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The Involvement of Membrane Vesiculation in ABCA1 Mediated Efflux

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
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Biochemistry, Microbiology and Immunology
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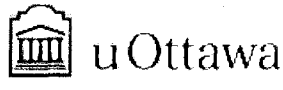

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Abstract

The mechanism of ATP binding cassette transporter A1 (ABCA1) mediated cholesterol efflux is presently unclear. Cells expressing ABCA1 reported to display distinctive membrane-protrusion-like morphology and apoA1 was also seen to bind to those structures. We hypothesized that cholesterol efflux may be in part originated from membrane shedding through membrane vesiculation and therefore may rely on relatively flexible plasma membrane. We tested several reagents known to modulate membrane fluidity and found that cholesterol efflux is indeed inversely correlated with membrane rigidity. Additionally, using differential ultracentrifugation, we provide evidence that, during ABCA1 mediated cholesterol efflux, cells produce a significant quantity of apoA1 free membrane vesicles along with apoA1 containing lipoproteins. This process can be inhibited by rigidifying the plasma membrane. We therefore conclude that flexibility of plasma membrane is necessary for ABCA1 mediated cholesterol efflux and speculate that ABCA1 and apoA1 work together to secrete membrane vesicles during efflux.

Acknowledgements

My thesis has been quite a journey through valuable experiences, which would not have been possible without the help and support from so many people. This is an opportunity to acknowledge this.

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Lastly, I would like to specially recognize my husband for his support, cooperation and patience, without which this work would not have been a reality.

Dedicated to my loving son
for his inspiring love to get me through the difficult times.

Dedicated to my parents and family
for their unconditional love and encouragement.

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List of Abbreviations

ABCA1	ATP binding cassette transporter A1
ApoA1	Apolipoprotein A1
apoE	Apoliprotein E
ATP	Adenosine triphosphate
BHK	Baby hamster kidney
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CH	Cholesteryl hemisuccinate
DMEM	Dulbecco's modified eagle's medium
DSP	Dithiobis succinimidyl propionate
EDTA	Ethylene diamine tetracetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
F-ACTIN	Filamentous actin
FOLCH	Chloroform methanol
HDL	High density lipoprotein
Hip	Hexane isoproponol
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSP-70	70 kilodalton heat shock protein
IP	Immunoprecipitation
kDa	Kilodalton
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoprotein
LGM	Lectin from glycine max
LXR	Liver X receptor
NBD	Nucleotide binding domain
Ox LDL	Oxidized LDL
PBS	Phosphate buffer saline
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PEST	Proline (P), Glutamic acid (E), Serine (S), Threonine (T).
PS	Phosphatidyl serine
PVDF	Polyvinylidene Difluoride
RCT	Reverse cholesterol transport
RXR	Retnoic X receptor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR-B1	Scavenger receptor B1
SREBP	Sterol response element binding protein
TM	Transmembrane domain
VLDL	Very low density lipoproteins
WGA	Wheat germ agglutinin

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Chapter 1

Introduction

Cholesterol is one of the vital components of the human body that is mainly used in forming cell membranes and hormones. However, a high level of cholesterol in blood is a major risk factor for coronary heart disease, potentially leading to heart attack. As the basic building block of the mammalian cell membrane, cholesterol not only provides proper rigidity to the membrane, but also plays an important role in signal transduction and membrane trafficking. Due to their hydrophobicity, cholesterol and other phospholipids cannot be dissolved in blood and have to be transported to and from the cells by lipoproteins. Lipoproteins are special carriers made up of lipids and proteins. There are several kinds of lipoproteins; the most important ones are low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Both LDL and HDL play key roles in cholesterol homeostasis.

Mammalian cells mainly synthesize cholesterol by a *de novo* pathway in the liver. Cholesterol is then transported to the peripheral cells to be incorporated to their membranes. Since most of the peripheral cells are unable to degrade cholesterol, surplus cholesterol is either stored as a cholesterol ester or is transported back to liver for degradation. The plasma membrane and sub-cellular compartments such as endosomes and lysosomes are the main storage locations of cholesterol in cells. Although the

endoplasmic reticulum (ER) is the main site for synthesis of cholesterol and its esterification, only 0.5% – 1% of cholesterol is found within this organelle (Lange *et al.*, 1997). Newly synthesized cholesterol is efficiently transported from the ER to the plasma membrane, where it is present at its highest concentration (about 50-90%) (Lange *et al.*, 1997). The pathway transporting cholesterol from the ER to the plasma membrane remains largely elusive. Some studies suggest that it follows a vesicular pathway, but disrupting the Golgi only inhibits 20% of cholesterol transport (Heino *et al.*, 2000; Urbani and Simoni, 1990). Therefore, vesicular transport is not likely to be the major pathway. Other studies suggest that cholesterol transport may follow a non-vesicular pathway. Intracellular cholesterol binding proteins, such as sterol-carrier protein 2 (SCP2) and caveolin may play major role in this process (Liu *et al.*, 2002; Puglielli *et al.*, 1996). This hypothesis is yet to be confirmed. It is not well understood currently how the uneven distribution of cholesterol among various intracellular organelles is maintained.

Cellular cholesterol content is tightly regulated; it involves cholesterol biosynthesis, uptake, transport, metabolism and secretion. Any defect in the distribution or transport of cholesterol may give rise to pathophysiological conditions. Often cholesterol related diseases and pathological conditions arise when excess cholesterol is accumulated in peripheral tissues. The process of removing cholesterol from these sites is called reverse cholesterol transport (RCT). In this process, the excess cholesterol from the peripheral cells is transported to the liver for degradation and conversion to bile acids.

1.1 Reverse Cholesterol Transport

Reverse cholesterol transport, or RCT, is a sequence of events starting with the generation of lipid-poor apolipoproteins, mainly apolipoprotein A1 (apoA1). As the major protein constituent of HDL, apoA1 is a 249-amino acid protein synthesized in the liver and small intestine. This exchangeable lipoprotein exhibits different structural conformations in the lipid-free and the lipid-bound states. The transition between the two states is extremely important for lipid binding and interaction. In the plasma, newly synthesized apoA1 undergoes proteolytic processing and post transcriptional modifications by the action of plasma proteases to become mature apoA1 (Bojanovski, 1985). The mature apoA1 is a 29 kDa polypeptide with 243 amino acids (Zannis *et al.*, 1982). The mature protein acquires phospholipids from hepatocyte during secretion and cholesterol from peripheral tissue to form small nascent or preβ-HDL particles through a process called cholesterol efflux. These nascent HDL particles are discoidal in shape and consist of a monolayer of phospholipids and apolipoproteins are inserted into the phospholipids. As these preβ-HDL take up more cholesterol from peripheral cells, their shape changes to spherical particle, named HDL_3. HDL_3 then become enriched in esterified cholesterol via an esterifying enzyme called lecithin cholesterol acyl transferase (LCAT) and, at the same time, gains more cholesterol/phospholipids to form mature large cholesterol ester (CE) rich HDL_2 (**Figure 1.1**).

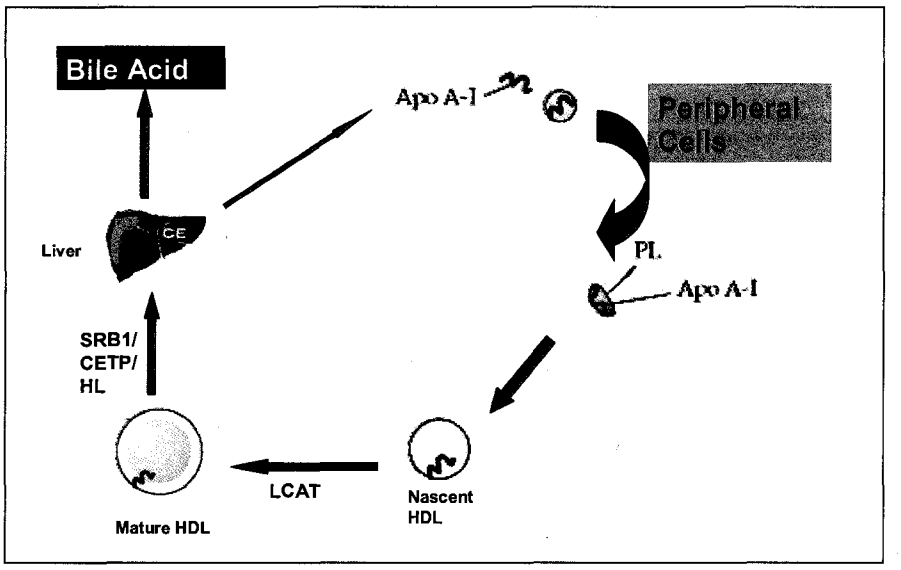

Figure 1.1: Reverse cholesterol transport pathway.

In the final step in the RCT pathway, cholesterol in the mature HDL is taken up by the liver. This final process involves participation of several enzymes and proteins such as hepatic lipase (HL), phospholipid transfer protein (PLTP) and scavenger receptor B1 (SR-B1). SR-B1 is primarily expressed in liver and steroidogenic tissues and mediates the uptake of cholesterol esters from HDL (Acton *et al.*, 1996; Wang *et al.*, 1996). In addition, cholesterol ester transfer protein (CETP) plays a major role in transferring CE from HDL to LDL or to very low density lipoprotein (VLDL) in the plasma (Deckelbaum *et al.*, 1979; Morton, 1990). CETP therefore removes HDL-CE in parallel to the SR-B1 pathway and influences the concentration, apolipoprotein content, and size of HDL particles in plasma. Cholesterol ester removed from the circulation through the reverse transport pathway is transformed and excreted by liver through bile acid pathway. Synthesis of bile acids is one of the predominant mechanisms for the excretion of excess cholesterol in the liver (Bergstrom, 1959; Suld *et al.*, 1962). However, when

dietary intake of cholesterol exceeds its normal limit, the degradation of cholesterol through the bile acid pathway becomes inadequate. Under such conditions excess of cholesterol removed from circulation is converted to CE and stored as the lipid droplets, leading to steatosis (fatty liver). To summarize, reverse cholesterol transport is the key process in humans to regulate cholesterol in the plasma. Nascent HDL formation begins with a process termed apoA1 mediated cholesterol efflux. For a long time it was unknown how apoA1 removes cholesterol from peripheral cells to HDL. A big breakthrough came from the discovery that links ATP binding cassette transporter A1, or ABCA1, to a rare recessive genetic disorder, Tangier disease (Bodzio, *et al.*, 1999; Rust *et al.*, 1999; Brooks-Wilson *et al.*, 1999). ABCA1 was identified to be the key molecular component that mediates cholesterol efflux to apoA1 to form nascent HDL.

1.2 ABCA1 & Cholesterol Efflux

In Tangier disease, patients have very low levels of HDL in the circulation. It has been reported that mutations in ABCA1 prevents either its expression on the cell surface or its ability to bind apoA1 (Bodzio *et al.*, 1999; Rust *et al.*, 1999) which diminishes the cholesterol efflux process. Poorly lipidated apoA1 is then rapidly catabolized and removed from the circulation by the kidney. As a result, Tangier patients have low apoA1 and low plasma HDL level (Marcil *et al.*, 1999). The inability to efflux cholesterol from peripheral cells causes accumulation of cholesterol esters in peripheral cells. Also, increased deposition of cholesterol esters in various tissues leads to increased prevalence for cardiovascular disease. In addition to apoA1, several lipoproteins, such as apoA2, apoA4, apoC1, apoC2, apoC3, and apoE, are reported to be capable of mediating

cholesterol efflux (Remaley *et al.*, 2001). The efflux mediated by these lipoproteins is also dependent on functional ABCA1 and none of them are able to rescue the cholesterol efflux in Tangier cells. ABCA1 therefore is absolutely required in human to maintain healthy levels of HDL. This was further confirmed through transgenic knockout studies in animal models. Mice that lack ABCA1 have virtually no HDL, reminiscent of Tangier disease in human (Orso *et al.*, 2000; Mcneish *et al.*, 2000). At the cellular level, ABCA1 expression markedly increases cellular cholesterol and phospholipids efflux to apoA1 in all the model systems tested to date. The exact mechanism by which ABCA1 mediates cholesterol efflux however is not well understood at present.

1.3 ABCA1 Structure, Expression and Function

ABCA1 belongs to the ABC transporter super family. This family is one of the largest protein families and is well conserved from lowest microorganisms to humans (Higgins, 1992). Almost all the ABC transporters bind and hydrolyze ATP and use the energy to transport many different substrates across the membrane (Higgins, 2001). The substrates include a wide variety of molecules, such as sugars, amino acids, metal ions, peptides, proteins and a large number of hydrophobic compounds and metabolites. To date 50 human ABC genes have been identified. A list of these is shown in **Table 1.1,** along with their subfamily, expressions, functions and associated diseases (Dean *et al.*, 2001).

Table 1.1: List of human ABC genes, their functions, phenotype and related diseases.

Gene	Sub-family	Expression	Function	Mendelian Disorder/ Disease
ABCA1	ABC1	Ubiquitous	Phospholipid & cholesterol efflux to apoA1	Tangier disease/ FHDLD
ABCA2	ABC1	Brain	Drug resistance	
ABCA3	ABC1	Lung		
ABCA4	ABC1	Photo receptors	N-retinylidene - PE efflux	Stargardt, FFM, RP, CRD, CD, AMD
ABCA5	ABC1	Muscle, Heart, Testes		
ABCA6	ABC1	Liver		
ABCA7	ABC1	Spleen, Thymus		
ABCA8	ABC1	Ovary		
ABCA9	ABC1	Heart		
ABCA10	ABC1	Muscles, Heart		
ABCA12	ABC1	Stomach		
ABCA13	ABC1	Low in all tissues		
ABCB1	MDR	Adrenal, Kidney, Brain	Multidrug resistance	Digoxin uptake
ABCB2	MDR	All cells	Peptide transport	Immunodeficiency
ABCB3	MDR	All cells	Peptide transport	Immunodeficiency
ABCB4	MDR	Liver	PC transport	PFIC-3, ICP
ABCB5	MDR	Ubiquitous		
ABCB6	MDR	Mitochondria	Iron transport	
ABCB7	MDR	Mitochondria	Fe/S cluster transport	XLSA/A
ABCB8	MDR	Mitochondria		
ABCB9	MDR	Heart, Brain		
ABCB10	MDR	Mitochondria		
ABCB11	MDR	Liver	Bile salt transport	PFIC-2
ABCC1	CF/MRP	Lung, Testes, PBMC	Drug resistance	
ABCC2	CF/MRP	Liver	Organic anion efflux	Dubin-johnson syndrome
ABCC3	CF/MRP	Lung, Intestine, Liver	Drug resistance	
ABCC4	CF/MRP	Prostrate	Nucleoside transport	
ABCC5	CF/MRP	Ubiquitous	Nucleoside transport	
ABCC6	CF/MRP	Kidney, Liver		Pseudoxanthoma elasticum
CFTR, ABCC7	CF/MRP	Exocrine tissues	Chloride ion channel	Cystic fibrosis, CBAVD, Pancreatitis
ABCC8	CF/MRP	Pancreas	Sulfonyl urea receptor	FPHHI
ABCC9	CF/MRP	Heart, Muscles		
ABCC10	CF/MRP	Low in all tissues		
ABCC11	CF/MRP	Low in all tissues		
ABCC12	CF/MRP	Low in all tissues		
ABCD1	ALD	Peroxisomes	VLCFA transport regulation	ALD
ABCD2	ALD	Peroxisomes		
ABCD3	ALD	Peroxisomes		

Gene	Sub-family	Expression	Function	Mendelian Disorder/ Disease
ABCD4	ALD	Peroxisomes		
ABCE1	OABP	Ovary, Testes, Spleen	Oligoadenylate binding protein	
ABCF1	GCN20	Ubiquitous		
ABCF2	GCN20	Ubiquitous		
ABCF3	GCN20	Ubiquitous		
ABCG1	WHITE	Ubiquitous	Cholesterol transport (?)	
ABCG2	WHITE	Placenta, Intestine	Toxin efflux, Drug resistance	
ABCG4	WHITE	Liver		
ABCG5	WHITE	Liver , Intestine	Sterol transport	Sitosterolesmia
ABCG8	WHITE	Liver , Intestine	Sterol transport	Sitosterolesmia

The ABC transporters in general comprised of either two transmembrane domains (TM) and two nucleotide binding domains (NBD) as full transporters, or one of each domain as half transporter (Dean *et al.*, 2001). The transmembrane domains are putatively rich in α helices, which is a characteristic of the super family. The NBD domain consists of three conserved domains namely Walker A, B and signature C motif (Hyde *et al.*, 1990). Depending upon the organization of NBD and TM as well as amino acid sequence, ABC transporters can be subdivided into seven different subfamilies. The NBD domain of most of the ABC transporters is located in the cytoplasm which helps these transporters to carry the substrate in one direction out of the cytoplasm. Almost all the members of the ABCA subfamily are mainly involved in lipid trafficking. ABCA1 is a full transporter of 240 kDa and has twelve transmembrane domains with two large extracellular loops and one cytoplasmic loop containing a hydrophobic region and two cytoplsmic NBD domains (**Figure 1.2**).

ABCA1 is expressed primarily in the plasma membrane with minor amounts in the Golgi, lysosomes and endosomes (Neufeld *et al.*, 2001; Landry *et al.*, 2006). ABCA1 expression is stringently regulated, mainly by the cholesterol status of the cells. The

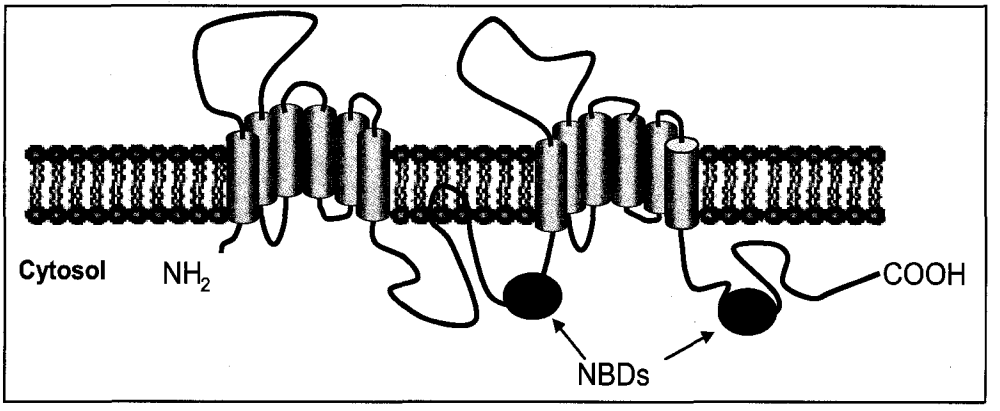

Figure 1.2: Structure of ABCA1. (Adapted from Ioannou, 2001)

expression is induced by cholesterol loading and is decreased by cholesterol efflux or depletion of cholesterol (Langmann *et al.*, 1999). In macrophages, ABCA1 expression is shown to be mediated by the liver X receptors, LXR and RXR, members of the nuclear hormone receptor family (Costet *et al.*, 2000; Schwartz *et al.*, 2000; Venkateswaran *et al.*, 2000). ABCA1 expression is also activated by oxysterol ligands such as 25-hydroxy cholesterol. In macrophages, ABCA1 expression can be stimulated by 9 cis-retnoic acid or cAMP, whereas in fibroblast cell lines cAMP is the main stimulator for ABCA1 expression. The turnover of ABCA1 is rapid, with a half life of about one hour (Goff *et al.*, 2004). This turnover is suggested to take place in lysosomes (Oram *et al.*, 2000). ABCA1 has a sequence rich in proline, glutamic acid, serine and threonine known as PEST sequence. Calpain proteases enhance degradation of ABCA1 through the PEST sequence and therefore control the expression of ABCA1 and cholesterol efflux activity. It has also been reported that the interaction of apoA1 with ABCA1 decreases the turnover of ABCA1. ApoA1 binding prevents phosphorylation of the PEST sequence in ABCA1, therefore protects ABCA1 against degradation by calpain proteolysis and increases surface expression of ABCA1 (Arakawa *et al.*, 2002; Wang *et al.*, 2003).

1.4 Mechanism of Action of ABCA1

The molecular mechanism of ABCA1-dependent cholesterol efflux remains poorly understood. ABCA1 expression has been shown to increase apoA1 binding to the cells, suggesting that apoA1 binds directly to ABCA1 (Wang *et al.*, 2000). A number of studies have indeed shown that ABCA1 may directly interact with apoA1 (Smith *et al.*, 2004; Wang *et al.*, 2000). This was also supported by the findings that natural mutations of ABCA1 that abolish cholesterol efflux also abolish crosslinking of ABCA1 with apoA1 (Panagotopulos *et al.*, 2002). The functional significance of such an interaction, however, is not entirely clear, since only a very small fraction of cell associated-apoA1 was found to be crosslinked with ABCA1. Furthermore, it has been observed that a mutation in the first loop (W590S) does not affect the apoA1 binding but diminishes efflux. This indicates that acceptor binding is necessary but is not sufficient for the efflux process to happen. Alternatively, ABCA1 may influence the plasma membrane through its ATPase related functions. ApoA1 then binds to the specific sites on the plasma membrane, which is generated by ABCA1 activity (Chambenoit *et al.*, 2001). This may initiate a signal for transferring cholesterol from internal compartment to plasma membrane. In this model, the physical interactions between ABCA1 and apoA1 may not be necessary. Instead, ABCA1 recruits cholesterol and phospholipids to the plasma membrane and makes them accessible to apoA1.

ABCA1 mediated efflux is known to lipidate apoA1 not only with cholesterol but also with phospholipids. It is unclear whether ABCA1 mediates apoA1 acquisition of phospholipids and cholesterol separately or simultaneously. In a "two-step model",

apoA1 first interacts with ABCA1 which facilitates apoA1 association with phospholipid-rich domains. This phospholipid-primed apoA1 then acquires cholesterol from cholesterol rich domains, a process thought to be independent of ABCA1. On the other hand, Smith *et al.* (2004) reported that ABCA1 mediates concurrent efflux of phospholipid and cholesterol to apoA1.

In addition to the plasma membrane, ABCA1 is also localized in the Golgi where it may facilitate vesicle budding from the Golgi (Orso *et al.*, 2000). Those vesicles may eventually fuse with the plasma membrane and transfer lipids to the plasma membrane. The majority of ABCA1 however is found in the plasma membrane and it is likely that the plasma membrane is the main loci where cholesterol efflux occurs.

1.5 Plasma Membrane and its Role in Efflux

Plasma membrane is composed of bilayer of lipids, and embedded proteins and carbohydrates moieties, which is supported by a meshwork of cytoskeleton (**Figure 1.3**).

Cytoskeleton elements have long been known to play a role in membrane traffic, not only by forming the structural scaffold and network over which membrane traffic flows, but also by directly deforming membranes. ABCA1 is mainly localized in the plasma membrane and ABCA1 expression also strongly influences the lateral organization of the plasma membrane.

Interestingly, phospholipids in the bilayer of the plasma membrane are asymmetrically distributed between two leaflets (Bretscher *et al.*, 1972). The majority of phosphatidyl

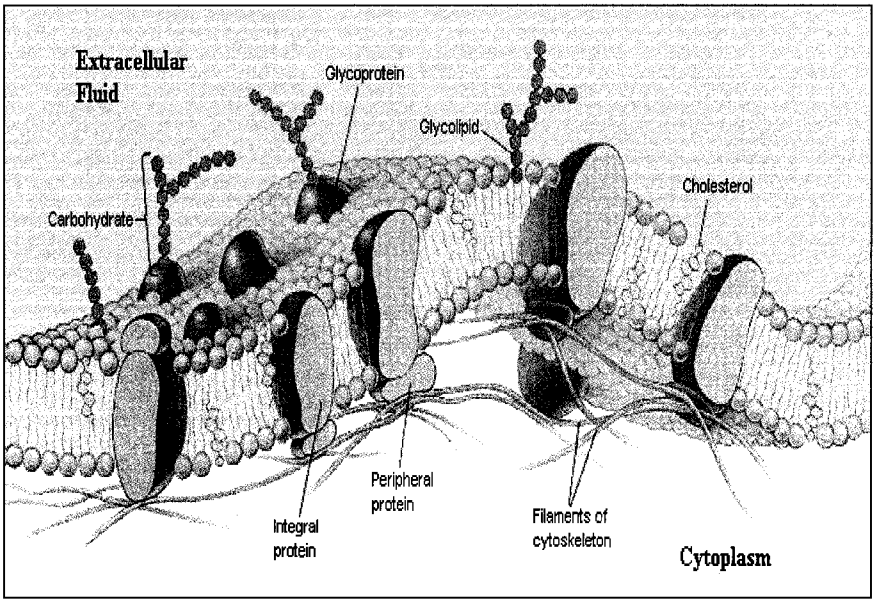

Figure 1.3: Structure of Plasma Membrane. (Geibel, 2003)

choline (PC) and sphingolipids, for example, are in the outer leaflet, and the aminophospholipids such as phosphatidylserine (PS) and phosphatidylethylamine (PE) are confined to the inner leaflet (Zachowski, 1993). This highly ordered asymmetry is thought to be established by many flippases, including aminophospholipid translocase, that consume metabolic energy. Aminophospholipid translocase flips PS or PE from the outer leaflet to the inner leaflet by hydrolyzing ATP. The purpose of such a phospholipid asymmetry on the plasma membrane is not clear. It is speculated that high level of phosphotidylcholine and cholesterol present in the outer leaflet of the plasma membrane provides an inert surface, essential for the stability and barrier function. By contrast, the preferential orientation of amino phospholipids in the inner leaflet forms an interface promoting membrane-membrane interactions and fusion. The presence of amino phospholipids in the cytosolic inner leaflet is thought to be important, because it prevents the exposure of PS on the cell surface which signifies apoptosis and results in

engulfment of the cells by macrophage bearing PS receptors (Marguet *et al.*, 1999). The detailed mechanism for maintaining such asymmetric distribution is not well understood. Membrane lipids do not spontaneously exchange between the two leaflets of lipid bilayer, since the polar head group cannot easily cross the hydrophobic interior of the membrane itself. It has been proposed that many of these flippases belong to the ABC transporter family (Borst and Elferink, 2002; Borst *et al.*, 2000; Daleke, 2003). ABCA1 has also been implicated in flipping phospholipids, particularly PS (Marguet *et al.*, 1999; Moynault *et al.*, 1998). Therefore an assumption was made on this basis that accumulation of cholesterol in the plasma membrane leads to the expression of ABCA1 which in turn flips the phospholipids to the outer leaflet and facilitates the efflux.

The plasma membrane lipids are thought to be organized into specialized membrane microdomains called lipid rafts. These are also referred as lipid-ordered domains. Non-raft domains are more loosely packed and soluble in Triton as compared to highly ordered and tightly packed raft domains which are insoluble in Triton. Therefore, the non-raft domains are more fluid in nature. Although the raft microdomains contain a high concentration of cholesterol (Brown and London, 1988; Simons and Ikonen, 1997), apoA1 was shown to acquire cholesterol preferably from loosely packed non-raft domains during ABCA1 mediated cholesterol efflux (Mendez *et al.*, 2001; Drobnik *et al.*, 2002). In addition to classical raft and non-raft domains, Ruper *et al.* recently identified novel cholesterol rich microdomains which are Triton soluble like non-raft domains but resistant to another reagent Lubrol WX. It has been suggested that these Lubrol-resistant microdomains have important roles in the formation of lamilipodia or filopodia by providing building blocks for membrane protrusions (Roper *et al.*, 2000).

ApoA1 is shown to inhibit filopodia formation in human monocyte cells in an ABCA1 dependent manner (Drobnik *et al.*, 2002). These lipid microdomains play an important role in membrane trafficking and signal transduction (Simons and Toomre, 2000), and potentially other important functions. Therefore these microdomains serve a wide range of cellular functions.

The asymmetric distribution of lipids in the plasma membrane also provides a platform for the endocytosis and exocytosis. In Tangier fibroblasts, an increased level of inward bending of the membrane or endocytosis has been observed in absence of functional ABCA1 (Zha *et al.*, 2001). ABCA1 thus appears to suppress inward membrane bending and promote outward movement (membrane protrusion). Interestingly, apoA1 treatment increases those protrusion-like structures (Lin and Oram, 2000) and this might be indicative of outward bending of the plasma membrane. There is very little homology in the primary amino acid sequence between apoA1 and the other apolipoproteins that are also capable of mediating cholesterol efflux in a ABCA1 dependent manner. They however all share common amphipathic helices in their secondary structures. Indeed, the key structural component required for cholesterol efflux is the amphipathic helix, shared by apoA1, apoA2, apoA4, apoC1, apoC2, apoC3, and apoE. Interestingly, many amphipathic proteins, such as epsin, are well known for their role in membrane bending (Ford *et al.*, 2002), thus generating invaginations or protrusions. ApoA1 could therefore further amplify the influence of ABCA1 on the plasma membrane by facilitating outward bending. Such an influence could be critically important for cholesterol efflux.

1.6 ABCA1 Mediated Cholesterol Efflux and Membrane Rigidity

Recently, it was shown that cells expressing ABCA1 displayed a distinctive morphology characterized by membrane protrusions resembling echinocytes (Wang *et al.*, 2000). Echinocytes are red blood cells containing plasma membrane protrusions and are spiky in appearance. According to bilayer-couple theory, such membrane protrusions occur when there are more phospholipids or amphipathic molecules on the outer leaflet of plasma membrane (Sheetz and Singer, 1974). It has been shown that platelets also form similar plasma membrane projections with the addition of phospholipids (Sune and Bienvenue, 1988; Ferrell *et al.*, 1985). This suggests that protrusions on ABCA1 expressing cells arise perhaps from an increased quantity of lipid, probably phospholipids and cholesterol, in the outer leaflet of the plasma membranes. Interestingly, it has been shown by electron microscopy that apoA1 frequently binds to similar protruding structures on the plasma membrane. Therefore, it is possible that apoA1 may acquire cholesterol and phospholipids from those protruding structures before dissociation. Furthermore, formation of such structures was impaired in Tangier fibroblast that lacks functional ABCA1 (Lin and Oram, 2000). This presents a possibility that such protrusions may be necessary for cholesterol efflux, either by creating particular membrane domains (high curvature) where apoA1 can acquire lipids or by facilitating pinch-off of these protrusions to release membrane vesicles.

Release of membrane vesicles or micro-particles from plasma membrane is indeed frequently observed in response to different types of cell stimulation. Depending upon

the type of stimulation, membrane vesicles from the same origin can have different lipid and protein composition (Hugel *et al.*, 2005). These micro-particles have been proposed to serve various purposes such as intercellular communication, angiogenesis, vascular function and immunity (Hugel *et al.*, 2005). Duong *et al.* (2006) observed that ABCA1 expressing cells are capable of generating membrane particles, and the production of such particles was stimulated by the addition of apoA1. Furthermore, ABCA1 knockout mice appear to have defective micro-particle production under various stimuli (Combes *et al.*, 2005). We therefore speculate that membrane particles or vesicles play a role in cholesterol efflux. Frequent membrane protrusions, however, require certain flexibility of the plasma membrane. It has been demonstrated by several researchers that there are differences in the fluidity within the bilayer of plasma membrane. The outer leaflet is thought to be more fluid (Cogan *et al.*, 1981; Schachter *et al.*, 1982; Schroeder *et al.*, 1988; Seigneuret *et al.*, 1984; Sweet and Schroeder, 1986). Recently it has also been shown that ABCA1 expression is associated with expansion of the non-raft domains on the plasma membrane (Drobnik *et al.*, 2002; Landry *et al.*, 2006). Non-raft membranes are flexible in nature, implying that ABCA1 expression and its normal functionality may depend on flexibility of the membrane. Membrane flexibility is likely one of the major requirements in forming membrane protrusions and, therefore one of the major determinants of cholesterol efflux.

Many reagents are known to have the capacity of specifically altering membrane rigidity. One of the best characterized reagents is wheat germ agglutinin (WGA) (Evans and Leung, 1984; Hochmuth and Waugh, 1987). WGA, a 36 kDa protein, belongs to the plant lectin family. It selectively recognizes and binds to sialic acid and N-

acetylneuraminic acid residues. It is a dimeric protein composed of two identical subunits (isolectin) with a molecular weight of 18 kDa (**Figure 1.4**). Amino acid analysis reveals that the polypeptide chain of WGA contains high amounts of glycine and cystine residues, the latter tend to form disulfide bonds.

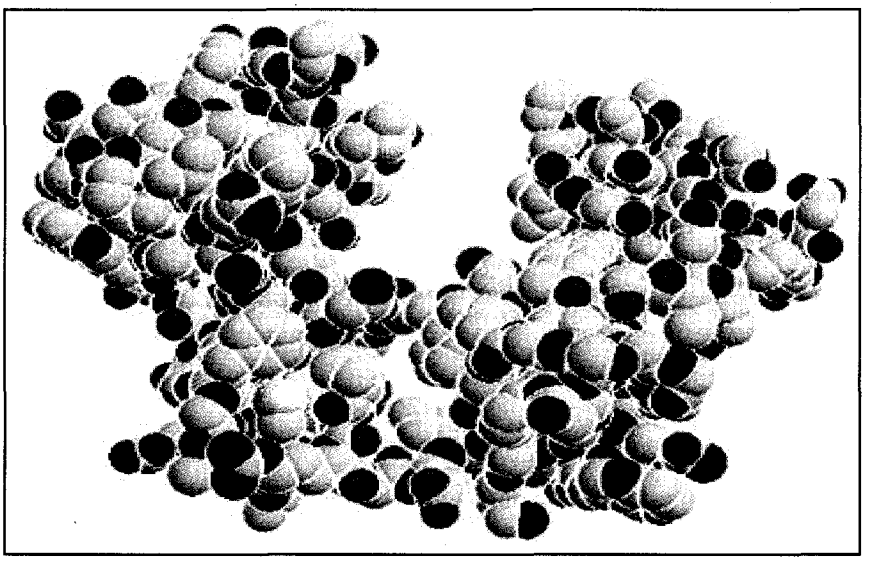

Figure 1.4: Structure of WGA. (Sengbusch, 2003)

Each of the subunits, namely wga1 and wga2, consists of four identical domains. These domains are composed of 42 or 43 amino acids, respectively, and are thought to play a role in sugar binding. WGA has particularly high affinity for oligosaccharides containing *N*-acetylglucosamine (Goldstein and Hayes, 1979; Evans and Leung, 1984), which are common to membrane glycoproteins. WGA also has high affinity for glycoconjugates in the plasma membrane. Therefore it binds multivalently to the glycoproteins and cross-links polysaccharides which lead to rigidification of plasma membrane (Nagata and Burger, 1974).

The effects of lectins are quite diverse. They are known to agglutinate blood cells and precipitate glycoconjugates. However the molecular basis of their interaction with the cell surface is still not clearly known. WGA is commonly used in cell biology to stain the Golgi complex in mammalian cells. Charge and avidity are thought to play an important role in WGA and glyco-conjuagate interactions. It has been shown that succinylated WGA, which is negatively charged, in contrast to positively charged native WGA, does not bind to cell surface glycoconjugates (Monsigny *et al.*, 1980).

It has been demonstrated by researchers that WGA treatment alters the physical properties of erythrocyte cells, and restricts changes in their shape (Evans and Leung, 1984; Hochmuth and Waugh, 1987). WGA makes the erythrocyte membrane stiff by interfering with the elastic property of erythrocytes. It has been predicted that it is mainly the membrane bound WGA which is responsible for the stiffening process (Evans and Leung, 1984). It induces stiffening of membrane cytoskeleton along with the membrane surface (Anderson and Lovrien, 1981). WGA treatment inhibits echinocytic conversion of erythrocytes, even in the presence of echinocyte inducing agents (Lovrien and Anderson, 1980).

In addition to WGA, cholesteryl hemisuccinate is another agent known to decrease membrane fluidity (Yuli *et al.*, 1981; Wang and Zhang, 2005). It is a derivative of cholesterol esterified at 3-hydroxyl group **(Figure 1.5)**. It is known to increase viscosity of lipid membrane. Cholesteryl hemisuccinate has a sterol ring-like structure (similar to cholesterol) which can be inserted in the monolayer of lipid bilayer and has a long tail that can penetrate deeply into the inner leaflet of the plasma membrane. This effectively

makes the membrane bilayer more compact and thus rigidifies the membrane (Ding *et al.*, 2005).

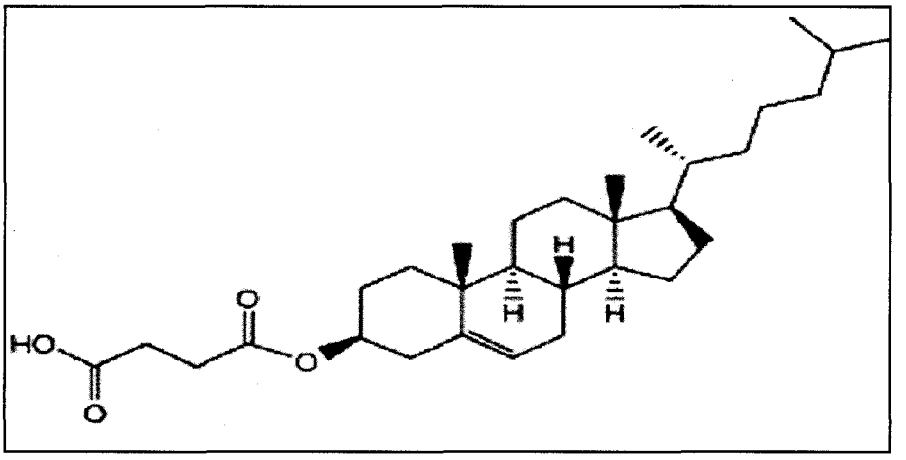

Figure 1.5: Structure of cholesteryl hemisuccinate. (Ding, 2005)

In contrast, benzyl alcohol and hexanol are two primary alcohols which are known to increase fluidity of the lipid bilayer (Gordon *et al.*, 1980; Meddings *et al.*, 1990; Wang and Zhang, 2005). Both of these primary alkanols, when inserted to membrane bilayer, interfere with packing of phospholipids in the plasma membrane because of the short length of their methylene chain (Pedersen *et al.*, 2007). Benzyl alcohol is a neutral compound and is a known local anesthetic. It can readily partition into membranes from aqueous solution and increase the fluidity of model phospholipid bilayers (Colley *et al.*, 1972). Benzyl alcohol is known to fluidize the exofacial leaflet of the plasma membranes preferentially (Dudeja *et al.*, 1991). Although the exact site of action of hexanol is not known, but it is reported to be more efficient as a membrane fluidizer than benzyl alcohol (Meddings *et al.*, 1990).

Taken together, the rigidity of the membrane can be effectively modulated by these well characterized reagents. Since these reagents can exert their effects on membrane rapidly, they can potentially be used to specifically manipulate the plasma membrane without significantly disturbing cells as a whole. Given the fact that most of the ABCA1 is localized on the plasma membrane, changes in the physical states of the plasma membrane induced by ABCA1 may have major impact on cholesterol efflux.

1.7 Research Hypothesis

We speculate that ABCA1 facilitates apoA1 acquisition of cholesterol/phospholipids at least partially by generating membrane deformation (**Figure 1.6**). This process produces both lipoprotein-containing and lipoprotein-free microparticles. The latter may be generated by membrane shedding. In particular, we proposed to test the following hypothesis:

- *The flexibility of the plasma membrane directly influences ABCA1 mediated cholesterol efflux.*
- *Membrane shedding is one of the mechanisms for removal of excess cholesterol and phospholipids.*

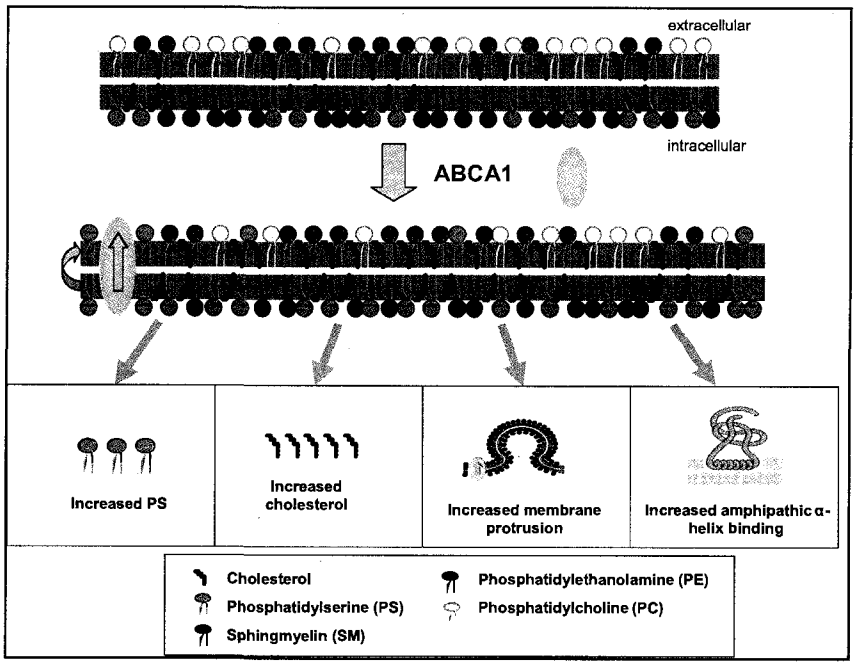

Figure 1.6: Proposed model of mechanism of ABCA1.

Chapter 2

Materials & Methods

2.1 Materials

Cell culture medium and reagents were supplied by Invitrogen Canada Inc. (Burlington, ON Canada). Mifepristone, cholesteryl hemisuccinate and all the lectins were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON Canada). ^{3}H cholesterol and ^{3}H choline chloride was bought from PerkinElmer LAS Canada Inc. (Woodbridge, ON Canada). The polyclonal antibody against ABCA1 was supplied by Novus Biological Inc. (Littleton, CO USA). Purified human apoA1 was obtained from Biodesign International (Saco, ME USA). ^{125}I and PD desalting columns sephadex G25 was purchased from GE-Healthcare Biosciences Inc. (ON Canada). Anti-apoA1 monoclonal and anti-mouse HSP-70 monoclonal antibodies were bought from BD Transduction (San Jose, CA USA). Complete protease inhibitor cocktail was purchased from Roche (Sainte-Foy, QC Canada). Scintillation liquid, chloroform and methanol were bought from Fisher Scientific (Ottawa, ON Canada). Potassium Bromide was purchased from Merck (Gibbstown, NJ USA) and finally the supersignal chemiluminescent kit and film were obtained from Pierce (Rockford, IL USA).

2.2 Cell Culture

Baby hamster kidney (BHK) cell lines were used in the present study. Transformed BHK cells were the generous gift from Dr. Jack Oram of University of Washington, Seattle. These cells carry a mifepristone inducible vector with or without an ABCA1 gene insert (Vaughan and Oram, 2003). Cells with the ABCA1 insert are termed as ABCA1 cells and cells without the ABCA1 insert as Mock cells. Without induction, ABCA1 cells express no ABCA1 protein. However, when induced by mifepristone, ABCA1 cells express high levels of ABCA1 protein, comparable to mouse macrophages and, as a result, can efflux cholesterol efficiently. Mock cells have no ABCA1 protein and therefore are not capable of cholesterol efflux even after induction with mifepristone.

For some of the experiments, an adherent mouse macrophage cell line, RAW 264.7 was used. The cell line was obtained from ATCC. This murine macrophage cell line has endogenous ABCA1 expression and treatment with cAMP analogues leads to a several fold induction of ABCA1 mRNA and protein expression. RAW macrophages can efficiently efflux free cholesterol and phospholipid to apoA1 (Zheng *et al.*, 2001). Cells without cAMP induction were used as negative controls.

Both BHK cells and macrophage cells were maintained in DMEM + 10% fetal calf serum at 37°C in a 5% CO_2 incubator. For BHK cells, induction medium consisted of DMEM with 1 mg/ml BSA and 10 nM mifepristone. In the case of macrophage cells, induction medium was comprised of DMEM with 1 mg/ml BSA and 0.5 mM of 8-bromo cAMP. For all experiments, both BHK and macrophage cells were incubated with induction medium for 18-20 h to induce the expression of ABCA1 prior to experiments.

2.3 Preparation of ApoA1

ApoA1 was purchased from Biodesign International in guanidine hydrochloride buffer solution. In order to re-nature the protein, apoA1 was dialyzed extensively in PBS using a 14 kDa molecular weight cut-off membrane with four changes of 4L of PBS at 4°C over 2 days. Dialyzed apoA1 was recovered; protein concentration was measured by Lowry's protein assay (Lowry *et al.*, 1951) and stored at 4°C.

2.3.1 Preparation of ^{125}I apoA1

1 mCi of ^{125}I and 500 µg of apoA1 were transferred to a tube containing Iodogen-coated beads under the fume hood and was incubated for 45 min at room temperature with manual agitation every 10 min. The reaction was stopped by adding one fifth volume of 5x solution of NaI. The reaction mixture was then transferred to a Sephadex G25 column (1.5 cm × 10 cm) and eluted with PBS. The radio-iodinated protein was separated from the free, non-reacted iodine. The eluted solution was collected as fractions and radioactivity of each fraction was determined in gamma counter. Usually the radiolabelled apoA1 will elute in fractions 5-12, while free iodine eluted in fractions 16-20. Fractions that contain radiolabelled apoA1 were combined and were dialyzed overnight in PBS. ^{125}I apoA1 was recovered and the protein concentration was measured by Lowry's protein estimation (Lowry *et al.*, 1951). ^{125}I apoA1 was aliquoted and stored at 4°C in lead container.

2.4 Methods

2.4.1 Cholesterol efflux assay

^{3}H cholesterol was directly added to the growing medium containing DMEM + 10% fetal calf serum and used for labeling cells in a concentration of 1 μCi/ml. Both BHK and macrophage cells were grown in this medium containing ^{3}H cholesterol for 1-2 days to label cellular cholesterol to equilibrium. After 2 days, the labeling medium was replaced by induction medium containing DMEM + 1 mg/ml BSA + 10 nM mifepristone for BHK cells. For macrophages, induction medium contained DMEM + BSA + 0.5 mM 8-bromo-cAMP. After about 18–20 h induction medium was replaced by fresh DMEM + BSA medium without inducting agent for efflux experiments. In most experiments, cholesterol efflux was carried out by incubating cells with or without 10 μg/ml apoA1 for 2 h at 37°C unless otherwise mentioned. At the end of the efflux assay, medium was collected and cells were washed twice with PBS. The collected medium was centrifuged at 3000 g for 5 min to remove any cells and cell debris. 0.3 ml of medium was mixed with scintillation liquid and counted for radioactivity. The cells were lysed in 0.5 ml of 0.5 N NaOH overnight with constant shaking. In separate vials, 0.2 ml of cell lysates were taken and mixed with scintillation liquid and also counted for radioactivity. Total cellular ^{3}H cholesterol was calculated as the sum of the radioactivity in the efflux medium plus the radioactivity associated with cells. Radioactivity in the medium was used to calculate the percentage of total ^{3}H cholesterol released from the cells. For the experiments involving WGA, cholesteryl hemisuccinate, benzyl alcohol, hexanol and LGM, cells were co-incubated with 10 μg/ml of apoA1 and respective reagents for various times. In

some experiments cholesterol efflux was performed in the absence of apoA1 and the medium was counted for radioactivity and the value was referred to as apoA1 independent cholesterol efflux.

Recovery of cells after exposure to WGA was conducted as follows; cells were pretreated with 10 μg/ml WGA for 2 h followed by recovery in WGA free medium. Recovery with glucoasmine was achieved by co-incubating cells with apoA1 and *N*-acetyl glucoasmine following WGA treatment and recovery in WGA free medium.

2.4.2 Plasma membrane rigidity measurement by Transferrin endocytosis

Cells were grown in glass bottom culture dishes to 80% confluency. ABCA1 expression was induced with the addition of mifepristone containing medium. Both ABCA1 and Mock cells were treated with and without WGA for a period of 2 h, and then cells were washed twice with PBS followed by incubation with Cy3-transferrin (20 μg/ml) for 15 min. Cells were then fixed with 4% paraformaldehyde, washed twice with PBS and visualized under a fluorescence microscope. Pictures were taken using a CCD camera attached to the microscope.

2.4.3 Plasma membrane rigidity measurement by micropipette aspiration method

To directly measure the effect of WGA, the membrane extensional rigidity on single cells was measured by a micropipette aspiration method. Cells were grown in glass bottom culture dish and ABCA1 expression was induced. BHK cells were treated with

WGA for 2 h before the membrane extensional rigidity on single cells was measured. In this experiment, a negative pressure (-10 mm Hg) was applied to a micropipette that contacts the plasma membrane. The negative pressure forced the plasma membrane to flow into micropipette and the rate of such flow was recorded by a video camera and compared between control and WGA treated cells.

2.4.4 Phospholipid efflux

BHK cells were grown in 12 well culture plates containing normal medium (DMEM plus 10% fetal calf serum) with 1 μCi/ml ^{3}H choline chloride for 2 days to label cellular phospholipids to equilibrium. Cells were then treated with the induction medium containing DMEM, 1 mg/ml BSA and 10 nM mifepristone for 18–20 h. Following day cells were treated with and without 10 μg/ml apoA1 and WGA (10 μg/ml) for 2 h at 37°C. At the end of the incubation, the medium was collected and phospholipids were extracted by the chloroform-methanol lipid extraction method. Briefly, medium was collected and centrifuged at 800 g for 10 min at 4°C to remove any floating cells. The supernatant was transferred into glass tubes and FOLCH solvent (Chloroform: methanol in a ratio 2:1) about 5x volumes were added to the glass tube. The mixture was vortexed and then shaken vigorously overnight at 4°C. On the following day, 250 μl of PBS was added to each tube and the mixture was spun at 1000 g for 20 min. The aqueous upper phase as well as the proteins which were in interphases between aqueous and organic phase was removed with glass Pasteur pipette and the lower organic phase was allowed to evaporate under a fume hood. Scintillation fluid was added, the mixture was vortexed vigorously and radioactivity was quantified. For lipid extraction from cells, the cells

were washed twice with PBS after removing the efflux medium. 0.5 ml of Hip solution (hexane:isopropanol in 3:2 ratio) was added and the mixture was incubated at room temperature for 30 min. The process was repeated and the mixture was combined to scintillation vial and was allowed to evaporate under fume hood. Scintillation fluid was added and radioactivity was counted. To each well 0.5 ml of NaOH was added and protein concentration was determined by Lowry's method (Lowry *et al.*, 1951). Phospholipid efflux was calculated as a percentage of ^{3}H choline released into the medium relative to the total ^{3}H choline.

2.4.5 Microscopic experiments

BHK cells were plated and grown in glass bottom culture dish up to 80% confluency followed by incubation for 18–20 h in DMEM containing 1 mg/ml BSA and 10 nM mifepristone. For immunofluorescent staining, cells were treated with WGA for 2 h followed by washing with PBS twice. Cells were fixed with 4% paraformaldehyde at room temperature for 10 min and washed twice with PBS. Cells were then permeabilized with 0.1 mg/ml saponin for 30 min at room temperature, followed by two wash with PBS. The cells were blocked with 50 mM NH_4Cl and 5% calf serum solution for 20 min. ABCA1 was visualized using a primary polyclonal antibody against ABCA1 (1:200 dilution) followed by a Alexa 488 conjugated secondary antibody.

Distribution of WGA in BHK cells was observed by treating cells with 10 μg/ml Alexa 488-WGA for 2 h. Following treatment, recovery from WGA was carried out by

incubating the cells in WGA free medium for another 2 hrs. Cells were then fixed and were studied under microscope.

2.4.6 Crosslinking & immunoprecipitation experiment

Spicific interactions between ABCA1 and apoA1 were studied using an immunoprecipitation approach. Cells were plated and grown in 10 cm dishes to 90% confluency. Cells were then treated with 10 μg/ml WGA and 10 μg/ml of ^{125}I apoA1 for 2 h followed by washing twice with PBS and incubation with DSP cross-linker for 30 minutes at room temperature. DSP cross-linker is a membrane permeable cross-linker that contains amine reactive esters that reacts with primary amine groups of apoA1 and ABCA1 and form stable amide bonds between these two proteins. At the end of 30 min, cells were washed twice with ice cold PBS and lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM Ethylene diamine tetracetic acid (EDTA), 0.5 mM Ethylene glycol tetraacetic acid (EGTA), 0.5% *N*-dodecylmaltoside and protease inhibitors at 4°C. Lysed samples were homogenized with a homogenizer with 40 strokes and centrifuged at 1000 g at 4°C for 10 min. The supernatant was recovered, protein concentration was measured by Bradford method (Bradford *et al.*, 1976) and the sample was immunoprecipited (IP). For IP, 200 μg cell lysate was combined with 10 μl anti-ABCA1 antibody and was shaken overnight at 4°C. The following day a 50 μl aliquot of 50% (v/v) slurry of protein-A Sepharose beads was added to each sample and was shaken for 2 h at 4°C. At the end of 2 h, samples were centrifuged at 1000 g for 10 min to pellet the beads. Supernatant was collected completely without disturbing the pellet. Both supernatant and pellet were counted separately for the presence of radioactivity using a

gamma counter. Results of crosslinking were reported as % CPM in the pellet/ (CPM_{Pellet} + CPM_{Sus}).

2.4.7 Purification of membrane vesicles by KBr density gradient centrifugation

In order to characterize cell derived membrane vesicles, ABCA1 and Mock cells were grown in 30 mm dishes and labeled with ^{3}H cholesterol (5 μCi/ml) for 2 days. Cells were induced with mifepristone in DMEM medium for 20 h before the vesicle purification. ApoA1 (5 μg/ml) mediated cholesterol efflux was carried out in the presence or absence of WGA for 4 h. Medium was collected at the end of the efflux period and was centrifuged for 10 min at 2,000 g in order to remove the cell debris. The medium was transferred to 10 kDa molecular cut off filters and centrifuged at 4,000 g for 20 min. The concentrated samples (0.5 ml) were then separated on a KBr gradient by ultracentrifugation. All of the KBr solutions were prepared in 0.9% NaCl solutions. For the KBr density gradient, 0.5 ml of concentrated sample was mixed with 0.95 g of KBr powder and volume was completed to 2 ml with PBS and loaded at the bottom of the tube. That will make the density of sample solution 1.3 g/ml. 2 ml KBr solution of density 1.26 g/ml was layered on top of the sample followed by another layer of 5 ml KBr solution of density 1.1 g/ml. Finally, 2 ml Tris-HCl (50 mM, pH 8.2) was layered at the top and subjected to ultracentrifugation for 22 h at 37,000 g in a Beckman SW70 rotor. At the end of 22 h, nine fractions, one ml each, were collected. 0.3 ml from each fraction was mixed with scintillation liquid and counted for total radioactivity. 40 μl from each collected sample was mixed with loading buffer, subjected to sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and probed for the presence of apoA1 using an anti-apoA1 primary antibody (1:2000 dilution) followed by horseradish peroxidase (HRP)-linked secondary antibody in 1:5000 dilution.

2.4.8 Immunoblot analysis of efflux medium

ABCA1 and Mock cells were plated in 30 mm dishes and grown to 90% confluency. Cells were induced with mifepristone for 18-20 h before the experiment. Cholesterol efflux was carried out by co-incubating cells with and without apoA1 (5 μg/ml) and also with and without WGA (10 μg/ml) for 4 h. Medium was collected at the end of efflux period and the cells were lysed in lysis buffer containing 25 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% *N*-maltosidase and protease inhibitors. The medium was centrifuged for 10 min at 2,000 g in order to remove the cell debris. Then medium was transferred to 10 kDa molecular cut off filters and centrifuged at 4,000 g for 20 min to concentrate the 15 ml sample to 0.5 ml. A 40 μl sample was run on a 12.5 % SDS-PAGE gel and immonoblot was performed. For the cell lysate, the protein concentration was measured by Lowry's method (Lowry *et al.*, 1951) and equal amount of proteins were loaded in the same gel along with concentrated medium. Electrophoresis was carried out and proteins were transferred overnight to Polyvinylidene Difluoride (PVDF) membrane. The membrane was blocked with 5% milk in PBS followed by exposure to either anti-apoE primary antibody (1:500) or anti-HSP-70 primary antibody (1:1000) followed by HRP-linked secondary antibody (1:4000 dilution). Finally membrane was developed using supersignal chemiluminescent kit. Each band was quantified using densitometry analysis.

2.4.9 Densitometry analysis

Densitometry is the quantitative measurement of optical density in a photographic film. A scanned image of immunoblot result was taken in gray scale prior to densitometry analysis. Image was then analyzed quantitatively by a densitometry. For this purpose a box was drawn in an area that has no band, this serve as background which was set to 100%. This gives background intensity of the image. Then same size boxes were drawn for each band of interest and background value was subtracted from each band value analyzed. Then result was represented in form of bar graph and was corrected for protein concentration.

Chapter 3

Results

3.1 ApoA1-independent Cholesterol Efflux

This research proposes that ABCA1 mediated efflux involves the shedding of plasma membrane vesicles. Our hypothesis is based on two observations. First, ABCA1 expression causes global changes in the plasma membrane such that there are more non-rafts membrane microdomains in comparison with cells not expressing any ABCA1 (Mock) (Mendez *et al.*, 2001; Landry *et al.*, 2006). Interestingly, this change occurs in the absence of apoA1. Secondly, cholesterol efflux experiments show that ABCA1 expressing cells always cause significant higher cholesterol efflux than Mock cells even in the absence of apoA1. This prompted us to examine the cholesterol efflux process more closely, particularly in the absence of apoA1. To do this, cholesterol efflux experiments were performed according to the routine protocol but without apoA1. Also, to measure the efflux more accurately, we prolonged the efflux process to 8 h. Upon ABCA1 expression, there was a substantial efflux in the absence of apoA1. This was observed in BHK cells that were induced to express ABCA1 (**Figure 3.1-A**) and in macrophages expressing endogenous ABCA1 (**Figure 3.1-B**). This apoA1-independent efflux entirely relies on ABCA1 expression as Mock BHK cells or non-induced macrophages cannot significantly efflux cholesterol under these conditions.

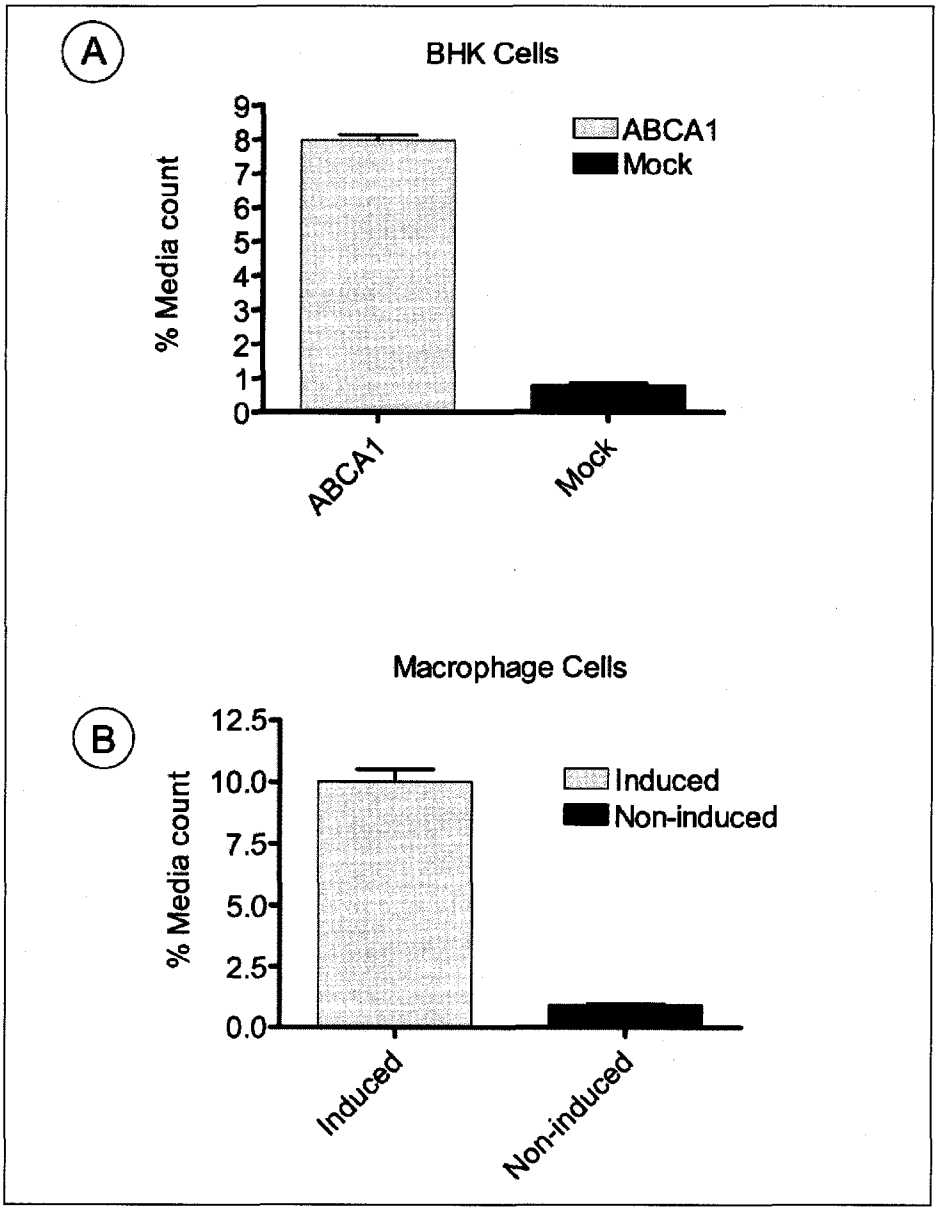

Figure 3.1-A, B: ApoA1-independent cholesterol efflux. BHK and macrophage cells were labeled and grown in (1 μCi/ml) ^{3}H cholesterol containing medium for 2 days. ABCA1 expression was induced by treating cells with **(A)** 10 nM mifepristone for BHK cells and, **(B)** 0.5 mM 8-bromo-cAMP in case of macrophage cells in DMEM+BSA medium for 20 h. Cells were changed into fresh DMEM+BSA medium without inducing agents. Medium was collected after 8 h and cells were lysed in 0.5 N NaOH and both medium and cell associated radioactivity was counted. Total cellular ^{3}H cholesterol was calculated as the sum of the radioactivity in the efflux medium plus the radioactivity in the cells and was used to calculate the percentage of total ^{3}H cholesterol released into the medium.

These two cell lines express similar levels of ABCA1 protein (data not shown). In addition, BSA, which is commonly used in the efflux medium, is not a significant contributor of such apoA1-independent efflux (data not shown). In terms of magnitude, the apoA1-independent efflux is about one third of the cholesterol efflux induced by apoA1 **(Figure 3.1-C)**. Although relatively small in magnitude, it reflected several fold increase in efflux in ABCA1 expressing cells over Mock background, suggesting a significant contribution to the overall efflux. Importantly, such apoA1-independent cholesterol efflux is not likely due to lipoprotein secretion. ABCA1 expression has been shown to promote apoA1 secretion and lipidation in hepatocytes (Kiss *et al.*, 2003). ApoE also has been associated with cholesterol efflux in macrophages (Zhang *et al.*, 1996). Had the similar processes occurred in BHK cells, it could have led to apparent cholesterol efflux without the presence of exogenous apoA1. We therefore checked the expression and secretion of apoA1 or apoE in BHK cells. We could not find evidence for such secretion or expression in BHK cells (**Figure 3.1-D**). Lipoprotein secretion is thus an unlikely explanation for the above observation. Given the overall morphological changes we have seen in ABCA1 cells, we speculate that ABCA1 expression alone would condition the plasma membrane and make it more flexible (more non-rafts). Such conditioning may promote a membrane vesiculation process or membrane shedding leading to cholesterol efflux to the medium. ApoA1 may further amplify the process to induce robust cholesterol efflux. If this is true, however, the flexibility of the plasma membrane should be an important factor influencing cholesterol efflux for both apoA1-dependent or independent efflux. We therefore decided to use reagents that are known to either rigidify or soften the plasma membrane and examine their effect on cholesterol efflux.

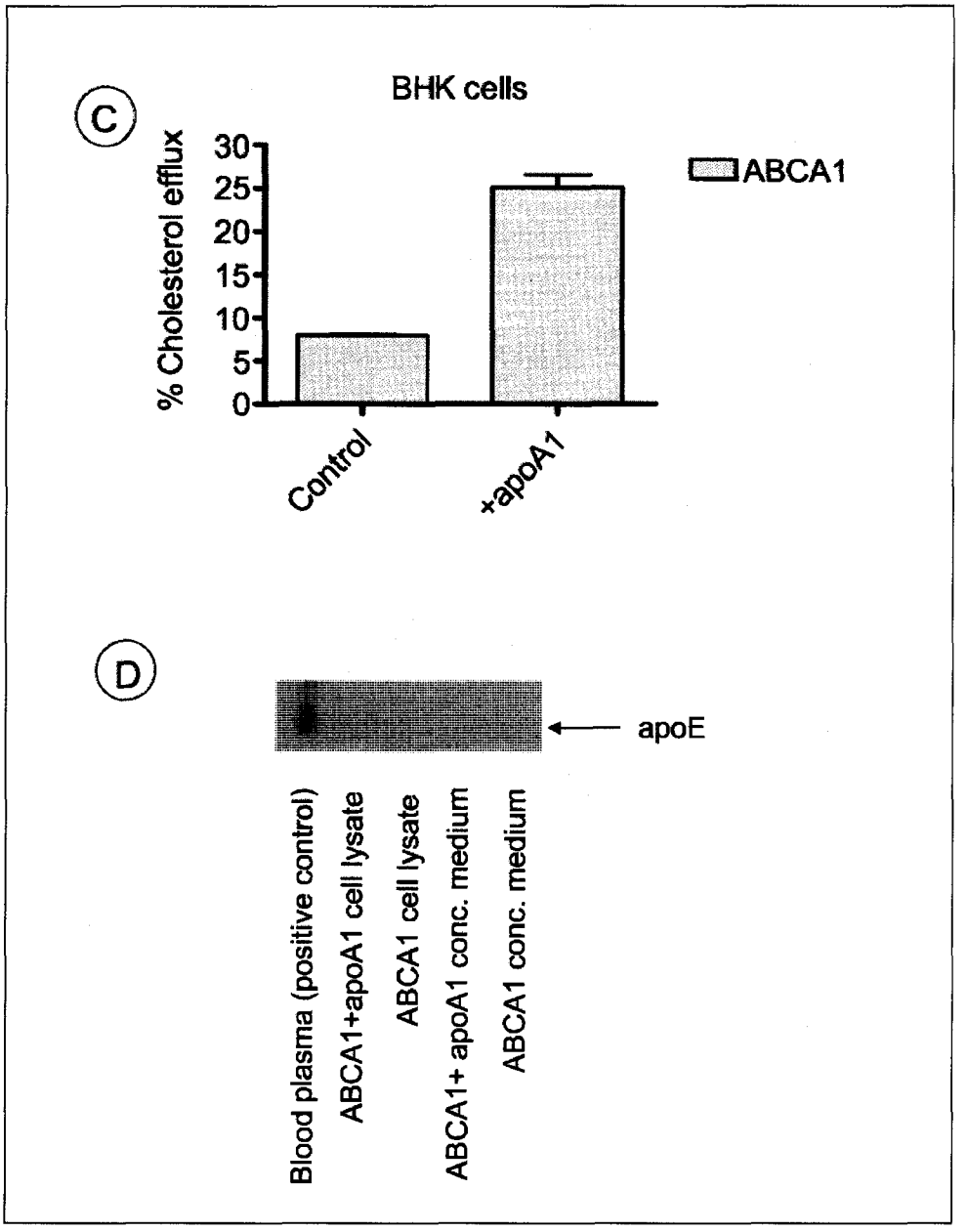

Figure 3.1-C, D: ApoA1-dependent cholesterol efflux and detection of apoE in the effluxed medium. **(C)** BHK cells were labeled and grown in (1 μCi/ml) ^{3}H cholesterol containing medium for 2 days. ABCA1 expression was induced by treating cells with 10 nM mifepristone. apoA1(10 μg/ml) mediated cholesterol efflux was carried out for 8 h. Medium was collected after 8 h and cells were lysed in 0.5 N NaOH and both medium and cell associated radioactivity was counted. Cholesterol efflux was calculated. **(D)** Efflux medium was concentrated by using centrifugal force through 10 kDa molecular weight cut off and loaded on SDS-PAGE gel and immunoblot was performed and presence apoE was detected using an Anti apoE primary antibody.

3.2 Effect of WGA on Plasma Membrane Rigidity

We first used WGA, a known reagent that rigidifies the membrane (Evans and Leung, 1984; Hochmuth and Waugh, 1987). We employed two protocols to verify the rigidifying effect of WGA in the plasma membrane. First, ABCA1 and Mock cells were treated with WGA and studied for transferrin endocytosis. Transferrin is a marker for endocytosis (Jing *et al.*, 1990) and its efficient uptake will require membrane flexibility. Endocytosis in WGA treated cells was diminished compared to the control cells **(Figure 3.2-A, First and Second row)** indicating a change in flexibility of the plasma membrane. This effect is highly specific to WGA binding to the cell surface, since transferrin endocytosis could be rescued if WGA is stripped off by a competitive sugar glucosamine **(Figure 3.2-A, Third row)**. Secondly, the rigidifying effect of WGA on BHK cells was measured by micropipette aspiration method Again, BHK cells were treated with WGA for 2 h before the membrane extensional rigidity on single cells was measured. In this experiment, a negative pressure (-10 mm Hg) was applied to a micropipette that contacts the plasma membrane **(Figure 3.2-B)**. The negative pressure forced the plasma membrane to flow into micropipette. The rate of such flow reflects the flexibility of the membrane and was recorded by a video camera. Under our experimental conditions, it was observed that the plasma membrane of control cells was aspirated into a micropipette at a speed 0.7 μm/sec. In WGA treated cells, when the same negative pressure was applied, the plasma membrane hardly moved at all **(Figure 3.2-C)**. Together, these experiments confirmed that WGA has a significant rigidifying effect on the plasma membrane of BHK cells, in agreement with previous studies (Evans and Leung, 1984; Hochmuth and Waugh, 1987).

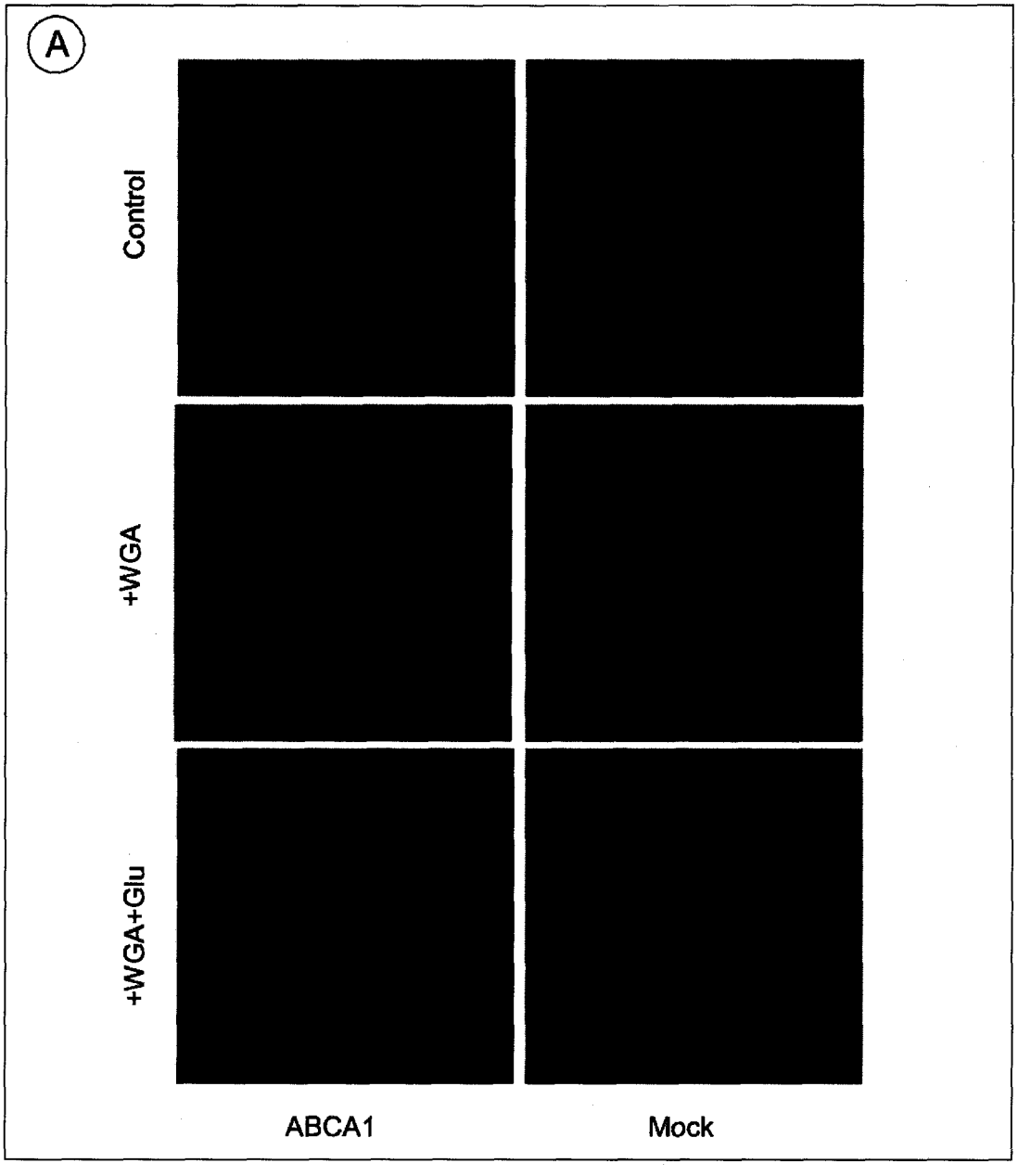

Figure 3.2-A: Effect of WGA on Tf-endocytosis. BHK cells were grown in microscopic dishes and ABCA1 expression was induced. Cells were treated with and without 10 μg/ml of WGA for 2 h. Then cells were either incubated with trasferrin for 15 min (**First & Second row**) or treated with 200 nM glucosamine for another 2 h and then incubated with transferrin for 15 min (**Third row**). Cells were fixed with paraformaldehyde and observed under a fluorescence microscope.

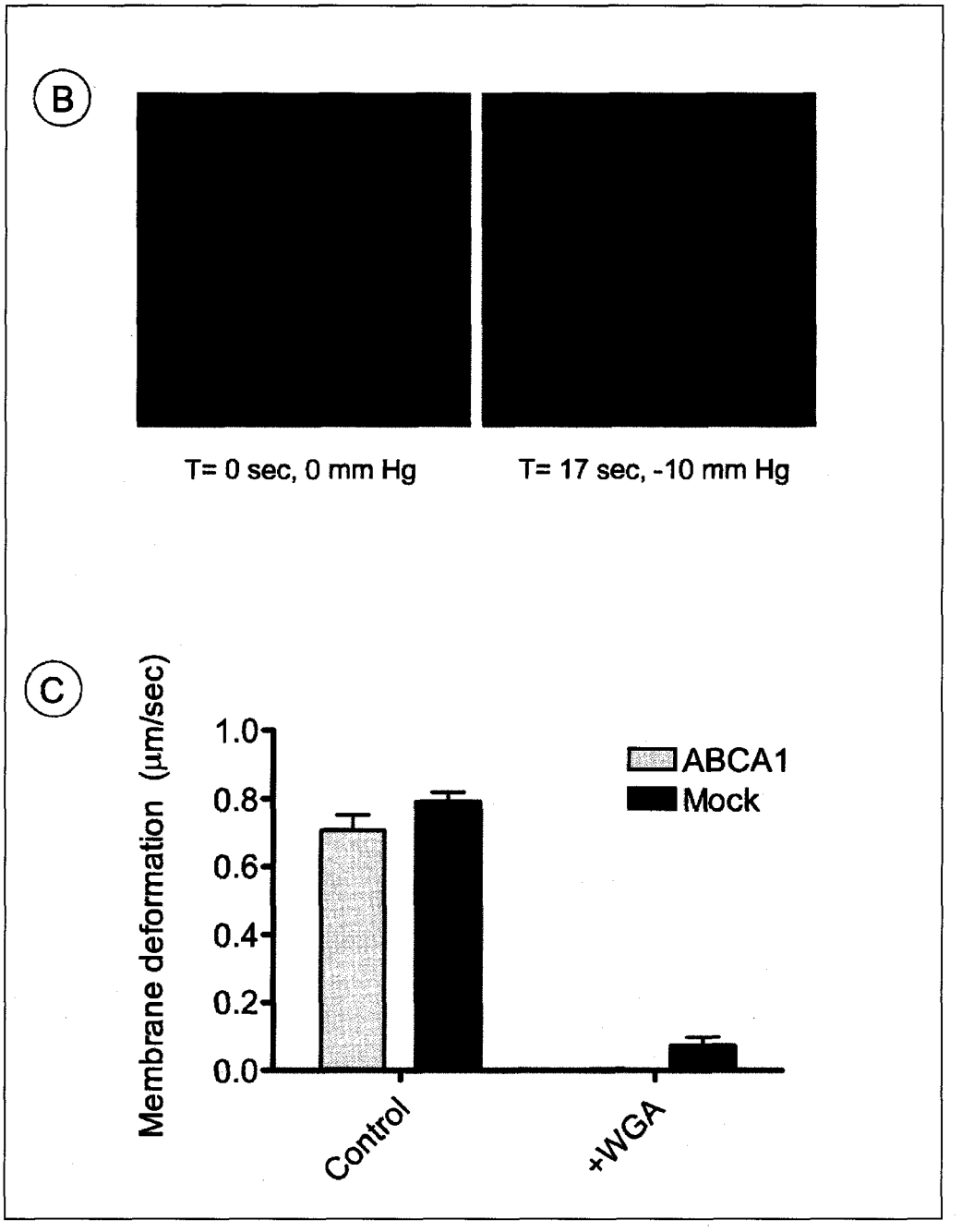

Figure 3.2-B, C: Effect of WGA on plasma membrane rigidity. BHK cells were pretreated with 10 µg/ml of WGA for 2 h then membrane extensional rigidity on live cells was measured by applying a negative pressure (-10 mm Hg) to a micropipette that contacts the plasma membrane. (**B**) Picture showing micropipette touching the plasma membrane. (**C**) Rate of membrane deformation was recorded by a video camera and was quantified.

3.3 Effect of Wheat Germ Agglutinin Treatment on Cholesterol Efflux

Having confirmed the rigidifying effect of WGA, we tested its effect on basal cholesterol efflux (apoA1-independent) in BHK cells. As described earlier, ABCA1 cells, when treated with mifepristone to induce ABCA1 expression, can produce cholesterol efflux even in the absence of apoA1. When we applied WGA in the BHK cells, we observed that WGA inhibited more than 70% of basal cholesterol efflux (apoA1-independent) relative to cells not exposed to WGA **(Figure 3.3-A)**. Mock cells were not affected by WGA.

We also tested the effect of WGA on apoA1 dependent cholesterol efflux. Addition of apoA1 to the medium boosts the efflux to about 10% in 2 h. When BHK cells were treated with WGA along with apoA1 for 2 h, WGA inhibited apoA1 mediated cholesterol efflux by more than 90% in ABCA1 cells **(Figure 3.3-B)**. WGA also inhibited basal cholesterol efflux (apoA1-independent). Mock cells were not capable of carrying out the efflux process in the absence or presence of apoA1. WGA also had no effect on these cells. Furthermore, the effect of WGA was tested on macrophages, a cell type that naturally expresses ABCA1 when induced with cAMP and effluxes cholesterol. A similar inhibition of cholesterol efflux by WGA was observed **(Figure 3.3-C)**, suggesting that the observed effect of WGA is not likely cell-type dependent.

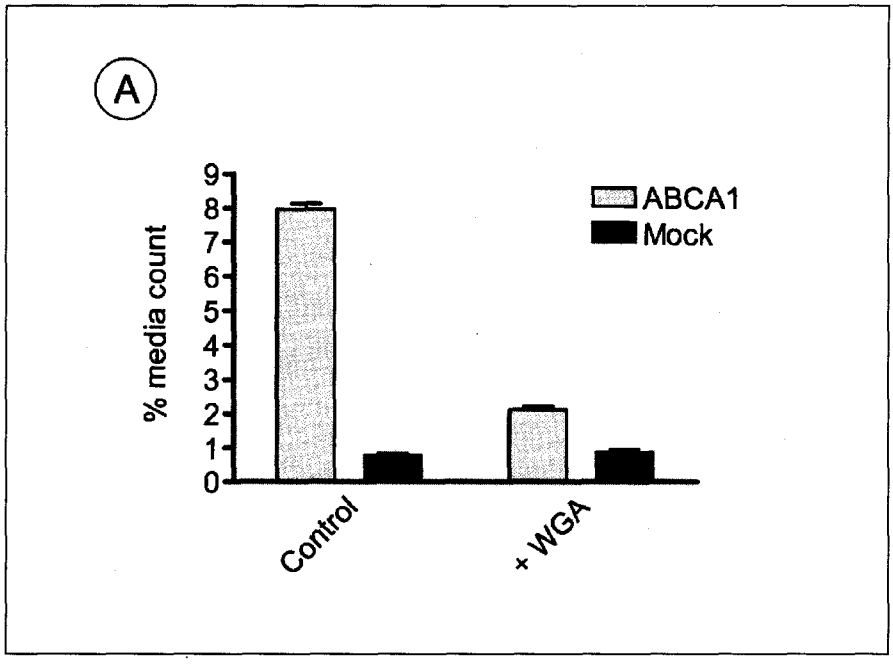

Figure 3.3-A: Effect of WGA on apoA1-independent cholesterol efflux. BHK cells were labeled with 1 μCi/ml ^{3}H cholesterol and were grown for 2 days. ABCA1 expression was induced and apoA1 independent cholesterol efflux was carried out in the presence or absence of WGA (10 μg/ml) for 2 h.

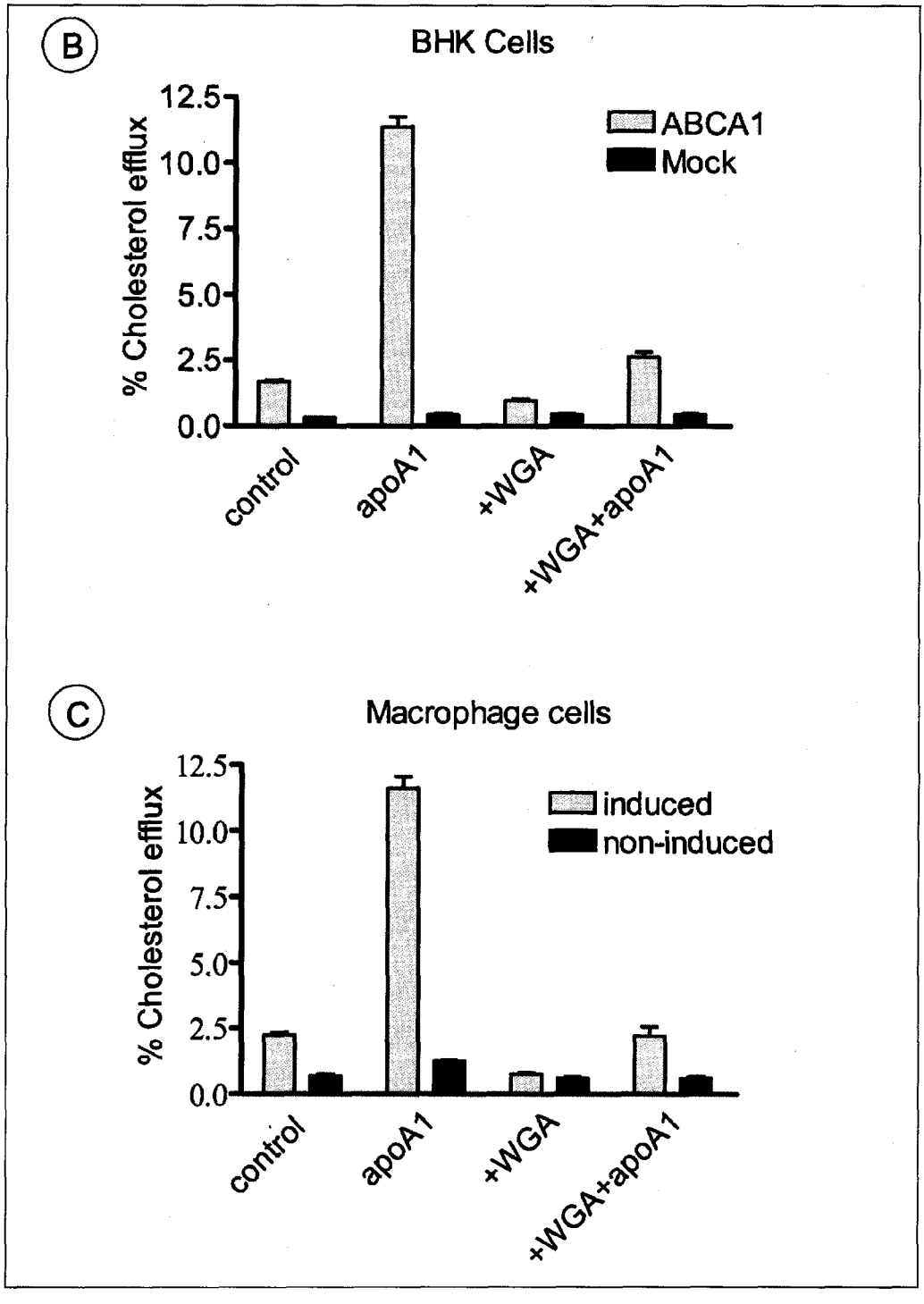

Figure 3.3-B, C: Effect of WGA on apoA1 mediated cholesterol efflux. ApoA1(10 μg/ml) mediated cholesterol efflux was carried out in (**B**) BHK cells and (**C**) Macrophage cells in presence or absence of 10 μg/ml WGA for 2 h.

The effect of different dosage of WGA on cholesterol efflux in BHK cells was then examined. WGA was able to inhibit cholesterol efflux in a dose-dependent manner **(Figure 3.3-D)**. Since 10 μg/ml of WGA was able to completely abolish the efflux, we chose this dose for further experiments. We also tested the time course of the process. As expected, apoA1 mediated cholesterol efflux was linearly increased with time **(Figure 3.3-E)**. However, when WGA was added in the medium, cholesterol efflux was completely inhibited at all time points examined **(Figure 3.3-E)**.

3.4 Recovery from WGA

To ensure that the effect of WGA on cholesterol efflux is due to its binding to the plasma membrane, the efflux recovery experiments were performed. Cells were treated with WGA for 2 h followed by recovery in WGA free medium for different time periods (data not shown) and then apoA1 mediated cholesterol efflux was carried out for 2 h. Initially, cholesterol efflux could not be fully recovered even after 12 h in WGA free medium **(Figure 3.4-A)**. We suspected that a significant amount of WGA might remain bound to the plasma membrane even after prolonged incubation in WGA free medium. To test whether WGA was still present in the membrane after its removal from the medium, cells were treated with a fluorescently labeled WGA, Alexa 488-WGA, for about 2 h and examined for cellular distribution of WGA. As expected, Alexa 488-WGA was found on the cells surface as well as intracellular compartments **(Figure 3.4-B, Upper panel)**. When these cells were extensively washed and incubated in Alexa 488 WGA free medium for 2 h, WGA was still seen to be predominantly bound to the plasma membrane even though there was much less overall fluorescence associated with cells **(Figure 3.4-B, Middle panel)**.

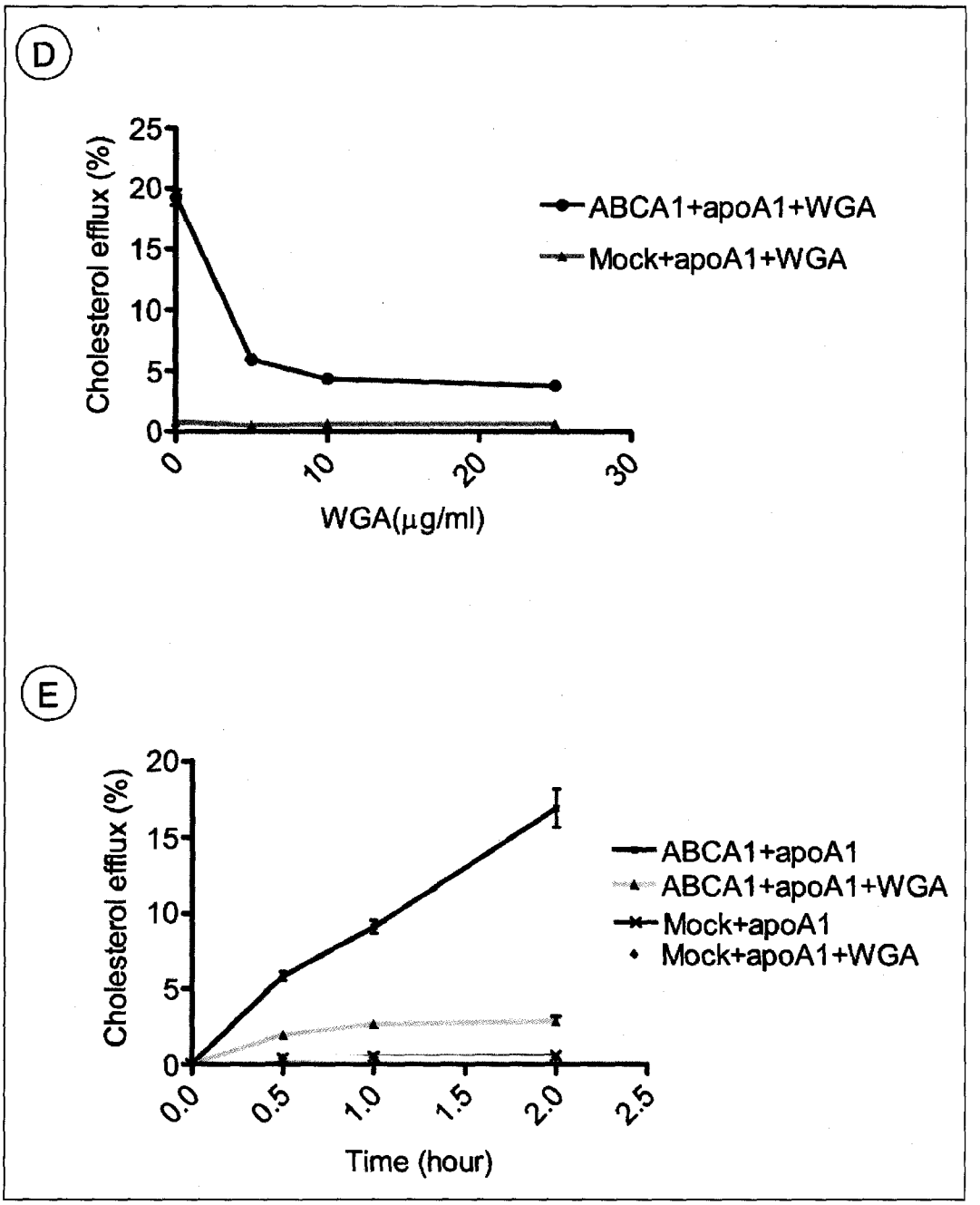

Figure 3.3-D, E: Dose and time dependent effect of WGA on cholesterol efflux. (D) ApoA1 (10 μg/ml mediated cholesterol efflux was performed with 5 μg/ml, 10 μg/ml and 25 μg/ml dose of WGA for 2 h in BHK cells. **(E)** ApoA1 mediated cholesterol efflux was carried out with 10 μg/ml WGA for ½ h, 1 h and 2 h.

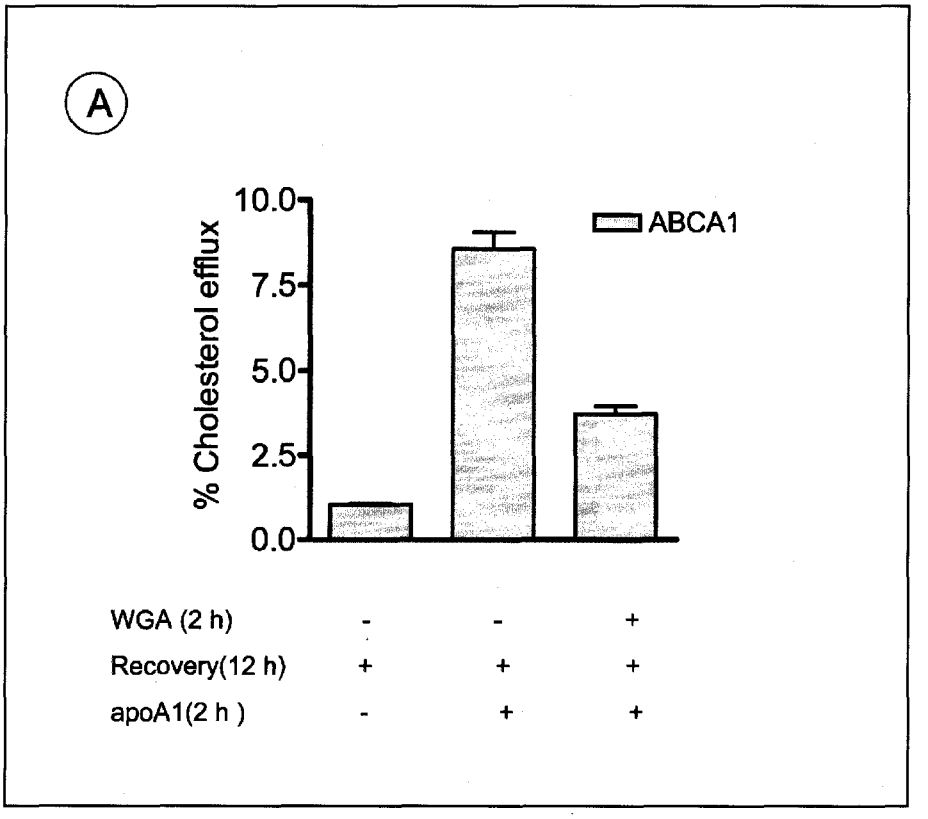

Figure 3.4-A: Recovery from WGA. BHK cells were labeled with 1 μCi/ml ^{3}H cholesterol and ABCA1 expression was induced. Cells were pretreated with 10 μg/ml of WGA for 2 h and then cells were washed with PBS twice to remove WGA. Cells were then recovered in WGA free medium for 12 h. Then apoA1 (10 μg/ml) mediated cholesterol efflux was carried out for 2 h.

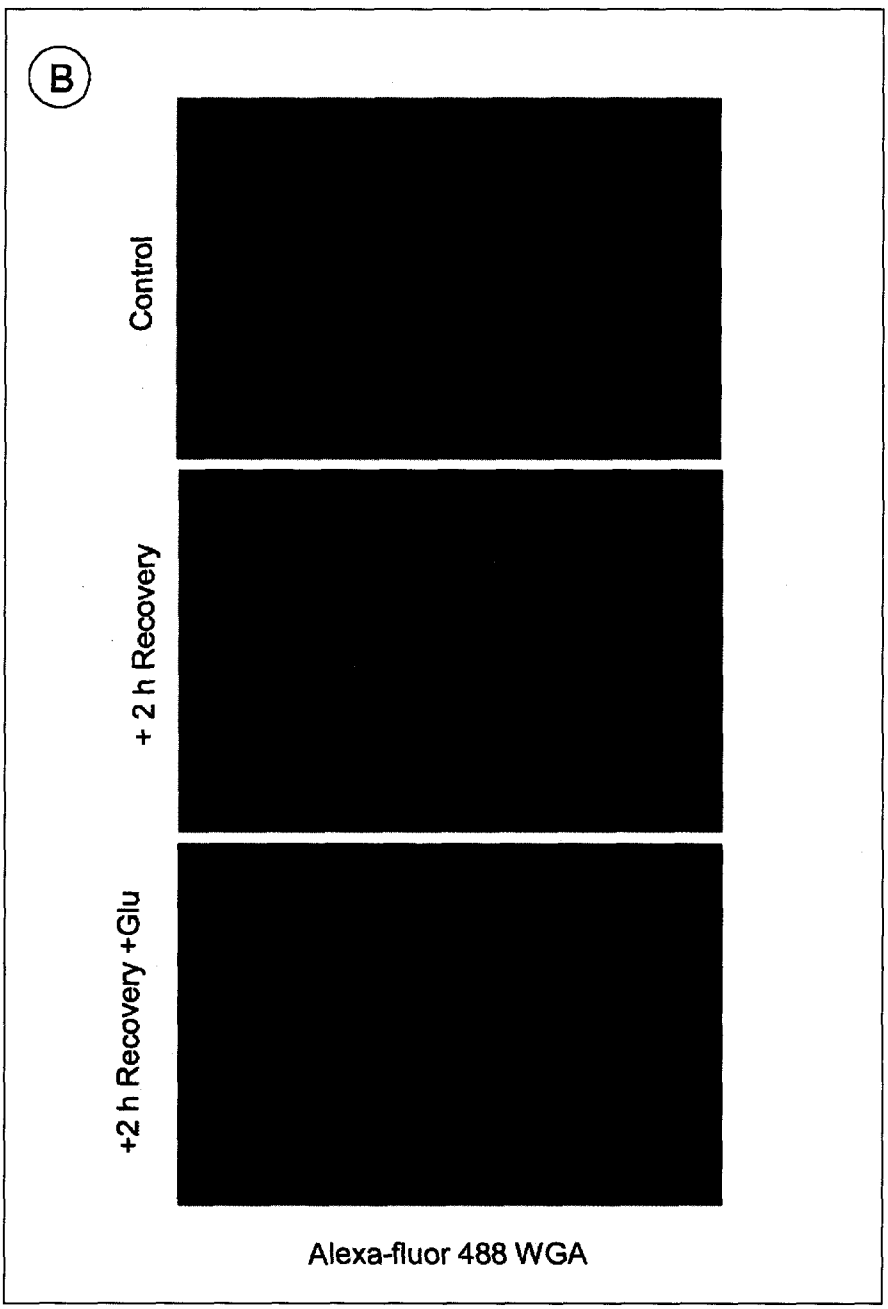

Figure 3.4-B: Distribution of WGA in ABCA1 cells. ABCA1 cells treated with Alexa-fluor 488 WGA (10 μg/ml) for 2 h and recovered for 2 h and then either fixed and observed under the microscope (Upper panel) or recovered in WGA free medium for another 2 h and then fixed and observed under the microscope (Middle panel) or following the recovery, treated with 200 mM glucosamine for another 2 h and then fixed and observed under the microscope (Lower panel).

This indicated that WGA could not be efficiently removed from the cell surface by simple washing, which would explain why the efflux could not be recovered. WGA is known to have a high affinity for *N*-acetylglucosamine (Goldstein and Hayes, 1979; Evans and Leung, 1984). Excess of a competitive sugar (*N*-acetylglucosamine) should be able to compete with cell surface for WGA binding. Therefore, *N*-acetylglucosamine was added in the medium in order to efficiently remove surface bound WGA. ABCA1 cells were treated with Alexa 488-WGA for 2 h followed by recovery in the Alexa 488-WGA free medium containing *N*-acetylglucosamine for another 2 h. We observed that Alexa 488-WGA was effectively removed from the plasma membrane by *N*-acetylglucosamine **(Figure 3.4-B, Lower panel),** although intracellular WGA was not significantly altered. We then tested whether it might be possible to rescue the efflux using *N*-acetylglucosamine. Cells were treated with WGA for 2 h followed by washing and incubation in WGA free medium for another 2 h. ApoA1 mediated cholesterol efflux was then carried out with and without *N*-acetylglucosamine. As expected we observed a full recovery in apoA1 mediated cholesterol efflux **(Figure 3.4-C)**, in agreement with our fluorescent observations. *N*-acetylglucosamine itself had no effect on efflux **(Figure 3.4-D)**. In addition, the above observations illustrated that even though WGA can be endocytosed and eventually processed intracellularly (Pugh and Kalia, 1982), these events are not likely to generate any significant impact on cholesterol efflux under our experimental conditions. *N*-acetylglucosamine was not able to remove intracellular WGA **(Figure 3.4-B, Lower panel)** and yet cholesterol efflux can be fully restored. These observations indicate that the key component that inhibited cholesterol efflux was WGA bound to the cell surface. Given the effect of WGA on rigidifying membrane, our observations so far are consistent with our hypothesis that membrane flexibility is

required for ABCA1 mediated cholesterol efflux via both apoA1-dependent and independent mechanism.

3.5 Effect of WGA on Phospholipid Efflux

Since WGA inhibits cholesterol efflux in BHK cells, WGA may also have an effect on phopholipid efflux. To test this, BHK cells were labeled with ^{3}H choline chloride for 2 days, ABCA1 expression was induced and apoA1 mediated efflux was carried out with and without WGA for 2 h. The medium was then collected and lipid contents were extracted. Lipids were also extracted from cells. Radioactivity was counted and efflux was calculated as percentage of ^{3}H-phospholipids in the medium versus total ^{3}H phospholipids.

Induction of ABCA1 expression produces efficient phospholipid efflux in ABCA1 cells in comparison to Mock cells **(Figure 3.5)**. Upon WGA treatment, we observed about 60% reduction in phospholipid efflux in ABCA1 cells in comparison to non-treated positive controls. WGA was also capable of inhibiting the basal phospholipid efflux (apoA1-independent) in this cell line. WGA treatment showed no effect in Mock cells. Taken together, these efflux results suggest that WGA is more effective in inhibiting cholesterol efflux (more than 90% inhibition), **(Figure 3.3-A)** in comparison to phospholipid efflux (about 60% inhibition). The reason for this discrepancy is not clear.

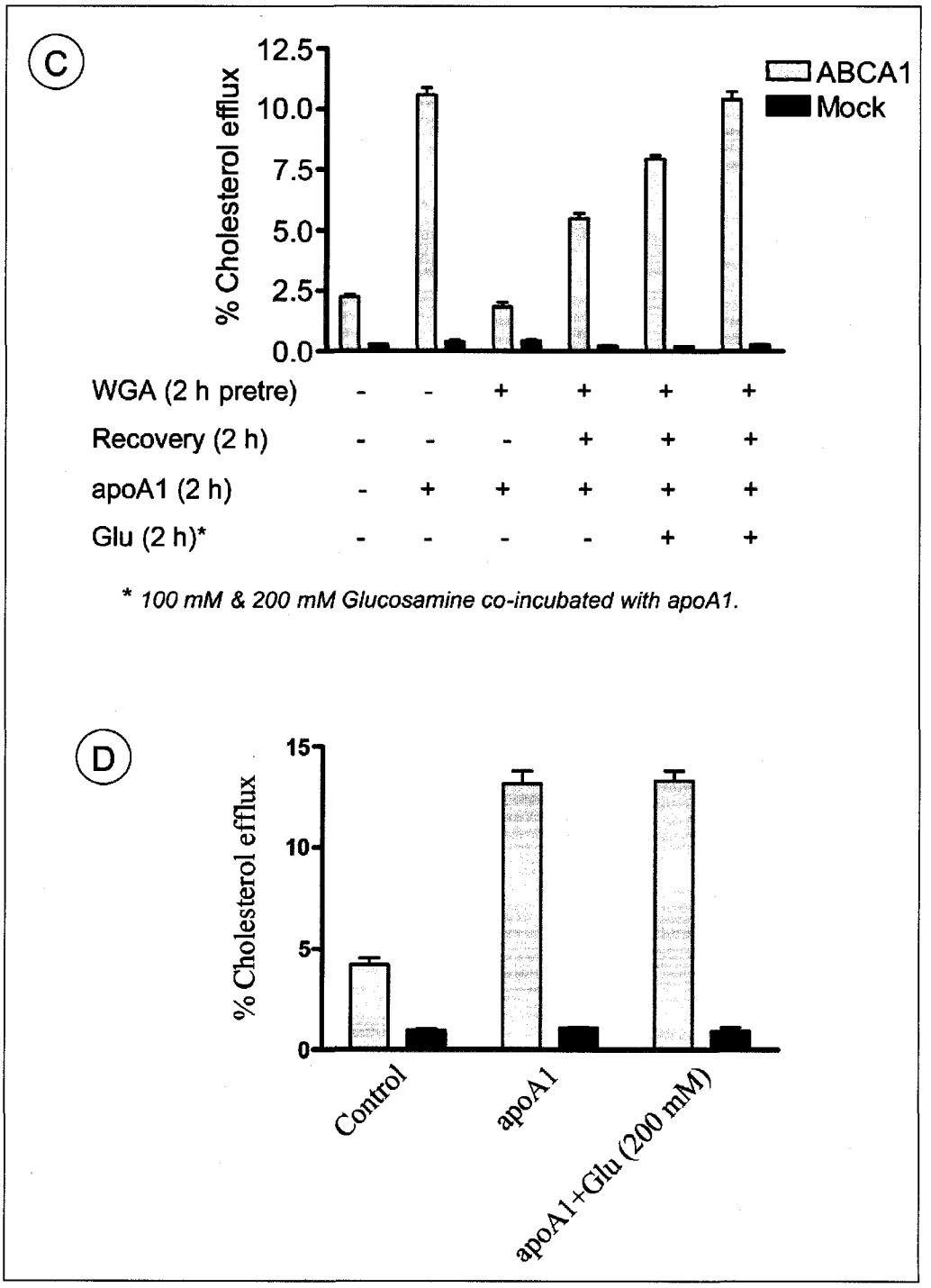

Figure 3.4-C, D: Recovery with glucosamine and efflux. **(C)** BHK cells were pretreated with WGA for 2 h followed by recovery in WGA free medium for another 2 h. Then apoA1 (10 μg/ml) mediated cholesterol efflux was then carried out with or without glucosamine for another 2 h. **(D)** ApoA1 (10 μg/ml) mediated cholesterol efflux was carried out with and without 200 mM of glucosamine for 2 h.

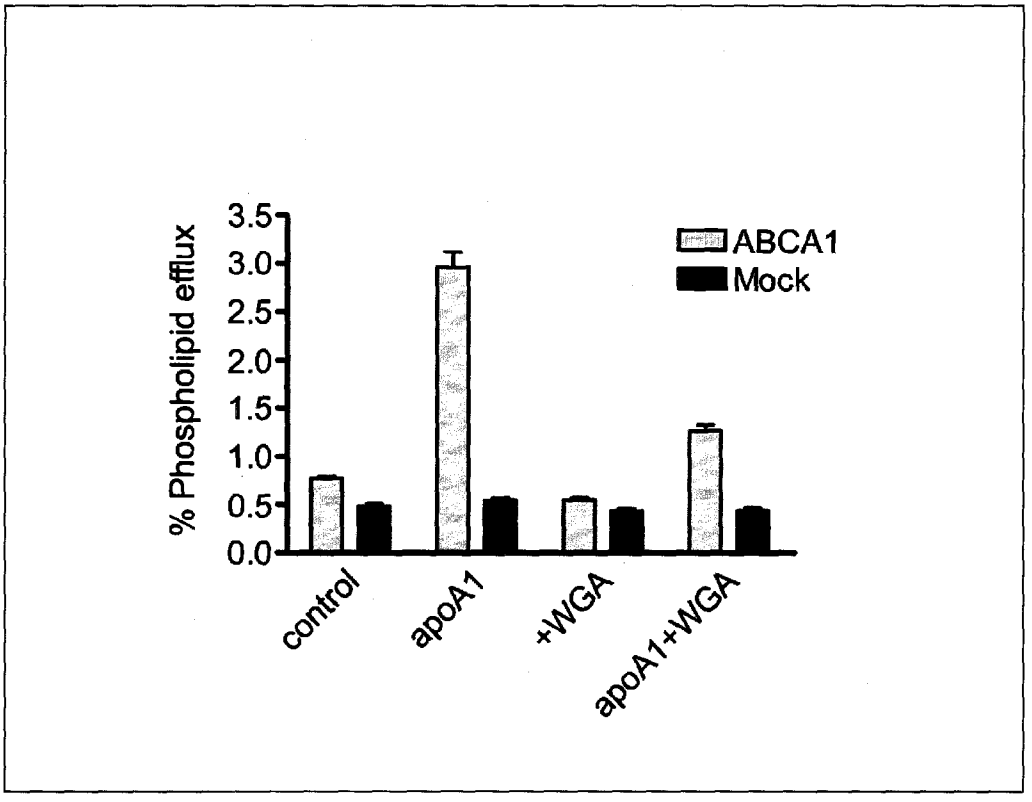

Figure 3.5: Effect of WGA on phospholipids efflux. BHK cells were labeled with 1 μCi/ml choline chloride for 2 days and ABCA1 expression was induced. Cells were treated with 10 μg/ml of WGA and 10 μg/ml of apoA1 for 2 h. Medium was collected and lipids were extracted. Cells were lysed and lipids were also extracted from cells. Phospholipid efflux was calculated as percentage of ^{3}H choline released into the medium of the total ^{3}H choline.

3.6 Effect of Membrane Flexibility on Cholesterol Efflux

WGA has been used as a membrane rigidifier so far, and demonstrated to effectively suppressed cholesterol efflux. Many other reagents are also known to decrease or increase the fluidity of the plasma membrane. Therefore those reagents were tested for their effects on cholesterol efflux.

Cholesteryl hemisuccinate, another known membrane rigidifying agent, was the first compound tested (Yuli *et al.*, 1981; Wang and Zhang, 2005). Cells were coincubated with and without cholesteryl hemisuccinate along with apoA1 (10 μg/ml) for 2 h. We observed that cholesteryl hemisuccinate was also able to effectively inhibit cholesterol efflux to apoA1 in a dose-dependent manner **(Figure 3.6-A)**. Cholesterol efflux was rescued upon removal of cholesteryl hemisuccinate was then tested. For this purpose, cells were pretreated with cholesteryl hemisuccinate followed by replacing the medium with cholesteryl hemisuccinate free medium. Then apoA1 mediated efflux was carried out. A full recovery in the efflux was observed **(Figure 3.6-B)**. This indicated that the inhibition of cholesterol efflux by cholesteryl hemisuccinate was not due to toxicity and cells were fully capable of cholesterol efflux once cholesteryl hemisuccinate was washed off. Thus, together with our observation from WGA, we concluded that rigidifying membrane has a general inhibitory effect on apoA1 mediated cholesterol efflux.

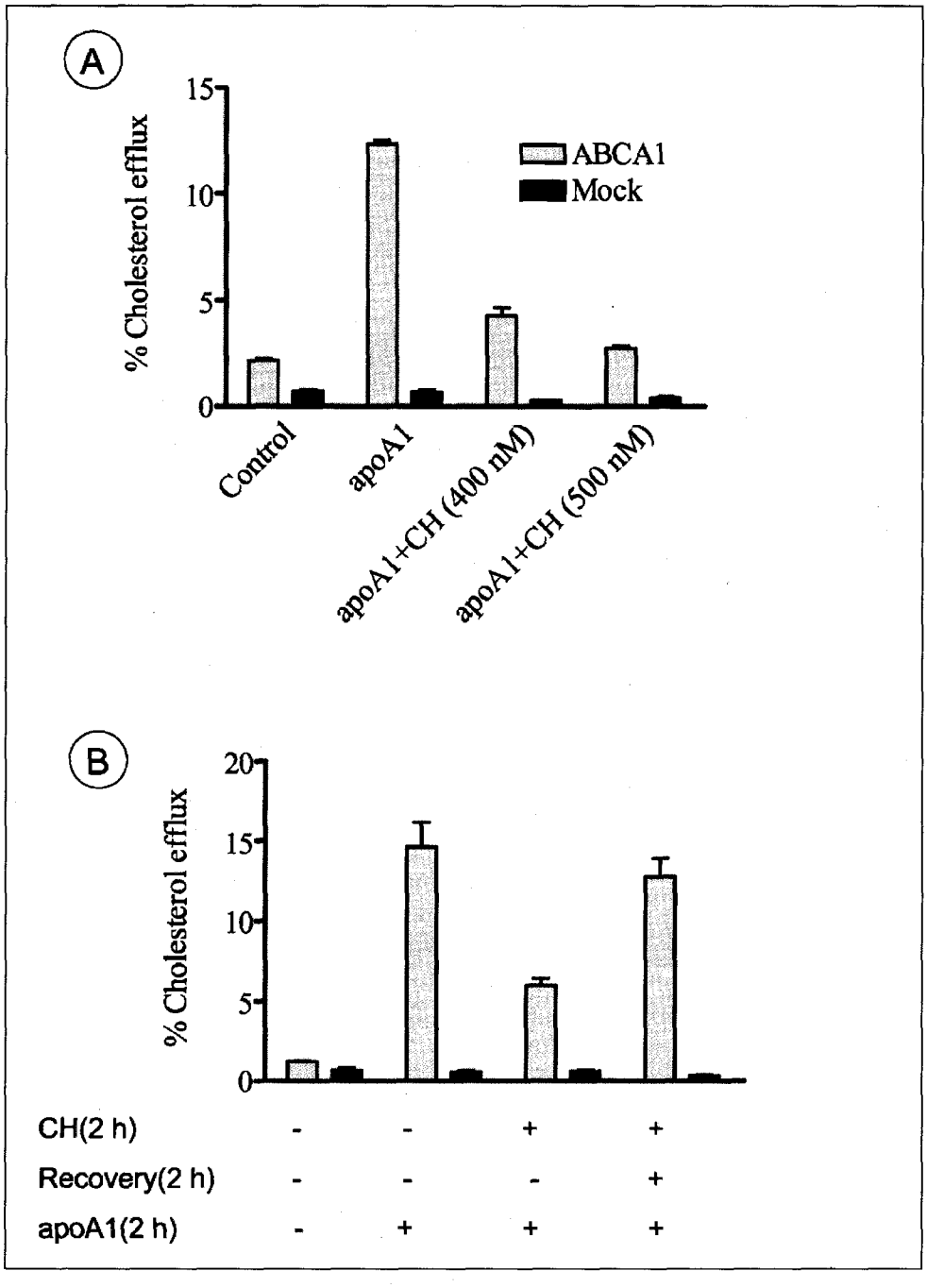

Figure 3.6-A, B: Effect of cholesteryl hemisuccinate on cholesterol efflux and recovery. BHK cells were labeled with 1 μCi/ml ^{3}H cholesterol for 2 days and ABCA1 expression was induced. **(A)** ApoA1 mediated cholesterol efflux was carried out with and without 400 nM, and 500 nM cholesteryl hemisuccinate for 2 h. **(B)** Cells were pretreated with 500 nM cholesteryl hemisuccinate for 2 h. Cells were washed twice with PBS to remove cholesteryl hemisuccinate and recovered in cholesteryl hemisuccinate free medium for 2 h. ApoA1 (10 μg/ml) mediated cholesterol efflux was carried out for another 2 h.

It was then tested whether softening the plasma membrane could enhance cholesterol efflux. For this purpose, we used well-known membrane fluidizers such as benzyl alcohol (Gordon *et al.*, 1980; Wang and Zhang, 2005) and hexanol (Ismaili *et al.*, 1999; Meddings *et al.*, 1990). First, apoA1 mediated cholesterol efflux was carried out with different doses of benzyl alcohol for 2 h. Benzyl alcohol was indeed able to significantly boost up cholesterol efflux **(Figure 3.6-C)**. Interestingly, such enhancement in cholesterol efflux was seen not only in apoA1 mediated efflux but also in apoA1-independent efflux. Benzyl alcohol had little effect on Mock cells. When hexanol was tested, it was observed that apoA1 mediated efflux was also significantly increased by hexanol. Hexanol was able to double the efflux in ABCA1 expressing cells while having little effect on Mock cells **(Figure 3.6-D)**. Once again, hexanol treatment increased cholesterol efflux even in the absence of apoA1. Our observation thus indicated that plasma membrane fluidity is related to ABCA1 mediated efflux. Together with our earlier observation, we conclude that plasma membrane flexibility is an important factor for ABCA1 mediated cholesterol efflux.

3.7 Efflux with another Lectin

One concern was whether the observed effect of WGA on the efflux is lectin specific. WGA belongs to a plant lectin family but not all the lectins can rigidify the membrane. Therefore another lectin from same family was tested. Lectin from glycine max, i.e. soybean (LGM) that has no known rigidifying effect in the membrane was used.

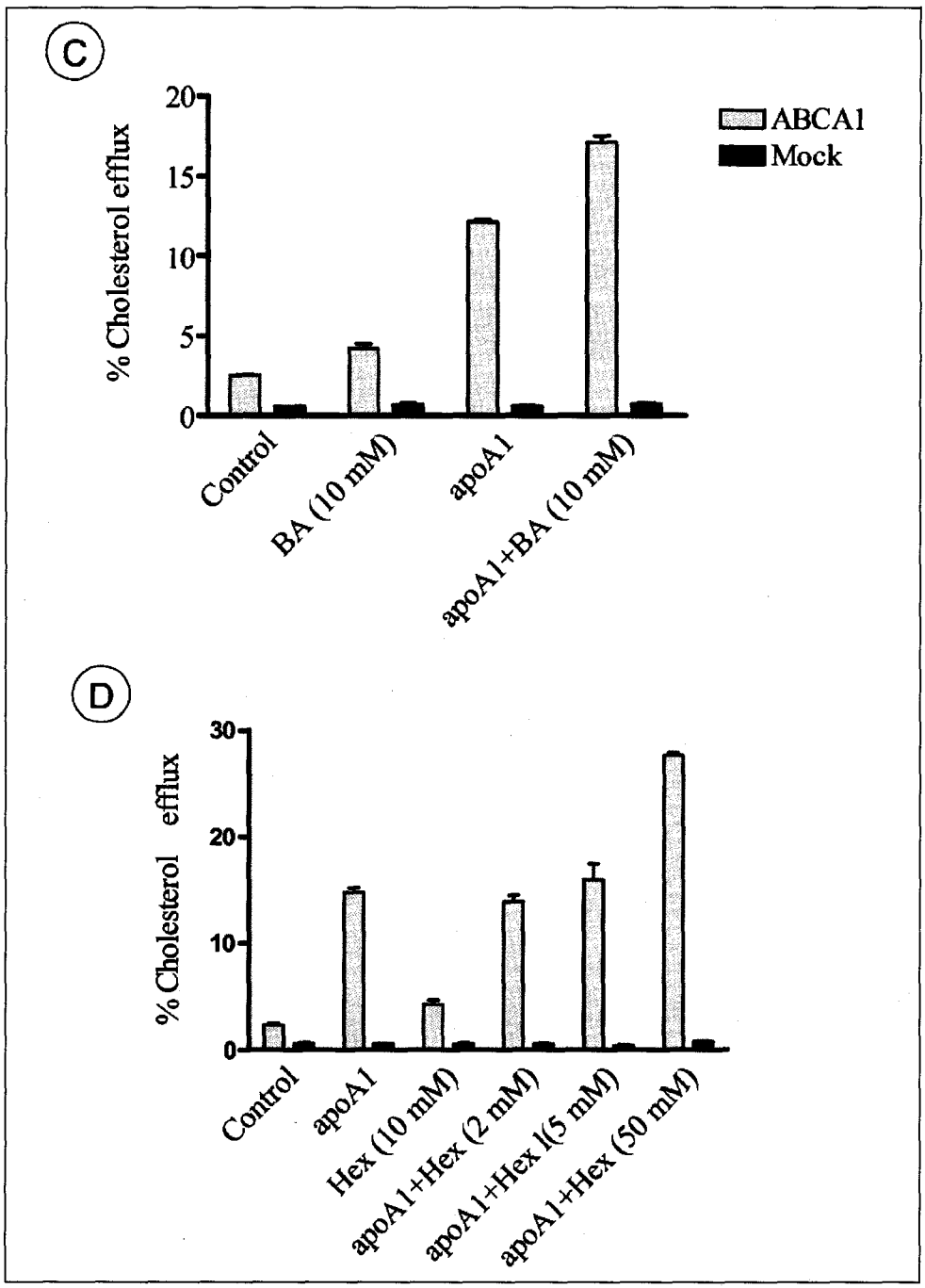

Figure 3.6-C, D: Effect of membrane fluidizers on cholesterol efflux. BHK cells were labeled with 1 μCi/ml ^{3}H cholesterol for 2 days and ABCA1 expression was induced. **(C)** ApoA1(10 μg/ml) mediated cholesterol efflux was carried out with and without 10 mM benzyl alcohol for 2 h. **(D)** ApoA1 mediated cholesterol efflux was carried out with and without 2 mM, 5 mM, and 50 mM of hexanol for 2 h.

When LGM at similar concentrations as WGA was included in the efflux medium, we observed little effect in apoA1 mediated cholesterol efflux **(Figure 3.7)**. This further clarified that inhibiting cholesterol efflux with WGA is not a lectin-specific phenomenon; rather, it is the rigidifying effect of WGA that inhibits cholesterol efflux.

3.8 Effect of Wheat Germ Agglutinin on ABCA1 Distribution

It is possible that, WGA treatment may have altered ABCA1 cellular distribution. The cellular distribution of ABCA1 is important since the presence of ABCA1 in the plasma membrane is absolutely necessary for cholesterol efflux (Oram *et al.*, 2000; Neufeld *et al.*, 2001). Cells were treated with or without WGA followed by immunofluorescence-staining to observe its distribution. ABCA1 cellular distributions were studied using confocal fluorescent microscopy. Images were taken from both basal membranes **(Figure 3.8, First row)** and middle sections **(Figure 3.8, Second row)**. It was observed that, in control cells **(Figure 3.8, First column)**, ABCA1 was mainly localized in the plasma membrane as well as in the Golgi, similar to previous reports (Neufield *et al.*, 2001; Landry *et al.*, 2006). WGA treatment **(Figure 3.8, Second column)** did not alter the ABCA1 distribution. Mock cells **(Figure 3.8, Third row)** showed no staining as expected and served as a negative control. It was therefore concluded that inhibition of cholesterol efflux by WGA was not caused by ABCA1 redistribution.

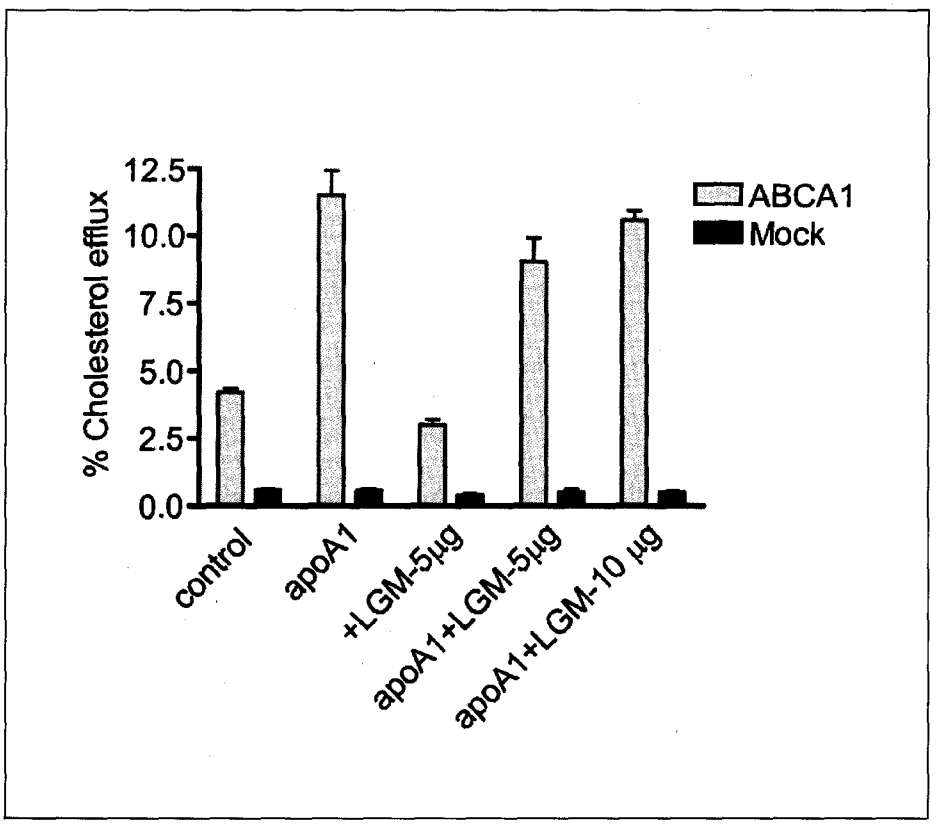

Figure 3.7: Efflux with another lectin. BHK cells were labeled with 1 µCi/ml ^{3}H cholesterol for 2 days and ABCA1 expression was induced. ApoA1(10 µg/ml) mediated cholesterol efflux was carried out with and without 5 µg/ml and 10 µg/ml of lectin from glycine max for 2 h.

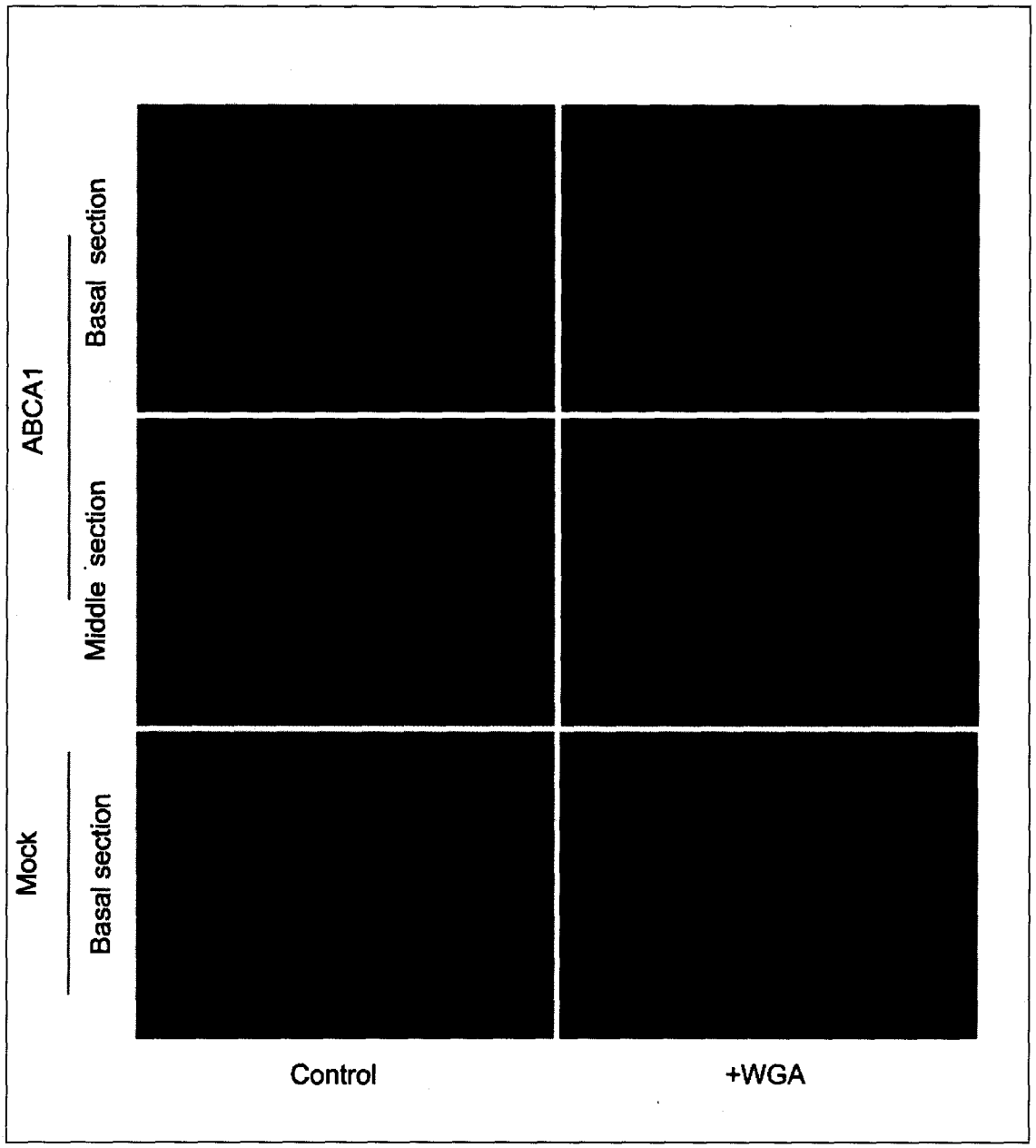

Figure 3.8: Effect of WGA on ABCA1 distribution. BHK cells were set up in microscopy dish and ABCA1 expression was induced. Cells were treated with and without WGA (10 μg/ml) for 2 h. Cells were fixed with paraformaldehyde and permebalized with saponin and ABCA1 expression was visualized by confocal microscopy by using a Anti-ABCA1 primary antibody (1:200 dilution) followed by Alexa 488 conjugated goat anti-rabbit IgG.

3.9 Effect of Wheat Germ Agglutinin on ApoA1 Binding

Sufficient apoA1 binding to the cells is also known to be necessary for efficient cholesterol efflux (Wang *et al.*, 2000). ABCA1 expression induces apoA1 binding to the cells, which is thought to be the first step in the efflux process. Therefore it was tested whether WGA disrupted binding of apoA1 to cells using ^{125}I apoA1. ABCA1 cells were coincubated with ^{125}I apoA1 and WGA followed by counting cell associated radioactivity. No significant difference was observed in the association of apoA1 between the control and WGA treated cells **(Figure 3.9-A)**. It was then concluded that WGA did not alter total association of apoA1 with cells.

Since an important aspect of the efflux process is the interaction of ABCA1 with apoA1 (Fitzgerald *et al.*, 2002; Wang *et al.*, 2000; Oram *et al.*, 2000), a detailed study of interaction between ABCA1 and apoA1 was required, even though WGA treatments neither changed the distribution of ABCA1 nor affected the cell association of apoA1. Cells were incubated with ^{125}I apoA1 with or without WGA, then washed and treated with DSP cross-linker for 30 min at room temperature. ABCA1 was then immunoprecipitated using a polyclonal antibody. The precipitants were then counted for radioactivity for associated ^{125}I apoA1. It was observed that IP of ABCA1 was able to pull down ^{125}I apoA1 in both treated and untreated cells. The amount of apoA1 co-precipitated with ABCA1 was slightly decreased in WGA treated cells **(Figure 3.9-B)**. WGA treatment inhibited interaction between ABCA1 and apoA1 by about 30%. Given that WGA inhibited more than 90% cholesterol efflux, this interruption could not be significant enough to count for the inhibition of cholesterol efflux by WGA.

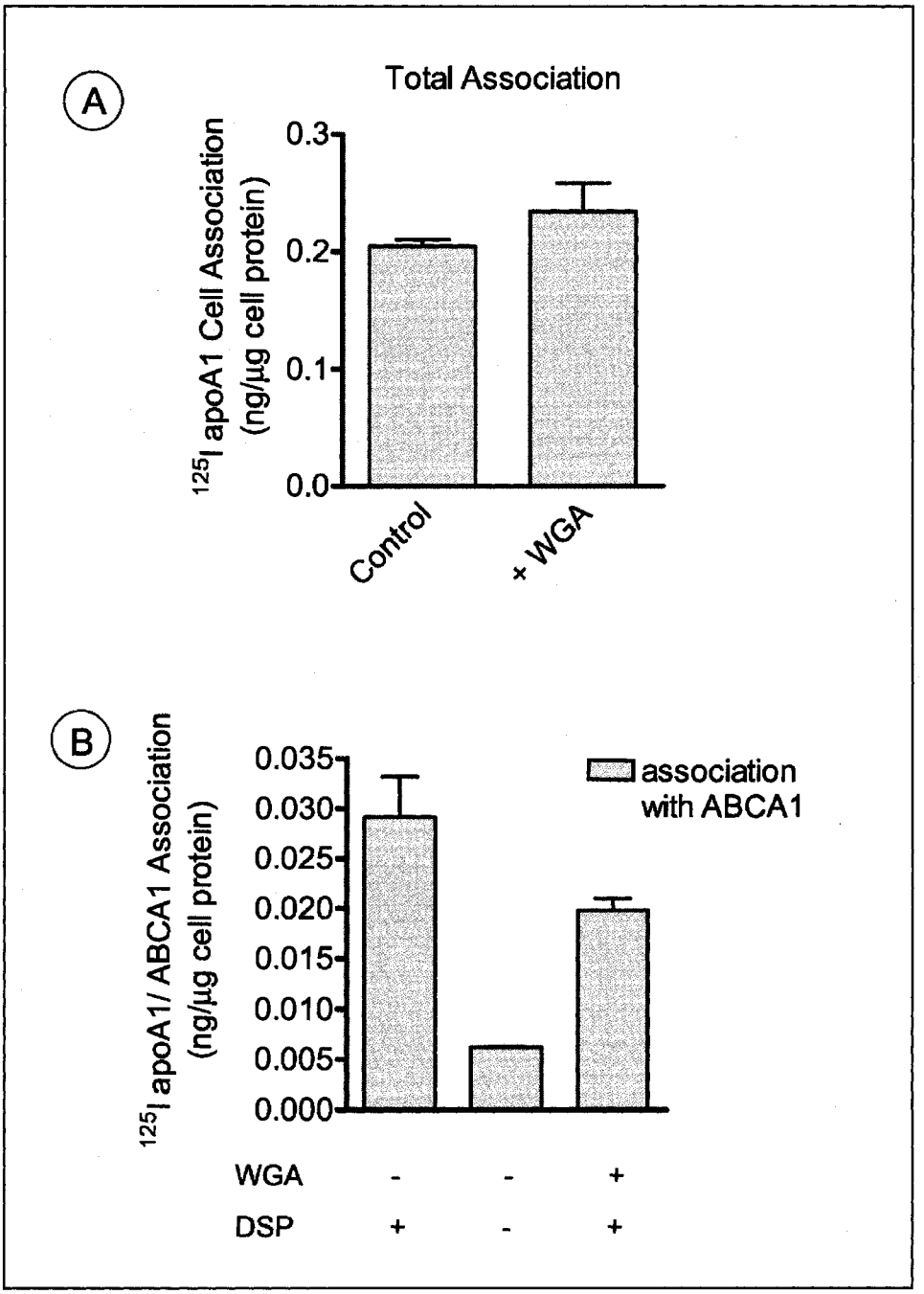

Figure 3.9-A, B: Effect of WGA on apoA1 binding. ABCA1 cells were treated with 10 µg/ml of WGA and ^{125}I apoA1 (10 µg/ml) for 2 h. **(A)** Cell associated apoA1 was calculated by counting radioactivity in a gamma counter. **(B)** Following incubation cells were washed twice with PBS and incubated with DSP cross-linker for 30 min at room temperature. IP was performed with anti ABCA1 antibody and cross linking of ABCA1 with apoA1was calculated.

Other influences by WGA such as rigidifying the membrane must play much more significant role in the process. We therefore concluded that inhibition of cholesterol efflux by WGA is not primarily due to interruption of interactions between ABCA1 and apoA1.

3.10 Detection of Membrane Derived Vesicles

Experimental evidences so far suggest that ABCA1 expression may facilitate the process of membrane shedding. WGA influences plasma membrane flexibility and therefore suppresses cholesterol efflux. In order to directly test this, we performed KBr density gradient ultracentrifugation on the medium collected at the end of cholesterol efflux experiments. KBr density gradient ultracentrifugation is a well known method for separating membrane/lipid particles based on their densities. When we performed KBr density gradient ultracentrifugation on the medium, generated from ABCA1 cells after 4 h incubation with apoA1, we observed that the majority of ^{3}H cholesterol was found in low density fractions (fraction 1-6) **(Figure 3.10-B)**. Interestingly, two major peaks were observed in the low density region. The first one was centered at D = (1.03) and the second one at D = (1.07). When apoA1 was probed for in each fraction, it was found that only the second peak contained apoA1, indicative of HDL particles. Non-lipidated apoA1 was also seen floating in higher density fractions. Most intriguingly, the first peak at low density contained more than 50% of total ^{3}H cholesterol, but was essentially free of apoA1. Since BHK cells do not express any apoE, this peak could not be apoE-containing particles. Therefore it is expected to be composed of membrane vesicles. Furthermore, these membrane vesicles can also be detected in the medium of ABCA1 cells that were not exposed to apoA1 **(Figure 3.10-A)**. As expected, WGA treatment abolished these low-density peaks **(Figure 3.10-C)**.

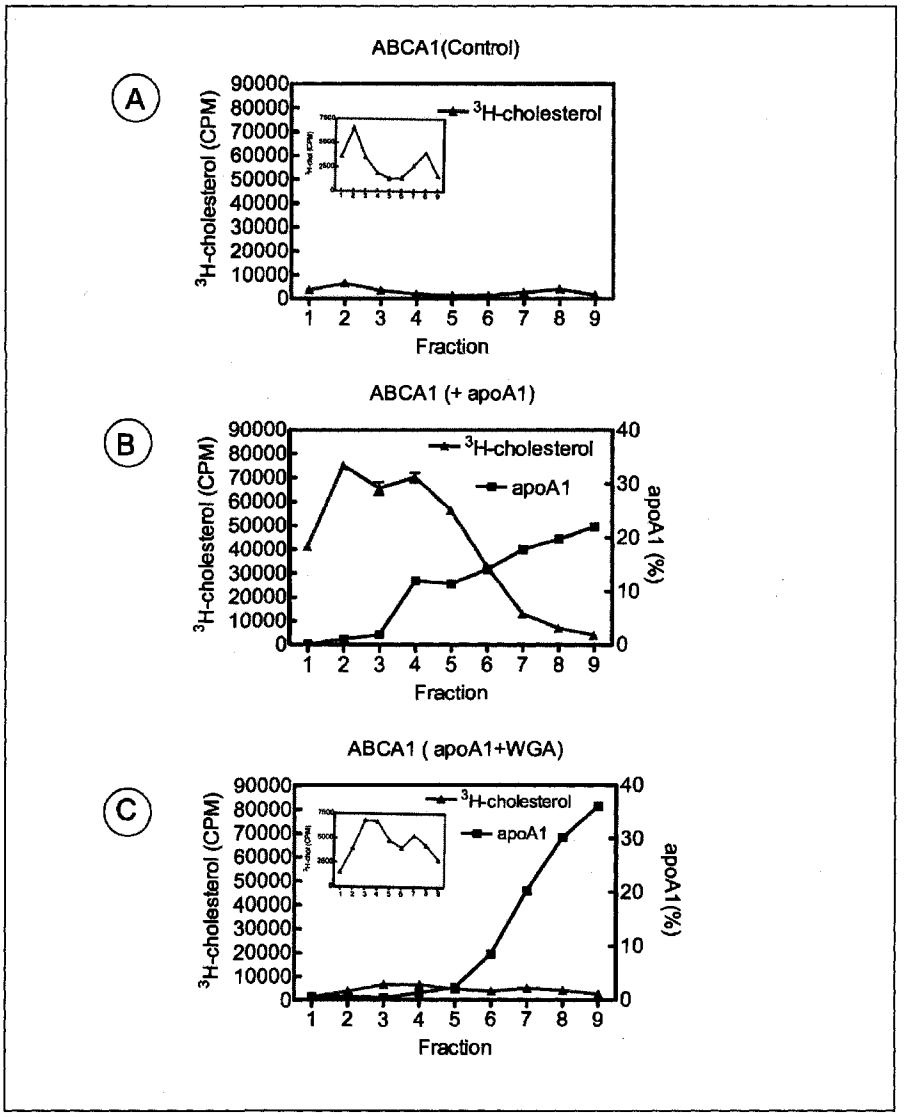

Figure 3.10-A, B, C: Detection of membrane derived vesicles from ABCA1 cells. BHK cells were labeled with ^{3}H cholesterol (5 μCi/ml) for 2 days and ABCA1 expression was induced. Cells were co-incubated with and without 10 μg/ml WGA and 5 μg/ml of apoA1 for 4 h. Medium was collected and concentrated using a 10 KD molecular weight cut off and loaded on linear KBr gradient and was spun for 22 h at 37,000g. Nine fractions were collected. 0.3 ml of each fraction was counted for the presence of radioactivity and 40 μl of each fraction was mixed with loading buffer and was run on 12% SDS-PAGE. Immunoblot was performed. ApoA1 was detected using an anti-apoA1 primary antibody and was quantified using densitometry and plotted in the graph. KBR gradient of **(A)** ABCA1 cells without any treatment, **(B)** apoA1 treated ABCA1cells and **(C)** apoA1 + WGA treated ABCA1 cells.

Mock cells also did not produce any significant low density peaks under all the conditions **(Figure 3.10-D, E, F)**. Our results therefore strongly suggest that an additional fraction of membrane vesicles are being produced during apoA1 mediated cholesterol efflux.

3.11 Detection of Cytosolic Protein in the Medium

If ABCA1 cells produce membrane vesicles by means of membrane shedding, cytosolic or membrane proteins may appear in the medium. To examine this, we probed for HSP-70, an abundant cytosolic protein. Both ABCA1 and Mock BHK cells were treated under various conditions, such as with and without apoA1 and with and without WGA. The medium were collected and concentrated as described before. Equal volumes of concentrated medium along with cell lysates were subjected to SDS PAGE and immunoblotted with HSP-70. We found that medium from ABCA1 cells incubated with apoA1 contained the most abundant HSP-70 **(Figure 3.11-A)**. WGA treatment abolished the appearance of HSP-70 in the medium, consistent with its role in inhibiting cholesterol efflux. Mock cells did not release significant amount of HSP-70 under any conditions. Interestingly, a small amount of HSP-70 was also present in the medium generated from ABCA1 cells without exposing to apoA1. Medium from Mock cells under the same conditions did not contain any detectable HSP-70. This is again in agreement with our earlier observation that ABCA1 cells can produce apoA1-independent efflux. All cells expressed similar levels of HSP-70 as we verified in cell lysates (data not shown). The bands of HSP-70 were quantified using densitometry and corrected for protein concentration under each condition and presented in arbitrary units.

Even after correcting for protein concentration we observed more protein in the medium in ABCA1 expressing cells than Mock cells **(Figure 3.11-B)**.

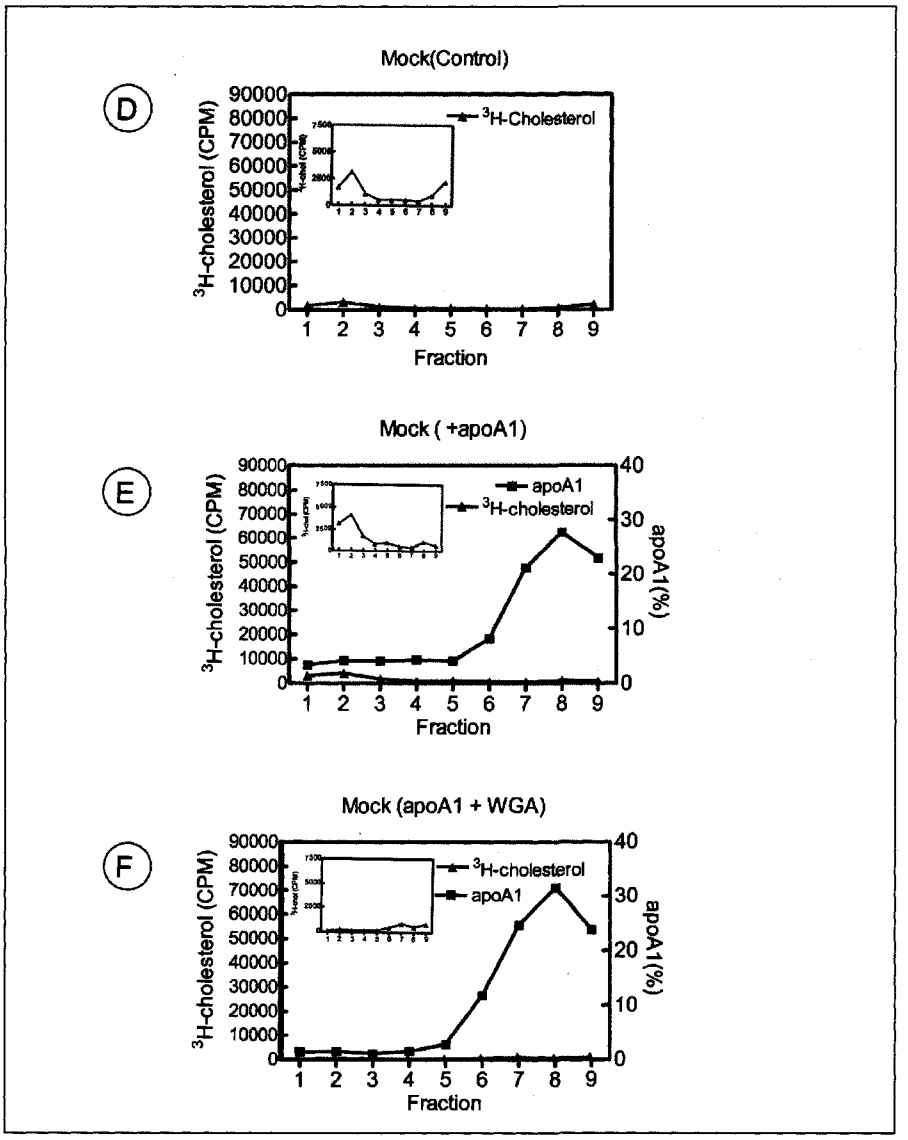

Figure 3.10-D, E, F: Detection of membrane derived vesicles from Mock cells. KBr gradient of **(A)** control Mock cells, **(B)** apoA1 treated Mock cells and **(C)** apoA1 + WGA treated Mock cells.

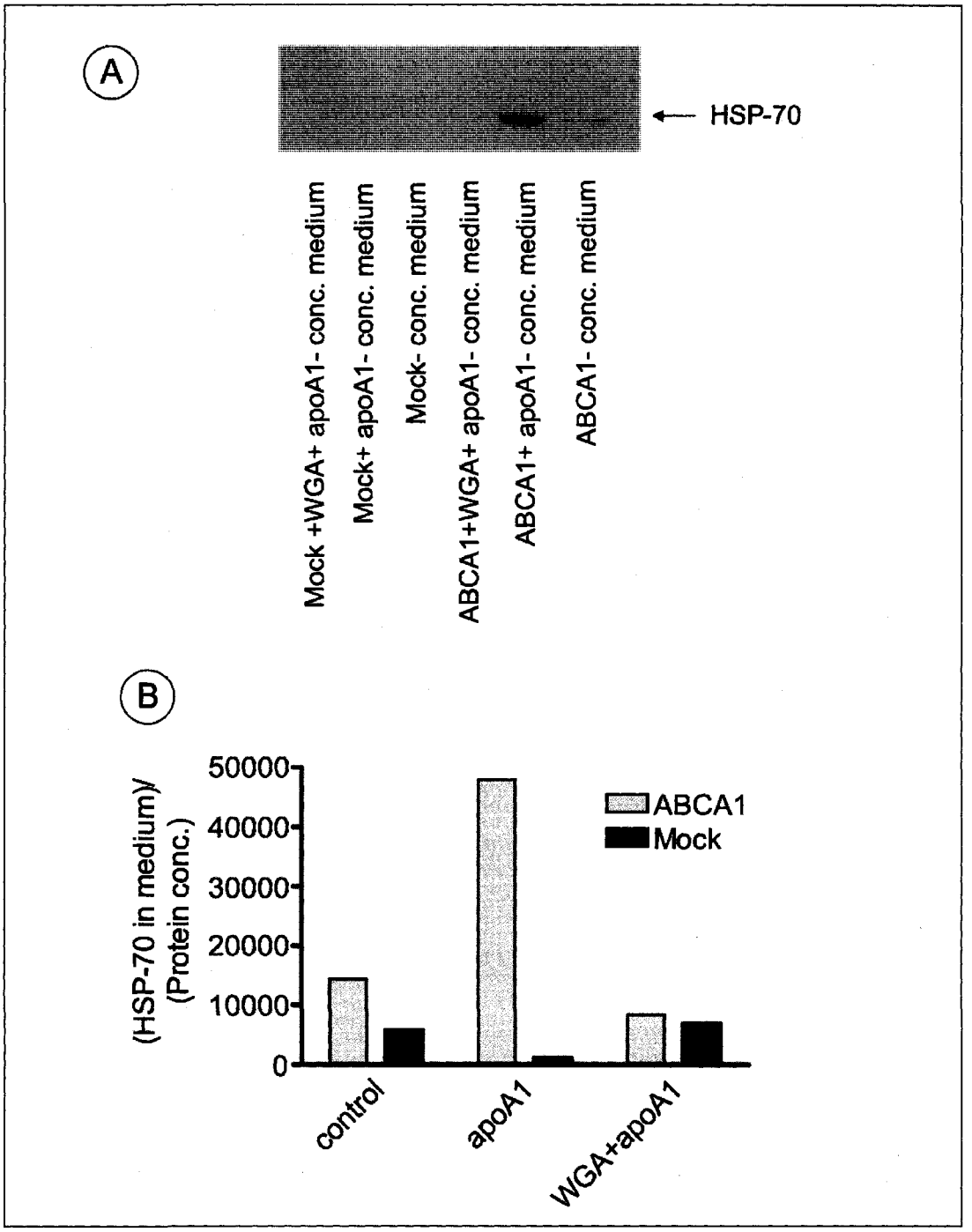

Figure 3.11-A, B: Detection of cytosolic protein in the media. (A) ApoA1 mediated cholesterol efflux was carried out with and without WGA and 5 μg/ml of apoA1 for 4 h. Medium was collected and concentrated by using centrifugal force through a 10 KD molecular weight cut off filter. 40 μl of concentrated medium was loaded in SDS-PAGE. Immunoblot blot was performed. Membrane was probed for HSP-70 using an Anti HSP-70 primary antibody followed by an HRP-linked secondary antibody. **(B)** Quantification of HSP-70 by densitometry and plotted and corrected for protein concentration, and is shown as a percentage.

Chapter 4

Discussion

The aim of this study was to test the effect of membrane flexibility on the cholesterol efflux in order to understand the molecular details of this process. In this study we reported for the first time that apoA1 mediated cholesterol efflux can be influenced by altering the flexibility of plasma membrane. We used a known membrane rigidifier WGA, to alter the cholesterol efflux process. WGA completely inhibited the apoA1 mediated cholesterol efflux at a relatively low concentration (10 μg/ml). We demonstrated that inhibition is completely reversible as long as WGA was removed from the plasma membrane. WGA was equally effective in inhibiting cholesterol efflux in macrophages, therefore the observed effect of WGA on efflux, is likely, a general phenomena and not dependent on specific cell types.

WGA is widely used to modulate membrane fluidity (Evans and Leung, 1984; Hochmuth and Waugh, 1987) and efflux inhibition by WGA is likely due to its function as a membrane rigidifier. We verified this in our cell model. Firstly, we observed inhibition of transferrin endocytosis by WGA. As endocytosis requires extensive membrane bending, we attribute such inhibition to a change in membrane rigidity. Importantly, we were able to rescue endocytosis using *N*-acetylglucosamine which efficiently and rapidly removes the plasma membrane bound WGA. Furthermore, the rigidifying effect of WGA

was directly confirmed by measuring membrane extension elasticity using micropipette aspiration method. The plasma membrane could no longer be aspirated into micropipette when bound with WGA, which further validated the rigidifying effect of WGA.

The analysis of the binding sites of WGA in BHK cells by microscopy with fluorescent WGA demonstrated that intracellular WGA has very little effect on the efflux. Rather, it is the plasma membrane bound WGA that plays the key role in inhibiting cholesterol efflux. WGA is known to bind to the plasma membrane and, at the same time, undergo active endocytosis, as confirmed by our fluorescence experiments. There are therefore two pools of WGA during the cholesterol efflux assay. To dissect the role of each WGA pool in cholesterol efflux, we took the advantage that WGA has a high affinity for *N*-acetylglucosamine. WGA has two binding sites for *N*-acetylglucosamine per subunit (Nagata and Burger, 1974). The binding of WGA with *N*-acetylglucosamine is thus highly specific and, when excess *N*-acetylglucosamine is present in the medium, it can reverse the binding of WGA with plasma membrane glycoconjugates (Goldstein and Hayes, 1979; Evans and Leung, 1984). We therefore added *N*-acetylglucosamine in the medium as a soluble competitor and were able to show an efficient removal of surface-bound WGA while leaving the intracellular WGA pool intact. This was sufficient to fully recover cholesterol efflux, demonstrating that the plasma membrane is the major platform for WGA action.

The impact of altering membrane rigidity was further confirmed using other structurally unrelated reagents. First, another membrane rigidifier, cholesteryl hemisuccinate, was equally capable of inhibiting apoA1 mediated cholesterol efflux in BHK cells. In

contrast, membrane fluidizers such as benzyl alcohol and hexanol were able to enhance cholesterol efflux. We also examined lectin specificity by using another plant derived lectin (LGM) in the efflux experiments and found no effect. Taken together, we demonstrated a general correlation between the rigidity of the plasma membrane and the efficiency of apoA1 mediated cholesterol efflux in ABCA1 expressing cells. Although we artificially modulated membrane fluidity in this study, our observations may be relevant to many physiological conditions. For example, it has also been shown by Favari *et al.* (2005) that oxidized LDL (ox LDL) can inhibit the ABCA1 mediated cholesterol efflux. Interestingly, oxidized LDL is also found to have a stiffening effect on the cells (Byfield *et al.*, 2006).

Furthermore, we tested several parameters known to influence cholesterol efflux in cells treated with WGA. Previously it has been suggested that the presence of ABCA1 in the plasma membrane is required for cholesterol efflux (Oram *et al.*, 2000; Neufeld *et al.*, 2001). Therefore we ensured normal ABCA1 localization by confocal fluorescence microscopy in WGA-treated cell. The direct interaction between apoA1 and ABCA1 has been postulated as an important factor for cholesterol efflux (Smith *et al.*, 2004; Wang *et al.*, 2000). Therefore we verified the effect of WGA on apoA1 binding. We confirmed that inhibition of efflux by WGA was not due to significant change in apoA1 binding to the cells. We also performed crosslinking and immunoprecipitation experiment to further authenticate the effect of WGA on ABCA1/apoA1 crosslinking. We found that there was a slight decrease of crosslinking between apoA1 and ABCA1 in the presence of WGA. The degree of inhibition on crosslinking by WGA, however, cannot explain the 90% abolishment of cholesterol efflux in cells treated under the same conditions with WGA.

Since the most important factors that facilitate cholesterol efflux, such as ABCA1 localization and apoA1 binding, were not altered by WGA treatment, we speculate that WGA most likely blocked the final steps of cholesterol efflux, such as membrane vesiculation (including nascent HDL dissociation) from the plasma membrane.

To test composition and properties of shed membrane vesicles during efflux, we used two approaches. One is the analysis of particles in the efflux medium by means of KBr density gradient ultracentrifugation. We also examined the appearance of cytosolic proteins in the efflux medium. By KBr density gradient ultracentrifugation, we were able to separate two populations of low density particles. HDL particles are 8-10 nm in diameter and floated between 1.063 and 1.21 g/ml density depending on the amount of lipids associated with it. The lipid-free apoA1 was detected in the bottom fractions, whereas lipidated apoA1 or HDL floats at the top fractions of the gradient. By KBr density gradient centrifugation we however detected a first peak which was free of apoA1 but highly enriched in cholesterol in the medium derived from ABCA1 expressing BHK cells. Interestingly, this peak was seen in the presence and absence of apoA1, though there was much less cholesterol associated with it when apoA1 was absent. We also observed a second peak which is highly rich in apoA1. All the peaks were suppressed by WGA treatment and Mock cells were unable to produce any such peaks. It is intriguing to see that ABCA1 expressing cells can produce particles absolutely free of apoA1 which consisted of more than 50% of cholesterol, in addition to generating classical HDL particles. A similar observation was made by Duong *et al.* (2006) in macrophages. A study from Yokoyama's laboratory also observed two

populations of particles in the efflux medium from ABCA1 expressing cells, although apoA1 was not analyzed (Hayashi *et al.*, 2005).

It is likely that membrane shedding or vesicles is a predominant process in ABCA1 expressing cells, independent of apoA1 treatment. This is supported by an earlier study by Landry *et al.* (2006) that ABCA1 expression alone induces re-organization of the plasma membrane. Such reorganization could condition cells in a way that would induce membrane shedding. With its amphipathic helix structure, apoA1 may merely amplify this process by inserting into outer leaflet of the plasma membrane and thus facilitating outward membrane bending. ApoA1 may acquire lipids including cholesterol during such interaction. By rigidifying the membrane, WGA suppresses this process and thus cholesterol efflux.

Since membrane shedding is likely accompanied by appearance of membrane proteins or cytosolic proteins, we examined the efflux medium for possible protein release during efflux. We probed for proteins namely vimentine and β-actin but found no correlation with cholesterol efflux (data not shown). However, we were also able to detect the enrichment of HSP-70, a cytoplasmic protein in the efflux medium from ABCA1 cells, particularly when cells were incubated with apoA1. On the contrary, very little HSP-70 can be detected in the Mock cells treated under identical conditions. Furthermore, WGA, at the experimented dose (10 μg/ml), completely abolished cholesterol efflux and also suppressed the secretion of HSP-70 in ABCA1 cells. The presence of cytosolic proteins, such as HSP-70, in the efflux medium of ABCA1 cells suggests a possibility that the cholesterol efflux may involve shedding of cytosolic proteins as a part of membrane

vesicles. The process is solely dependent on ABCA1 as there is no detectable amount of HSP-70 in the efflux medium from Mock cells. Interestingly addition of apoA1 amplifies process, indicating the significance of membrane shedding in cholesterol efflux. WGA as a potent inhibitor of cholesterol efflux, also inhibit the process of membrane shedding and therefore appearance of intracellular proteins in the medium.

Collectively, the experimental evidence presented in this study support the notion that membrane shedding may be involved in ABCA1 mediated cholesterol efflux. The definitive proof of such scheme is still however elusive at present, since each experimental approach described above may have its own limitation. For example, ultracentrifugation may alter the composition of the particles found in the efflux medium, especially with proteins such as apoA1 that can be readily exchanged among different particles. Ultracentrifugation force may influence such an exchange or simply dissociate apoA1 from particles. Analyses of the efflux medium by high performance liquid chromatography (HPLC), like the one used by Yokoyama's laboratory, may be a better approach. Also, we need to analyze more membrane and cytosolic proteins as markers for membrane shedding. A profiling of these proteins can potentially provide clues about the origin of the lipids including cholesterol that appeared in the efflux medium.

In summary, we demonstrated in this study the relationship between membrane rigidity and cholesterol efflux. We also provide evidence that cholesterol efflux may be originated from membrane shedding. However neither the exact origin of membrane vesicles nor the mechanism involved in their release is known at present. We speculate that membrane vesicles could be originated from the plasma membrane. Activation of

ABCA1 may promote pinching off from the plasma membrane to form membrane vesicles. Alternatively, those membrane vesicles could be of intracellular origin; they may fuse with plasma membrane and release their content of lipids and cholesterol into extracellular space. Obviously more definitive evidence is required in order to understand the exact origin of membrane vesicles as well as the mechanism involved in the process.

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SHILPI NANDI

Career Objective: A challenging position in Biological Research.

Skill Summary

- In-depth knowledge and experience in scientific theories and procedures
- Skilled in independent research and analysis
- Proficient in computing
- Highly energetic, efficient and sincere
- Responsible, organized and dedicated worker

Work Experience

2007- Present: Research Assistant (Dept. of Hormones, Growth & Development; Ottawa Health Research Institute, Ottawa, Canada)

- Performed cell biology and tissue culture techniques regularly
- Developed expertise in microscopy
- Optimized conditions for cholesterol efflux technique
- Efficiently managed laboratory, organized equipment and materials.
- Ordered supplies and maintained inventory for laboratory

2004 – 2007: Graduate Student (Ottawa Health Research Institute, Ottawa, Canada)

- Worked on cholesterol homeostasis and membrane trafficking in mammalian cells
- Skilled in immunoblot, TLC, gradient fractionation, immuno-precipitation, cholesterol efflux.
- Proficient in several tissue culture technique and microscopy.
- Performed lipid extraction and purification.

1999 - 2000: Medical Representative (Glaxo India Limited, India)

- Supported field sales of cardiac medicines.
- Promoted company products to the medical practitioners.
- Received special training on mode of actions of cardiac and diabetic medicines on body.

Academic Qualification

2004 – 2007: M.Sc. in Biochemistry (University of Ottawa, Canada)

Thesis: The involvement of membrane vesiculation in A1 mediated efflux.

Courses: Advanced topics in structure and function of plasma lipoproteins (BCH 8107), Physical and chemical methods in biochemistry (BCH 8101), Macromolecules (BCH 4122).

2000-2001: Computer Training (NIIT, India)

- **Programming Language:** Java, UML, HTML
- **Database:** Microsoft SQL Server 7.0
- **Software:** MS Word, Excel, PowerPoint
- **Operating System:** Windows

1996 - 1998: M.Sc. in Biochemistry (First Class; Rani Durgavati University, India)

Dissertation: Level of serum creatinine and cholesterol in carcinoma cervix patients.

Courses: Metabolisms and Regulations, Genetics and Microbial Genetics, Clinical Biochemistry, Immunology and Immunochemistry, Enzymology, Molecular Biology, Physiology and Nutrition, Vitamins and Hormones, General Microbiology, Biostatistics, Techniques in Biology, Chemistry of Biomolecules.

1993 - 1996: B.Sc. in Biochemistry (First Class; Hawabagh Women's College, India)

Courses: Microbiology, Biochemistry, Pathology etc.

Publications:

- Nandi, S., Denis, M., and Zha, X. 2006. "The involvement of membrane vesiculation in ABCA1-mediated efflux." Canadian lipoprotein conference. Gimli, Manitoba. (Poster presentation)
- Karwatsky, J., Nandi, S., Feng, Y., Zha, X. 2006. "ABCA1-mediated cholesterol efflux is modulated by ion-channels." 46th Annual Meeting of the American Society for Cell Biology. San Diego, California. (Poster presentation)
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References are available upon request.