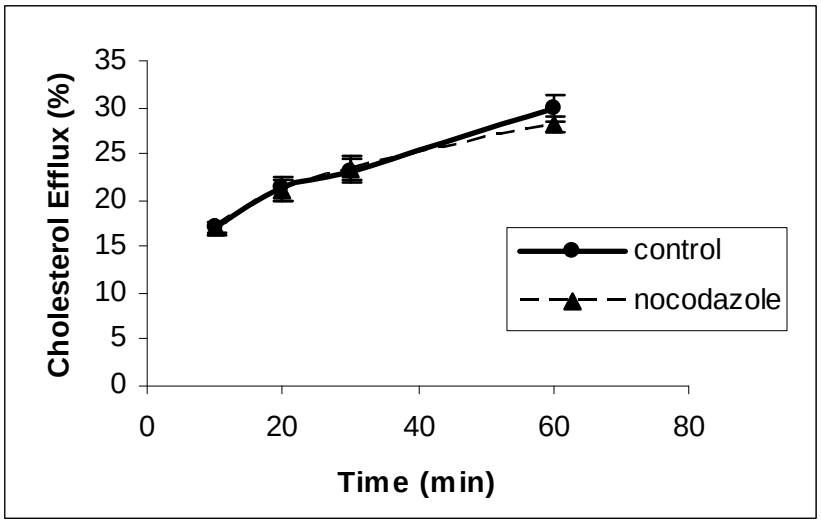


Fig. 3.14 Effect of nocodazole on efflux of plasma membrane derived cholesterol to MβCD. Cells were labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 20 µM nocodazole for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

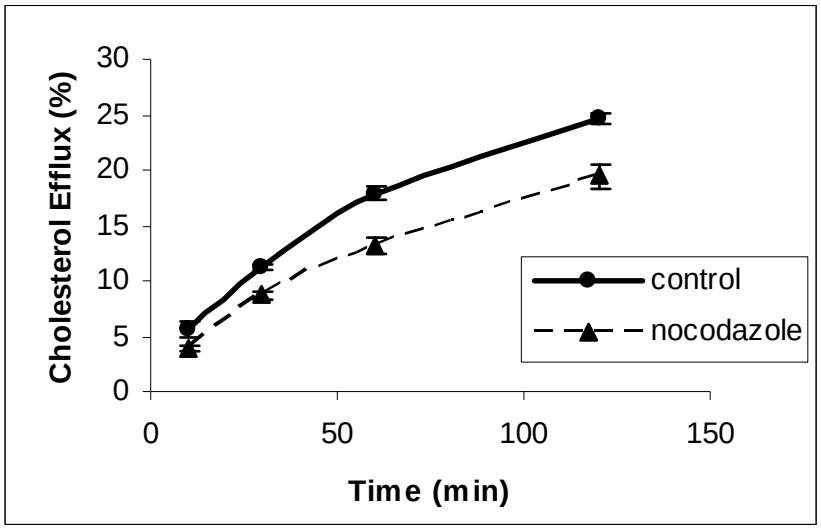
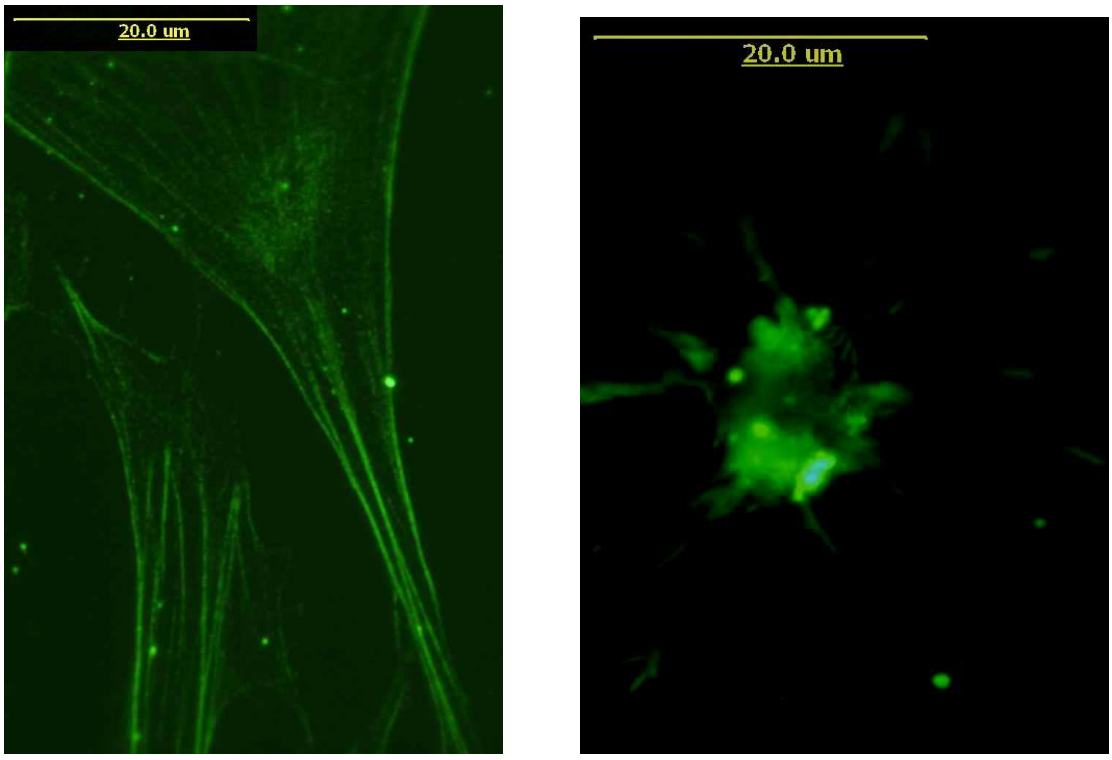


Fig. 3.15 Effect of nocodazole on efflux of de novo synthesized cholesterol to MβCD. Cells were cultured in delipidated EMEM medium for 48 hours and then labeled with 10 μCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 20 μM nocodazole for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.3.3. Jasplakinolide

Jasplakinolide is a drug that promotes and stabilizes actin polymerization (Bubb et al., 1994). Fig. 3.16 shows that jasplakinolide destabilized the actin microfilaments and changed the cells' morphology dramatically. The fibriform cells became starfish-like. Most of the actin subunits concentrated in the center of the cells and a small part of actin subunits distributed in the dendrites of the cells. Meanwhile, jasplakinolide increased cholesterol efflux to apoA-I whether the cholesterol is derived from plasma membrane or

newly synthesized (Fig. 3.17 and Fig.3.18). However, jasplakinolide did not change the cholesterol efflux to MβCD regardless of the origin of cholesterol. These results suggest that actin filaments may play an important role in cholesterol efflux to apoA-I.



Control cells Jasplakinolide treated cells

Fig. 3.16 Effect of jasplakinolide on polymerization of actin microfilaments. Cells were seeded in 24 well plates with clean cover slips. When cells were about 50% confluence, complete EMEM medium was replaced by serum-free EMEM medium containing 100 nM jasplakinolide and incubated for 24 hours. The fluorescence immune staining was performed according to materials and methods.

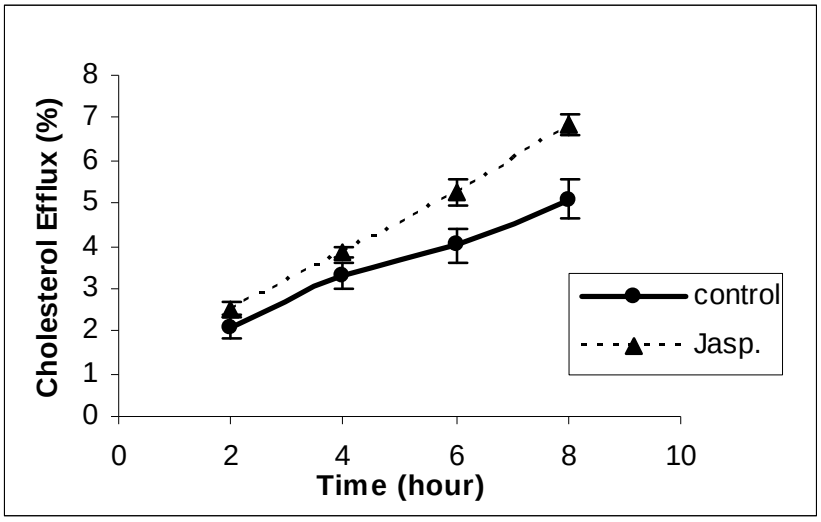


Fig. 3.17 Effect of jasplakinolide on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 µM TO-901317 for 24 hours and then labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 100 nM jasplakinolide for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. Each value is the mean ± S.D. of triplicates.

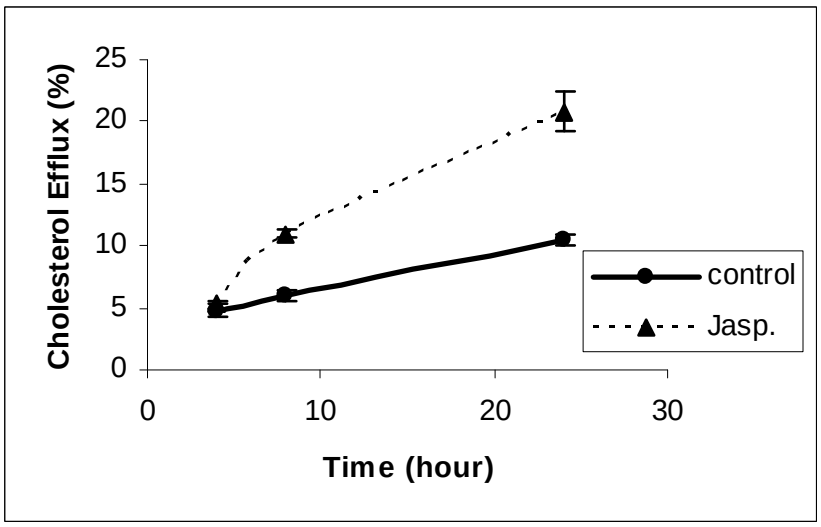


Fig. 3.18 Effect of jasplakinolide on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with 5 µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 100 nM jasplakinolide for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. Each value is the mean ± S.D. of triplicates.

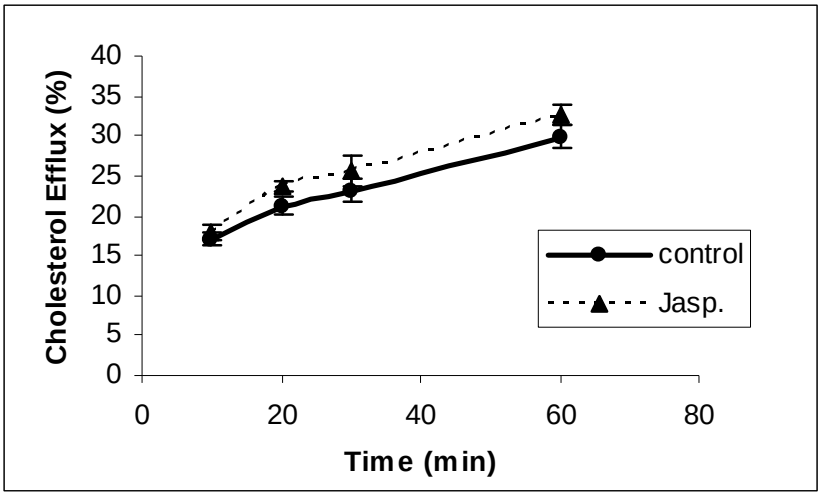


Fig. 3.19 Effect of jasplakinolide on efflux of plasma membrane derived cholesterol to MβCD. Cells were labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 100 nM jasplakinolide for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

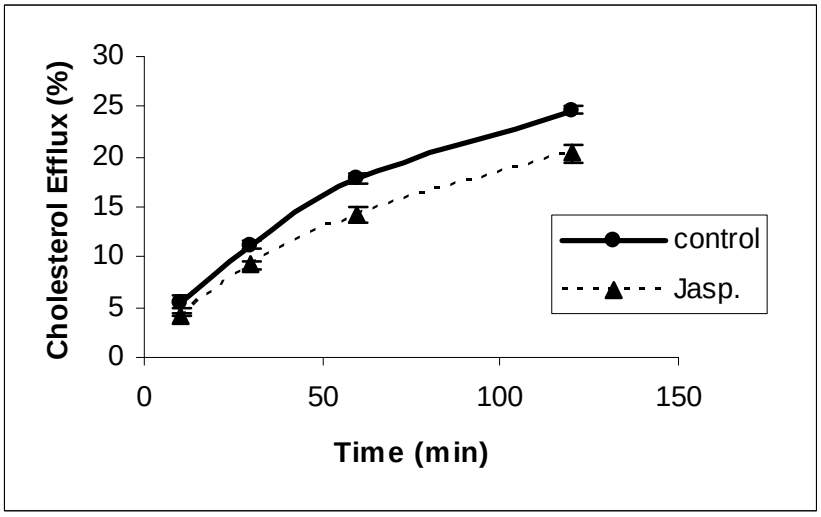
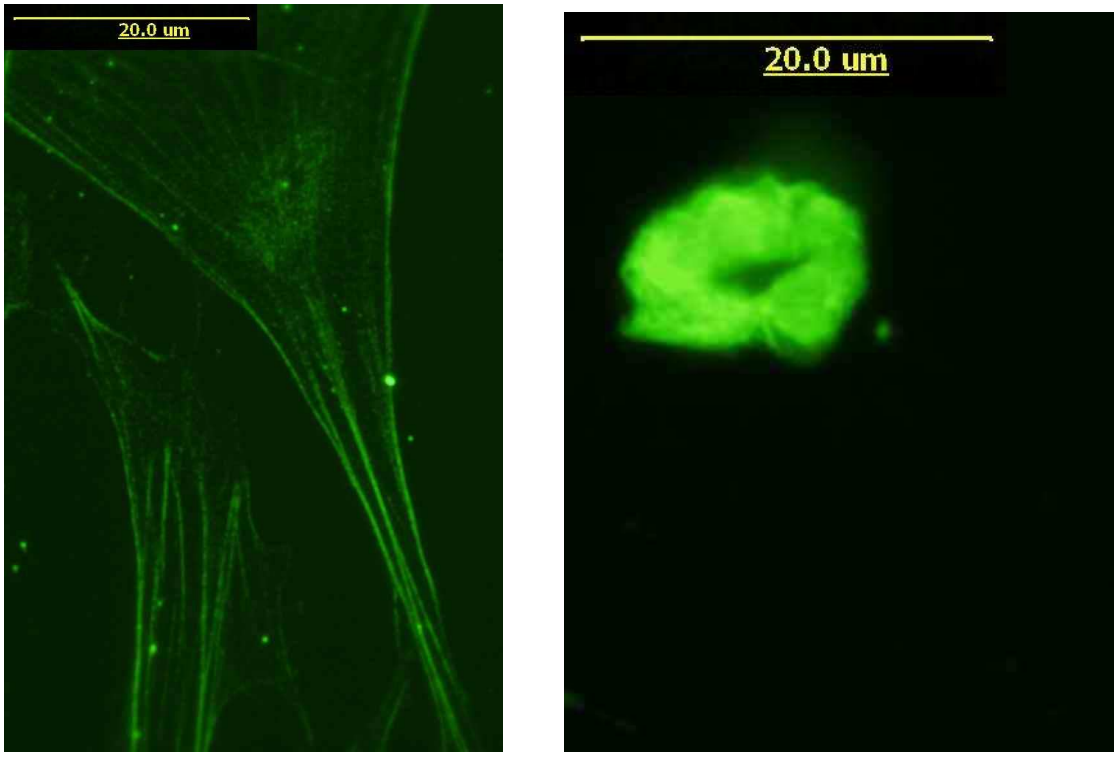


Fig. 3.20 Effect of jasplakinolide on efflux of de novo synthesized cholesterol to MβCD. Cells were cultured in delipidated EMEM medium for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 100 nM jasplakinolide for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.3.4. Cytochalasin D

Cytochalasin D is known to inhibit actin polymerization (Casella et al., 1981). Fig. 3.21 shows that cytochalasin D completely changed the cells' morphology and inhibited polymerization of actin microfilaments. The fibriform cells became round. The actin subunits distributed in the round cells uniformly. Fig. 3.22 and Fig. 3.23 show that cytochalasin D significantly increased cholesterol efflux to apoA-I regardless of the cholesterol was derived from the plasma membrane or newly synthesized. However, cytochalasin D did not change the cholesterol efflux to MβCD under similar conditions (Fig. 3.24 and Fig. 3.25). These results imply that action filaments are involved in cholesterol efflux to apoA-I.



Control cells

Cytochalasin D treated cells

Fig. 3.21 Effect of cytochalasin D on actin microfilaments. Cells were seeded in 24 well plate with clean cover slips. When cells were about 50% confluence, complete EMEM medium was replaced by serum-free EMEM medium containing 2 µM cytochalasin D and incubated with cells for 24 hours. The fluorescence immune staining was performed according to materials and methods.

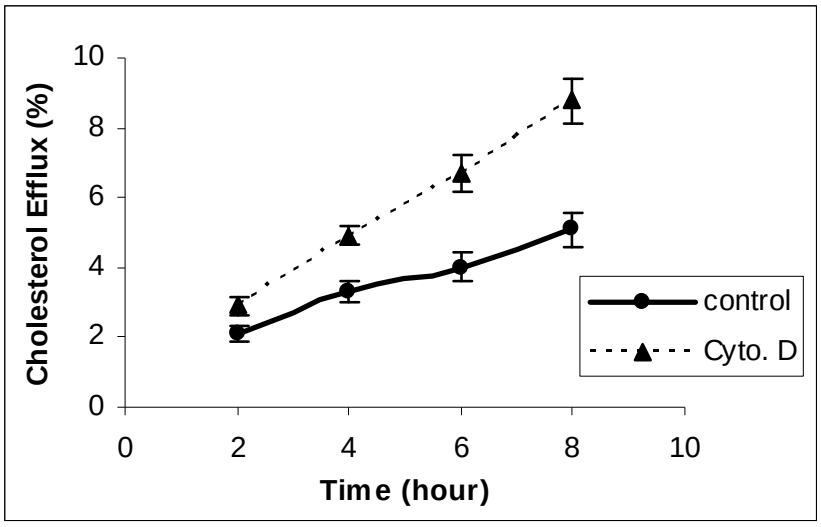


Fig. 3.22 Effect of cytochalasin D on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 µM TO-901317 for 24 hours and then labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 2 µM Cytochalasin D for two hours. Thereafter 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

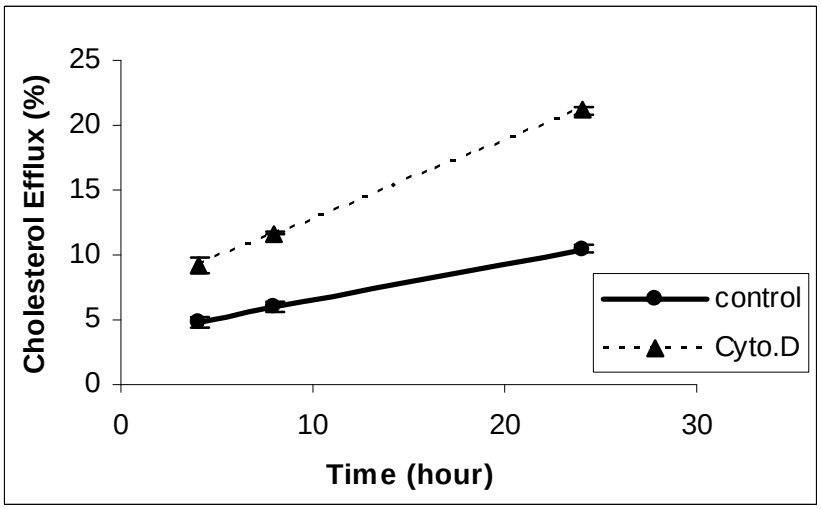


Fig. 3.23 Effect of cytochalasin D on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with 5 µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 2 µM cytochalasin D for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

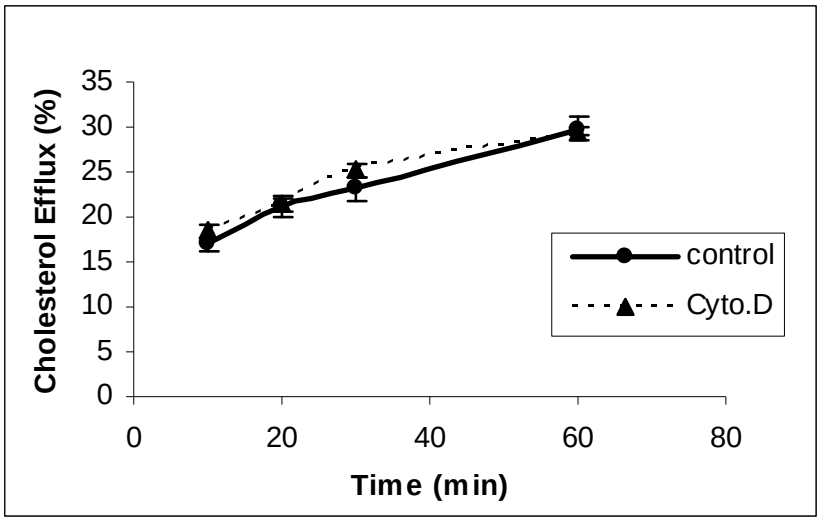


Fig. 3.24 Effect of cytochalasin D on efflux of plasma membrane derived cholesterol to MβCD. Cells were labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 2 µM cytochalasin D for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

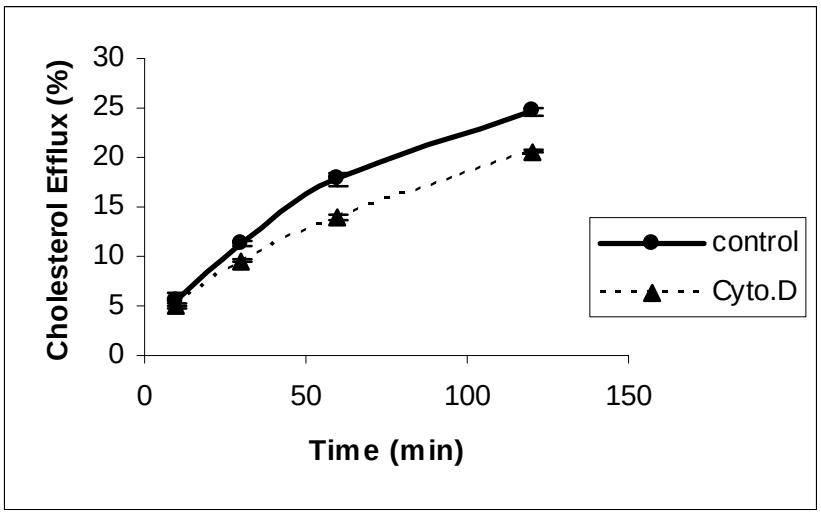


Fig. 3.25 Effect of cytochalasin D on efflux of de novo synthesized cholesterol to MβCD. Cells were cultured in delipidated EMEM medium for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 2 µM cytochalasin D for two hours. Thereafter 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.3.5. Deep blue dyed latex beads

To examine if the ER-plasma membrane contacts will affect cholesterol efflux to apoA-I or MβCD, the fibroblasts were incubated with 1% deep blue dyed latex beads for 2 hours before the efflux was started. The results show that treatment with latex beads did not change the cholesterol efflux under any conditions tested. These results indicate that ER-plasma membrane contacts might not contribute to efflux of cholesterol derived either from ER or from the plasma membrane.

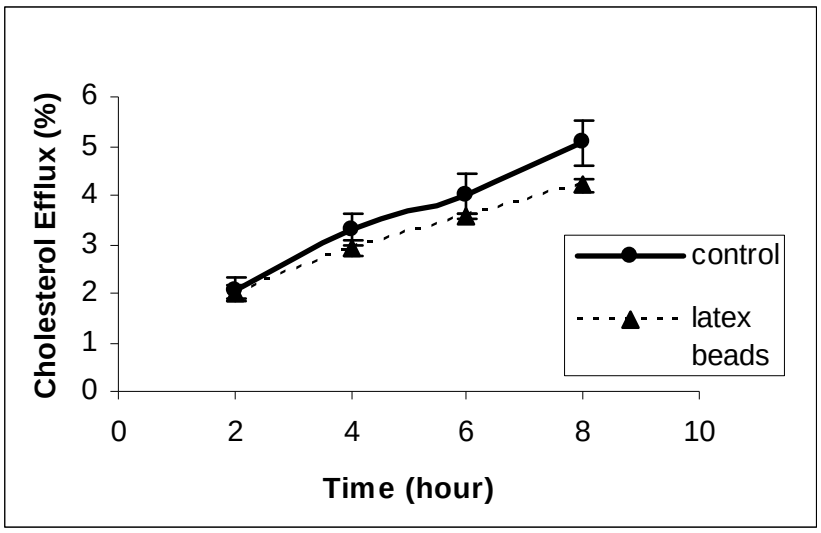


Fig. 3.26 Effect of deep blue dyed latex beads on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 µM TO-901317 for 24 hours and then labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 1% deep blue dyed latex beads for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

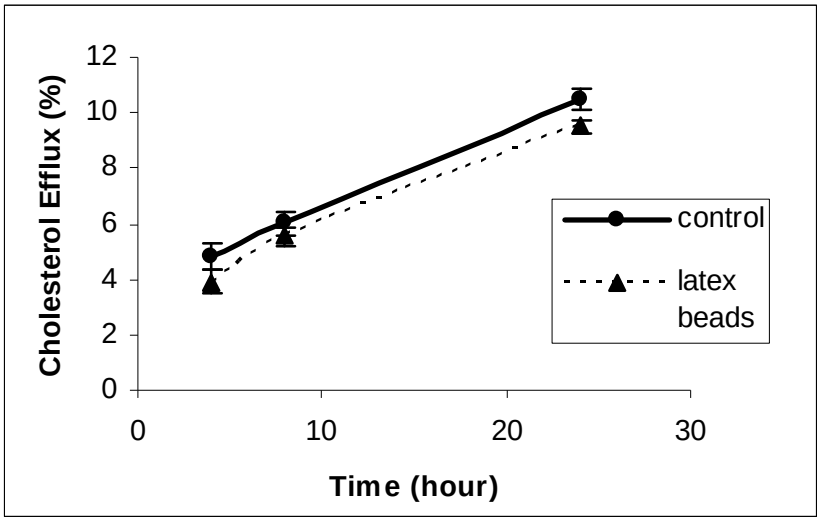


Fig. 3.27 Effect of deep blue dyed latex beads on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with 5µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 1% deep blue dyed latex beads for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate the cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

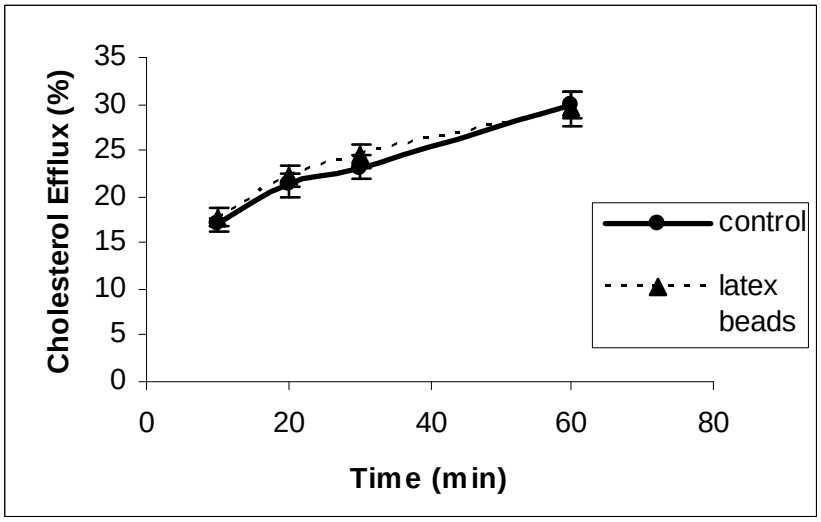


Fig. 3.28 Effect of deep blue dyed latex beads on efflux of plasma membrane derived cholesterol to MβCD. Cells were labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium with or without 1% deep blue dyed latex beads for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

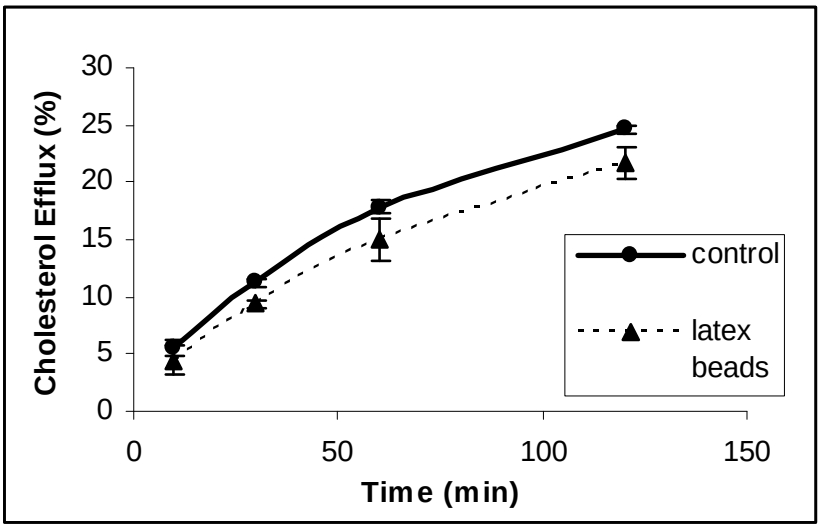


Fig. 3.29 Effect of deep blue dyed latex beads on efflux of de novo synthesized cholesterol to MβCD. Cells were cultured in delipidated EMEM medium for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium with or without 1% deep blue dyed latex beads for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

3.3.6. U18666A

U18666A at nanomolar concentration selectively interferes with cholesterol trafficking to the ER without substantively affecting cholesterol transfer to the plasma membrane, while micromolar concentration of U18666A inhibits multiple pathways of cholesterol trafficking from late endosomes (Underwood et al., 1996).

In order to study the potential effect of such cholesterol intracellular trafficking on cholesterol efflux, fibroblasts were treated with 70 nM or 2 µM U18666A before

cholesterol efflux was initiated. The results showed in Fig. 3.31-3.33 indicate that efflux of cholesterol, whether it was plasma membrane derived or freshly synthesized, was not affected when the acceptor as MβCD. However, when apoA-I acted as acceptor, the efflux rate was reduced by U18666A for plasma membrane derived cholesterol but not the de novo synthesized cholesterol in a doze dependent manner, more retardations being observed at higher U18666A concentration.

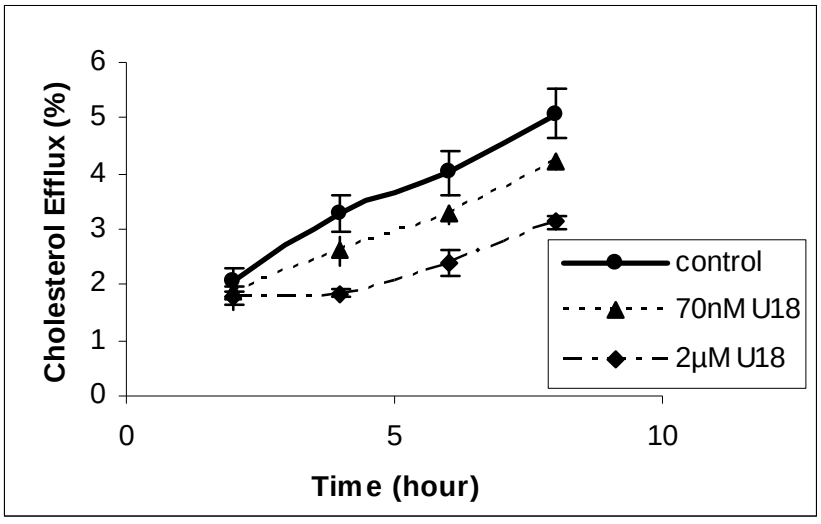


Fig. 3.30 Effect of U18666A on efflux of plasma membrane derived cholesterol to apoA-I. Cells were cultured in complete EMEM medium with 5 μM TO-901317 for 24 hours and then labeled with 1 μCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium without or with 70 nM U18666A or 2 μM U18666A for two hours. Thereafter, 10 μg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

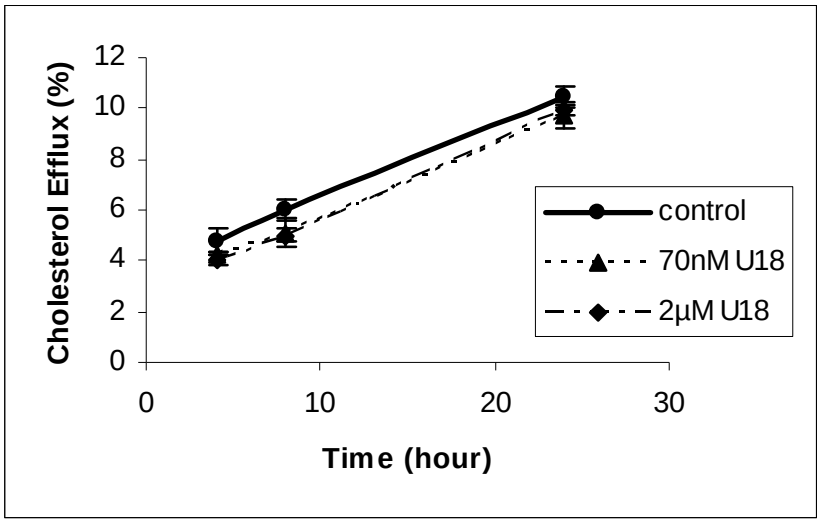


Fig. 3.31 Effect of U18666A on efflux of de novo synthesized cholesterol to apoA-I. Cells were cultured in delipidated EMEM medium with 5 µM TO-901317 for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium without or with 70 nM U18666A or 2 µM U18666A for two hours. Thereafter, 10 µg/ml apoA-I in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

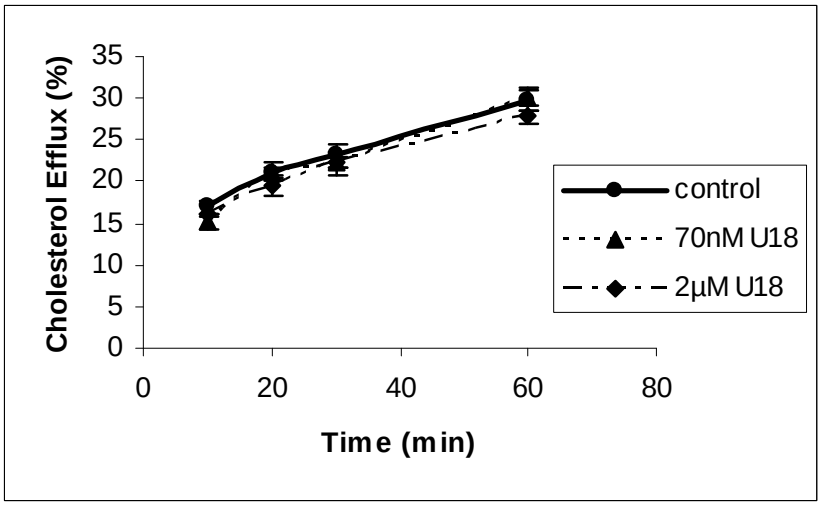


Fig. 3.32 Effect of U18666A on efflux of plasma membrane derived cholesterol to MβCD. Cells were labeled with 1 µCi/ml ^{3}H-cholesterol for 24 hours. After washing, cells were incubated in serum-free EMEM medium without or with 70 nM U18666A or 2 µM U18666A for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

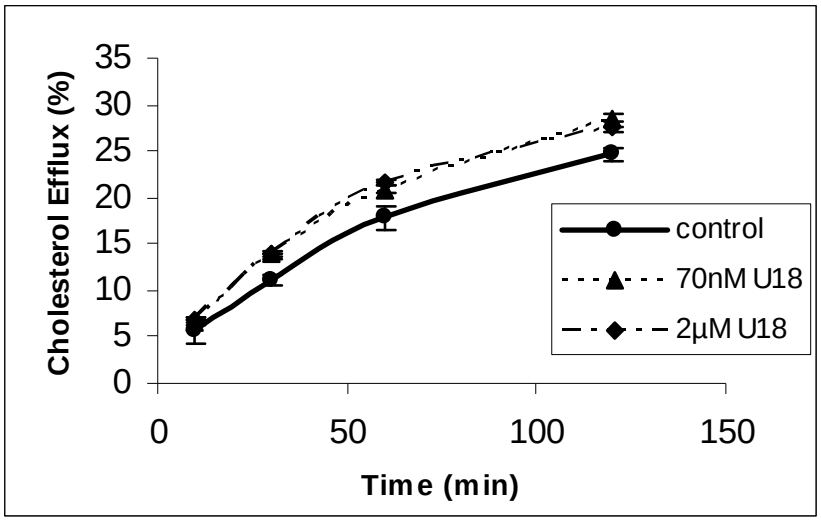


Fig. 3.33 Effect of U18666A on efflux of de novo synthesized cholesterol to MβCD. Cells were cultured in delipidated EMEM medium for 48 hours and then labeled with 10 µCi/ml ^{3}H-acetate for 2 hours. After washing, cells were incubated in serum-free EMEM medium without or with 70 nM U18666A or 2 µM U18666A for two hours. Thereafter, 0.1% MβCD in serum-free EMEM medium was added to initiate cholesterol efflux. The experiments were performed at 37 °C and each value is the mean ± S.D. of triplicates.

CHAPTER 4. DISCUSSION

4.1. Effect of TO-901317 on ABCA1 expression and cholesterol efflux: cholesterol efflux to apoA-I does involve ABCA1

The liver X receptors (LXRs) are members of the nuclear receptor and transcription factor superfamily and have two isoforms including LXRα and LXRβ. LXRα is highly expressed in liver, intestine, kidney and macrophages. LXRβ is ubiquitously expressed in nearly every tissue. Two isoforms of LXRs have a central DNA-binding domain consisting of a zinc-finger module and a large ligand-binding domain with a lipophilic core that binds specific small lipid molecules. After ligands bind, LXRs form heterodimers with the retinoic acid receptor (RXR) which binds to characteristic DNA sequence of target genes including ABCA1, ABCG5, ABCG8 and apolipoprotein E (apoE). The heterodimers can be activated by ligands for either receptor (Repa and Mangelsdorf, 2002).

ABCA1 belongs to the ABC family of genes encoding transmembrane proteins which are believed to transport a specific set of molecules across the membrane. ABCA1 is believed to play a key role in the transfer of cholesterol and phospholipids to apoA-I, the initial step in HDL formation and RCT. Expression of ABCA1 which is dependent on the activation of LXR α/β is transcriptionally controlled and increases in response to cholesterol loading. However, neither FC nor CE can bind and activate LXRs. It is

speculated that increased intracellular cholesterol induces the production of oxysterols that serve as LXR ligands (Repa and Mangelsdorf, 2002). On the other hand, addition of oxysterols or synthetic LXR agonists such as TO-901317 induces ABCA1 expression and cholesterol efflux independent of cholesterol loading in macrophages (Rowe et al., 2003).

It has been demonstrated that activation of the nuclear receptor LXRα by oxysterol ligands up-regulates expression of ABCA1 in mouse NIH 3T3 fibroblasts or RAW264.7 macrophages. The data also show that ligand activation of LXRα is sufficient to promote cholesterol efflux to extracellular acceptor apoA-I through ABCA1 (Venkateswaran et al., 2000). These results suggest that cellular cholesterol efflux is controlled, at least partly, by a nuclear receptor signaling pathway. The ability of oxysterol ligands of LXR to stimulate cholesterol efflux is dramatically reduced in Tangier cells with known mutations of ABCA1. In addition, treating macrophages with the LXR nonsteroidal ligand TO-901317 also induces LXR-dependent up-regulation of ABCA1 expression. However, TO-901317 treatment did not affect the intracellular sterol status of these macrophages (Repa et al., 2000). The up-regulation of ABCA1 is attributed to the presence of a unique DR4 element in the ABCA1 promoter. Activated LXR forms a heterodimer with RXR and binds to the DR4 element, activating transcription of the ABCA1 gene (Gan et al., 2001).

In this study, treating human fibroblast with TO-901317 increased ABCA1 expression and the cholesterol efflux to apoA-I, whether cholesterol was derived directly from the plasma membrane or newly synthesized. However, cholesterol efflux to MβCD was not

affected. These results have confirmed the previous conclusion, largely obtained in macrophage, in human fibroblasts. Furthermore, these results imply that cholesterol efflux to MβCD may not be, while cholesterol efflux to apoA-I is clearly ABCA1 dependent. In other word, ABCA1 does play a key role in cholesterol efflux to apoA-I.

4.2. Role of caveolae in cholesterol efflux

Caveolae are glycosphingolipids/cholesterol rich, clathrin-free plasma membrane invaginations with a characteristic diameter of 50 to 100 nm (Smart et al., 1999). The core structure of caveolae is enriched in cholesterol, gangliosides, ceramide, diacylglycerol (Liu and Anderson, 1995), phosphatidylinositol diphosphate (Pike and Casey, 1996), and the integral membrane protein caveolins which are cholesterol binding proteins with three isoforms (Murata et al., 1995). Although representing only 1-2% of cell plasma membrane, caveolae contain 6-7% of the total cellular cholesterol. If plasma membrane contains 90% of the cellular cholesterol, the cholesterol concentration in caveolae membranes is 4 to 8-fold higher than the surrounding non-caveolae plasma membrane. In living cells, only FC in caveolae was found accessible to filipin and cholesterol oxidation (Smart et al., 1994). The properties of this FC pool in caveolae are different from those in non-caveolae plasma membrane domains, while have led to the hypothesis that caveolae may play a role in cellular cholesterol metabolism.

Previous observations indicate that caveolae play a role in cholesterol transport. (i) Caveolin, structure protein of caveolae, is a cholesterol-binding protein (Smart et al.,

1999). (ii) The nascent cholesterol synthesized in ER first appears in the caveolae domain of the plasma membrane and then non-caveolae membrane. It was proposed that the newly synthesized cholesterol was transported by a heat-shock multiprotein complex consisting of caveolin, heat-shock protein 56, cyclophilin 40, cyclophilin A, and cholesterol (Uittenbogaard et al., 1998). (iii) Progesterone, which blocks cholesterol transport, causes caveolin accumulation in the lumen of the ER simultaneously (Smart et al., 1996). (iv) Cholesterol oxidase treatment causes caveolin to dissociate from plasma membrane and redistribute to intracellular vesicles that co-localize with Golgi apparatus markers (Smart et al., 1994). (v) The caveolin mRNA levels and caveolin expression and cellular FC content are intimately related and may display reciprocal regulation. An increase in LDL–FC internalization was associated with proportional cellular FC and up-regulation of caveolin (Fielding et al., 1997). Depletion of caveolar FC by CD, led to down-regulation of caveolin mRNA and cell surface protein levels (Hailstones et al., 1998). On the other hand, lymphocytes expressing caveolin had a 4.4-fold higher concentration of cholesterol in the caveolae fraction and about 4 times higher cholesterol transport speed to plasma membrane than control cells lacking caveolin (Smart et al., 1996). (vi) SR-BI which mediates cholesterol flux between cells and lipoproteins is concentrated in caveolae. This suggests that caveolae are the sites in plasma membrane for cholesterol exchange (Graf et al., 1999). (vii) ApoA-I (or pre-β-HDL) could bind directly to the FC-rich exofacial face of the caveolae and enhance the transport of cholesterol to the caveolae in a dose- and time- dependent manner (Sviridov et al., 2001). (viii) When ^{3}H-labeled FC from labeled LDL was selectively transferred into the cells to increase cellular FC, the cholesterol concentration in caveolae increased dramatically.

Subsequent incubation of these cells with unlabeled native plasma or high density lipoprotein selectively unloads FC from such caveolae (Fielding and Fielding, 1995). All these studies suggest that caveolae play an essential role in cholesterol metabolism. However, the direct evidence about this process is absent. In addition, some data available remain controversial, partly due to the different cell types used and the different analytical methods adopted.

For instance, it is pointed out that caveolin-1 down-regulation enhanced cellular cholesterol efflux (Frank et al., 2001). In such experiments, the cholesterol efflux in cells that contained less caveolae could transfer intracellular stored cholesterol to acceptor faster via a pathway not involving caveolin-1 compared to control cells. This is apparently inconsistent with the results of other studies.

To investigate the role of caveolae in intracellular cholesterol trafficking and efflux in human fibroblast, the cells were treated with 10 mM MβCD for 30 min to disrupt caveolae structure (Fig. 3.6). Cholesterol depletion by MβCD treatment should cause an entire change in the morphology of caveolae. After cholesterol depletion, caveolae invaginations might have either disappeared or become shallow with wide openings. Immunogold labeling of caveolin showed that the caveolin remained clustered, indicating that underlying caveolar structures were still present in the membrane after 50% reduction of the amount of plasma membrane cholesterol by 10 mM MβCD treatment, perhaps reflecting the existence of flattened caveolar patches or “rafts” in the membrane after the extensive but partial cholesterol depletion (Parpal et al., 2001). In this study, it

has been found that 10 mM MβCD treatment reduced cellular cholesterol by 75%, resulting in increase in cholesterol efflux to apoA-I for about 4 fold. Although the cholesterol concentration in caveolae is high, only a small quantity of cholesterol exits in the exofacial face of the caveolae. ApoA-I could bind directly to the exofacial face of the caveolae to facilitate FC desorption (Saito et al., 1997). In other words, the cholesterol concentration on the exofacial face of caveolae partially determinates the rate of the cholesterol efflux mediated by apoA-I. Such dramatic increase in cholesterol efflux observed in this study may be explained by the following considerations. MβCD treatment extracts cholesterol from cell membrane, disrupts caveolae structure and thus probably changes the membrane environment of caveolae and surrounding non-caveolae membrane. It is hence possible that some cholesterol transfer from cytofacial face of the membrane to the exofacial face of the membrane and the higher cholesterol concentration on the exofacial face of plasma membrane induces higher cholesterol efflux rate. Secondly, previous study pointed out that sphingomyelin which is rich in caveolae preferentially interacts with cholesterol and can decrease sterol transfer between membranes. Cells containing less caveolae or reduced sphingomyelin/cholesterol-rich plasma domains can transfer cholesterol to an acceptor faster than control cells. In that case, cholesterol could be transferred from intracellular cholesterol pool(s) to the plasma membrane via a pathway bypassing caveolin-1 (Frank et al., 2001). If this is the case, disassembly of caveolae caused by MβCD extraction will free cholesterol from sphingomyelin and the cholesterol transport between membranes will be enhanced, resulting in higher cholesterol efflux to apoA-I from MβCD-treated fibroblasts compared

to that of the control cells. More studies are needed to verify if these two hypotheses are correct.

4.3. How does cholesterol intracellular trafficking affect its efflux?

4.3.1. Golgi apparatus

When cells were labeled with ^{3}H-cholesterol directly, the majority of cellular ^{3}H-cholesterol was maintained in the plasma membrane and only a minority of cellular ^{3}H-cholesterol recycled between plasma membrane and cell interior. Although this process is inhibited by various drugs, inhibition induced by BFA was not reported. So it is reasonable to find that cholesterol efflux was not affected by BFA treatment in cells labeled with ^{3}H-cholesterol whether the efflux was to apoA-I or MβCD (Fig. 3.8 and Fig. 3.10).

It has been reported that Golgi apparatus plays an active role in the movement of LDL-derived cholesterol from lysosomes to plasma membrane (Coxey et al., 1993). Treatment with BFA resulted in enhanced cholesterol delivery to ACAT. This could be due to LDL-derived cholesterol destined for the plasma membrane being redistributed to ER by blocking the Golgi dependent pathway (Neufeld et al., 1996).

On the other hand, newly synthesized cholesterol was found to be transported from ER to plasma membrane via a vesicular system (Kaplan and Simoni, 1985). An efficient alternative pathway for nascent cholesterol movement was proposed because severe disruption of Golgi apparatus did not alter the kinetics of cholesterol arrival at the plasma membrane (Urbani and Simoni, 1990). According to this hypothesis, the movement of a portion of the newly synthesized cholesterol from ER to plasma membrane occurs by a Golgi apparatus-independent pathway. It was reported that newly synthesized cholesterol is transported from ER to plasma membrane caveolae for efflux through a heat-shock protein-immunophilin chaperone complex consisting of caveolin, heat-shock protein 56, cyclophilin 40, cyclophilin A, and cholesterol (Uittenbogaard et al., 1998). Recently, it has been found that both raft-poor and raft-rich vesicular transport from the Golgi apparatus increased during ABCA1 mediated cholesterol efflux (Zha et al., 2003). Taken together, both Golgi-dependent and –independent pathways may be involved in the intracellular cholesterol transport and efflux from ER.

In this study, BFA treatment partly inhibited nascent cholesterol efflux to apoA-I (Fig. 3.9). This is consistent with the notion that the Golgi apparatus plays an active role in cholesterol transport from ER to the plasma membrane sites available for efflux to apoA-I. Disruption of Golgi by BFA treatment would hamper the trafficking of cholesterol to the plasma membrane for efflux. The significance of Golgi-apparatus in cholesterol efflux appears only to the process mediated by ABCA1 as efflux of newly synthesized cholesterol to MβCD was not affected by BFA treatment (Fig. 3.11).

4.3.2. Cytoskeleton

4.3.2.1. Microtubule network

In many cell types, microtubules (MTs) radiate outward from a perinuclear MT-organizing center (MTOC) and serve as intracellular “highways” along which tubulovesicular transport intermediates of the secretory and endocytic pathways travel between the cell centre and the plasma membrane. Due to their radial organization in the cell and their involvement in membrane transport, MTs are responsible for maintaining the normal intracellular locations and organization of the membrane systems such as ER, the Golgi apparatus, endosomes and lysosomes (Conrad et al., 1995). So far, the role of microtubule in several aspects of cholesterol metabolism has been investigated:

(i). Caveolin cycles between the plasma membrane and the Golgi apparatus through a multi-step process. One step, ER/Golgi intermediate compartment (ERGIC)-to-Golgi transport, is microtubules-dependent. This bidirectional pathway may indicate roles for microtubules in maintaining caveolae and for caveolin in shuttling cholesterol between the plasma membrane and the ER/Golgi system (Conrad et al., 1995).

(ii). Are microtubules involved in the transport of LDL-derived cholesterol? Some researchers pointed out that disruption of microtubules had no effect on movement of LDL-derived cholesterol to ER (Underwood et al., 1998) or plasma membrane (Liscum and Dahl, 1992; Liscum, 1990). On the other hand, the contrary conclusion has also been

reported that the transport of intracellular ^{3}H-FC to the cell surface was reduced by nocodazole which disrupts microtubules network (Fielding and Fielding, 1996).

(iii). Microtubules play a role in the transport of cholesterol both to and from the mitochondria (Crivello and Jefcoate, 1980).

(iv). It has been observed that in intestinal cells the transport of newly synthesized cholesterol from ER to the plasma membrane did not require microtubules. These authors used cholesterol oxidase to estimate the amount of cholesterol reached the cell surface (Field et al., 1998). However, nocodazole treatment inhibited the transport of newly synthesized cholesterol to the plasma membrane by about 25% when MβCD was used to extract cholesterol from cell surface (Heino et al., 2000).

In this study, the efflux of plasma membrane derived cholesterol was not affected by nocodazole treatment whether the efflux was to apoA-I or MβCD (Fig. 3.12 and Fig. 3.14). However, about 25% of the efflux of newly synthesized cholesterol to MβCD was inhibited by nocodazole (Fig. 3.15). This is consistent with the conclusion of Heino et al (2000). However, the efflux of newly synthesized cholesterol to apoA-I was not affected too much by nocodazole treatment. As MβCD and apoA-I acquire cholesterol from cell surface via different mechanisms and may even from different membrane domains; the different efflux rate caused by different acceptors may suggest the existence of separate intracellular cholesterol transport pathways or cholesterol pools. It appears that the transport of cholesterol to the membrane domains which provide cholesterol for efflux to

MβCD involves microtubules, while the transport to the domains which provide cholesterol for efflux to apoA-I is not affected by microtubules.

4.3.2.2. Actin microfilament

Actin filaments determine the shape of the cells. The dynamic behavior of this network is necessary for many of the cell motility processes. Microfilament is a dynamic structure, maintained by a rapid and continual exchange of compact and globular subunits between the soluble and filamentous forms and this subunit flux is necessary for normal cytoskeletal function. Some drugs, such as cytochalasin and jasplakinolide, have a rapid and profound effect on the organization of the microfilament in living cells (Alberts et al., 2002).

The involvement of actin filaments in the transport process was demonstrated by treatments of three specific inhibitors, cytochalasins, anti-actin antibodies and DNase I. Actin-depolymerizing drug cytochalasin reduced the uptake of ^{3}H-LDL from coated pits by an average of 65% at 40 μM, probably by preventing the polymerization of actin into microfilaments required for effective invagination (Fielding and Fielding, 1996). It was reported that jasplakinolide, which promotes actin polymerization and stabilizes the actin filaments (Bubb et al., 1994), inhibited nascent cholesterol transport slightly but reproducibly. This inhibition effect was apparently additive with that of BFA, suggesting jasplakinolide affected the Golgi-independent cholesterol transport pathway (Lusa et al., 2003). Disruption of actin filaments in macrophages leads to a specific inhibition of

cholesterol esterification. The inhibition effect can not be explained by reduced cellular cholesterol content or general inhibitory effects on cholesterol trafficking or the ACAT enzyme in these cells. Rather, the data imply that an intact actin cytoskeleton plays a key role in the process that occurs specifically when lipoproteins interact with macrophages (Tabas et al., 1994).

Further more, actin filaments are essential for caveolae integrity which may play a key role in cholesterol trafficking and efflux. Ultrastructural and biochemical analyses have indicated an association of caveolae with the actin filaments (Chatenay-Rivauday et al., 2004). Treatment with actin-depolymerizing drug cytochalasin D or high concentration (>5mM) CD increases the mobility of caveolin and caveolae in the plasma membrane. Corresponding to the treatment, the number of caveolin located near the cell surface declines markedly and caveolae move laterally and cluster in the plane of the membrane. These results strongly indicate that both cholesterol and an intact actin cytoskeleton are required for the integrity and immobility of caveolae which is very important for cholesterol trafficking and efflux (Deurs et al., 2003; Thomsen et al., 2002).

In this study, human fibroblasts were treated with cytocalasin D or jasplakinolide, respectively, to investigate the role of actin microfilaments in cholesterol trafficking and efflux. As shown in the results, both cytocalasin D (Fig. 3.21) and jasplakinolide (Fig. 3.16) treatments changed cell morphology dramatically. Most protrusions of the cells disappeared and the fibrous network labeled with fluorescence also disappeared. The cells treated with cytocalasin D became round and the fluorescence distributed in the cell

uniformly. However, the shape of cells treated with jasplakinolide became starfish-like. The majority of the fluorescence labeled actin subunits concentrated in the center of the cell body and a small part of them distributed in the dendrites of the cells. Both cytocalasin D and jasplakinolide did not change cholesterol efflux to MβCD whether cholesterol is derived directly from the plasma membrane or from do novo synthesized pool. It is interesting to find that both cytocalasin D and jasplakinolide dramatically increased cholesterol efflux to apoA-I under similar conditions. In these experiments, the nascent cholesterol was synthesized for 2 hours and subsequently treated with the drugs for 2 hours at 37°C. Some of the nascent cholesterol may have been transported to the plasma membrane at the time when efflux was initiated. Thus, the apparent efflux observed may reflect more on the property of the plasma membrane rather than the intracellular trafficking. As previously discussed, caveolae play a key role in cholesterol trafficking and efflux regulation. Actin filaments and cholesterol are essential for caveolae integrity and function (Chatenay-Rivauday et al., 2004). It may therefore be assumed that change of caveolae caused by actin filament disruption increased the cholesterol efflux to apoA-I. How disruption of caveolae would increase cholesterol efflux to apoA-I has been discussed before. Moreover, cholesterol efflux to MβCD did not change by treatment with cytocalasin D and jasplakinolide may be explained by its non-specific extraction of cholesterol from the cell plasma membrane.

It is noted that both cytocalasin D and jasplakinolide, which affect actin filaments in opposite directions, enhanced cholesterol efflux to apoA-I (Fig. 3.17, Fig. 3.18, Fig. 3.22 and Fig. 3.23). How can cytocalasin D and jasplakinolide have the same effect on

cholesterol efflux to apoA-I? The actin cytoskeleton is a dynamic filament network which polymerizes and depolymerizes continuously. This dynamic equilibrium is essential for multiple cellular functions involving actin filaments. Depolymerizing of actin filaments by cytocalasin D or stabilizing of actin filaments by jasplakinolide both change the structure and function of the filaments at equilibrium which may explain why both cytocalasin D and jasplakinolide increased cholesterol efflux to apoA-I. This explanation is supported by the fact that treatment of adipocytes with actin-depolymerizing agent cytochalasin D or the actin stabilizing agent jasplakinolide both inhibit insulin-stimulated GLUT4 translocation (Kanzaki and Pessin, 2002).

4.3.3. Plasma-ER membrane contact

It was pointed out that the movement of newly synthesized cholesterol from ER to plasma membrane might occur by a Golgi apparatus-independent pathway although the mechanism has not been identified (Urbani and Simoni, 1990). Three candidates have been proposed including specialized transport vesicles, transport by soluble sterol-binding proteins and transport at regions of close apposition of the ER and plasma membrane (Prinz, 2000). The third mechanism is suggested by studies on the transport of phospholipids to mitochondria, where membrane association between mitochondria and the ER of the yeast *Saccharomyces cerevisiae* is probably required for phospholipid translocation between these two organelles (Achleitner et al., 1999).

Lusa et al. (2003) demonstrated that promotion of ER-plasma membrane contacts induced by latex beads did not affect efflux of nascent cholesterol and prelabeled cholesterol to serum in baby hamster kidney (BHK) cells (Lusa et al., 2003). Here I tested if ER-plasma membrane contacts would affect the cholesterol efflux in human fibroblast. It is consistent to the previous study in BHK cells that latex beads treatment does not change the efflux of nascent cholesterol and preexisting cholesterol to apoA-I or MβCD (Fig. 3.26 – Fig. 3.29). From the results, I know that promotion of ER-plasma membrane contacts did not affect cholesterol efflux. However, we could not draw the conclusion that ER-plasma membrane was not involved in the intracellular cholesterol transport and efflux. It is possible that the original ER-plasma membrane contacts without latex beads treatment are already sufficient for cholesterol movement from ER to plasma membrane. More investigations are needed to verify if ER-plasma membrane contacts play a role in cholesterol trafficking and efflux.

4.3.4. Effect of U18666A on intracellular cholesterol transport

The pharmacological agent U18666A has been reported to inhibit multiple intracellular cholesterol transport pathway. Most studies about U18666A demonstrate that U18666A inhibits the transport of LDL-derived cholesterol from late endosomes/lysosomes to plasma membrane and causes LDL-derived cholesterol accumulating at high levels in late endosomes/lysosomes (Liscum and Faust, 1989). U18666A also inhibits both the basal movement of plasma membrane cholesterol to ACAT and the accelerated delivery of

plasma membrane cholesterol to ACAT that responds to plasma membrane sphingomyelin degradation (Underwood et al., 1996). In addition to affecting the transport of cholesterol from the plasma membrane to the ER, U18666A was also reported to inhibit the transfer of cholesterol to the mitochondria. In steroidogenic cells, cholesterol is used as a precursor for synthesis of steroid hormones and the transport of cholesterol to mitochondria plays an important role in the cellular cholesterol homeostasis. A time-course study pointed out that the inhibition induced by U18666A of plasma membrane cholesterol to cell interior was rapid (within l0-15 min) and reversible if U18666A was removed from the incubation mixture (Härmälä et al., 1994). However, the mechanism by which U18666A affects various intracellular cholesterol transport pathways is unclear.

It was also found that U18666A inhibits cholesterol esterification in ER. This effect is probably not a result of a direct inhibition of ACAT activity by U18666A because this drug cannot inhibit the esterification of oleoyl-CoA to cholesterol by ACAT in cell-free homogenates. It was supposed that the inhibition of cholesterol esterification by U18666A arose from the effect of U18666A on cholesterol transfer steps (Härmälä et al., 1994). On the contrary, Underwood et al. (1996) pointed out that this inhibition involves more than the simple retardance of cholesterol movement from lysosomes to the plasma membrane and then from the plasma membrane to ER and it must also inhibit a previously unknown pathway or a signal event (Underwood et al., 1996). Another verified effect of U18666A on cholesterol metabolism is that U18666A reduces

cholesterol de novo synthesis dramatically by inhibiting a step in cholesterol synthesis distal to HMG-CoA reductase (Chen et al., 1993).

In this study, I treated cells with 70 nM or 2 μM U18666A to examine if different concentration of U18666A would affect cholesterol efflux differently. The results show that U18666A treatment didnot affect the efflux to MβCD whether the cholesterol is plasma membrane-derived or newly synthesized (Fig 3.32 and Fig. 3.33). When apoA-I was used as the acceptor, U18666A reduced the efflux of plasma membrane-derived cholesterol in a dose-dependent manner, while the efflux of nascent cholesterol was not affected significantly.

Underwood et al. (1998) demonstrated that U18666A inhibits movement of FC from late endosomes/lysosomes into a cholesterol oxidase accessible pool. Further study pointed out that when concentration of U18666A increased, the size of the cholesterol oxidase accessible pool of cholesterol decreased (Kellner-Weibel et al., 1999). In living cells, only FC in caveolae was found accessible to cholesterol oxidation (Smart et al., 1994). In other word, U18666A treatment will reduce the cholesterol concentration in caveolae. From previous discussion, we know that cholesterol efflux to apoA-I is closely correlated with caveolae. Taken together, it is reasonable that the lower cholesterol concentration in caveolae induced by U186666A treatment will reduce cholesterol efflux to apoA-I, as observed in this study. However, the mechanism of U18666A treatment is very complicated and unclear until now. More investigation is certainly needed in this area.

4.5. Summary of cholesterol intracellular cholesterol trafficking and cholesterol efflux

To maintain cellular cholesterol homeostasis, HDL or its apolipoproteins remove excess FC from cells. This process prevents the excessive accumulation of cholesterol in the vessel wall and the development of atherosclerosis. In this study, we investigated the mechanism of intracellular cholesterol trafficking and its effect on cholesterol efflux to extracellular cholesterol acceptors, namely, apoA-I and MβCD, in human fibroblast. Here, the cholesterol for efflux was from two different cholesterol pools: plasma membrane derived cholesterol, which was directly labeled with ^{3}H-cholesterol, and de novo synthesized cholesterol labeled by using ^{3}H-actate as the precursor. Before cholesterol efflux, the cells were treated with different drugs which would affect the microtubules, the actin network, the Golgi apparatus and the ER, respectively to examine if these factors would contribute to cholesterol trafficking and efflux.

ApoA-I is the main protein of HDL that plays a key role in cholesterol efflux in vivo. ApoA-I could bind directly to the exofacial face of the caveolae to facilitate FC desorption (Saito et al., 1997). It has been pointed out that apoA-I can stimulate the translocation of intracellular cholesterol to the plasma membrane (Oram and Yokoyama, 1996) and enhance the efflux of intracellular cholesterol (Sviridov and Fidge, 1995). On the other hand, MβCD is a non-specific acceptor for cholesterol. It gets cholesterol from both caveolae and non-caveolae membrane domains. The results obtained in this study confirm that the kinetics of cholesterol effluxes to apoA-I or MβCD were clearly

different in most cases, probably due to the fact that apoA-I and MβCD take cholesterol from different cholesterol pools.

Also from the results of this study, it is know that caveolae are the key regulator of intracellular cholesterol trafficking and efflux. Disassembly of caveolae by cholesterol depletion markedly increased the cholesterol efflux. Disruption of actin microfilaments which are necessary for caveolae integrity also significantly enhanced cholesterol efflux. One of the other conclusions can be drawn through this study is that Golgi apparatus appears to play a minor role in the movement of nascent cholesterol from ER to plasma membrane. It seems that microtubules, U18666A-inhibited cholesterol intracellular trafficking and ER-plasma membrane contacts did not affect cholesterol efflux at any significant level. However, more investigations are needed to verify these observations.

5. Reference

Achleitner G., Gaigg B., Krasser A., Kainersdorfer E., Kohlwein S. D., Perktold A., Zellnig G., and Daum G. (1999) Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. *Eur J Biochem* **264**, 545-553

Alberts B., Johnson A., Lewis J., Raff M., Roberts K., and Walter P. (2002) *Molecular Biology of the Cell* **4**th edition

Angelin B. (1995) Studies on the regulation of hepatic cholesterol metabolism in humans. *Eur J Clin Invest* **25**, 215-224

Babitt J., Trigatti B., Rigotti A., Smart E. J., Anderson R. G. W., Xu S., and Krieger M. (1997) Murine SR-B1, a high density lipoprotein receptor that mediates selective lipid uptake, is *N*-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. *J Biol Chem* **272**, 13242-13249

Blanchette-Mackie E. J. (2000) Intracellular cholesterol trafficking: role of the NPC1 protein. *Biochim Biophys Acta* **1486**, 171-183

Brasaemle D. L. and Attie A. D. (1990) Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts. *J Lipid Res* **31**, 103-112

Brooks-Wilson A., Marcil M., Clee S. M., Zhang L. H., Roomp K., van Dam M., Yu. L., Brewer C., Collins J. A., Molhuizen H. O. F., Loubser O., Ouelette B. F. F., Fichter K., Ashbourne-Excoffon K. J. D., Sensen C. W., Scherer S., Mott S., Denis M., Martindale D., Frohlich J., Morgan K., Koop B., Pimstone S., Kastelein J. J. P., Genest J. Jr and Hayden M. R. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet* **22,** 336-345.

Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W., Barlage S., Büchler C., Porsch-Özcürümez M., Kaminski W. E., Hahmann H. W., Oette K., Rothe G., Aslanidis C., Lackner K. J. and Schmitz G. (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet* **22**, 347-351

Brown M. S. and Goldstein J. L. (1980) Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J Lipid Res* **21**, 505-517

Brown M. S. and Goldstein J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* **232**, 34-47

Bubb M. R., Senderowicz A. M. J., Sausville E. A., Duncan K. L. K., and Korn E. D. (1994) Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competively inhibits the binding of phalloidin to F-actin. *J Biol Chem* **269**, 14869-14871

Butler J. D., Blanchette-Mackie J., Goldin E., O'Neill R. R., Carstea G., Roff C. F., Patterson M. C., Patel S., Comly M. E., Cooney A., Vanier M. T., Brady R. O., and Pentchev P. G . (1992) Progesterone blocks cholesterol translocation from lysosomes. *J Biol Chem* **267**, 23797-23805.

Casella J. F., Flanagan M. D., and Lin S. (1981) Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. *Nature* **293**, 302-305

Chambenoit O., Hamon Y., Marguet D., Rigneault H., Rosseneu M. and Chimini G. (2001) Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. *J Biol Chem* **276**, 9955-9960

Chang T.Y., Chang C. C.Y., and Cheng D. (1997) Acyl coenzyme A: cholesterol acyltransferase. *Annu Rev Biochem* **66**, 613-638

Chao W. T., Fan S. S., Chen J. K., and Yang V. C. (2003) Visualizing caveolin-1 and HDL in cholesterol-loaded aortic endothelial cells. *J Lipid Res* **44**, 1094-1099

Chatenay-Rivauday C., Cakar Z. P., Jenö P., Kuzmenko E. S., and Fiedler K. (2004) Caveolae: biochemical analysis. *Molecular Biology Reports* **31,** 67-84

Chen H., Born E., Mathur S. N., and Field F. J. (1993) Cholesterol and sphingomyelin syntheses are regulated independently in cultured human intestinal cells, CaCo-2: role of membrane cholesterol and sphingomyelin content. *J Lipid Res* **34**, 2159-2167

Chinetti G., Lestavel S., Bocher V., Remaley A. T., Neve B., Torra I. P., Teissier E., Minnich A., Jaye M., Duverger N., Brewer H. B., Fruchart J. C., Clavey V., and Staels B. (2001) PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med* **7,** 53-58

Conrad P. A., Smart E. J., Ying Y. S., Anderson R. G., and Bloom G. S. (1995) Caveolin cycles between plasma membrane caveolae and the Golgi complex by microtubule-dependent and microtubule-independent steps. *J Cell Biol* **131**, 1421-1433

Coxey R. A., Pentchev P. G., Campbell G., and Blanchette-Mackie E. J. (1993) Differential accumulation of cholesterol in Golgi compartments of normal and Niemann-Pick type C fibroblasts incubated with LDL: a cytochemical freeze-fracture study. *J Lipid Res* **34**, 1165-1176

Crivello J. F. and Jefcoate C. R. (1980) Intracellular movement of cholesterol in rat adrenal cells. Kinetics and effects of inhibitors. *J Biol Chem* **255**, 8144-8151

Daniel J. R. MD. (2003) Regulation of reverse cholesterol transport and clinical implications. *Am J Cardiol* **92**(suppl), 42J-49J

Dean M., Hamon Y., and Chimini G. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res* **42**, 1007-1017

de la Llera-Moya M., Connelly M. A., Drazul D., Klein S. M., Favari E., Yancey P. G., Williams D. L., and Rothblat G. H. (2001) Scavenger receptor class B type I affects cholesterol homeostasis by magnifying cholesterol flux between cells and HDL. *J Lipid Res* **42**, 1969-1978

de la Llera-Moya M., Rothblat G. H., Connelly M. A., Kellner-Weibel G., Sakr S. W., Phillips M. C., and Williams D. L. (1999) Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. *J Lipid Res* **40**, 575-580

DeGrella R. F. and Simoni R.D. (1982) Intracellular transport of cholesterol to the plasma membrane. *J Biol Chem* **257**, 14256-14262

Deurs B. V., Roepstorff K., Hommelgaard A. M., and Sandvig K. (2003) Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol* **13**, 92-100

Dietschy J. M., Turley S. D., and Spady D.K. (1993) Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species including humans. *J Lipid Res* **34**, 1637-1659

Evans R. M. (1994) Intermediate filaments and lipoprotein cholesterol. *Trends Cell Biol* **4**, 149-151

Field F. J., Born E., Murthy S., and Mathur S. N. (1998) Transport of cholesterol from the endoplasmic reticulum to the plasma membrane is constitutive in CaCo-2 cells and differs from the transport of plasma membrane cholesterol to the endoplasmic reticulum. *J Lipid Res* **39**, 333-343

Fielding C. J., Bist A., and Fielding P. E. (1997) Caveolin mRNA levels are up-regulated by free cholesterol and down-regulated by oxysterols in fibroblast monolayers. *Proc Natl Acad Sci U S A* **94**, 3753-3758

Fielding C. J. and Fielding P. E. (1997) Intracellular cholesterol transport. *J Lipid Res* **38,** 1503-1521

Fielding C. J. and Fielding P. E. (2001) Cellular cholesterol efflux. *Biochim Biophys Acta* **1533**, 175-189

Fielding P. E., and Fielding C. J., (1996) Intracellular transport of low density lipoprotein derived free cholesterol begins at clathrin-coated pits and terminates at cell surface caveolae. *Biochemistry* **35**, 14932-14938.

Fielding P. E. and Fielding C. J. (1995) Plasma membrane caveolae mediate the efflux of cellular free cholesterol. *Biochemistry* **34,** 14288-14292

Fielding P. E., Nagao K., Hakamata H., Chimini G., and Fielding C.J. (2000) A two-step mechanism for free cholesterol and phospholipid efflux from human vascular cells to apolipoprotein A-I. *Biochemistry* **39**, 14113-14120

Fitzgerald M. L., Morris A. L., Rhee J. S., Andersson L. P., Mendez A. J. and Freeman M. W. (2002) Naturally occurring mutations in the largest extracellular loops of ABCA1 can disrupt its direct interaction with apolipoprotein A-I. *J Biol Chem* **277**, 33178-33187

Francis G. A., Knopp R. H., and Oram J. F. (1995) Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier disease. *J Clin Invest* **96**, 78-87

Frank P. G., Galbiati F., Volonte D., Razani B., Cohen D. E., Marcel Y. L., and Lisanti M. P. (2001) Influence of caveolin-1 on cellular cholesterol efflux mediated by high-density lipoproteins. *Am J Physiol Cell Physiol* **280**, C1204-C1214

Fredrickson D. S. (1964) The inheritance of high density lipoprotein deficiency (Tangier disease). *J Clin Invest* **43**, 228-236

Fu Y., Hoang A., Escher G., Parton R. G., Krozowski Z., and Sviridov D. (2004) Expression of caveolin-1 enhances cholesterol efflux in hepatic cells. *J Biol Chem* **279**, 14140-14146

Gan X., Kaplan R., Menke J. G., MacNaul K., Chen Y., Sparrow C. P., Zhou G., Wright S. D., and Cai T. Q. (2001) Dual mechanisms of ABCA1 regulation by geranylgeranyl pyrophosphate. *J Biol Chem* **276**, 48702-48708

Gillotte K. L., Davidson W. S., Lund-Katz S., Rothblat G. H., and Phillips M. C. (1998) Removal of cellular cholesterol by pre-β-HDL involves plasma membrane microsolubilization. *J Lipid Res* **39**, 1918-1928

Gillotte K. L., Zaiou M., Lund-Katz S., Anantharamaiah G. M., Holvoet P., Dhoest A., Palgunachari M. N., Segrest J. P., Weisgraber K. H., Rothblat G. H., and Phillips M. C. (1999) Apolipoprotein-mediated plasma membrane microsolubilization: role of lipid affinity and membrane penetration in the efflux of cellular cholesterol and phospholipid. *J Biol Chem* **274**, 2021-2028

Glomset, J. A. (1968) The plasma lecithin:cholesterol acyltransferase reaction. *J Lipid Res* **9,**155-167

Graf G. A., Connell P. M., van der Westhuyzen D. R., and Smart E. J. (1999) The class B, type I scavenger receptor promotes the selective uptake of high density lipoprotein cholesterol ethers into caveolae. *J Biol Chem* **274**, 12043-12048

Graf G. A., Matveev S. V., and Smart E. J. (1999) Class B scavenger receptors, caveolae and cholesterol homeostasis. *Trends Cardiovasc Med* **9**, 221-225

Hailstones D., Sleer L. S., Parton R. G., and Stanley K. K. (1998) Regulation of caveolin and caveolae by cholesterol in MDCK cells. *J Lipid Res* **39**, 369-379

Härmälä Ann-Sofi., Isabella Pörn M., Mattjus P., and Slotte J. P. (1994) Cholesterol transport from plasma membranes to intracellular membranes is inhibited by 3β-[2-(diethylamino) ethoxy]androst-5-en-17-one. *Biochim Biophys Acta* **1211**, 317-325

Härmälä A-S., Isabella Pörn M., and Slotte J. P. (1993) Sphingosine inhibits sphingomyelinase-induced cholesteryl ester formation in cultured fibroblasts. *Biochim Biophys Acta* **1210**, 97-104

Heino S., Lusa S., Somerharju P., Ehnholm C., Olkkonen V. M., and Ikonen E. (2000) Dissecting the role of the Golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface. *Proc Natl Acad Sci U S A* **97**, 8375-8380

Horton J. D., Goldstein J. L., Brown M. S. (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* **109,** 1125-1131

Ikonen E. (2001) Roles of lipid rafts in membrane transport. *Curr Opin Cell Biol* **13**, 470-477

Ji Y., Jian B., Wang N., Sun Y., Moya M. L., Phillips M. C., Rothblat G. H., Swaney J. B., and Tall A. R. (1997) Scavenger receptor B1 promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem* **272**, 20982-20985

Jian B, de la Llera-Moya M., Ji Y., Wang N., Phillips M. C., Swaney J. B., Tall A. R., and Rothblat G. H. (1998) Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J Biol Chem* **273**, 5599-5606

Johnson W. J., Chacko G. K., Philips M. C., and Rothblat G.H. (1990) The efflux of lysosomal cholesterol from cells. *J Biol Chem* **265**, 5546-5553

Johnson W. J., Mahlberg F. H., Rothblat G. H., and Phillips M. C. (1991) Cholesterol transport between cells and high-density lipoproteins. *Biochim Biophys Acta* **1085**, 273-298

Kanzaki M. and Pessin J. E. (2002) Caveolin-associated filamentous actin (Cav-actin) defines a novel F-actin structure in adipocytes. *J Biol Chem* **277**, 25867-25869

Kaplan M. R. and Simoni R.D. (1985) Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. *J Cell Biol* **101**, 446-453

Kellner-Weibel G., de la Llera-Moya M., Connelly M. A., Stoudt G., Christian A. E., Haynes M. P., Williams D. L., and Rothblat G. H. (2000) Expression of scavenger receptor BI in COS-7 cells alters cholesterol content and distribution. *Biochemistry* **39**, 221-229

Kellner-Weibel G., Geng Y. J., and Rothblat G. H. (1999) Cytotoxic cholesterol is generated by the hydrolysis of cytoplasmic cholesteryl ester and transported to the plasma membrane. *Atherosclerosis* **146**, 309-319

Kellner-Weibel G., Luke S. J., and Rothblat G. H. (2003) Cytotoxic cellular cholesterol is selectively removed by apoA-I via ABCA1. *Atherosclerosis* **171**, 235-243

Knight B.L. (2004) ATP-binding cassette transporter A1: regulation of cholesterol efflux. *Biochem Soc Trans* **32**, 124-127

Krieger M. (1999) Charting the fate of the "good cholesterol": identification and characterization of the high-density lipoprotein receptor SR-BI. *Annu Rev Biochem* **68**, 523-558

Lange Y. (1994) Cholesterol movement from plasma membrane to rough endoplasmic reticulum. *J Biol Chem* **269**, 3411-3414

Lange Y., Strebel F., and Steck T. L. (1993) Role of the plasma membrane in cholesterol esterification in rat hepatoma cells. *J Biol Chem* **268**, 13838-13843

Lange Y., Ye J., Rigney M., and Steck T. L. (1999) Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. *J Lipid Res* **40**, 2264-2270

Liscum L. and Munn N. J. (1999) Intracellular cholesterol transport. Biochim Biophys Acta **1438**, 19-37

Langmann T., Klucken J., Reil M., Liebisch G., Luciani M. F., Chimini G., Kaminski W. E., and Schmitz G. (1999) Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. *Biochem Biophys Res Commun* **257**, 29-33

Lin G. and Oram J. F. (2000) Apolipoprotein binding to protruding membrane domains during removal of excess cellular cholesterol. *Atherosclerosis* **149**, 359-370

Liscum L. (1990) Pharmacological inhibition of the intracellular transport of low-density lipoprotein-derived cholesterol in Chinese hamster ovary cells. *Biochim Biophys Acta* **1045**, 40-48

Liscum L. and Dahl N. K. (1992) Intracellular cholesterol transport. *J Lipid Res* **33**, 1239-1254

Liscum L. and Faust J. R. (1989) The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3- beta-[2-(diethylamino)ethoxy]androst-5-en-17-one. *J Biol Chem* **264**, 11796-11806

Liscum L. and Munn N. J. (1999) Intracellular cholesterol transport. *Biochim Biophys Acta* **1438**, 19-37

Liu P. and Anderson R. G. W. (1995) Compartmentalized production of ceramide at the cell surface. *J Biol Chem* **270**, 27179-27185

Lusa S., Heino S., and Ikonen E. (2003) Differential mobilization of newly synthesized cholesterol and biosynthetic sterol precursors from cells. *J Biol Chem* **278**, 19844-19851

Mendez A. J. and Uint L. (1996) Apolipoprotein-mediated cellular cholesterol and phospholipid efflux depend on a functional Golgi apparatus. *J Lipid Res* **37**, 2510-2524

Michael L. F., Kei-ichiro O., Short G. F. III, Manning J. J., Bell S. A., and Freeman M. W., (2004) ATP-binding cassette transporter A1 contains a novel C-terminal VFVNFA motif that is required for its cholesterol efflux and apoA-I binding activities. *J Biol Chem* **279**, 48477-48485

Millard E. E., Srivastava K., Traub L. M., Schaffer J. E., and Ory D. S. (2000) Niemann-Pick Type C1 (NPC1) overexpression alters cellular cholesterol homeostasis. *J Biol Chem* **275**, 38445-38451

Mukherjee S., Zha X., Tabas I., and Maxfield F. (1998) Cholesterol distribution in living cells: fluorescence imaging using dehydroergosterol as a fluorescent cholesterol analog. *Biophys J* **75**, 1915-1925

Murata M., Peranen J., Schreiner R., Wieland F., Kurzchalia T. V., and Simons K. (1995) VIP21/Caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci U S A* **92**, 10339-10343

Neufeld E. B., Cooney A. M., Pitha J., Dawidowicz E. A., Dwyer N. K., Pentchev P. G., and Blanchette-Mackie E. J. (1996) Intracellular trafficking of cholesterol monitored with a cyclodextrin. *J Biol Chem* **271**, 21604-21613

Oram J. F., Lawn R. M., Garvin M. R., and Wade D. P. (2000) ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem* **275**, 34508-34511

Owen J. S. and Mulcahy J. V. (2002) ATP-binding cassette A1 protein and HDL homeostasis. *Atheroscler Supp* **3,** 13-22

Panousis C. G. and Zuckerman S. H. (2000) Interferon-gamma induces downregulation of Tangier disease gene (ATP- binding-cassette transporter 1) in macrophage-derived foam cells. *Arterioscler Thromb Vasc Biol* **20**, 1565-1571

Parpal S., Karlsson M., Thorn H., and Stralfors P. (2001) Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *J Biol Chem* **276**, 9670-9678

Pentchev P. G., Brady R. O., Blanchette-Mackie E. J., Vanier M. T., Carstea E. D., Parker C. C., Goldin E., and Roff C. F. (1994) The Niemann-Pick C lesion and its relationship to the intracellular distribution and utilization of LDL cholesterol. *Biochim Biophys Acta* **1225**, 235-243

Pike L. J. and Casey L. (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. *J Biol Chem* **271**, 26453-26456

Phillips M. C., Johnson W. J., and Rothblat G. H. (1987) Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochim Biophys Acta* **906**, 223-276

Pittman R. C., Knecht T. P., Rosenbaum M. S., and Taylor, C. A. J. (1987) A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J Biol Chem* **262**, 2443-2450.

Pohl P., Saparaov S. M., and Antonenko Y. N. (1998) The size of the unstirred layer as a function of the solute diffusion coefficient. *Biophys J* **75**, 1403-1409

Prinz W. (2002) Cholesterol trafficking in the secretory and endocytic systems. *Semin Cell Dev Biol* **13**, 197-203

Raggers R. J., Pomorski T., Holthuis J. C. M., Kälin N., and Meer G. (2000) Lipid traffic: the ABC of transbilayer movement. *Traffic* **1**, 226-234

Repa J. J. and Mangelsdorf D. J. (2002) The liver X receptor gene team: potential new players in atherosclerosis. *Nat Med* **8**, 1243-1248

Repa J. J., Turley S. D., Lobaccaro J. A. Medina J., Li L., Lustig K., Shan B., Heyman R. A., Dietschy J. M., and Mangelsdorf D. J. (2000) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* **289**, 1524-1529

Rodriguez-Lafrasse C., Rousson R., Bonnet J., Pentchev P. G., Louisot P., and Vanier M. T. (1990) Abnormal cholesterol metabolism in imipramine-treated fibroblast cultures. Similarities with Niemann-Pick type C disease. *Biochim Biophys Acta* **1043**, 123-128

Rothblat G. H., Llera-Moya M., Atger V., Kellner-Weibel G., Williams D. L., and Phillips M. C. (1999) Cell cholesterol efflux: integration of old and new observations provides new insights. *J Lipid Res* **40**, 781-796

Rothblat G. H. and Phillips M. C. (1982) Mechanism of cholesterol efflux from cells effects of acceptor structure and concentration. *J Biol Chem* **257**, 4775-4782

Rowe A. H., Argmann C. A., Jane Y., Edwards J. Y., Sawyez C. G., Morand O. H., Hegele R. A., and Huff M. W. (2003) Enhanced synthesis of the oxysterol 24(S),25-epoxycholesterol in macrophages by inhibitors of 2,3-oxidosqualene:lanosterol cyclase: a novel mechanism for the attenuation of foam cell formation. *Circ Res* **93**, 717-725

Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze J. F., Brewer H. B., Duverger N., Denèfle P. and Assmann G. (1999) Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet* **22**, 352-355

Sasahara T., Nestel P., Fidge N., and Sviridov D. (1998) Cholesterol transport between cells and high density lipoprotein subfractions from obese and lean subjects. *J Lipid Res* **39**, 544-554

Saito H., Miyako Y., Handa T., and Miyajima K. (1997) Effect of cholesterol on apolipoprotein A-1 binding to lipid bilayers and emulsions. *J Lipid Res* **38**, 287-294

Schroeder F, Gallegos A. M., Atshaves B. P., Storey S. M., McIntosh A. L., Petrescu A. D., Huang H., Starodub O., Chao H., Yang H., Frolov A., Kier A. B. (2001) Recent advances in membrane microdomains: rafts, caveolae, and intracellular cholesterol trafficking. *Exp Biol Med* **226**, 873-890

Simons K. and Ikonen E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569-572

Skiba P. J., Zha X., Maxfield F. R., Schissel S. L., and Tabas I. (1996) The distal pathway of lipoprotein-induced cholesterol esterification, but not sphingomyelinase-induced cholesterol esterification, is energy-dependent. *J Biol Chem* **271**, 13392-13400

Smart E. J., Graf G. A., McNiven M. A., Sessa W. C., Engelman J. A., Scherer P. E., Okamoto T., and Lisanti M. P. (1999) Caveolins, liquid-ordered domains, and signal transduction. *Mol Cell Biol* **19**, 7289-7304

Smart E. J., Ying Y. S., Conrad P. A., and Anderson R. G. W. (1994) Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. *J Cell Biol* **127**, 1185-1197

Smart E. J., Ying Y. S., Donzell W. C., and Anderson R. G. W., (1996) A Role for Caveolin in Transport of Cholesterol from Endoplasmic Reticulum to Plasma Membrane. *J Biol Chem* **271**, 29427-29435

Stocker R. and Keaney J. F., JR. (2004) Role of oxidative modifications in atherosclerosis. *Physiol Rev* **84**, 1381-1478

Sviridov D. and Fidge N. (1995) Efflux of intracellular versus plasma membrane cholesterol in HepG2 cells: different availability and regulation by apolipoprotein A-I. *J Lipid Res* **36**, 1887-1896

Sviridov D., Fidge N., Beaumier-Gallon G., and Fielding C. (2001) Apolipoprotein A-I stimulates the transport of intracellular cholesterol to cell-surface cholesterol-rich domains (caveolae). *Biochem J* **358**, 79-86

Sviridov D. and Nestel P. (2002) Dynamics of reverse cholesterol transport: protection against atherosclerosis. *Atherosclerosis* **161**, 245-254

Tabas I., Zha X., Beatini N., Myers J. N., and Maxfield F. R. (1994) The actin cytoskeleton is important for the stimulation of cholesterol esterification by atherogenic lipoproteins in macrophages. *J Biol Chem* **269**, 22547-22556

Tall A. R. (1990) Plasma high density lipoproteins metabolism and relationship to atherogenesis. *J Clin Invest* **86**, 379-384

Thomsen P., Roepstorff K., Stahlhut M., and Deurs B. V., (2002) Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol Biol Cell* **13**, 238-250

Yancey P. G., Bortnick A. E., Kellner-Weibel G., Llera-Moya M., Phillips M. C., Rothblat G. H., (2003) Importance of different pathways of cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* **23**, 712-719

Uittenbogaard, A., Ying, Y.S., and Smart, E. J., (1998) Characterization of a cytosolic heat-shock protein-caveolin chaperone complex. Involvement in cholesterol trafficking. *J Biol Chem* **273**, 6525-6532

Underwood K. W., Andemariam B., McWilliams G. L., and Liscum L. (1996) Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport. *J Lipid Res* **37,** 1556-1568

Underwood K. W., Jacobs N. L., Howley A., and Liscum L. (1998) Evidence for a cholesterol transport pathway from lysosomes to endoplasmic reticulum that is independent of the plasma membrane. *J Biol Chem* **273**, 4266-4274

Urbani L. and Simoni R. D. (1990) Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane. *J Biol Chem* **265**, 1919-1923

Vedhachalam C., Liu L., Nickel M**.**, Dhanasekaran P., Anantharamaiah G. M., Lund-Katz S., Rothblat G. H., and Phillips M. C. (2004) Influence of ApoA-I structure on the ABCA1-mediated efflux of cellular lipids. *J Biol Chem* **279**, 49931-49939

Venkateswaran A., Laffitte B. A., Joseph S. B., Mak P. A., Wilpitz D. C., Edwards P. A. and Tontonoz P. (2000) Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXRα. *Proc Natl Acad Sci U S A* **97**, 12097-12102

Walker J. E., Saraste M., Runswick M. J., and Gay N. J. (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1**, 945-951

Wang N., Silver D. L., Costet P. and Tall A. R. (2000) Specific Binding of ApoA-I, Enhanced Cholesterol Efflux, and Altered Plasma Membrane Morphology in Cells Expressing ABC1. *J Biol Chem* **275**, 33053-33058

Wang N., Silver D. L., Thiele C., and Tall A. R. (2001) ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem* **276**, 23742-23747

Zha X., Gauthier A., Genest J., and McPherson R. (2003) Secretory vesicular transport from the Golgi is altered during ATP-binding cassette protein A1 (ABCA1)-mediated cholesterol efflux. *J Biol Chem* **278**, 10002-10005