uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Andrea McKenzie
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biochemistry)
GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Effect of Caveolin-1 on the Synthesis and Efflux of Cholesterol in Hepatoma Cells and Hepatocytes

TITRE DE LA THÈSE / TITLE OF THESIS

Yves Marcel
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dan Sparks

Zemin Yao

Gary W. Slater
LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES /
DEAN OF THE FACULTY OF GRADUATE AND POSTDOCORAL STUDIES

The Effect of Caveolin-1 on the Synthesis and Efflux of Cholesterol in Hepatoma Cells and Hepatocytes

Andrea McKenzie

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry, Microbiology and Immunology
University of Ottawa
Ottawa Heart Institute

Your file Votre référence
ISBN: 0-494-14928-0
Our file Notre référence
ISBN: 0-494-14928-0

Abstract

The effect of caveolin-1 on synthesis and efflux of cholesterol was studied using three cell systems. The McA-RH7777 cells were stably transfected with caveolin-1 previously. HepG2 cells were infected with a caveolin-1 adenovirus, and primary hepatocytes were isolated from homozygous and heterozygous caveolin-1 deficient and control mice.

Overexpression of caveolin-1 resulted in an increase of cholesterol synthesis, and the absence of caveolin-1 resulted in a decrease in cholesterol synthesis. Cholesterol ester and phospholipid synthesis are not affected. The influence of caveolin-1 on efflux to HDL was small in the three cell models. Efflux of cholesterol to apoA-I was not significantly affected by caveolin-1 over expression.

Caveolin-1 deficient hepatocytes had reduced cholesterol synthesis. Cholesterol efflux to HDL and exogenously added apoA-I decreased in the absence of apoA-I. Phospholipid and cholesterol lipidation of endogenously synthesized apoA-I was reduced in the absence of caveolin-1.

Overall, caveolin-1 deficiency had more consistent effect on cholesterol metabolism than over expression of caveolin-1, suggesting that the basal level of caveolin-1 in hepatocytes is probably sufficient to regulate cholesterol homeostasis and cellular transport.

Dedication

This work is dedicated to my parents.

Acknowledgements

I would like to thank my supervisor Dr. Yves Marcel for the opportunity to work on this project, and for his support and patience. I would like to thank my advisory committee, Dr. Heidi McBride and Dr. Ruth McPherson for their support and advice. I would like to thank Dr. Philip Links for the training in the skills and techniques necessary for this project, as well as the tools and procedures he has developed. I would like to thank Peter Rippstein for his work and assistance in the electron microscopy preparation and analysis. I want to acknowledge the present and former members of Dr. Marcel's lab, who have taught, discussed and contributed much to this project. I would especially like to thank my family for thir support and encouragement throughout this project. Lastly, I would like to thank the members of Fellowship Baptist Church for their prayers and encouragement.

Table of Contents

List of Tables

List of Figures

Abreviations

ABCA1	ATP binding cassette A1
ABCG1	ATP binding cassette G1
Ad5	Adenovirus 5
ADRP	adipocytes differentiation related protein
Apo	apolipoprotein
ApoA-I	apolipoprotein A-I
ApoE	apolipoprotein E
ApoE(-/-)	apoE deficient mouse
C-MAD	C-terminal membrane attachment domain
CA-repeat	Cytosine-Adenosine repeat
CAV1	human gene of caveolin-1
cav1	caveolin-1
cav1(+/+)	caveolin-1 control mouse
cav1(+/-)	heterozygous caveolin-1 deficient mouse
cav1(-/-)	homozygous caveolin-1 deficient mouse
CAV2	human gene of caveolin-2
CBD	caveolin binding domain
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CSD	caveolin scaffolding domain
DNA	deoxyribonucleic acid

EGF	endothelial growth factor
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FC	unesterified (free) cholesterol
GFP	green fluorescent protein
GLUT-4	glucose transporter 4
GPI-linked	glycosylphosphatidylinositol-linked
HAdv	species C human adenovirus
HDL	high density lipoprotein
HDL-C	HDL associated cholesterol
HepG2	hepatoma derived cell line G2
HMGCoA Reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
HSP 56	heat shock protein 56
HSP 60	heat shock protein 60
IDL	intermediate density lipoprotein
IR-β	insulin receptor β
kb	kilobase
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDL-C	LDL derived cholesterol
LDLr	low density lipoprotein receptor
LOH	loss of heterozygosity
LPDS	lipoprotein deficient serum

LPL	lipoprotein lipase
Luc	lucifierase
MAP kinase	mitogen activated protein kinase
mβCD	methyl β cyclodextrin
McA-RH7777	McArdle rat hepatoma cell line
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
N-MAD	N-terminal membrane attachment domain
oxLDL	oxidized low density lipoprotein
PDGF	platelet-derived growth factor
PKC	protein kinase C
PL	phospholipids
PLTP	phospholipid transfer protein
PMA	phorbol myristate 3-acetate
PM	plasma membrane
SMC	smooth muscle cells
SRE	sterol responsive element
SREBP-1	SRE binding protein 1
SR-BI	scavenger receptor class BI
SV40	simian virus 40
TLC	thin layer chromatography
TG	triglycerides
tTA	tetracycline transactivator

VCAM-1	vascular cell adhesion molecule-1
VIP21	vesicular integral protein 21 (caveolin-1)
VLDL	very low density lipoprotein

Chapter One: Introduction

Heart disease and stroke are the primary causes of death in North America, and atherosclerosis is a major element of these two diseases (1;2). There are many risk factors associated with atherosclerosis including a correlation between low density lipoprotein (LDL) and atherosclerosis, and an inverse correlation between high density lipoprotein (HDL) and atherosclerosis (3). The absence of caveolin-1, a protein involved in cellular transport of cholesterol, in the apolipoprotein E (apoE) deficient mice increases their atherogenic lipid profile. This is one of the reasons for our interest in the cellular function of caveolin-1 and cholesterol traffic (4).

A. Atherosclerosis

Atherosclerosis is a disease of chronic inflammation and is characterized by accumulation of lipids and fibrous elements in lesions called atherosclerotic plaques (1;2;5-7). Key cells involved in plaque formation include endothelial cells, macrophages and smooth muscle cells; all express caveolin-1, though at various levels (8). There are three stages of plaque formation. Early lesions, known as fatty streaks, consist of a subendothelial accumulation of macrophages engorged with cholesterol, called foam cells (5). More advanced lesions have a fibrous cap composed of smooth muscle cells (SMC), and an extracellular matrix with a lipid-rich necrotic core (1). In the final stages of lesion formation, the plaque becomes complex, including calcification. The plaque can become unstable due to thinning of the fibrous cap and can rupture, causing formation of blood clots (1;6).

Many studies have shown that HDL is inversely correlated with atherosclerosis. However, the mechanisms involved in HDL's atheroprotection are poorly understood

(9;10). "Reverse cholesterol transport" may be a key mechanism by which HDL can inhibit atherosclerosis (11). In this model, HDL scavenges cholesterol from peripheral tissues, including foam cells found in the subendothelial space. The HDL transfers the cholesterol to apoB-containing lipoproteins, and these lipoproteins return the cholesterol to the liver so it can be eliminated in the bile (11;12).

B. Lipoproteins

Macromolecular complexes called lipoproteins are the vehicles of lipid transport in the blood. They are made up of amphipathic apolipoproteins and lipids. A neutral core of cholesteryl esters (CE) and triglycerides (TG) is stabilized by a monolayer of phospholipids (PL) and unesterified cholesterol (FC)(13). The apolipoproteins are divided into two classes: the non-exchangeable apolipoproteins (apoB-100 and apoB48), and the exchangeable apolipoproteins (apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III and apoE). The interaction between exchangeable apolipoproteins and lipids is reversible, and the interaction between nonexchangeable apolipoproteins and lipids is irreversible (14;15). The apolipoproteins are involved in regulating the metabolism and structural stability of lipoproteins.

Lipoproteins are identified and purified by their buoyant density. Chylomicrons, the largest lipoproteins, are secreted from the intestine, containing a large core of TG. They enter circulation through the lymphatic system, and transport the TG to the muscle and adipose tissue where fatty acids are hydrolysed from TG by lipoprotein lipase (LPL)(16). LPL is found in the endothelial cells lining the capillaries. A similar protein, hepatic lipase (HL), found on hepatic cells also hydrolyses TG and PL from lipoproteins,

including chylomicron remnants (1). Chylomicron remnants, containing mostly cholesterol, apoB-48 and apoE, bind to and are taken up by the chylomicron remnant

Table 1: Classes of Human Lipoproteins and Their Physical Properties

Lipoproteins	Density (g/ml)	Diameter (nm)	Molecular Weight (KDa)	Associated Apolipoproteins	Electrophoretic Mobility
Chylomicrons	>0.93	75-1200	$50\text{-}100X10^3$	A-I, A-II, B-48, C-I, C-II, C-III, E	Stays at origin
VLDL	0.93-1.006	30-80	$10\text{-}80X10^3$	A-I, B-100, C-I, C-II, C-III, E	Pre β migration
IDL	1.006-1.019	25-35	$5\text{-}10X10^3$	B-100, C-I, C-II, C-III, E	Slow pre β migration
LDL	1.019-1.063	18-25	$2.3X10^3$	B-100	β migration
HDL_2	1.063-1.125	9-12	360	A-I, A-II, A-IV, C-I, C-II, C-III, E	α migration
HDL_3	1.125-1.21	5-9	175	A-I, A-II, A-IV, C-I C-II, C-III, E	α migration
Pre β HDL	1.28	~5	67	A-I	Pre β migration

Adapted from Havel, R.J. and Kane, J.P. 2001. Introduction: Structure and Metabolism of Plasma Lipoproteins. In Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D., editors. *The Metabolic and Molecular Basis of Inherited Disease,* McGraw-Hill Companies Inc., New York.

receptor, which recognizes apoE (17).

The liver is the other major site of apoB synthesis, and where very low density lipoprotein (VLDL) is formed (18;19). VLDL contains TG and CE at the core with some cholesterol and various lipoproteins. VLDL enters the circulatory system to deliver TG to peripheral tissues for energy production or storage, and LPL again hydrolyses the TG to fatty acids. As VLDL releases its contents to adipose and muscle tissues, the core becomes smaller, and the lipoprotein becomes the intermediate density lipoprotein,

(IDL). As more TG is hydrolysed IDL becomes low density lipoprotein (LDL), through two pathways. As more TG is removed from the IDL, CE is added from HDL by action of cholesterol ester transfer protein (CETP), IDL can be taken up by the liver through an interaction with the LDL receptor (LDLr), through apoB-100 (1;20;21).

LDL, containing apoB100, circulates to the peripheral tissues, where the lipids associated with LDL are removed from circulation by hepatic LDLr-mediated endocytosis (1).

HDL, the least buoyant class of lipoproteins, is a heterogeneous group of lipoproteins and contains only exchangeable apolipoproteins. The primary apolipoprotein present on HDL is apolipoprotein A-I (apoA-I). There can be two to five apoA-I molecules per HDL particle. HDL mediates the transport of cholesterol from peripheral cells to the liver and steroidogenic tissues in a process called reverse cholesterol transport. Excess cellular cholesterol can be taken up by HDL, and esterified by lecithin:cholesterol acyltransferase (LCAT), which is associated with the HDL. The CE can then be transferred to VLDL and LDL by CETP, another protein associated with HDL. The LDLr-mediated pathway of the liver internalizes the LDL. The HDL is also internalized by receptors, including scavenger receptor class BI (SR-BI), a process which leads to selective uptake of cholesteryl esters but not other HDL constituents, called selective uptake. The cholesterol in the liver can then be used to synthesize lipoproteins or bile acids (22;23).

C. HDL

HDL is the smallest class of lipoproteins. This class has a diverse population with differing structures. HDL is dense and has a protein-rich surface with a small apolar core. There are three subclasses of HDL, preβ HDL, HDL_2 and HDL_3, which are characterized by lipid composition, charge, shape, and density. The primary protein present is apoA-I, which is synthesized in the small intestine, and secreted on chylomicrons, and in the liver (9), where it is secreted as HDL particles (14;24).

HDL precursors may be produced by the liver or intestine, and also include surface lipid and exchangeable apolipoproteins that dissociate from chylomicrons and VLDL during hydrolysis of TG by LDL (1;24). HDL precursors can also be produced by the conversion of HDL_2 to HDL_3 by CETP (13;26). As the size of the lipoprotein core decreases the outer surface also can decrease.

ApoA-I and HDL mediate cholesterol efflux from the membrane by several possible pathways. One of these pathways is named the two-step model of cholesterol efflux. In this model, lipid-poor and lipid free apoA-I interacts with ATP Binding Cassette A1

Table 2: HDL Subclasses

HDL Subclass	Protein	Composition PL	(%w/w) FC	CE	TG
Pre β HDL	70	25	5	nd	nd
HDL_2	43	30	5	20	2
HDL_3	55	25	3	16	1

PL, phospholipid; FC, free cholesterol; CE, cholesteryl ester; TG, triacylgycerol; nd, not detectable.

Adapted from Fielding, P.E. and Fielding, C.J. 1996. *Biochemistry of Lipids, Lipoproteins and Membranes*, Vance, D.E. and Vance, J.E., eds. New York, New York: Elsevier.

(ABCA1) to bind phospholipid. In step two, the preβ HDL accumulates cholesterol from the plasma membrane, including caveolae (22;25).

The resulting product of the efflux is HDL_3. The FC is then esterified by LCAT, and other components of HDL are remodelled by phospholipid transfer protein (PLTP), resulting in HDL_2, a larger particle. Another efflux pathway is that mediated by ATP Binding Cassette G1, (ABCG1) (201), or scavenger receptor BI (SR-BI). This pathway effluxes lipids specifically to HDL but not to apoA-I (26).

D. History of Caveolae

1. Discovery of Caveolae

Caveolae were observed long before caveolin. In 1953, Palade (27) reported small invaginations on the plasma membrane (PM) of endothelial cells of blood capillaries. Yamada (28) described a morphologically identical structure in gall bladder epithelium. He named these structures caveolae because they were like "little caves". Caveolae have since been morphologically described as omega, flask-shaped invaginations connected to the PM by a neck-like structure. By electron microscopy, these caveolae appear to be smooth-coated compared to the electron dense coat of clathrin-coated pits.

2. Discovery of Caveolin-1

Caveolin was first reported as a 22kD protein in an antibody screening for tyrosine phosphorylated substrates in Rous sarcoma virus-transformed cells by Glenney and Zokas (29). The tyrosine phosphorylation of this protein correlated with transformation potential in cells transformed by Rous sarcoma virus. Antibodies to this protein stained cells in a punctate pattern that was concentrated at the PM (29).

The same 22kD protein was linked to caveolae by immunogold electron microscopy using the antibody previously developed (30). Using a rapid freeze technique, the protein was seen as a series of striated rings. Treatment of the cells with nystatin and filipin, both cholesterol binding agents, caused the caveolae to flatten, and this protein to dissociate from the PM (30). This 22kD protein was called caveolin because of its association with caveolae. Its concentration on caveolae, and the correlation between caveolin dissociation and the change of caveolae morphology led to the hypothesis that caveolin was an important structural protein of caveolae (30).

At the same time another group found and cloned a protein they named vesicular integral protein 21 (VIP21) as part of the cellular machinery sorting vesicles to the apical and basolateral PM. They noted that VIP21 was a component of vesicles derived from the trans Golgi network (31).

Cloning of caveolin led to the discovery that VIP21 and caveolin had identical sequences (32;33). The three fields in which caveolin was discovered has directed research into the functions of caveolin in signalling and cancer, in endocytosis and in the structure and role of caveolae.

3. Discovery of caveolins –2 and –3

Since other caveolin proteins have been discovered, the first caveolin protein is called caveolin-1.

Microsequencing of purified adipocyte caveolae revealed a peptide sequence closely related to caveolin-1. The cloning of the novel protein revealed similarities between this 162 amino acid protein and caveolin-1, even though they differed in several key conserved residues (34). This new protein was called caveolin-2.

Caveolin-3 was cloned from a cDNA library using a sequence related to caveolin-1 that was found downstream of the rat oxytocin receptor (35).

E. The Caveolin Gene Family

1. Caveolin Genes

Human genes for caveolin-1 and caveolin-2 (CAV1 and CAV2) are found at the locus 7q31, downstream of D7S522, a CA-repeat microsatellite that maps to 7q31.1 (36;37). CAV2 is located approximately 67 kilobases (kb) downstream from the D7S522 marker and is followed by CAV1 about 19 kb further downstream. CAV1 and CAV2 both have

Table 3: The chromosomal location of the caveolin gene family in human DNA

Gene	*Locus*	*# of exons*	*Markers*
CAV1	7q31.1	3	D7S522
CAV2	7q31.1	3	D7S22
CAV3	3p25	2	D3S18, D3S4163, D3S4539

3 exons, and are highly conserved across species (38). CAV3 is located on a different chromosome at 3p25 (39).

2. Promoter Region of CAV1

Regulation of CAV1 transcription was first noted in relation to levels of cellular cholesterol. When fibroblasts were loaded with unesterified cholesterol (FC) by 15%, caveolin levels increased 3 fold (40). If these cells were depleted of FC by cyclodextrin or incubation in lipoprotein deficient serum (LPDS) resulting in a 60% decrease of FC, caveolin-1 mRNA decreased by 4 to 5 fold (40;41). A 924bp CAV1 gene promoter

fragment was ligated to a luciferase gene expression vector pGL3 (42). The caveolin promoter was directly responsive to cellular FC levels. Transcriptional activity increased in response to increased cellular FC. From this observation, three putative sterol response elements (SRE) were identified. Selective mutational deletion revealed that two of the SRE's, 640 and 395 bp upstream from the caveolin-1 translation start site, were essential for the transcriptional response to FC (42). The SRE at –640bp binds SRE binding protein 1 (SREBP-1), and the other site binds an unknown transcription factor. The caveolin-2 gene is reported to contain similar SRE's (43).

Two other *cis* elements regulate caveolin-1 transcription; c-Myc, an oncogene, represses caveolin-1 transcription, and p53, a tumor suppressor, stimulates caveolin-1 expression (44;45). The result of the activity of these elements is the repression of caveolin-1 expression in transformed cells.

Just as the down regulation of caveolin-1 by oncogenes is desirable to decrease regulation of signalling molecules involved in transformation and survival of cancer cells, the removal of caveolin-1 by mutation also promotes tumour formation. Galbiati et al. (46) found that antisense downregulation of caveolin-1 in NIH 3T3 cells causes cellular transformation due to hyperactivation of the Ras p42/44 MAP kinase signalling cascade. These observations suggest that caveolin-1 suppresses tumor formation.

7q31 has a high deletion frequency around the D7S522 in many epithelial cancers including primary breast cancers (47), prostate (48;49), colon (50), ovarian (51) and renal cell carcinomas (52). The D7S522 maps to 7q31.1 and is upstream from caveolin-1 and caveolin-2 (36;37). The role of caveolin-1 as a tumor suppressor is contested, as

caveolin-1 is also upregulated in some cancers. Recent research suggests that another protein, ST7 , is the tumor suppressor proximal to D7S522 (61).

3. Caveolin Isoforms

Caveolin-1 has two isoforms, α and β. The α isoform is the full length 22kD protein and caveolin-1β is 3kD shorter (53). The caveolin-1β isoform is missing 30 amino acids from the N-terminus and is created either by the alternate translation site at M32, or by a shorter mRNA variant (53;54). Both caveolin-1 isoforms can drive caveolae formation. However, caveolin-1α appears to create deeper caveolae and drives caveolae formation more efficiently (53;54).

Caveolin-2 has three isoforms: α, β and γ by alternate translation sites as well as splice variants of mRNA (34;55;56). Caveolin-2α is the most prominently studied

Table 4: Isoforms of the Caveolin Family

Protein	Isoform	Length (amino acids)
Caveolin-1	α	178
	β	147
Caveolin-2	α	162
	β	149
	γ	Unknown
Caveolin-3		151

isoform and is targeted to caveolae with coexpression of caveolin-1. The β and γ isoforms have not been studied much. However, caveolin-2β has been observed on lipid droplets.

4. Tissue Specificity of Caveolins

Caveolin-1 and caveolin-2 are expressed at their highest levels in adipocytes, endothelial cells, pneumocytes and fibroblasts. However, lower levels of both proteins are found in most cell types (34;57;58). They are not expressed in the majority of cells expressing caveolin-3 except for smooth muscle cells. Caveolin-3 is expressed in cardiac, skeletal and smooth muscle cells (59). Expression of all three caveolins is highest in cells that are terminally differentiated (60).

While caveolin-1 and caveolin-2 have a similar tissue distribution pattern, the sequence of caveolin-1 is only 58% similar and 38% identical to caveolin-2, but 85% similar and 65% identical to caveolin-3 (58;61).

F. Caveolin-1 Protein Topology

Caveolin-1 is a 22kD protein that is highly conserved. An 8 amino acid sequence (FEDVIAEP) is conserved in all caveolin proteins both in the three proteins of the caveolin gene family and among all species containing the caveolin proteins. This sequence is called the "caveolin signature motif" (61;62). Deletion of this sequence traps caveolin-1 in the endoplasmic reticulum (ER) (62). However, no other role is known for this sequence yet.

1. Membrane Binding Domain

Early studies showed that caveolin-1 is a nonconventional membrane-spanning protein. Caveolin-1 was resistant to extraction by sodium carbonate, indicative of an integral membrane protein (31;63).

Antibodies against caveolin-1 were incubated with cells to determine whether caveolin-1 had an extracellular domain. The antibodies bound to caveolin-1 in lysed cells but not in intact ones, indicating that it may not have any extracellular domain (64). As well, caveolin-1 is sensitive to proteolysis, but only in lysed cells; the C-terminus is palmitoylated, and the N-terminus is phosphorylated, indicating that both ends of the protein are cytoplasmic (65-67).

Surface biotinylation of cells revealed that caveolin-1 was not accessible to biotinylation, and so not present on the extracellular side of the plasma membrane (68). The analysis of the caveolin-1 sequence revealed a hydrophobic region 32 amino acids long (102-134). This hydrophobic region itself acts as a signal peptide to insert into the membrane cotranslationally.

2. Membrane Attachment Domains

While the hydrophobic region of caveolin is inserted into the membrane, two other regions are capable of binding tightly to the membrane (69;70). Deletion analysis showed that amino acid residues 82-101 of the N-terminus and 135-150 of the C-terminus mediate membrane attachment (70). These two regions, labelled N-terminal and C-terminal membrane attachment domains (N-MAD and C-MAD), flank the transmembrane domain.

C-MAD contains a Golgi targeting sequence. A C-MAD-GFP fusion protein was localized to the *cis*-Golgi. This GFP was also resistant to alkaline carbonate buffer, which is consistent with an integral membrane protein, and TritonX-100 at 4 ^{O}C (70).

An N-MAD-GFP fusion protein localized to caveolae on the PM. A shorter sequence in the N-MAD, KYWFYR, targeted GFP to the PM, but not to caveolae (70). Amino acids 82-101 are in the same region called the scaffolding domain, and can have protein-protein interactions with signalling molecules localized in caveolae (71-73). This domain allows caveolin-1 to hold signalling molecules in caveolae or regulate signalling activity by inhibition or activation (74-76).

3. Oligomerization Domain

Caveolin-1 forms a highly stable complex of 14-16 monomers. This complex of 350-400 kD must be dissociated with harsh detergent treatment at high temperatures for disassembly (77;78). The region responsible for oligomerization was mapped to residues 61-101 by deletion mutagenesis (78). This region is also called the caveolin scaffolding domain, and binds proteins found in caveolae (61).

A second stage of oligomerization occurs by interaction of the C-terminal domain of the caveolin-1 proteins, creating a network of caveolin that can drive caveolae formation (78). Caveolin-2 binds the caveolin-1 membrane binding domain.

4. Posttranslational Modifications

Caveolin-1 is palmitoylated at cysteine 133, 143 and 156. These sites are thought to stabilize the structure of caveolin-1 at the plasma membrane (65;79). Two of the palmitoylation sites, 143 and 156, are also required for association of caveolin-1 with hsp56 and cyclophilin A in the cytosolic complex containing caveolin-1, cholesterol, and

Figure 1: The 7q31.1 locus. The caveolin-2 gene (CAV2) is 67 kb downstream of the D7S522 marker and the caveolin-1 gene (CAV1) is 19 kb downstream of CAV2. Exons of CAV1 and CAV2, and D7S522 are represented by blue boxes. The numbers above the boxes are the distance between the exons, and the numbers below the boxes are the length of each exon.

Fig. 1.

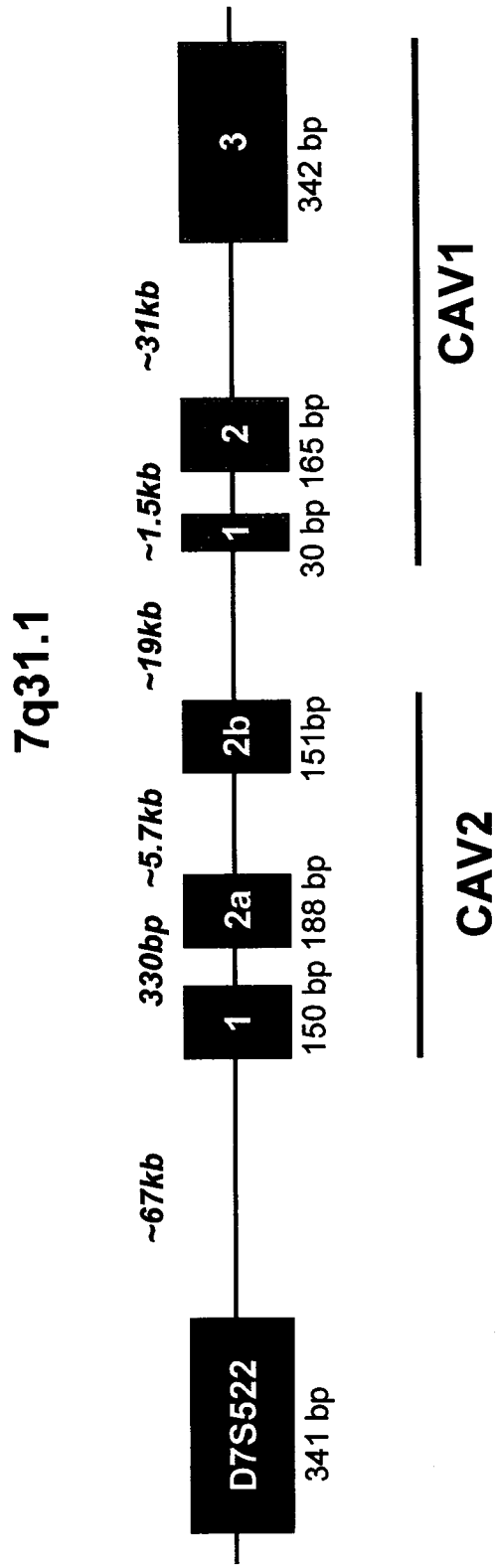
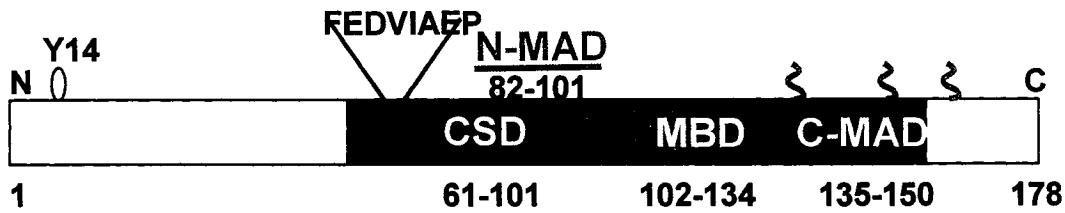

7q31.1
~67kb
330bp
~5.7kb
~19kb
~1.5kb
~31kb
D7S522
341 bp
1
150 bp
2a
188 bp
2b
151bp
1
30 bp
2
165 bp
3
342 bp
CAV2
CAV1

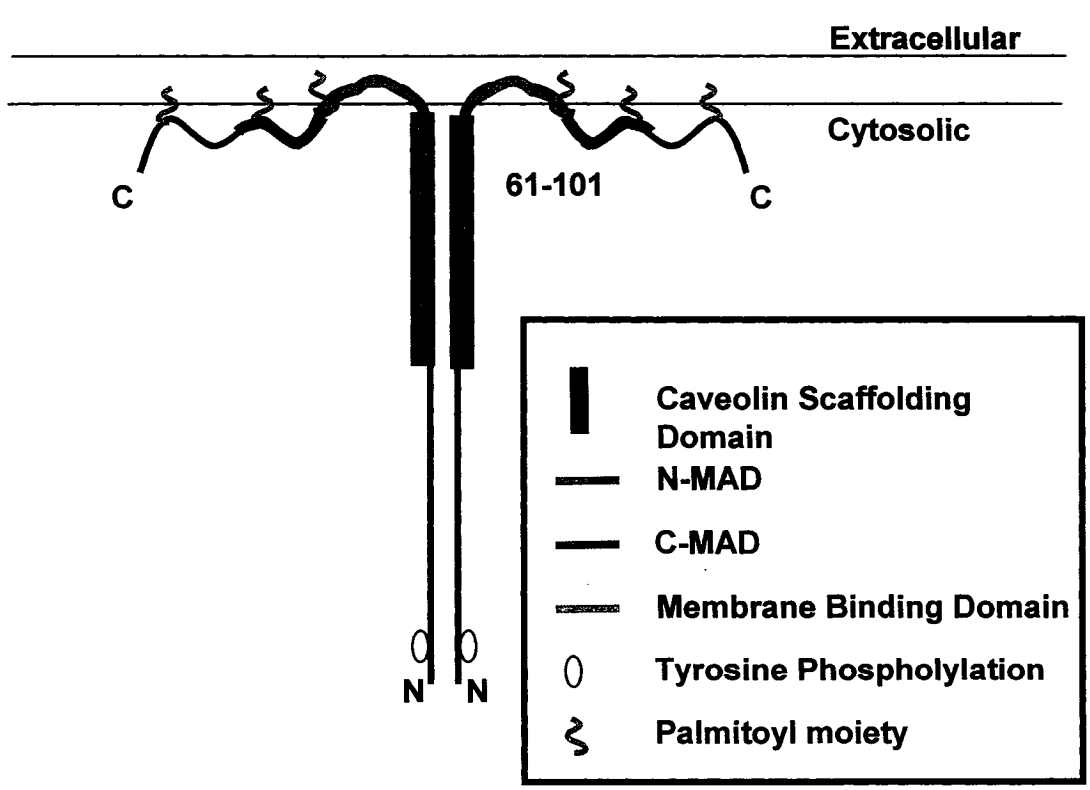
Figure 2: Protein Domains and Topology of Caveolin-1. A. A schematic diagram of caveolin-1. The membrane binding domain (MBD) is flanked by two membrane attachment domains (N-MAD and C-MAD). The caveolin scaffolding domain (CSD) interacts with caveolin-1 and other proteins found in the caveolae. The caveolin signature motif (FEDVIAEP) is found in the CSD. Caveolin-1 has one tyrosine phosphorylation site (Y14) and three putative palmitoylation sites (C133, C143, C156). B. Proposed topology of caveolin-1 in the plasma membrane.

Fig. 2.

A


FEDVIAEP
Y14
N-MAD
82-101
N
C
CSD
MBD
C-MAD
1
61-101
102-134
135-150
178

B


Extracellular
Cytosolic
C
61-101
C
N
N
Caveolin Scaffolding Domain
N-MAD
C-MAD
Membrane Binding Domain
Tyrosine Phospholylation
Palmitoyl moiety

chaperone proteins (80). A third palmitoylation site is not required in this complex, but may be related to localization of caveolin to lipid droplets.

Phosphorylation at Y14 is involved in the interaction with signalling molecules (81).

G. Caveolin Transport

Caveolin-1 is cotranslationally inserted into the ER membrane (79). Caveolin-1 does not have an N-terminal signal peptide to cause insertion into the membrane, but the transmembrane domain acts as a signal peptide. Caveolin-1 homooligomerizes with itself in the ER (77;79). The amino acid sequence IDFED (66-70) is required for transport of caveolin-1 to the Golgi (62). In the Golgi, caveolin-1 undergoes the second stage of oligomerization and becomes detergent insoluble (77;79;82). Binding of caveolin-2 through the membrane binding domain also occurs in the Golgi (83). From the Golgi, the caveolin-1 network is transported in a vesicle to the PM (72). Caveolin on the PM are found in caveolae. Internalized caveolae, called caveosomes, may move back to the Golgi (30).

Caveolin-1 may also enter the cytosol as a soluble complex with cholesterol, and chaperone proteins HSP56 and cyclophilin A (84-86). This cytosolic complex appears to be the size and density of HDL, and has multiple intracellular destinations (87). This includes transport to and from the ER to acquire de novo synthesized cholesterol to transport back to the caveolae. Little is known about this soluble complex.

H. Functions of Caveolin

1. Cholesterol Homeostasis

I. Structure of Caveolae

Caveolae are defined as detergent-insoluble plasma membrane domains containing caveolin proteins. The morphology of caveolae is classically described as a flask-shaped membrane invagination with a diameter of 50-100 nm (28). This description has been expanded to add other morphologies. Caveolae can also be flat in the plane of the membrane, detached from the membrane as vesicles, caveosomes (88), and fused to form tubules (89-91), or grape-like clusters (57;92).

Biochemically caveolae lipids are primarily cholesterol and sphingolipids (sphingomyelin and glycosphingolipids) and contain few phospholipids (93). The cholesterol and sphingolipids form a detergent insoluble membrane termed a liquid ordered domain (94-97).

Caveolin is not necessary for the formation of liquid ordered domains. Combining cholesterol and sphingolipids *in vitro* results in the formation of liquid ordered domains. Lipid rafts are, in fact, liquid ordered domains without the presence of any caveolin proteins. Lipid rafts and caveolae are both liquid ordered domains found on the PM. Caveolae are considered a subclass of lipid raft (94;95). Liquid ordered domains are resistant to mild non-ionic detergents and have a low density. These two biochemical properties allow purification and characterization of lipid raft/caveolar membranes through incubation with detergent and buoyancy in sucrose ultracentrifugation.

II. Cholesterol, Caveolin-1 and Caveolae

The sensitivity of caveolae to changes in cellular cholesterol concentration was demonstrated in early studies (30). Cholesterol binding agents, such as nystatin, filipin and methyl β cyclodextrin (mβCD) can flatten the caveolae invaginations on the cell surface and "unravel" the caveolin-1 coat, causing caveolin-1 to move to an ER/Golgi complex location. The influence of cholesterol on caveolin-1 is evident by its intracellular translocation with cholesterol depletion. Caveolin-1 only moves back to the PM when the cholesterol levels are restored. *In vitro* caveolin-1 will only bind to liposomes containing cholesterol (98). Caveolin-1 directly binds cholesterol in a 1:1 ratio. Loading cells with cholesterol resulted in a stabilization and increase of caveolae at the PM (99).

Caveolin-1 synthesis is directly affected by unesterified cholesterol (FC) levels in the cell. Depletion of cholesterol reduces caveolin-1 expression in MDCK cells and fibroblasts (40;41). In contrast, loading smooth muscle cells with low density lipoprotein (LDL) derived FC results in an increase of caveolin-1 expression and mRNA (40).

Cholesterol can regulate caveolin-1 expression through the two SRE's present in its promoter region. SRE binding protein-1 (SREBP-1) binds one of the SRE's in the caveolin-1 promoter region (42;99). SREBP-1 also activates the transcription of HMG CoA Reductase and the LDL receptor.

The expression of caveolin-1 in cells without any endogenous caveolin-1 in the basal state is sufficient to drive caveolae formation, and downregulation of caveolin-1 results in a decrease of caveolae at the PM (46;100;101). Caveolin-1 binds both cholesterol and sphingolipids (98;100;102), creating a detergent resistant domain. The caveolin-1

oligomeric complexes interact to form the caveolin network resulting in the cave-shaped morphology (70;72).

The role of caveolin-1 in cholesterol regulation was studied directly in synchronized primary skin fibroblasts. Immediately after S phase, caveolin-1, caveolar FC and efflux to lipoproteins decreased in order to increase the cellular FC mass in preparation for cell division. When cells were transfected with caveolin-1 cDNA, cholesterol efflux increased and the FC accumulation was significantly inhibited (103).

III. Cholesterol Efflux to HDL

The influence of caveolin-1 on HDL mediated cholesterol efflux is poorly understood, and the field is filled with conflicting reports. It has been suggested that caveolin-1 promotes efflux (104-107), caveolin-1 does not affect efflux, and caveolin-1 decreases efflux (108).

A large portion of the studies of the relationship between cholesterol and caveolin-1 used fibroblasts as the working model since they have high caveolin-1 expression and many caveolae. Fielding et al. (109), have studied the relationship between caveolin-1 and cholesterol efflux to HDL, plasma and apoA-I. In fibroblasts, cholesterol efflux occurs by a diffusional method and by a receptor mediated method. Cholesterol efflux to pre β HDL or apoA-I was defined as receptor mediated efflux, and cholesterol efflux to plasma HDL or native plasma was considered to be diffusional (109). LDL derived cholesterol was found enriched in caveolae. The cholesterol content of the caveolae was correlated to the rate of cholesterol efflux to HDL (40). Labelling cells with LDL cholesterol increased the level of caveolin-1 mRNA. Inhibiting caveolin-1 with okadaic acid decreased the efflux to HDL and native plasma (104). Antisense caveolin-1 also

decreased the efflux of LDL derived cholesterol (110). Conversely, overexpression of caveolin-1 cDNA increased efflux of cholesterol to HDL (40), and decreased the cholesterol mass of the fibroblasts (103). Caveolar cholesterol can be transferred to apoA-I as well (179). The transfer of newly synthesized cholesterol to the caveolae was stimulated by incubation with apoA-I.

THP-1 cells differentiated into macrophages by phorbol myristate 13-acetate (PMA), show a large increase in cholesterol mass, an increase in caveolin-1 expression and an increase in HDL mediated cholesterol efflux. Transfection of these cells with a caveolin-1 antisense oligomer causes a decrease in cholesterol incorporation into HDL, but phospholipid efflux is unchanged (106). In HepG2 cells stably transfected with caveolin-1, efflux to human plasma was 280% higher than negative controls and efflux to apoA-I was 45% higher (105).

The influence of caveolin-1 on plasma HDL was studied using mice infected with Ad5-caveolin-1 in which caveolin-1 expression was confined to the liver. This expression resulted in a two-fold increase of total HDL cholesterol mass in the plasma. Hepatocytes infected with caveolin-1 have an increased apoA-I secretion (107).

In RAW and J774 macrophage cell lines (111-113) caveolin-1 was not detected, nor did caveolin-1 mRNA respond to cholesterol loading. Transfection of caveolin-1 cDNA resulted in no changes in efflux to HDL. The authors thought that the influence of caveolin-1 on cholesterol efflux may be dependent on cholesterol mass as caveolin-1 expression in THP-1 cells was correlated with a large increase in cellular cholesterol mass (111).

In another conflicting report about caveolin-1 and cholesterol efflux, NIH 3T3 cells were used. NIH 3T3 cells express high levels of caveolin-1. When they are transformed by H-ras or v-abl, caveolin-1 expression decreases. HDL-mediated cholesterol efflux was lower in the parental cell line than the transformed cells. The parental cells were transfected with a caveolin-1 antisense or sense oligomer, and HDL mediated efflux was once again increased with the decrease of caveolin-1 expression (108). Caveolin-1 expression and activity of the caveolin-1 promoter region were also decreased with chronic exposure to HDL.

*i) **Facilitated Diffusion***

Cholesterol can transfer by simple diffusion between the PM and FC sequestering molecules like albumin and lipoproteins along the concentration gradient. This allows a bi-directional flux of cholesterol between the PM and lipoproteins in an energy-independent manner. This process is slow and depends on the proximity of the lipoproteins to the PM. By interacting with receptors, such as SR-BI or ABCG1, on the PM this process is faster and more efficient. This process, called facilitated diffusion, is still energy independent, and bi-directional. SR-BI binds to HDL to transfer cholesterol esters to the cell and facilitate the diffusion of FC between HDL and the PM (114-118). In both the mouse overexpressing SR-BI and the SR-BI null mice, there were changes to the plasma HDL (56;119).

SR-BI is localized to the caveolae in several cell lines (120;121). In CHO cells transfected with SR-BI cDNA, 80% of the SR-BI expressed was localized to PM caveolae (121). Coexpression of caveolin-1 and SR-BI in THP1 cells also resulted in a localization of SR-BI to caveolae (122) and negatively regulated cholesterol ester uptake.

Transfection of fibroblast cells with SR-BI increased HDL-mediated cholesterol efflux. Decreased expression of caveolin-1 by okadaic acid, or antisense caveolin-1 resulted in inhibition of the efflux facilitated by SR-BI (110).

SR-BI, an HDL receptor, stabilizes and increases the caveolae at the PM (99). In caveolae, CD36, a receptor for oxidized LDL (oxLDL), promotes sterol efflux to oxLDL, and causes caveolin-1 and endothelial NO synthase (eNOS), to translocate to an intracellular compartment (123). HDL protects against this translocation in an SR-BI-dependent manner by replacing the sterol removed by oxLDL (124).

ii) The Two-Step Model of Efflux

ATP-binding cassette A1, ABCA1, is a protein whose involvement in apoA-I specific efflux has only been studied recently. Defects in ABCA1 cause Tangier disease, in which efflux of cholesterol from macrophages is impaired, resulting in low concentrations of HDL and apoA-I, and an accumulation of foam cells. Tangier disease fibroblasts have impaired efflux of both phospholipid and cholesterol (125;126). In the absence of ABCA1 in hepatocytes, efflux to apoA-I is inhibited.

Two cell lines were used to identify the role of ABCA1 in efflux (25). HUVEC cells have low ABCA1 expression and very high caveolin-1 expression, while aortic smooth muscle cells (SMC) have high ABCA1 expression and low caveolin-1 expression. Incubation of apoA-I with HUVEC cells resulted in little efflux of phospholipid or cholesterol to apoA-I. Incubation of SMC with apoA-I resulted in a phospholipid rich, cholesterol poor complex with apoA-I. This efflux was not inhibited by vanadate, (Vanadate is an inhibitor of H-type ATPases and protein phosphatases, and causes caveolae to internalize) (127). If the media containing the

phospholipid rich apoA-I complex from aortic SMC was then incubated with the HUVEC cells, cholesterol was incorporated into the lipoprotein complex with the phospholipid and apoA-I (25). This efflux of cholesterol was inhibited by vanadate. Cholesterol efflux to preformed HDL was possible in Tangier fibroblasts, but not to lipid-free apoA-I (128). These observations led to the "two-step model" of efflux (25). In this model ABCA1 interacts and lipidates apoA-I with phospholipids. This preβ-apoA-I then acquires cholesterol from the PM, including caveolae. In endothelial cells and human SMC, 80% of cholesterol efflux can be inhibited by vanadate (127) and is filipin sensitive. Cells without caveolin and caveolae, such as J774 cells are still capable of efflux. However, this efflux is cAMP dependent, and unaffected by either filipin or vanadate. This highly specific efflux of cholesterol is dependent on ABCA1, not caveolin. Fielding et al. (110), suggest that this ABCA1-dependent efflux of cholesterol may be specific to transformed and immortalized cell lines. However, this alternate pathway of cholesterol efflux may be valid in other cells deficient in caveolin.

IV. Caveolin-1 and Intracellular Lipid Transport

The identification of caveolin-containing vesicles suggests that caveolin is involved in intracellular transport as well as efflux. Caveolin-1 associates with liquid ordered domains in the Golgi complex before moving to the PM. With the depletion of cholesterol, caveolin-1 moves to the ER, and then to the Golgi until cholesterol levels are restored. The cycling of caveolin-1 between the PM and the Golgi network may be involved in the cycling of cholesterol to the PM from intracellular stores or endosomal vesicles. The cholesterol from LDL can be selectively internalized independently from

the LDL receptor pathway (129). Cholesterol label was traced from the PM to endosomal vesicles, then to vesicles with markers of the *trans* Golgi network, and caveolin-1. Finally this cholesterol was found in the caveolae (130).

The transport of cholesterol from the ER to the PM occurs independently from the Golgi compartment. Pulse labelling of the cholesterol with [^{3}H]-mevalonate or [^{3}H]-acetate resulted in cholesterol label rapidly entering the caveolae with a half-life of 10 min. From the caveolae, FC was either transferred rapidly to HDL, or, in the absence of HDL, slowly dispersed to the rest of the membrane ($t_{1/2}$=30-40min) (131). This de novo cholesterol was isolated in a cytosolic complex containing caveolin-1, heat shock protein 56 (HSP56), cyclophilin A and cyclophilin 40. Inhibition of HSP56 or the cyclophilins blocked the transport of cholesterol to the PM (84). This complex is the same size and buoyant density as HDL. Mutational analysis revealed that palmitoylation of caveolin-1 is required for the formation of this complex. Cyclophilin A is unable to bind with caveolin-1 mutant cav(cys143ala) and resulted in a 50% decrease of cholesterol binding. HSP56 cannot bind cav(cys156ala), and cholesterol binding decreased 75% (80). Since cells containing little or no caveolin-1 can still transport de novo cholesterol, caveolin-1 may only facilitate cholesterol transport. In cells transfected with caveolin-1 cDNA, caveolar cholesterol levels were enhanced 4-5 fold (131).

V. Lipid Droplets

Recently caveolins were found on lipid droplets (132-134). Lipid droplets are found in all cells, but are most prominent in adipocytes. They are used for storage of excess lipids and cholesterol in the forms of triglycerides (TG) and cholesterol esters (CE). The core of TG and CE is surrounded by a phospholipid monolayer derived from

the ER and the surface is covered with proteins. The major proteins on the lipid droplet are perilipins in adipocytes and steroidogenic cells (135-137), and adipocyte differentiation related protein (ADRP) in preadipocytes and other cells (138).

Fujimoto and colleagues (132), found that the β isoform of caveolin-2 localized to the lipid droplet while caveolin-2α localized to the Golgi, and, with caveolin-1 expression, moved to form caveolae on the plasma membrane.

The caveolin-3 mutant cav-3 (DGV) is distributed to the lipid droplets (133;134). All of the caveolins can also distribute to the lipid droplets when they were retained in the ER by tagging an ER retrieval signal (KKSL) onto caveolin transfected into culture cells, or by brefeldin A treatment of cells already expressing caveolin-1. Caveolin targeting to the lipid droplets was irreversible (133;134;139). The conclusion of these results is that excess caveolin in the ER is targeted to lipid droplets. While this targeting appears to be physiological for caveolin-2β, the other caveolins only appear to target lipid droplets when there is excess caveolin in the ER.

Recent work by Pol and colleagues (140), suggests that caveolin-1 and caveolin-2 redistribute to lipid bodies in cells loaded with lipids, and leave the lipid droplets with lipid depletion. This adds to the idea that caveolin plays a critical role in cholesterol homeostasis within the cell, and needs further examination to determine the role caveolin does play with lipid droplets.

2. <u>Endocytosis</u>

Clathrin coated pits are the best characterized mechanism for internalizing specific proteins and bulk membrane by endocytosis (141). However, another pathway of endocytosis has also been reported (142;143). This clathrin independent

pathway is cholesterol sensitive (144;145), and inhibited by okadaic acid (146). This pathway uses both lipid rafts and caveolae. Antibodies for proteins found exclusively in caveolae accumulated interstitially 50 fold more than non-specific antibodies (147). This showed that caveolin-1 is involved in endocytosis.

Caveosomes are defined as endosomes containing caveolin-1, and caveolin-1 remains an effective marker of endosomes involved in the clathrin-independent endocytic pathway. Caveosomes are likely involved with the transport of sphingolipid and GPI-linked proteins from the PM to the Golgi (148;149).

3. Cell Signalling

Through both biochemical and morphological studies many of the signalling molecules isolated in both the caveolae and lipid rafts are at a higher concentration than found in total plasma membrane samples (61). This led to the hypothesis that there is a raft/caveolae compartment on the plasma membrane that concentrates and organizes signalling molecules (150). This would increase the efficiency of signalling, and also explain the cross talk between signalling pathways. Proteins localized to caveolae include G-proteins (151;152), insulin receptor (153), PDGF receptor (154),and EGF receptor (155), eNOS (156), GLUT-4 (157), nonreceptor tyrosine kinases (63;158), and adaptor proteins (159). The roles of both lipid rafts and caveolae as signalling compartments cannot be separated at this time, and new methods need to be developed to look at the separate roles of these two membrane domains.

i) Caveolin Scaffolding Domain (CSD)

The CSD binds directly to proteins found in the caveolae. ϕXϕXXXXϕ, ϕXXXXϕXXϕ, and ϕXϕXXXXϕXXϕ are the sequences that bind the CSD, where ϕ is Phe, Tyr or Trp, and X is any amino acid (71). These sequences are called the caveolin binding sequence (CBD). In all of the proteins known to interact with caveolin-1, a CBD was found at least once in their primary structure, but not all proteins with putative CBD's bind caveolin-1.

The interaction of caveolin-1 in several cases inhibits the basal level of the signalling molecule. In some of those cases, this is due to the location of the CBD in the active catalytic domain of the protein. The CSD is sufficient to inhibit signalling activity (160) including eNOS, endothelial nitric oxide synthase (74;161-164).

I. Caveolin-1 Knockout Mice

To examine the overall effects of caveolin-1 in vivo, caveolin-1 deficient mice were created (165;166). Initially these mice appeared normal and healthy. On closer examination abnormalities were found in adipose tissue, cardiac function and lung differentiation. Life span was reduced by 50%. The caveolin-1 deficient mice suffer from abnormalities in both the heart and pulmonary organs (165;167-169). In both cases these defects stem from fibroblast and endothelial cells, both normally abundant in caveolae.

No caveolae were found in adipose or endothelial tissue in these mice, where normally 5000 to 10 000 caveolae are found in endothelial cells (27). Caveolin-2 protein levels were reduced by approximately 90% due to protein destabilization and proteasomal degradation. Remaining caveolin-2 was retained in the Golgi (61). Cardiac

and striated muscle cells, expressing caveolin-3 retained their caveolae, but smooth muscle cells did not. While caveolae were absent in most cells, the GPI-anchored protein and lipid compositions of detergent-insoluble membranes were unchanged in cells isolated from the caveolin-1 deficient mice (61).

1. Lipid Homeostasis

The caveolin-1 null mouse has hypertriglyceridemia, and elevated chylomicrons and VLDL. The adipocytes of these mice were poorly differentiated and hypercellular, and the adipocyte cell diameters were reduced. The mice were lean and resistant to diet induced obesity. Lipid droplets were smaller in caveolin-1 null adipocytes, and responded poorly to lipolytic agonists (170;171). These mice are insulin resistant, and the insulin receptor levels in the adipocytes were severely reduced (170). The decrease of HDL in the caveolin-1 null mouse was small (171).

2. Signalling

Many important signalling molecules do not localize to caveolae exclusively, the absence of caveolae still changes the activation of some signalling molecules (61;166;167).

eNOS (endothelial nitric oxide synthase) signalling is upregulated in the caveolin-1 null mouse (165;172). eNOS causes the vasodilation of blood vessels (165;169) and is active in adhesion molecule expression on endothelial cells (173).

3. Cardiac function

Cardiac myocytes do not express caveolin-1, but do express caveolin-3. Cardiac endothelial cells and fibroblasts do express caveolin-1. They have a 50% reduction in life span, living for about 27 to 62 weeks and typically dying from sudden cardiac death

syndrome (169). The hearts of caveolin-1 null mice are abnormal with the right ventricular cavity enlarged and the left ventricular wall thickened. Caveolin-1 null mice develop cardiac hypertrophy (167). The absence of caveolin-1 causes an upregulation of p42/44 MAP kinase activity, hyperproliferation and fibrosis. The activity of eNOS, which regulates the contraction of the vessels and the heart, is increased in the caveolin-1 null mouse. The increased permeability of the blood vessels is also attributed to increased eNOS activity.

4. Lungs

Caveolin-1 null mice showed a lack of lung capacity and endurance by a swimming test in which the wild-type mice had a longer endurance than the caveolin-1 knockout mice (165;172). On closer inspection, the lung parenchymal cells were poorly differentiated, and there was a thickening of the alveolar wall due to an increase in the number of cells present. This phenotype was not due to the absence of caveolin-1, however, but the inability of caveolin-2 to form caveolae in the absence of caveolin-1. This phenotype was also seen in caveolin-2 knockout mice (174), and this phenotype is seen in caveolin-1 deficient mice due to degradation of caveolin-2.

5. $ApoE^{-/-}$ $caveolin\text{-}1^{-/-}$ mice

The caveolin-1 deficient mouse has a decrease in atherosclerotic lesion incidence and size. In order to look at the activity of caveolin-1 in an atherosclerotic background, the caveolin null mouse was crossed with the apoE deficient mouse (4). Caveolin-1 has both pro and anti-atherogenic properties.

Firstly, in the $cav1^{-/-}apoE^{-/-}$ mouse, there is an increase in plasma cholesterol levels with both the normal chow diet and the western-type diet. The non-HDL

cholesterol plasma levels are two-fold higher with the western-type diet (4). This lipid profile indicates an anti-atherogenic role for caveolin-1.

In contrast measurement of lesion size and quantity shows that caveolin-1 has an overall proatherogenic effect. The area of the lesions was reduced by ~70% in the absence of caveolin-1 in the apoE-/- mice with either the normal chow diet or the western-type diet.

The influence of caveolin-1 on the lipid profile of these mice suggests that caveolin-1 influences the metabolism of lipoproteins (4;8). The absence of caveolin-1 in an apoE deficient background results in hypercholesterolemia, whether the animals are fed a low fat, low cholesterol diet or a Western diet (4). The lipid profile shows that this is largely due to high levels of VLDL and IDL. Mice fed the control diet have no change in HDL levels or apoA-I expression. However, when caveolin-1 $^{(-/-)}$ apoE $^{(-/-)}$ mice are fed a western diet plasma apoA-I plasma apoA-I drops by more than 80% (4).

Hypothesis

Our hypothesis is that caveolin-1 influences the metabolism of cholesterol in hepatic cells. We will show the effect of caveolin-1 expression on two areas of cholesterol metabolism: cholesterol synthesis and efflux, using three different cell systems.

Chapter Two: Materials and Methods

Materials

The adenovirus vectors Ad5-GFP (green fluorescent protein), Ad5-cav1 (caveolin-1) and Ad5-tTA (tetracycline transactivator) were a gift from the lab of Dr. Lisanti, and the caveolin-1 deficient mice were from the same lab (175). Ad5-caveolin-2 and Ad5-caveolin-1 adenovirus vector were a generous gift from the lab of Dr. Sessa. [^{3}H]cholesterol, [^{3}H]mevalonate, [^{3}H]acetate, and [^{3}H]choline were purchased from PerkinElmer Life and Analytical Sciences. Anti-caveolin polyclonal antibody was purchased from Transduction labs, anti human apoA-I antibody from Calbiochem, and anti murine apoA-I antibody from Biodesign International. Sepharose G beads were ordered from Amersham Biosciences. Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Modified Essential Medium (EMEM), Hepatozyme, fetal bovine serum (FBS) and bovine serum albumin (BSA) were from Sigma. Trypsin, L-glutamine and penicillin-streptomycin were purchased from Gibco. Methyl β cyclodextrin was obtained from Cerestar.

Adenovirus systems

Caveolin-1, green fluorescent protein, and tetracycline transactivator recombinant ΔE1ΔE3 adenoviruses (Ad5-cav1, Ad5-GFP and Ad5-tTA respectively) were donated by Dr. Lisanti and generated as described previously (175). The C-terminal Myc-tagged canine caveolin-1 cDNA or GFP cDNA was placed under the control of a CMV immediate early (IE) minimal promoter preceded by a heptamer of tetO sequences. Protein expression in cells was then induced by the co-infection of caveolin-1 (or GFP)

adenovirus with a second adenovirus containing a constitutive CMV-driven transactivator (Ad5-tTA) whose expression was suppressed in the presence of tetracycline.

Cells were infected with 25 moi of Ad5-cav1 or Ad5-GFP, accompanied by the 25 moi of Ad5-tTA for 1 h at 37 ^{0}C.

The caveolin-1 and caveolin-2 adenoviruses were from the lab of Dr. William Sessa(176). These vectors were not tetracycline inducible, but gene transcription was controlled by a CMV immediate early minimal promoter. Cells were infected with 30 moi of Ad5-caveolin-1 or –2 for 1 h at 37 ^{0}C.

Ad5-Luciferase (Ad5-luc), and Ad5-apoA-I were generated in this lab by Dan McManus and colleagues (177). Luciferase and apoA-I were also under the control of the CMV promoter(177). Cells were infected with 75 moi of Ad5-apoA-I for 1 h at 37 ^{0}C. McA-RH7777 and HepG2 cells were infected with 30 moi of Ad5-luc for 1 h at 37 ^{0}C, and hepatocytes were infected with 75 moi of Ad5-luc for 1 h at 37 ^{0}C.

Viral Scale-up and Titering

Virus was grown in 293 cells starting with 1 x 10 cm dish up to 30 x 100 cm dishes. Cells were scraped. Cells were frozen at –80 °C in PBS $^{2+}$ -glycerol, and then thawed three times. Cell lysates were fractionated on a cesium chloride gradient. Cesium chloride was solubilized in 10mM Tris-HCl to make three solutions at densities of 1.4 g/ml, 1.35 g/ml and 1.2 g/ml. In 2 tubes, 2 ml of the 1.4 g/ml cesium chloride solution was added first, and then 4 ml of the 1.2 g/ml solution. The viral stock was added gently on the top, and balanced. Tubes were centrifuged for 90 min at 100 000 x g at 4 °C. The white band of viral particles was completely removed. The 1.35 g/ml cesium chloride solution was added to the tube and balanced with another tube. The viral

particles taken from the first spin were combined. The tubes were centrifuged for 18 h at 100 000 x g at 4 °C and the lower white band was taken. Viruses were dialyzed in 3 x 4L of PBS^{2+} with 10% glycerol at 4 °C, and titer was determined by plaque assay (177).

Cell culture of McA-RH7777 cells and HepG2 cells

McA-RH7777 cells were grown in DMEM, 10% FBS, 1% L-glutamine and 100 μg G418. Cells were seeded 1 x 10^5 cells into 6-well Primaria plates, and cultured for 24 h in a CO_2 incubator at 37 °C.

HepG2 cells were grown in EMEM, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded 8 x 10^4 cells into 6-well Primaria plates, and grown for 24 h in a CO_2 incubator at 37 °C.

Hepatocyte culture

Hepatocyte cells were prepared by Vivian Franklin, and seeded on 6-well plates coated with 20 μg/well of fibronectin. Hepatocytes from mouse liver, both C57Bl6 and caveolin-1 deficient mice were seeded at 2 x 10^6 cells/well and incubated for 5 h at 37 °C. Radioactivity was added at 5 μCi/ml of [^{3}H]choline or [^{3}H]cholesterol, or 10 μCi/ml of [^{3}H]mevalonate for a total of 40 h.

Lipid Synthesis

Cells were labelled with 10 μCi/ml of [^{3}H]acetate or [^{3}H]mevalonate in DMEM containing 2 mg/ml BSA (DMEM-BSA) for 1 h up to 24 h. Cells were rinsed in PBS and scraped into 2 ml H_2O:methanol (1:1 v/v). Lipids were extracted as described previously.

Cells were extracted in 4:1:1:1:0.1 (v/v/v/v) of chloroform: methanol, H_2O: water saturated NaCl:acetic acid. The organic phase was removed and the aqueous phase was discarded.

Thin Layer Chromatography (TLC)

Samples in the organic phase were dried down under N_2 gas. To each sample, 50 µl of chloroform was added and then loaded onto a silicon-coated glass plate, and run in polar and nonpolar solvent systems: chloroform:methanol:acetic acid:formic acid:H_2O (105:45:7.5:3:1.5 v/v/v/v/v) and hexane:diethyl ether:acetic acid (105:45:1.5 v/v/v). Band locations were determined by iodine. Bands were scraped and radioactivity was determined by scintillation counting.

HDL and apoA-I mediated efflux from McA-RH7777 and HepG2 cells labelled by [^{3}H]cholesterol

Cells were seeded in 6-well plates for 24 h before labelling. [^{3}H]cholesterol was incubated with HDL or LDL isolated human plasma for 30 min at 37°C in 500µl of DMEM-BSA. The volume was increased so that there is 5 µg/ml of LDL or HDL and 1 µCiml [^{3}H]cholesterol in the labelling media. Labelling media was added to the cells for 24h. Cells were equilibrated for 4 h with DMEM-BSA in order to allow the [^{3}H]cholesterol to label the recycling endosomes. Efflux media containing 50 µg/ml of either HDL isolated from human plasma, or 15 µg/ml of recombinant his-tagged human apoA-I was then added to the cells. Media was collected for scintillation counting and cells were solubilized in 0.5N NaOH.

HDL and apoA-I mediated efflux of newly synthesized cholesterol from McA-RH7777 and HepG2 cells

To label cells with newly synthesized cholesterol the label media was either 10 μCi/ml of [^{3}H]acetate or 5 μCi/ml of [^{3}H]mevalonate. Efflux occurred as in the previous efflux assays. However, media and cell samples were collected in 1:1 H_2O:MeOH and samples were processed as described in lipid synthesis.

Cholesterol Depletion with Methyl β Cyclodextrin

Methyl β cyclodextrin (mβCD) was solubilized in DMEM to a concentration of 10 mM. Cells were labelled as described above in HDL and apoA-I mediated efflux. Cells were labelled with [^{3}H]cholesterol, and incubated for 30 min at either 4 °C or 37 °C to bring the cells to the temperature required for cholesterol depletion. The mβCD solution was added to cells for 15 min at either 4 °C or 37 °C. The media was collected for scintillation counting, and cells were scraped into 0.5 N NaOH for scintillation counting and protein determination.

ApoA-I Specific Efflux from Hepatocytes

The cells were labelled for 40 h as described in hepatocyte culture. To quantify the lipidation of secreted apoA-I, 20h into the labelling period, hepatocytes were infected with 75 moi of either Ad5-apoA-I or Ad5-Luc for 1 h. Cells were equilibrated for 1 h in Hepatozyme media, and then cells are incubated in the presence or absence of human apoA-I for 5 h at 37 °C either by adenovirus infection or exogenously adding 15 μg/ml of

apoA-I. Excess cells and cell debris in the media were removed by centrifugation. The media was incubated with anti-human apoA-I antibody for 18 h and then with Sepharose G beads to precipitate the labelled lipids associated with apoA-I. Cells were also collected for protein determination and cholesterol extraction. Determination of synthesized lipids is described above. All samples were scintillation counted to determine radioactivity.

Electron Microscopy

McA-RH7777 and HepG2 cells were fixed in 1.6% glutaraldehyde in 0.1 M cacodylate buffer, and post fixed with osmium tetroxide. Cells were stained with uranyl acetate, and dehydrated in consecutive solutions of increasing ethanol concentration. Cells were embedded in Spurr resin, stained with uranyl acetate and lead citrate, and cut into sections by Peter Rippstein. The sections were examined with a Jeol 1230 transmission electron microscope.

Western Blot Analysis

Cell proteins were solubilized in 1 ml/well of SDS loading buffer. The samples were loaded into a 12% SDS PAGE gel and run at 90 V. They were transferred to nitrocellulose membrane at 125 V for 2 h. The membrane was incubated for 1h in blocking buffer (5% skim milk, and 0.2% Tween in PBS). The membrane was probed with either anti-caveolin-1 antibody or anti-HSP 60 antibody overnight, or with the biotinylated anti-apoA-I antibody, 4H1, for 1 h at 4 °C. The membrane was subsequently probed with the secondary antibody coupled to horse radish peroxidase (HRP): anti-

rabbit IgG, anti-mouse IgG or streptavidin antibody respectively. The proteins were detected using Pierce West Pico SuperSignal substrate.

Statistical Analysis

Each data point was calculated as the average +/- SD of triplicate or quadruplicate samples. All experiments were reproduced between 2 and 5 times, and the experiments shown are representative. In order to determine significance the two-tailed t test was used.

Chapter Three: Results

A. Stably Transfected McA-RH7777 cells

Electron Microscopy

The ultrastructure of the McA-RH7777 cells was examined for caveolae. No caveolae were formed in the MH cells, (expressing no caveolin-1), but caveolae were found attached to the membrane of both the M3-31 and M3-35 stably transfected cells. Thirty-one percent of M3-31 sections contained caveolae, and 49% of M3-35 sections contained caveolae (Fig 3).

Lipid Synthesis

Cells were labelled with 1 μCi/ml of [^{3}H]acetate for 1 h. After lipid extraction the lipids were separated by TLC as described in the *Materials and Methods* section. The synthesis of cholesterol from cells labelled with [^{3}H]acetate was increased by 30% in the presence of caveolin-1. However, the synthesis of triglycerides, cholesterol esters, and phosphatidyl choline was not significantly increased with caveolin-1 expression (Fig 4). This was previously observed in the lab by Philip Links (178).

Earlier findings suggested that apoA-I is capable of stimulating cholesterol synthesis (85;179). The synthesis of cholesterol, triglycerides, cholesterol esters, and phosphatidyl choline was measured again in repeated experiments, but in the presence of 15 μg of apoA-I during 1 h of lipid synthesis. We found no significant increase in the synthesis of any lipids when apoA-I was present (Fig 4 B, C, D).

Figure 3. **Electron Microscopy pictures of McA-RH7777 cells show that stable transfection with caveolin-1 results in caveolae formation.** Stably transfected McA-RH7777 cells are shown at 15000-fold magnification. MH (A) are mock transfected, M3-31 (B) are an intermediate, and M3-35 are high expressers of caveolin-1. These data are representative of the examination of 50 cells where caveolae were counted and this experiment was repeated twice.

Fig. 3.

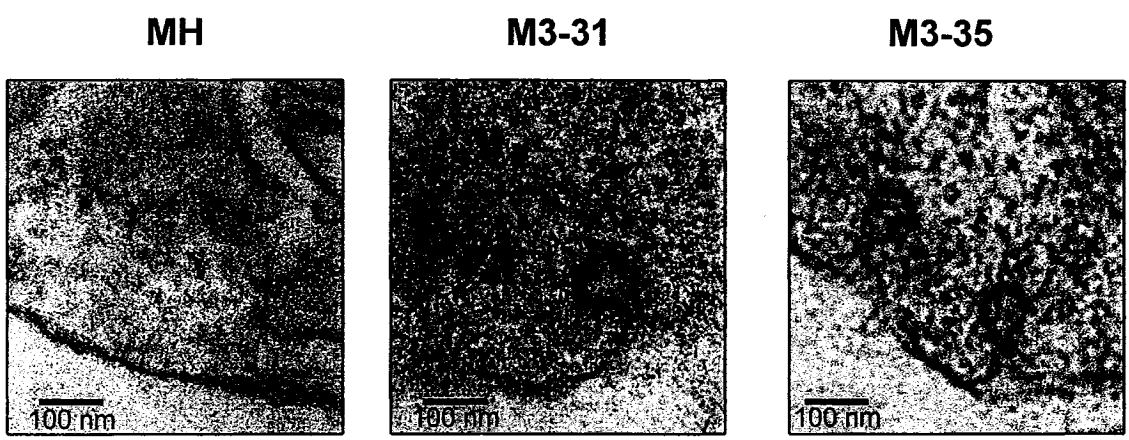
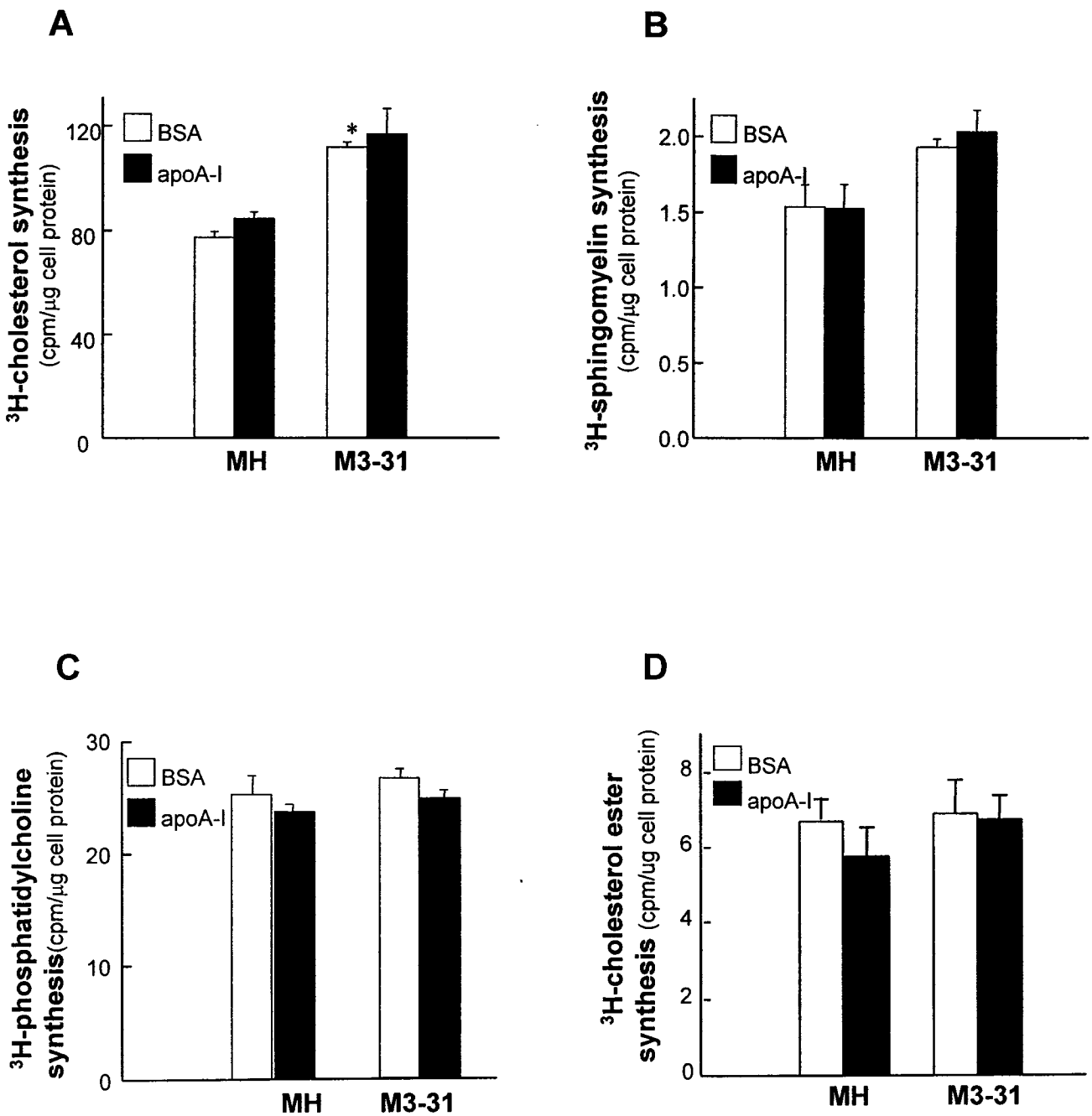


Figure 4. **Caveolin-1 expression in McA-RH7777 cells increases cholesterol synthesis.** Stably transfected caveolin-1 McA-RH7777 cells were labelled with 10 μCi/ml [^{3}H]acetate for 1 h, and then incubated for 3 h in the presence or absence of 15 ug of apoA-I. Synthesis of [^{3}H]cholesterol (A), [^{3}H]sphingomyelin (B), [^{3}H]phosphatidyl choline (C), and cholesterol esters (D) from the [^{3}H]acetate was measured. Cell lysate was collected for lipid extraction and TLC to separate the lipids. Cholesterol synthesis increases significantly in M3-35 cells compared with MH cells (* = $P<0.01$)

Fig. 4.


A
B
C
D
BSA
apoA-I
^{3}H-cholesterol synthesis (cpm/μg cell protein)
^{3}H-sphingomyelin synthesis (cpm/μg cell protein)
^{3}H-phosphatidylcholine synthesis(cpm/μg cell protein)
^{3}H-cholesterol ester synthesis (cpm/ug cell protein)
MH
M3-31
*

Cholesterol efflux to apoA-I, HDL

Mock (MH), M3-31, and M3-35 cells were labelled for 24 h with [^{3}H]cholesterol as described in *Materials and Methods*. After a 4-h equilibration period, HDL was added to the cells for 0, 3, 6 and 24 h (Fig 5A). At 6 h and 24 h, there were significant increases in the amount of cholesterol effluxed from the cells with the highest level of caveolin-1 expression, $p<0.01$ and $p<0.005$, respectively. At 24 h there was an increase in the amount of cholesterol efflux to HDL from the cells with intermediate expression of caveolin-1. This is consistent with previous work from our lab (178).

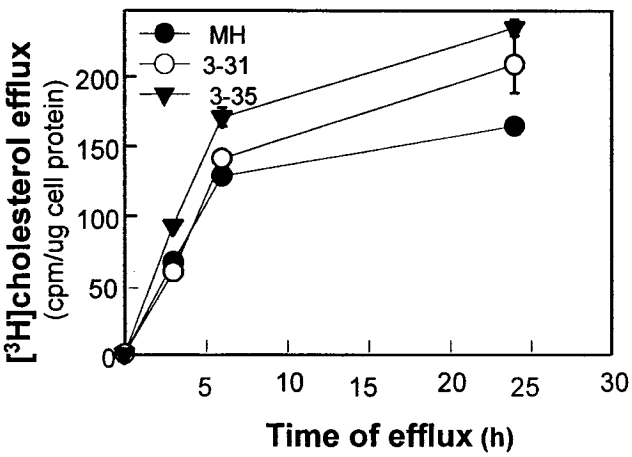
In order to examine efflux of the newly synthesized cholesterol from caveolin-1 expressing cells, mock and M3-35 cells were labelled with [^{3}H]mevalonate for 24 h and the cells were treated as described previously (Fig 5B). After incubation for 0, 3, 6 and 24 h in the presence of HDL, ^{3}H-cholesterol in the medium was measured as described in *Materials and Methods*. ApoA-I mediated efflux of newly synthesized sterol significantly increased by 20% ($p<0.01$) with the expression of caveolin-1 when immunoprecipitated by human apoA-I antibody. This amount does correlate with the increase of cholesterol synthesis observed in these cells observed previously by Philip Links (178).

To understand how caveolin-1 influences efflux, cells were incubated with methyl β cyclodextrin, a cholesterol binding agent at temperatures preventing intracellular trafficking (4 °C). Cells labelled with exogenous cholesterol were incubated with 10 mM mβCD for 15 min at 4 °C or 37 °C (Fig 6). The overexpression of caveolin-1 did

Figure 5. **HDL mediated efflux of exogenous cholesterol and newly synthesized cholesterol is increased in the presence of caveolin-1.** (A) MH, and M3-35 cells were labelled with 1 μCi/ml of [^{3}H]cholesterol for 24 h, equilibrated for 4 h and effluxed to 50 μg of HDL for 3, 6 and 24 h. Media and cells were collected and counted by scintillation counting. (B) MH and M3-35 cells were labelled with 5 μCi/ml of [^{3}H]mevalonate for 24 h, equilibrated for 4 h, and effluxed to 15 μg of apo for 3, 6 and 24 h. Media was collected and immunoprecipitated using anti human apoA-I antibody. Cells were collected for lipid extraction and isolation of cholesterol by TLC. Each point is an average +/- SD, n=4. There were significant differences in efflux between MH and M3-35 cells at 6 and 24 h, (* $P<0.01$, ** <0.005).

Fig. 5.

A


MH
3-31
3-35
[3H]cholesterol efflux
(cpm/ug cell protein)
0
50
100
150
200
5
10
15
20
25
30
Time of efflux (h)

B

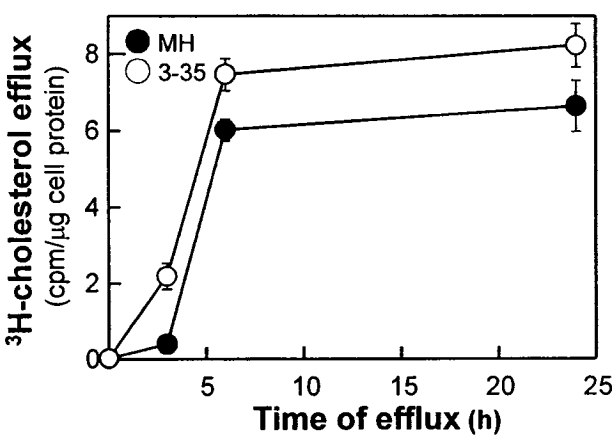
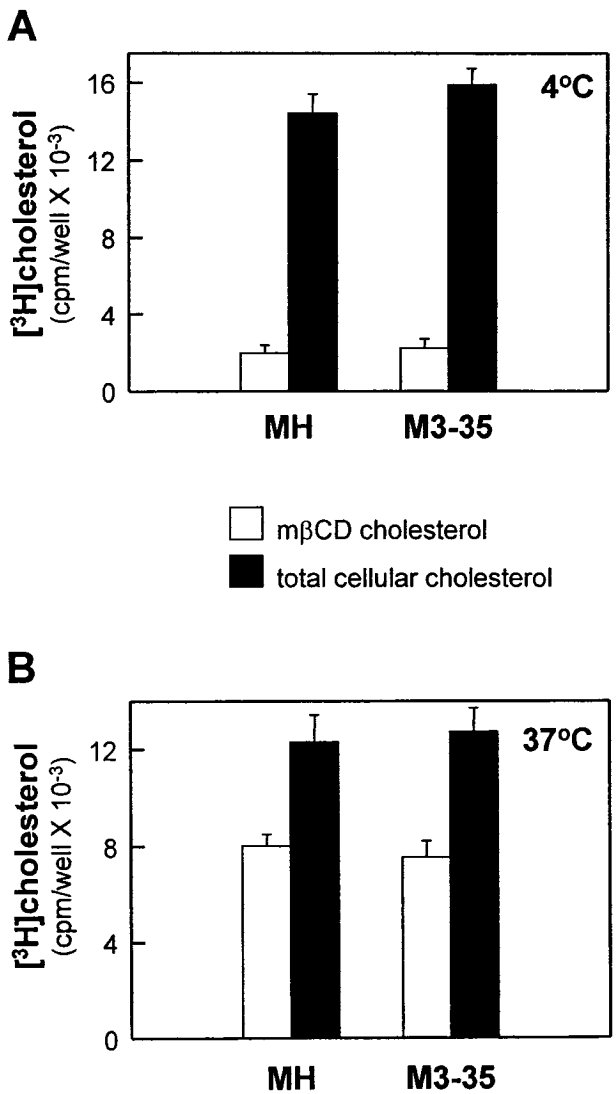

MH
3-35
3H-cholesterol efflux
(cpm/μg cell protein)
0
2
4
6
8
5
10
15
20
25
Time of efflux (h)

Figure 6. **Overexpression of caveolin-1 does not change the accessibility of mβCD to [^{3}H]cholesterol in McA-RH7777 cells.** Efflux of [^{3}H]cholesterol was mediated by 10 mM methyl β cyclodextrin (mβCD) at 4 °C (A), and 37 °C (B) for 15 min. Mock (MH) and M3-35 cells were incubated with [^{3}H]cholesterol associated with LDL for 24 h, and equilibrated to the temperature for efflux (4 °C or 37 °C). Each bar is an average +/- SD, n=3. The difference between MH and M3-35 cells was not significant (P>0.01)

Fig. 6.


A
[3H]cholesterol (cpm/well X 10-3)
16
12
8
4
0
4°C
MH
M3-35
mβCD cholesterol
total cellular cholesterol
B
[3H]cholesterol (cpm/well X 10-3)
12
8
4
0
37°C
MH
M3-35

not change the amount of cholesterol accessible to mβCD at 4 °C or 37 °C, and therefore, caveolin-1 did not increase the amount of cholesterol accessible on the PM.

B. HepG2 cells infected with Caveolin-1 tetracycline Sensitive Adenovirus

In order to evaluate the effect of short-term caveolin-1 expression on cholesterol efflux, we selected an inducible adenovirus tetracycline off system of caveolin-1 expression (175). First, McA-RH7777 cells were infected with 25 moi of both Ad5-tTA and Ad5-cav1 or Ad5-tTA and Ad5-GFP. Even though infection efficiency was high and cells were healthy as determined by trypan blue, the cells did not adhere to the plate effectively and were washed off of the plate during the efflux assay. McA-RH7777 cells were then abandoned as a model for these transfections and replaced by HepG2 cells.

HepG2 cells also represent another hepatoma cell line, which, like the McA-RH7777 cells, are easily infected with adenovirus. They are good models, because they do not express detectible levels of caveolin-1 (Fig 7B), and do not show caveolae (data not shown).

Caveolin-1 expression

HepG2 cells were infected with 5 to 50 moi of Ad5-tTA and Ad5-cav1, respectively for 24 h and the expression of caveolin-1 was verified by western blot analysis (Fig 7B). As more adenovirus is added to the cells, the level of protein expression is increased. Cells infected with 5 moi of adenovirus had low infection efficiency. With 10

Figure 7. **Caveolin-1 expression occurs in HepG2 cells infected with Ad5-caveolin-1.** (A) This model demonstrates the tetracycline sensitive adenovirus system used to express caveolin-1 in HepG2 cells. (B) The western blot shows the expression of caveolin-1 with rabbit anti-caveolin in HepG2 cells infected with Ad5-GFP, Ad5-tTA, and Ad5-caveolin-1.

Fig. 7.

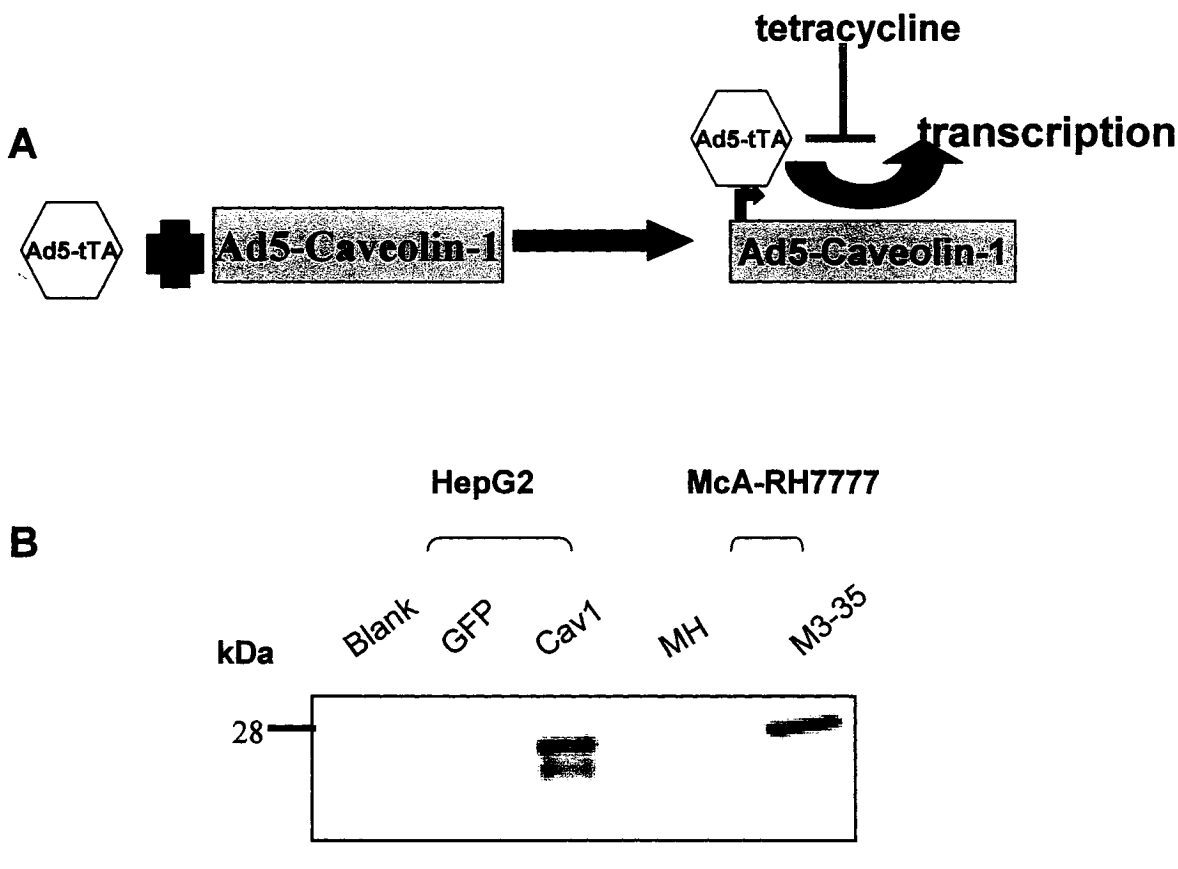
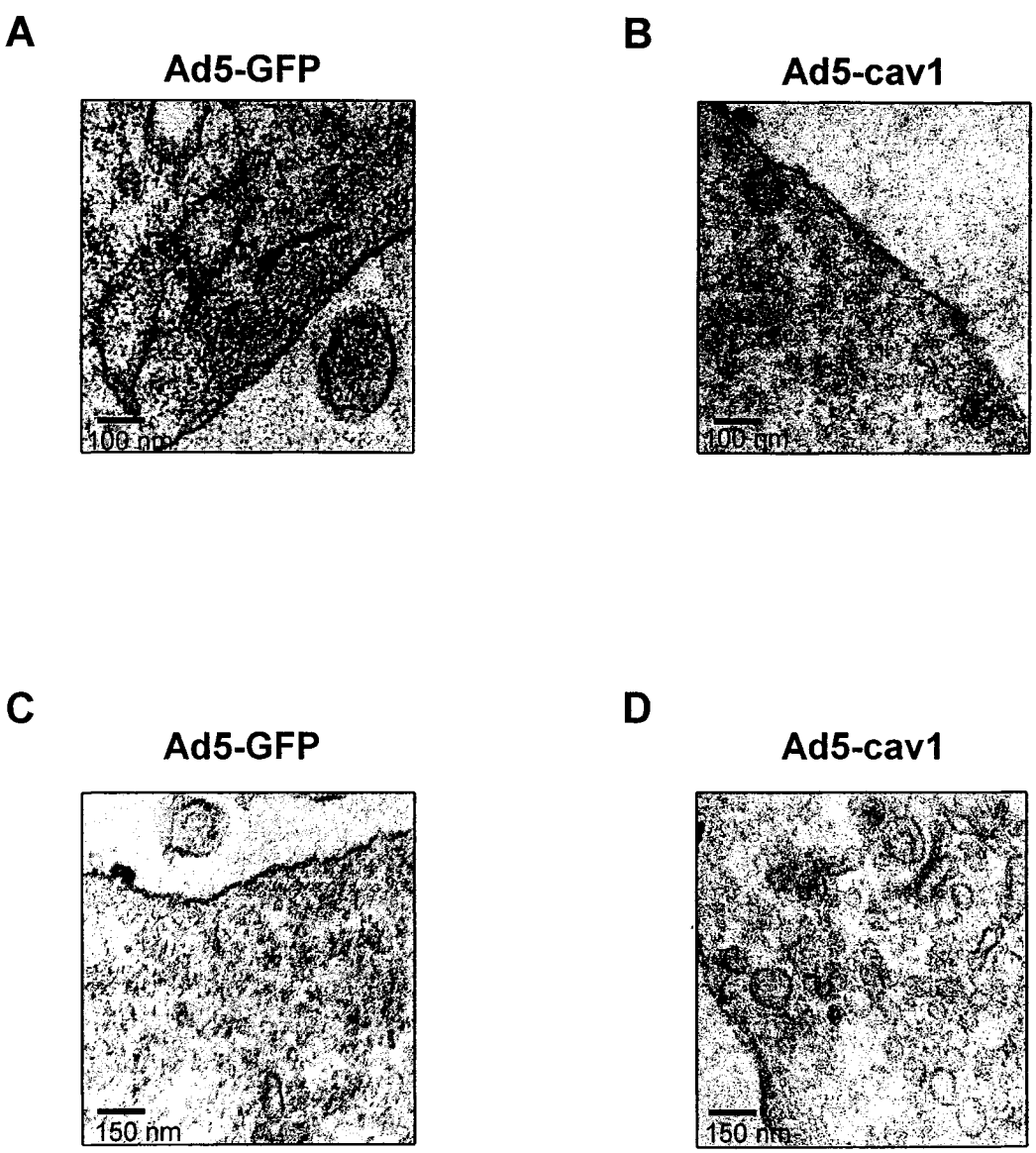

A
tetracycline
Ad5-tTA
transcription
Ad5-tTA
Ad5-Cavcolin-1
Ad5-Caveolin-1
B
HepG2
McA-RH7777
Blank
GFP
Cav1
MH
M3-35
kDa
28

Figure 8. **Caveolae are present in HepG2 cells overexpressing caveolin-1.** HepG2 cells expressing GFP show no caveolae (A) and caveolin-1 expressing cells have caveolae present (B) at 15000-fold magnification. Cells expressing caveolin-1 have an increase in the number of vesicles present (D) compared to GFP expressing cells (C). Cells were infected with 25 moi of Ad5-caveolin-1 or Ad5-GFP accompanied by infection with 25 moi of Ad5-tTA. Cells were fixed and prepared for electron microscopy as described in *Materials and Methods*.

Fig. 8.


A
Ad5-GFP
100 nm
B
Ad5-cav1
100 nm
C
Ad5-GFP
150 nm
D
Ad5-cav1
150 nm

moi of virus there was about 25 % infection efficiency. With 25 moi of Ad5-cav1 and Ad5-tTA there was 96% infection efficiency. Trypan blue dye was added to the cells to verify the viability of the cells infected with adenovirus. At 100 moi of both Ad5-tTA and Ad5-cav1, the cell viability decreased by 9%.

Electron Microscopy

HepG2 cells were infected with adenovirus to express GFP or caveolin-1 for 24 h. Cells were then fixed and prepared for examination of the ultrastructure. Caveolae were not found in cells with GFP, but 83% of cells expressing caveolin-1 contained caveolae (Fig 8B). On average, most cell sections containing caveolin-1 contained two or more caveolae. There was also an increase in small vesicles in the cytosol in caveolin-1 expressing cells that were not visible in cells expressing GFP (Fig 8 C, D).

Lipid Synthesis

After 1-h co-infection of the HepG2 cells with 25 moi of both Ad5-tTA and Ad5-caveolin-1 we incubated them with [^{3}H]acetate. We found a ~48% increase in cholesterol synthesis when caveolin-1 was expressed (Fig 9). This was consistent with the results found in the McA-RH7777 cells stably transfected with caveolin-1.

Cholesterol efflux mediated by HDL and apoA-I

Cells were labelled with HDL [^{3}H]cholesterol for 24 h, and caveolin-1 was expressed for 3, 7 or 24 h (Fig 9A). After 4-h equilibration, cells were incubated with HDL for 3 h. No increase in HDL mediated efflux was observed between cells with caveolin-1 expression and GFP expression (Fig 10B).

To examine the influence of caveolin-1 on the efflux of newly synthesized cholesterol to apoA-I and HDL, cells were labelled with [^{3}H]mevalonate, and infected to

Figure 9. **Short-term caveolin-1 expression in HepG2 cells increases cholesterol synthesis.** HepG2 cells were infected with Ad5-tTA and Ad5-cav1 or Ad5-tTA and Ad5-GFP. Cells were rinsed and incubated with EMEM-BSA for 2 h before the addition of 10 μCi/ml [^{3}H]acetate. The cells were labelled for 2 h at 37 °C. Cellular cholesterol was extracted and measured as described under *Materials and Methods*. (A) Western blot of HepG2 cells infected with the adenovirus was performed. (B) ^{3}H-cholesterol synthesized from [^{3}H]acetate was increased in cells expressing caveolin-1. Each bar is the average cholesterol per cell protein +/- SD (n=3). The difference in cholesterol synthesis between GFP and caveolin-1 expressing cells was significant (* $p<0.0005$).

Fig. 9.

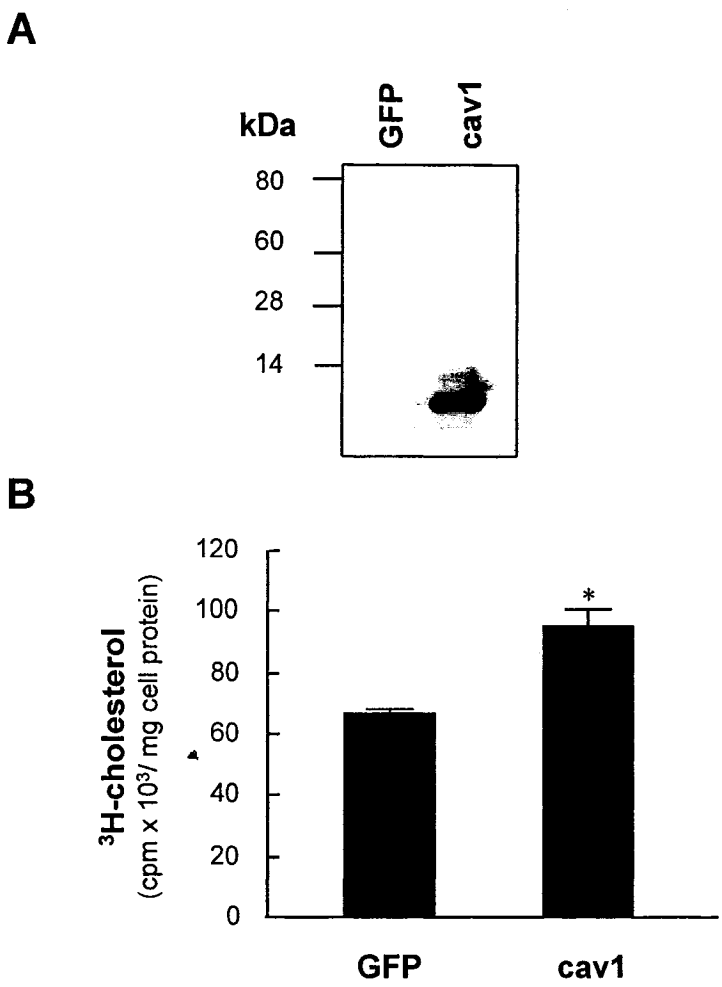

A
kDa
GFP
cav1
80
60
28
14
B
^{3}H-cholesterol
(cpm x 10^3/ mg cell protein)
120
100
80
60
40
20
0
*
GFP
cav1

Figure 10. **HDL and apoA-I mediated efflux from HepG2 cells infected with Ad5-caveolin-1 was unchanged.** A. 24 h after seeding, the cells were infected with 25 moi each of Ad5-cav1 and Ad5-tTA or with 25 moi or Ad5-GFP and Ad5-tTA for 1 h Tetracycline was used to suppress expression of GFP and caveolin-1 until the desired time. The cells were washed and incubated with 1 μCi/ml [^{3}H]cholesterol associated with HDL for 23 h. After labelling and washing, the cells were switched to medium without tetracycline, and for the last 3 h to an efflux medium containing 50 μg HDL and no tetracycline. B. Cholesterol was effluxed to 50 μg HDL for 3h with 3, 7 and 24 h caveolin-1 or GFP protein expression. C. Efflux of newly synthesized cholesterol was mediated by HDL or apoA-I in the presence of 24 h caveolin-1 or GFP protein induction. Each bar is the average percent efflux +/- S.D. (n = 3) and is representative of three independent experiments. The difference in efflux to HDL between GFP and caveolin-1 expressing HepG2 cells was significant in B (* = $p<0.005$).

Fig. 10.

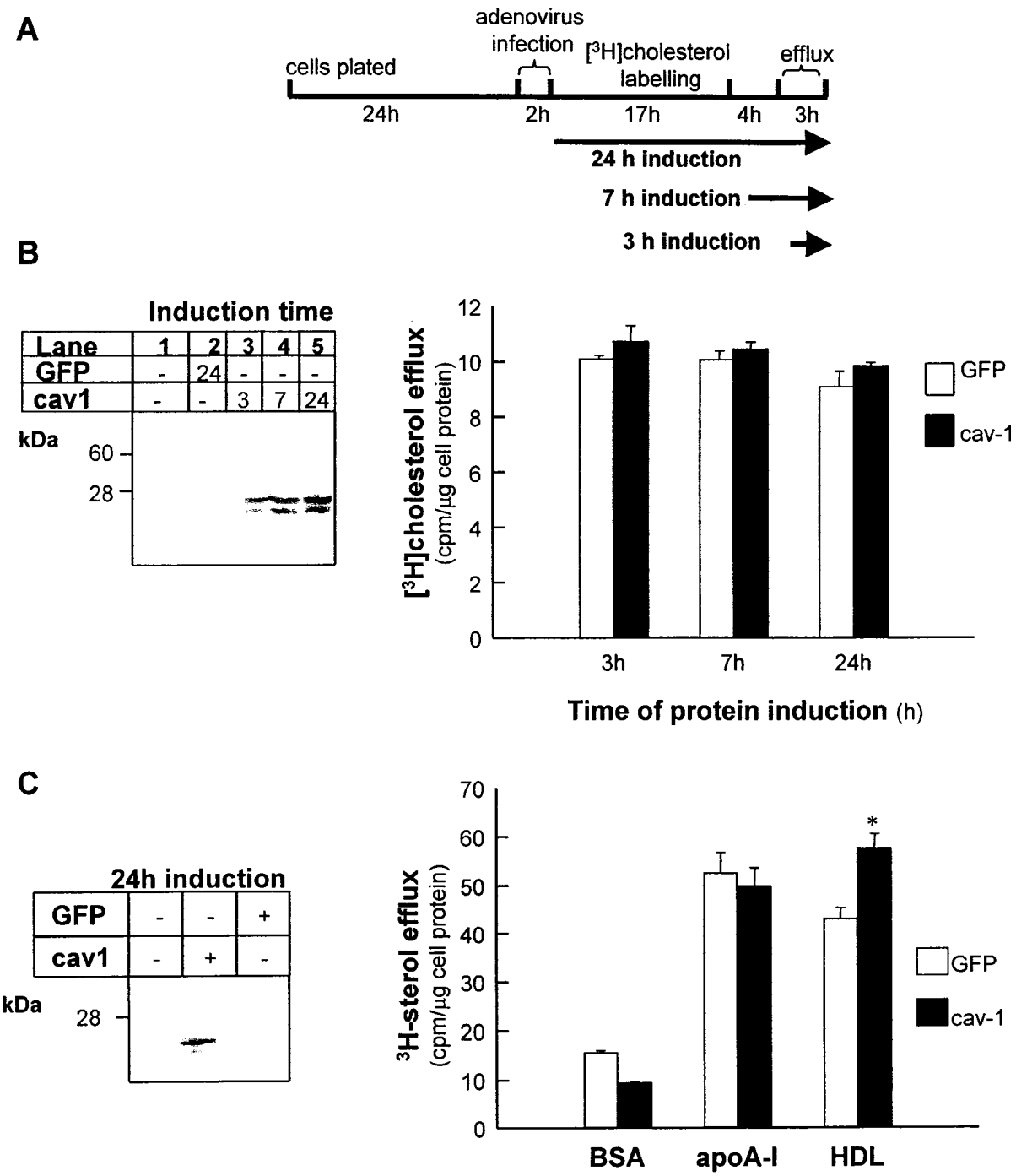
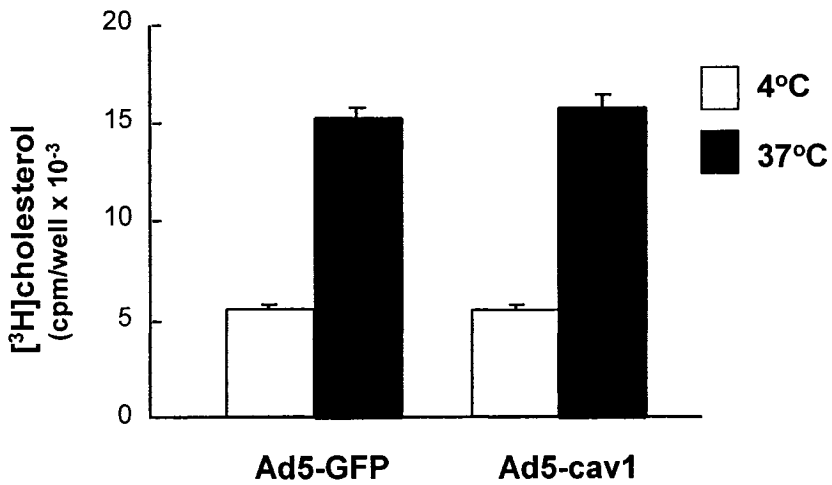

A
adenovirus infection
[3H]cholesterol labelling
efflux
cells plated
24h
2h
17h
4h
3h
24 h induction
7 h induction
3 h induction
B
Induction time
Lane 1 2 3 4 5
GFP - 24 - - -
cav1 - - 3 7 24
kDa
60
28
[3H]cholesterol efflux (cpm/µg cell protein)
12
10
8
6
4
2
0
3h
7h
24h
GFP
cav-1
Time of protein induction (h)
C
24h induction
GFP - - +
cav1 - + -
kDa
28
3H-sterol efflux (cpm/µg cell protein)
70
60
50
40
30
20
10
0
*
BSA
apoA-I
HDL
GFP
cav-1

Figure 11. **Adenoviral vector mediated caveolin-1 expression does not increase [^{3}H]cholesterol accessibility to mβCD in HepG2 cells.** HepG2 cells were infected with Ad5-tTA and either Ad5-GFP or Ad5-caveolin-1 and then labelled with [^{3}H]cholesterol for 18 h. Cells were equilibrated to the correct temperature for efflux, and then efflux of [^{3}H]cholesterol was mediated by 10 mM methyl β cyclodextrin (mβCD) at either 4 °C or 37 °C for 15 min. Each bar is an average +/- SD, n=3. The difference in cholesterol removal between cells expressing caveolin-1 or GFP was not significant (P>0.01)

Fig. 11.


20
15
10
5
0
[3H]cholesterol
(cpm/well x 10-3
4°C
37°C
Ad5-GFP
Ad5-cav1

expressed either GFP or caveolin- for 24 h. There was no increase in ^{3}H-sterol efflux to BSA or apoA-I, but efflux to HDL increased by 30% when cells expressed caveolin-1 instead of GFP (Fig 10C).

HepG2 cells overexpressing caveolin-1 or GFP were incubated with methyl β cyclodextrin (mβCD). Cells labelled with exogenous cholesterol were incubated with 10 mM mβCD at 4°C, to prevent intracellular trafficking, or 37°C for 15 min. We found that there was no significant difference in [^{3}H]cholesterol accessibility to mβCD between cells overexpressing GFP or caveolin-1 (Fig. 11)

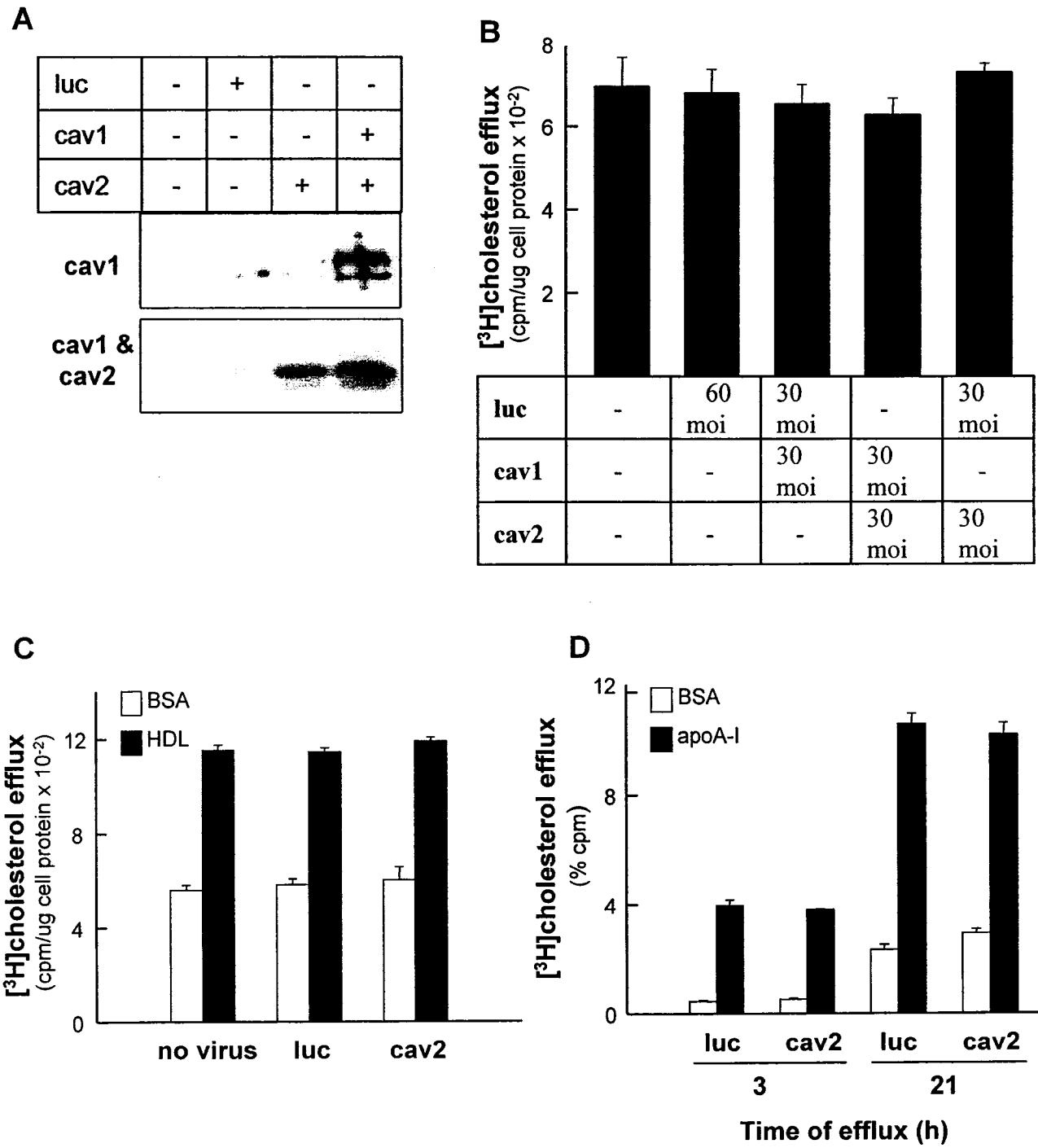
Influence of Caveolin-2 on Cholesterol Efflux to HDL and ApoA-I in Hepatocytes and McA-RH7777 Cells

Fujimoto et. al (54) found that caveolae were stabilized when caveolin-1 and caveolin-2 were coexpressed in HepG2 cells compared to cells expressing only caveolin-1. We obtained a caveolin-2 adenoviral vector (Ad5-cav2) from Dr. William Sessa. We infected HepG2 cells with 30 moi of Ad5-luc, Ad5-cav-1 and Ad5-cav-2 (Fig 12A), and Found that caveolin-2 was expressed in the HepG2 cells. We wanted to look at the result of coexpressing caveolin-1 and caveolin-2 on efflux of exogenous cholesterol to apoA-I.

Cells were infected with a total of 60 moi of adenovirus for 1 h before labelling cells with LDL-cholesterol for 24 h. After 1-h equilibration of the label, 15μg of apoA-I was added to the cell for a 6 h efflux. One group of cells was not infected in order to observe the effect of the adenovirus itself on efflux. No changes in efflux were detected. The cells were infected with Ad5-luc, Ad5-luc and Ad5-cav-1, Ad5-cav-1 and Ad5-cav-2or Ad5-luc and Ad5-cav-2. There was no change to efflux of exogenous cholesterol

Figure 12: **Coexpression of caveolin-1 and caveolin-2 does not change efflux of exogenously added cholesterol mediated by HDL or apoA-I in HepG2 or McA-RH7777 cells.** A. Western blots of HepG2 cells infected with Ad5-luciferase, Ad5-caveolin-1, or Ad5-caveolin-2 show expression of caveolin-1 or caveolin-2. The first nitrocellulose membrane was probed with a caveolin-1 polyclonal antibody, and the second membrane was probed with a monoclonal caveolin-2 antibody. HepG2 cells were infected with 30 moi of Ad5-luciferase, Ad5-caveolin-1 or Ad5-caveolin-2 for 1 h and then grown for 24 h before collection in SDS loading buffer. Samples were normalized to cell protein. B. Efflux assay of HepG2 cells expressing luciferase, caveolin-1, caveolin-2 or caveolin-1 and caveolin-2. Cells were infected with adenovirus for 1 h before labelling. After 1 h equilibation, cells were incubated with apoA-I for 6 h. C and D show an efflux assay of M3-35 cells expressing luciferase or caveolin-2. Cells were infected with adenovirus for 1 h before labelling for 24 h with 1 μCi/ml of [^{3}H]-cholesterol equilibrated with LDL. After 1 h equilibration of the label, cells were incubated for 6 h with 50 μg HDL (C), or 3 and 21 h with 15 μg apoA-I (D). Each bar is an average +/- SD (n=3)

Fig. 12.


A
luc - + - -
cav1 - - - +
cav2 - - + +
cav1
cav1 & cav2
B
[3H]cholesterol efflux (cpm/ug cell protein x 10-2)
8
6
4
2
luc - 60 moi 30 moi - 30 moi
cav1 - - 30 moi 30 moi -
cav2 - - - 30 moi 30 moi
C
BSA
HDL
[3H]cholesterol efflux (cpm/ug cell protein x 10-2)
12
8
4
0
no virus
luc
cav2
D
BSA
apoA-I
[3H]cholesterol efflux (% cpm)
12
8
4
0
luc
cav2
luc
cav2
3
21
Time of efflux (h)

with the expression of caveolin-2 or the coexpression of caveolin-1 and caveolin-2 (Fig. 12B).

Since McA-RH7777 cells were more responsive to the effects of caveolin-1 on efflux, we decided to see if caveolin-2 would change efflux of exogenous cholesterol in a different cell model. We infected M3-35 caveolin-1 expressing cells with 30 moi of caveolin-2. In order to ensure that the virus itself did not affect the efflux of cholesterol, we also performed the experiment with cells not infected with the virus. We used the same method of efflux that we used above with the HepG2 cells, except that we used 50 μg of HDL instead of the apoA-I. We found no change in HDL mediated efflux of exogenously added cholesterol (Fig. 12C). Lastly, we looked at efflux to apoA-I from M3-35 cells transiently expressing caveolin-2 (Fig. 12D). We used the same efflux method as we used above in the previous experiment, but we changed the efflux time to 3 and 21 h (Fig. 12D). We found no change in efflux of exogenous cholesterol to apoA-I.

Thus, despite significant expression of caveolin-2, we conclude that caveolin-1 and caveolin-2 coexpression does not result in augmented efflux of cholesterol.

C. Hepatocytes from Caveolin-1 deficient C57Bl6 mice

The introduction of caveolin-1 into two hepatoma cell lines has shown that caveolin-1 increases cholesterol efflux gradually, and also increases the synthesis of cholesterol. In order to confirm the significance of these findings, caveolin-1 deficient mice were obtained from Jackson laboratories. Previous work by Lisanti's lab shows that no caveolin-1 is present in these mice, caveolae are not present with the exception of striated and cardiac muscles (172).

Cholesterol Synthesis

Homozygous caveolin-1 deficient (cav-/-), heterozygous caveolin-1 deficient (cav+/-) and control (cav+/+) hepatocytes were incubated with 5 μCi/ml of [^{3}H]choline or 10 μCi/ml of [^{3}H]mevalonate for 40 h to examine the influence of caveolin-1 on lipid synthesis in hepatocytes. To determine phospholipid synthesis, cells labelled with [^{3}H]choline were subjected to lipid extraction with dH_2O:methanol:chloroform (2:1:4 v/v). No significant differences in phospholipid synthesis were detected (Fig 13). Cholesterol in the cell lysate was isolated by TLC. Cholesterol synthesis was decreased ~40% in the absence of caveolin-1. LDL-mediated cholesterol influx was unchanged in the absence of caveolin-1.

ApoA-I and HDL mediated efflux

Hepatocytes from the cav-/-, cav+/- and cav+/+ mice were labelled with [^{3}H]-mevalonate and LDL derived cholesterol (LDL-C). They were incubated with 50 μg/ml of HDL for 5 h and the media was collected. Cholesterol bound to human apoA-I was measured after immunoprecipitation. HDL-mediated efflux of LDL-C decreased by approximately 25%, and efflux of newly synthesized cholesterol decreased by over 74% in the absence of caveolin-1 (Fig 14). This experiment was repeated with efflux mediated by apoA-I instead of HDL. The absence of caveolin-1 resulted in a similar decrease in apoA-I mediated efflux of both LDL-C (36%), and newly synthesized cholesterol (83%) (Fig. 15). ApoA-I needs to acquire phospholipid before cholesterol, so we labelled the phospholipids of the cell with [^{3}H]-choline. Cells were incubated for 5 h with apoA-I, and media was collected for immunoprecipitation. The apoA-I mediated

efflux of PL was decreased by approximately 68% in the caveolin-1 deficient hepatocytes (Fig 15A).

Hepatocytes secrete lipoproteins, including apoA-I. To look at the influence of caveolin-1 on the secretion of lipids with apoA-I, the cav-/-, cav+/- and cav+/+ mice were infected with Ad5-apoA-I. Cells were labelled with tritiated choline, mevalonate or LDL-derived cholesterol. The impairment of the lipidation of endogenously synthesized apoA-I was more pronounced than efflux to exogenous apoA-I. Lipidation of newly synthesized apoA-I by phospholipid was decreased by over 90% in caveolin-1 null mice, and lipidation by newly synthesized cholesterol decreased by 90% (Fig 16). Efflux of LDL-C decreased to 73% in the caveolin-1 null mouse compared to the wild type. To understand this difference, hepatocytes were infected with Ad5-apoA-I and the cells were collected to quantify the amount of human apoA-I present in the hepatic cells. The samples were transferred to nitrocellulose and probed for human apoA-I, caveolin-1 and HSP60. We were concerned that caveolin-1 deficiency was impairing the ability of the adenovirus to infect the hepatocytes and synthesize apoA-I. Cellular human apoA-I was equal between all three types of hepatocytes, despite varying levels of caveolin-1 synthesis (Fig 17A). Intensity of the signal of apoA-I was determined through Quantity One, and compared, relative to HSP 60 (Fig 17B). No difference in cellular apoA-I was found. Secretion of apoA-I into the media was not tested.

Figure 13. **Caveolin-1 deficient hepatocytes have decreased cholesterol synthesis.** Hepatocytes were labelled with [^{3}H]choline (A), [^{3}H]mevalonate (B), or exogenously added [^{3}H]cholesterol for 48 h before cells were collected. (A) Phospholipid synthesis from [^{3}H]choline was not significantly decreased. (B) Synthesis of cholesterol from [^{3}H]mevalonate is decreased significantly in caveolin-1 deficient cells compared to wild type hepatocytes (* = $P<0.001$). (C) Influx of exogenous LDL cholesterol was unchanged by the absence of caveolin-1. Each bar is the average percent efflux +/- S.D. ($n = 3$).

Fig. 13.

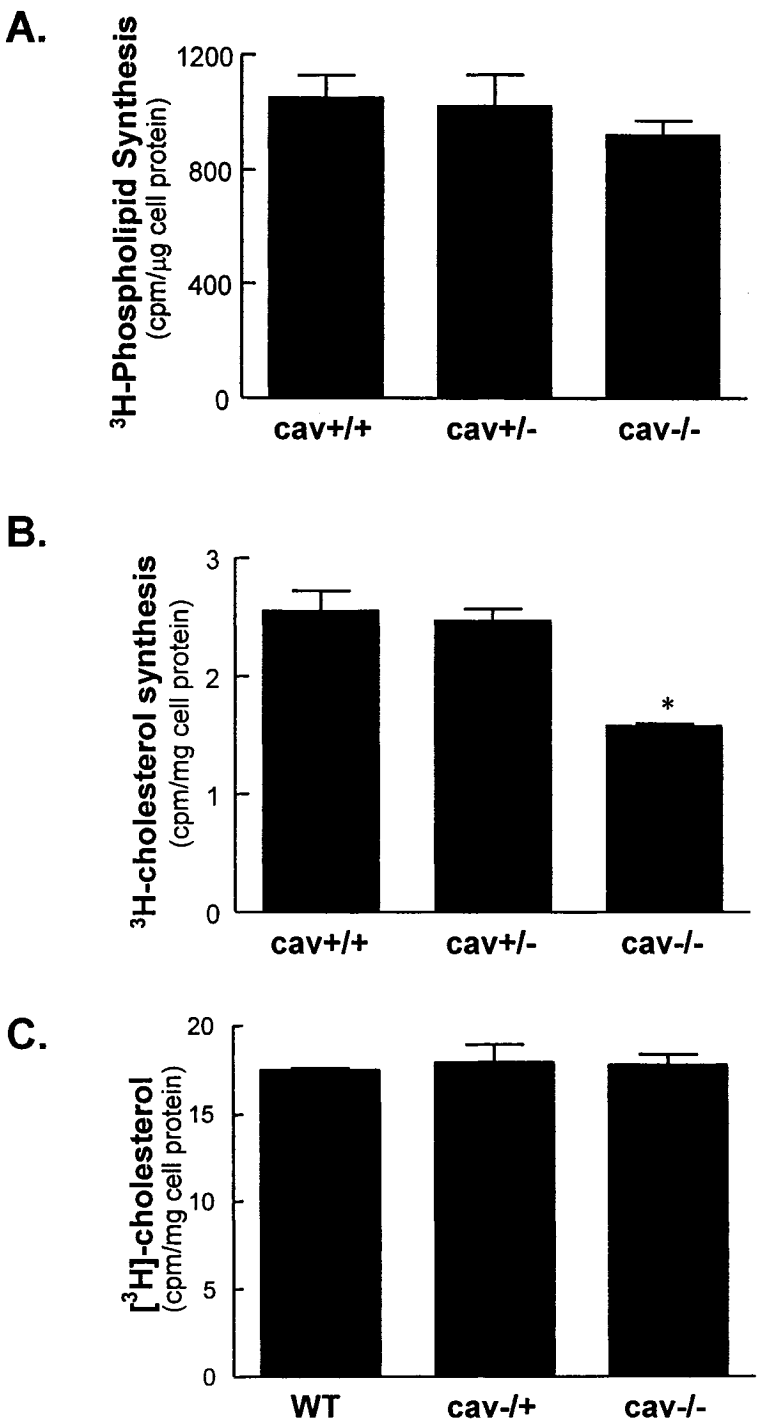

A.
^{3}H-Phospholipid Synthesis
(cpm/μg cell protein)
1200
800
400
0
cav+/+
cav+/-
cav-/-
B.
^{3}H-cholesterol synthesis
(cpm/mg cell protein)
3
2
1
0
*
cav+/+
cav+/-
cav-/-
C.
[^{3}H]-cholesterol
(cpm/mg cell protein)
20
15
10
5
0
WT
cav-/+
cav-/-

Figure 14. **Caveolin-1 deficient hepatocytes have decreased HDL mediated cholesterol efflux.** HDL mediated efflux of sterol synthesized from [^{3}H]mevalonate (A), and exogenously added cholesterol (B) is significantly decreased in the absence of caveolin-1. Cells were incubated with 10 μCi/ml of [^{3}H]mevalonate or 5 μCi/ml of [^{3}H]cholesterol-LDL in Hepatozyme for 40 h. After a 1 h equilibration, 50 μg/ml of HDL was added for 5 h. Media was incubated with anti human apoA-I antibody and precipitated with Sepharose G beads. Each bar is the average percent efflux +/- S.D. (n =3). The difference in efflux was significant compared to wild type hepatocytes (** = $P<0.005$, * = $P<0.05$).

Fig. 14.

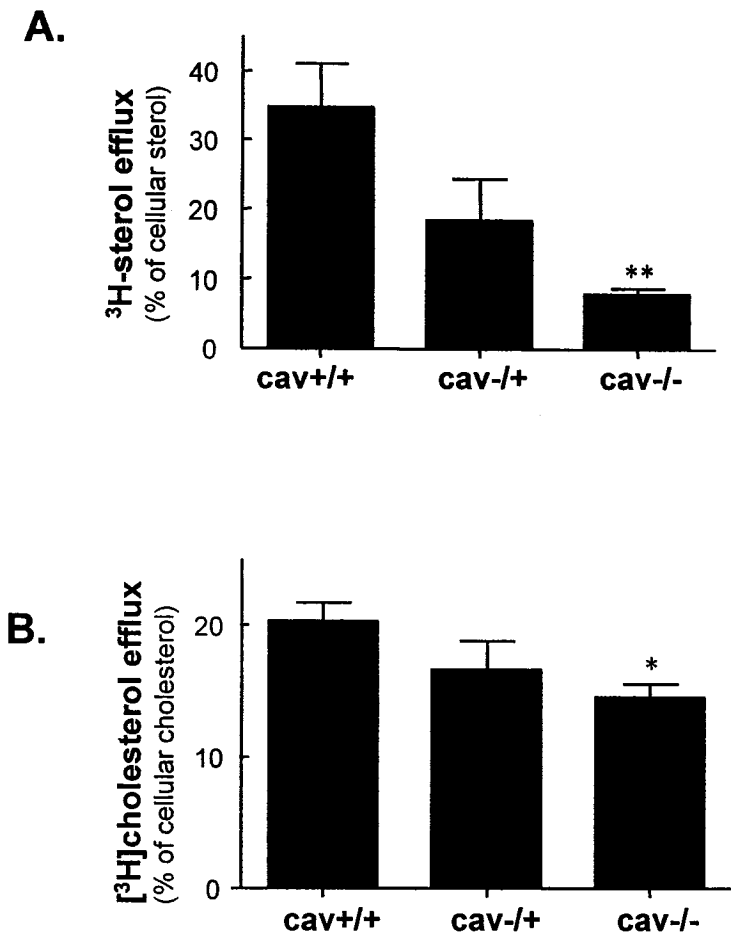

A.
^{3}H-sterol efflux
(% of cellular sterol)
40
30
20
10
0
**
cav+/+
cav-/+
cav-/-
B.
[^{3}H]cholesterol efflux
(% of cellular cholesterol)
20
10
0
*
cav+/+
cav-/+
cav-/-

Figure 15. **Caveolin-1 deficient hepatocytes have decreased apoA-I mediated efflux.** ApoA-I mediated efflux of phospholipid synthesized from [^{3}H]choline (A), sterol synthesized from [^{3}H]mevalonate (B), and exogenously added cholesterol (C) is significantly decreased in the caveolin-1 deficient hepatocytes compared to wild-type hepatocytes. Cells were incubated with 5 μCi/ml of [^{3}H]choline, 10 μCi/ml of [^{3}H]mevalonate or 5 μCi/ml of [^{3}H]cholesterol-LDL in Hepatozyme for 40 h. After a 1 h equilibration, 15μg/ml of apoA-I was added for 5 h. Media was incubated with anti human apoA-I antibody and precipitated with Sepharose G beads. Each bar is the average percent efflux +/- S.D. (n = 4). The difference in efflux was significant compared to wild type hepatocytes (* = $P<0.05$, ** = $P<0.005$, *** = $P<0.0001$).

Fig. 15.

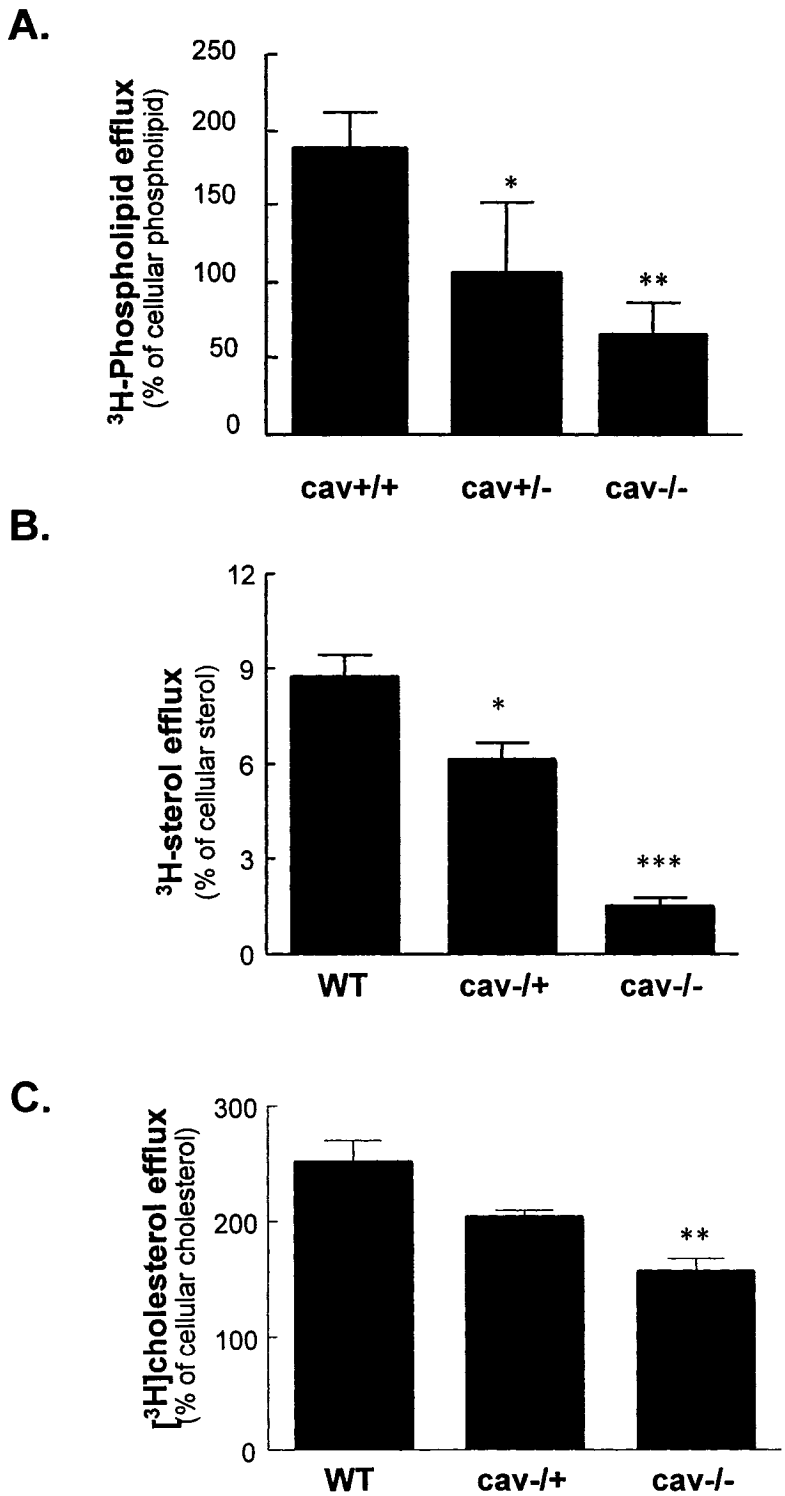

A.
^{3}H-Phospholipid efflux
(% of cellular phospholipid)
250
200
150
100
50
0
*
**
cav+/+
cav+/-
cav-/-
B.
^{3}H-sterol efflux
(% of cellular sterol)
12
9
6
3
0
*
***
WT
cav-/+
cav-/-
C.
[^{3}H]cholesterol efflux
(% of cellular cholesterol)
300
200
100
0
**
WT
cav-/+
cav-/-

Figure 16. **Caveolin-1 deficient hepatocytes have decreased cholesterol and phospholipid lipidation of endogenously synthesized apoA-I.** (A) Phospholipid secretion with apoA-I is abolished in the absence of caveolin-1. (B) Newly synthesized sterol secreted with apoA-I is decreased with the absence of caveolin-1. (C) Exogenously added cholesterol secreted with apoA-I is decreased with the absence of caveolin-1. Cells were incubated with [^{3}H]choline (A), [^{3}H]mevalonate (B) or [^{3}H]cholesterol-LDL (C) in Hepatozyme for 40 h, and infected with 75moi of Ad5-apoA-I or Ad5-Luc 18 h prior to efflux. After a 1 h equilibration, Media was added for 5 h. Media containing the secreted human apoA-I was incubated with anti human apoA-I antibody and precipitated with Sepharose G beads. Each bar is the average percent efflux +/- S.D. (n = 3). The difference in efflux was significant compared to wild type hepatocytes (* = $P<0.005$, ** = $P<0.0001$).

Fig. 16.

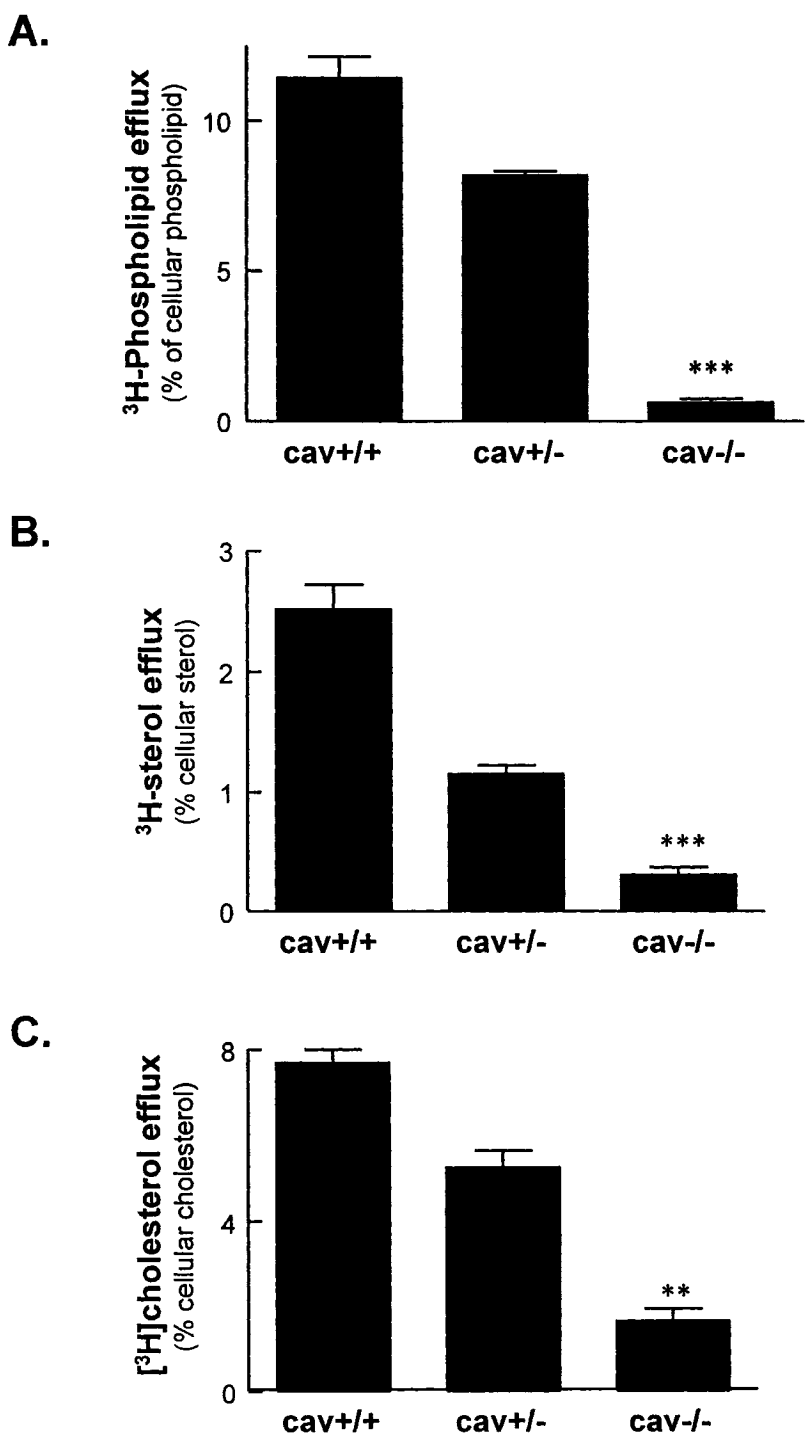
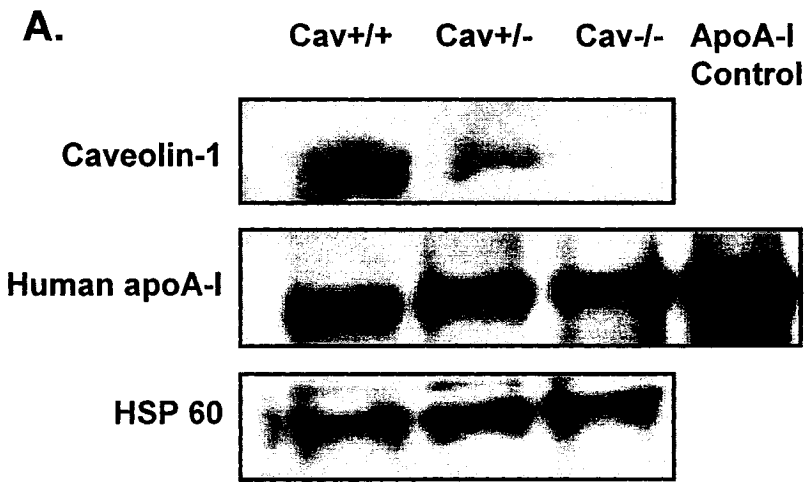
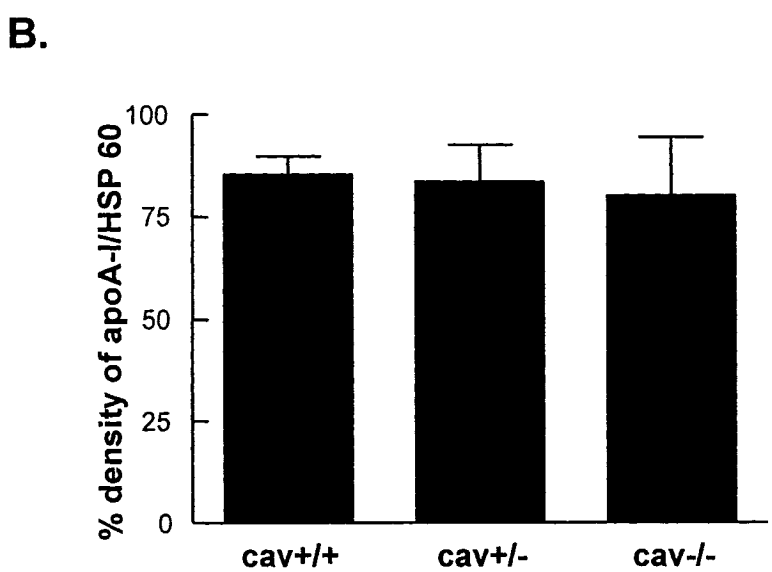

A.
^{3}H-Phospholipid efflux
(% of cellular phospholipid)
10
5
0
***
cav+/+
cav+/-
cav-/-
B.
^{3}H-sterol efflux
(% cellular sterol)
3
2
1
0
***
cav+/+
cav+/-
cav-/-
C.
[^{3}H]cholesterol efflux
(% cellular cholesterol)
8
4
0
**
cav+/+
cav+/-
cav-/-

Figure 17. Expression of caveolin-1 and human apoA-I in primary hepatocytes shows that caveolin-1 deficiency does not effect adenovirus infection. A. Primary hepatocytes were harvested from wild type (cav+/+), heterozygous (cav+/-), and knockout (cav-/-) mice, and infected with 75 moi Ad5-apoA-I for 18 h. Cells were collected in loading buffer, and loaded on a 12% Novex gel. The samples were transferred to nitrocellulose and probed for caveolin-1, human apoA-I and HSP 60. B. Relative expression of apoA-I detected compared to HSP 60.

Fig. 17.


A.
Cav+/+
Cav+/-
Cav-/-
ApoA-I Control
Caveolin-1
Human apoA-I
HSP 60


B.
% density of apoA-I/HSP 60
100
75
50
25
0
cav+/+
cav+/-
cav-/-

Chapter Four: Discussion

We used three hepatocyte models to examine the role of caveolin-1 in cholesterol efflux and synthesis. Two cell models overexpressed caveolin-1, McA-RH7777 cells were stably transfected with caveolin-1, and HepG2 cells were infected with adenovirus caveolin-1. Primary hepatocytes deficient in caveolin-1 provided the third cell model.

Several McA-RH7777 cell lines stably transfected with caveolin-1 have been developed in our lab by Philip Links. We used three of these cell lines: the MH cell line was developed by transfecting cells with the plasmid containing no caveolin-1, to act as a control. The M3-31 and M3-35 cell lines were transfected with the caveolin-1 vector, and expressed intermediate and high levels of caveolin-1, respectively.

The original purpose of this cell model was to study the influence of caveolin-1 on efflux, but we found that the influence of caveolin-1 on cholesterol efflux to HDL was limited. This may be due to adaptation of the cells to high caveolin-1 expression to preserve cholesterol homeostasis. While this may be due to the few caveolae presented on the cell surface of the hepatoma cells, Fu et. al (105) found cholesterol efflux did increase with caveolin-1 expression, and presented a comparable number of caveolae.

Cells may adapt quickly to inbalances in cellular cholesterol, whether as a change in the net content or as a result of transfer to a specific pool. This would minimize changes to efflux. Therefore, another system was obtained to evaluate the effect of inducible caveolin-1 expression in cholesterol efflux to HDL. Lisanti et. al (175) developed a caveolin-1 adenovirus vector where expression was inhibited through tetracycline. This system allowed us to express caveolin-1 for short periods of time, and keep the cells from adapting to changes to cholesterol homeostasis. The expression times

we used for caveolin-1 expression coincide with labelling the cells with cholesterol (24 h), equilibration of the labelled cholesterol (7 h), and the start of the efflux period (3 h).

Originally we intended to use this system in McA-RH7777 cells. However, two different adenovirus vectors were necessary for this system, (Ad5-caveolin-1 and Ad5-tetracycline transactivator), and while the McA-RH7777 cells were still viable they were not able to remain attached during the subsequent washing necessary in an efflux experiment. Therefore, we used another hepatoma cell line. We did not detect caveolin-1 in the HepG2 cell line, (Fig. 7B), in agreement with Fujimoto et. al, who found no caveolin-1 in HepG2 cells (54) . Infection of the HepG2 cells with Ad5-caveolin-1 and Ad5-tTA was effective and did not affect cell viability or the attachment of the cells to the plate. Exposure of HepG2 cells for 24 h to the adenovirus system resulted in caveolin-1 expression similar to the high expression in McA-RH7777 cells. Tetracycline could effectively inhibit caveolin-1 expression, which allowed us to induce caveolin-1 expression independently of adenoviral infection.

In both the McA-RH7777 and HepG2 cell models, there was a consistent increase of cholesterol synthesis. The increase in efflux of exogenously added cholesterol to HDL was small in both cells systems in the presence of caveolin-1. The increase in efflux only started to become significant at 6 h.

We had the opportunity to test these observations from a different angle. We used hepatocytes from caveolin-1 deficient mice obtained from the lab of Dr. Lisanti. We wanted to determine if the removal of caveolin-1 from hepatocytes would result in the opposite effect to that seen in caveolin-1 overexpressing cells. In hepatocytes, caveolin-1 expression is low, and there are few caveolae (107). This system allowed us to observe

the effect of physiological levels of caveolin-1 in primary hepatocytes compared to no caveolin-1. We were able to examine cholesterol and phospholipid synthesis, and the efflux of LDL-derived cholesterol and newly synthesized cholesterol to HDL and apoA-I.

1. Caveolae Formation

The necessary components for caveolae formation are cholesterol, sphingolipids and caveolin (93;180;181). Caveolin-1 expression is sufficient to induce caveolae formation (100). We wanted to ensure that caveolin-1 overexpression resulted in functional caveolin-1 that could move to the PM. Fu et. al (105) found that stable transfection of HepG2 cells with caveolin-1 could induce caveolae formation. In the McA-RH7777 cell model, we fixed the MH, M3-31 and M3-35 cells and identified caveolae by electron microscopy. We found no caveolae in the MH cells (Fig. 3A), while in the M3-31 cells, approximately 1 caveola per cell could be seen (Fig. 3B). In the M3-35 cells, 1-2 caveolae were found per cell (Fig. 3C). To look for caveolae formation in the HepG2 cells, we exposed them to the caveolin-1 adenovirus 24 h before fixing. We found 2-4 caveolae per cell and no caveolae were seen in untransfected HepG2 cells (Fig. 8).

The caveolae found in the HepG2 cells and McA-RH7777 cells were not reflective of cells expressing high levels of caveolin-1 such as fibroblasts, endothelial cells and adipocytes (43), but Fu et al (105) also found that caveolae formed in low numbers in their stably transfected HepG2 cells. They also found that cholesterol efflux to plasma and apoA-I increased in the presence of caveolin-1.

2. Cholesterol Synthesis

Caveolin-1 is known to be involved in cholesterol homeostasis, and conversely cholesterol regulates caveolin-1 synthesis (42) and localization (30). Here also we found that caveolin-1 influences the synthesis of cholesterol.

In McA-RH7777 cells M3-31, cholesterol synthesis increased by 30% compared to MH cells (Fig. 4). These caveolin-1 overexpressing cells were incubated with [^{3}H]acetate for 1 h. Similarly, when we incubated HepG2 cells with [^{3}H]acetate for 24 h after infection with caveolin-1 adenovirus for 1 h, we observed a 48% increase increase in cholesterol synthesis (Fig. 9). The same trend is found in both cell lines and with similar proportions. Fu et. al (105) found that cholesterol synthesis doubled in HepG2 cells stably transfected with caveolin-1. The difference between our results and these other results in HepG2 cells may be due to the method of overexpressing caveolin-1. Since we infected the cells with adenovirus shortly before labelling them with [^{3}H]acetate, at the beginning of the period of cholesterol synthesis, caveolin-1 expression would have been low (Fig. 10B), but the stably transfected HepG2 cells would have had a constant level of caveolin-1 expression. If this is the case, then we can conclude that the effect of caveolin-1 is greater in HepG2 cells than in McA-RH7777 cells. If the difference of cholesterol synthesis is due to the amount of caveolin-1 expression, then the increase of cholesterol synthesis is related to the levels of caveolin-1 in the cell.

We also incubated the primary hepatocytes with [^{3}H]mevalonate to directly examine the effects of caveolin-1 deficiency on cholesterol synthesis (Fig 13B). We found that cholesterol synthesis decreased in the absence of caveolin-1 by approximately 40%, a difference parallel to the results found with the overexpression of caveolin-1.

The observed impact of caveolin-1 on cholesterol synthesis suggests that caveolin-1 is important in the homeostasis of cholesterol in these cells. Fu et. al (105) observed the same influence of caveolin-1 on cholesterol synthesis in HepG2 cells. They also observed a 67% increase in cholesterol transport (105).

Smart et. al (131) observed that caveolin-1 could transport newly synthesized cholesterol directly from the ER to the PM. They also observed that cells lacking caveolin-1 could transport newly synthesized cholesterol, but at approximately one fourth of the control rate (131). Further work by Uittenbogaard et. al showed that caveolin-1 and cholesterol moved from the ER to the PM as part of a chaperone complex (84).

Because caveolin-1 increased the rate of cholesterol transport four fold (131), the resulting decrease of cholesterol in the ER would cause an increase in cholesterol synthesis. SREBP resides in the ER and regulates the synthesis of proteins involved in cholesterol homeostasis, such as HMG CoA Reductase (182). Future studies into caveolin-1 should include examining how caveolin-1 expression changes the expression of proteins involved in cholesterol homeostasis.

Sviridov et. al (183) observed an increase in transport of newly synthesized cholesterol to the PM in fibroblast cells as a result of stimulation by apoA-I. They suggested that the caveolin-1 chaperone complex responds to stimulation by apoA-I. To examine this possibility in the McA-RH7777 cell model, we incubated cells with apoA-I for 1 h, while cells were synthesizing cholesterol from [^{3}H]acetate (Fig. 4). We found no significant increase in cholesterol synthesis due to apoA-I. Our results indicate a connection between caveolin-1 transport of newly synthesized cholesterol and cholesterol synthesis, but caveolin-1 transport of cholesterol from the ER to the PM is probably not

stimulated by apoA-I in the McA-RH7777 cell system. Fibroblasts have many more caveolae than what we observed in the McA-RH7777 cells stably transfected with caveolin-1 (Fig. 3). This probably explains why apoA-I did not stimulate cholesterol transport, since less caveolin-1 was on the PM.

In McA-RH7777 cells we also examined the synthesis of sphingomyelin, phosphatidyl choline and cholesterol ester (Fig. 4B, C, D), but we found no significant change in the synthesis of any of these lipids. As well, in the caveolin-1 deficient hepatocytes, we found no change in phospholipid synthesis from [^{3}H]choline (Fig. 13A). The increase of synthesis is only evident for cholesterol, confirming the role caveolin-1 plays in cholesterol homeostasis.

3. Efflux of exogenously added and equilibrated cholesterol

The majority of cell cholesterol is on the PM (184). However, cholesterol is also located in the recycling endosomes and Golgi network (185), and synthesized in the ER (186), and lipid droplets contain cholesterol, mainly as cholesteryl esters (187). These can all be sources of cholesterol for efflux. In order to analyze the efflux from these different pools of cholesterol, specific labelling procedures need to be used. Various labs have already used [^{3}H]acetate and [^{3}H]mevalonate to study the pool of newly synthesized cholesterol (131;179;188), which also enables tracking of the transport of this newly synthesized cholesterol from the ER to the PM (80;84;131;179). Fielding et. al (130) reported that LDL-derived, unesterified cholesterol enters the cell via the clathrin coated pits and is found first colocalized with Golgi markers and then in caveolae. Hui Zheng and colleagues in our lab (188) used three methods to preferentially label specific pools of cholesterol in hepatocytes. The first method involved labelling the pool of newly

synthesized cholesterol by incubating with [^{3}H]mevalonate, a cholesterol precursor. In the second method, [^{3}H]cholesterol was pre-equilibrated with LDL or HDL to label the PM and recycling endosomes. Lastly, [^{3}H]cholesterol was delivered by mβCD to the PM at 4°C. Zheng et. al (188) used mβCD to bind cholesterol and determine the proportion of [^{3}H]cholesterol associated with the three different pools. At 4 °C mβCD removes [^{3}H]cholesterol from the PM exclusively, and at 37 °C mβCD removes [^{3}H]cholesterol from both the PM and the recycling endosomes. The radioactivity that is not accessible to mβCD at 37 °C was considered associated with intracellular pools (188).

In our study, we labelled two pools of cholesterol. We found that the uptake of cholesterol into these pools was not modified by overexpression or deficiency of caveolin-1 (Fig. 12C). To label intracellular compartments, we used newly synthesized cholesterol derived from [^{3}H]acetate or [^{3}H]mevalonate. The PM and recycling endosomes were labelled with exogenously added [^{3}H]cholesterol.

SR-BI is a receptor known to bind HDL, and has been shown to facilitate diffusion of cholesterol to HDL (116;117). Matveev et. al (122) demonstrated that when SR-BI and caveolin-1 are coexpressed, SR-BI is localized in caveolae, and Malerod et. al (189) reported SR-BI in the caveolae of several cell lines. Because hepatocytes have a high level of SR-BI, efflux to HDL is mainly mediated by SR-BI in these cells (189). Graf et. al (190) suggest that caveolin-1 plays an important role in SR-BI mediated efflux of cholesterol due to the localization of SR-BI in caveolae. Though caveolae presentation on the PM is low in the hepatoma cells, we wanted to look at the influence of caveolin-1

on diffusional efflux. We used HDL as a cholesterol acceptor to examine the influence of caveolin-1 on diffusional efflux.

With McA-RH7777 cells labelled at the PM and recycling endosomes, we observed a gradual increase in cholesterol efflux to HDL with increasing caveolin-1 expression (Fig. 4A). This diffusional cholesterol efflux was directly proportional to the concentration of caveolin-1, in agreement with previous work done in our lab by Philip Links.

Using cholesterol pre-equilibrated with LDL, we labelled the PM and recycling endosomes of cells overexpressing caveolin-1 with exogenous cholesterol. Then we used mβCD at 4 °C to remove cholesterol from the PM (Fig. 6A, 11). And we used mβCD at 37°C to remove cholesterol from the PM and recycling endosomes (Fig. 6B, 11). If caveolin-1 influenced the concentration of cholesterol at the PM, we would have expected to see higher levels of cholesterol removed from there at 4 °C. We did not. This suggests that there was no change in the amount of cholesterol located at the PM, and that partition of cholesterol between the PM and recycling endosomes was independent of the presence of caveolin-1. Fu et. al (105) also found that HepG2 cells stably transfected with caveolin-1 had no increase of mβCD accessible cholesterol. They did note that there was an increase of cholesterol accessible to cholesterol oxidase, and suggest that this is a pool of detergent insoluble membrane (105). We did not examine the accessibility of cholesterol to cholesterol oxidase in the caveolin-1 overexpressing cells, nor did we investigate the amount of cholesterol in detergent insoluble membranes.

Fujimoto et. al (54) suggested that caveolin-2 can stabilize caveolae. *In vivo* caveolin-1 and caveolin-2 are coexpressed (54). In order to see if caveolin-2 would influence the efflux of exogenous cholesterol, we used an adenoviral vector to transiently express caveolin-2 in the stable caveolin-1 overexpressing cell system (Fig. 7). Cells labelled with exogenously added cholesterol were incubated with HDL. Efflux of cholesterol was unchanged by caveolin-2 expression.

Because the McA-RH7777 cells were stably transfected, the cells may have adapted to caveolin-1 expression in order to keep cholesterol homeostasis. We decided to see if a transient expression of caveolin-1 by adenovirus infection would increase the effect of caveolin-1 on efflux of exogenously added cholesterol to HDL (Fig. 10A, B). By varying the infection time, caveolin-1 protein expression was induced 24, 7 and 3 h before the media was collected (Fig. 10A). We found no significant increase in efflux of the exogenously added cholesterol to HDL with any of the time points of protein induction (Fig. 10B). The effects of caveolin-1 on HDL mediated efflux gradual in the McA-RH7777 stably transfected cell line. Fu et. al (105) found that overexpression of caveolin-1 in HepG2 cells resulted in a 280% increase of efflux to human plasma, and a 45% increase of efflux to apoA-I. Both of these results are higher than what we observed in either of our caveolin-1 overexpressing cell models. The differences in cholesterol efflux between the two labs may be due to differences in experimental procedures, such as labelling, and the cholesterol acceptors. The difference of caveolin-1 expression levels may be a significant factor in the increase of cholesterol efflux.

To further confirm that caveolin-1 does stimulate HDL mediated efflux of exogenously added cholesterol, we used primary hepatocytes labelled with LDL-derived

cholesterol (Fig. 14B). We found that efflux to HDL was increased by 25% in the presence of caveolin-1. This confirmed our previous results with McA-RH7777 cells, that overexpression of caveolin-1 does moderately increase efflux of exogenously added cholesterol.

The colocalization of SR BI and caveolin-1 has led researchers to believe that caveolin-1 may facilitate SR BI mediated cholesterol efflux (190;191). Matveev et. al (191) reported that caveolin-1 enhances cholesterol efflux to HDL in THP-1 cells. Two other labs have shown that caveolin-1 does not regulate SR BI mediated cholesterol efflux in other cell lines (99;192). Despite the reported localization of SR-BI in caveolae, we observed only a small increase in cholesterol efflux to HDL, so we can conclude that caveolin-1 only plays a small role in SR-BI mediated efflux in hepatocytes.

ApoA-I, the primary protein component of HDL (26;193), and its interaction with hepatic ABCA1 results in the primary lipidation which is essential for HDL formation (194;195). Results from the ABCA1 deficient mice, and Tangier patients show that ABCA1 is necessary for the lipidation of apoA-I, and thus the formation of HDL (194). Efflux to HDL still occurs in the absence of ABCA1, but efflux to apoA-I does not (25). ABCA1 is highly expressed in hepatic cells. Zheng et. al (188) found that ABCA1 deficiency in hepatic cells results in significant decrease in efflux of exogenously added cholesterol. At later timepoints cholesterol efflux was ABCA1 dependent. We used apoA-I to look at ABCA1 dependent efflux in our three cell models.

Previous work by Philip Links in our lab with McA-RH7777 cells overexpressing caveolin-1 showed that apoA-I mediated efflux of exogenous cholesterol increased in response to caveolin-1 overexpression in the same manner as HDL mediated efflux. In

HepG2 cells, however, we found that there was no significant increase of apoA-I mediated efflux of exogenously added cholesterol (Fig. 10C). This also contradicted the results of Fu et. al (105) who reported that cholesterol efflux to apoA-I increased by 45% with the overexpression of caveolin-1. The differences between these systems of caveolin-1 overexpression and those of Links and colleagues and of Fu and colleagues are two-fold. First, this may reflect differences between cell lines. However, Fu et. al (105), also contradicted our results and reported that cholesterol efflux to apoA-I increased by 45% with the stable overexpression of caveolin-1 in HepG2 cells. The other possibility is that the different methods of caveolin-1 expression account for the inconsistency in efflux to apoA-I. The McA-RH7777 cells stably overexpressed caveolin-1, and the HepG2 cells studied by Fu et. al (105) also were stably transfected with caveolin-1, whereas our HepG2 cells were infected with Ad5-caveolin-1. This difference in either the method of caveolin-1 expression or the length of time caveolin-1 was expressed may account for the differences divergent effects of cholesterol efflux to apoA-I. The results from the knockout mouse were also consistent with the results from the caveolin-1 stable transfections.

In caveolin-1 deficient hepatocytes, the efflux of exogenously added cholesterol mediated by exogenously added apoA-I decreased by 36% in caveolin-1 deficient hepatocytes (Fig. 15C), and also the cholesterol efflux to endogenously synthesized apoA-I decreased by 73% in the absence of caveolin-1 (Fig. 16C).

As seen in the efflux to HDL, caveolin-1 had only a small increase on efflux to exogenous apoA-I. However, cholesterol lipidation of endogenously synthesized apoA-I is very low in the absence of caveolin-1. This suggests that apoA-I lipidation before

leaving the hepatocytes is critical, since efflux to exogenously added apoA-I was not affected as much. The ABCA1 is involved in the lipidation of endogenously synthesized apoA-I (188), but ABCA1 is not localized in caveolae. To determine how caveolin-1 may be involved in the intracellular lipidation of apoA-I more experiments are needed.

4. Efflux of Newly Synthesized Cholesterol

Work by Smart et. al (131) shows that newly synthesized cholesterol is transported from the ER directly to caveolae where it can be effluxed to HDL, or dispersed to the rest of the plasma membrane. Transport of newly synthesized cholesterol occurs in the absence of caveolin-1, but transport of this pool of cholesterol is 4-5 times faster in the presence of caveolin-1 (131). This means that this pool of newly synthesized cholesterol is implicated in intracellular transport of cholesterol as well as efflux.

In HepG2 cells caveolin-1 overexpression resulted in an increase of newly synthesized cholesterol efflux to HDL, but this did not reflect in efflux to apoA-I (Fig.10C). Efflux of newly synthesized cholesterol to HDL also decreased by 74% in caveolin-1 deficient hepatocytes compared to control cells (Fig. 16A). This large decrease of cholesterol efflux, which is significantly greater than the decrease in efflux of cell surface cholesterol, probably reflects a decrease in newly synthesized cholesterol transport to the cell surface.

We labelled MH and M3-35 cells with [^{3}H]mevalonate to study the efflux of newly synthesized cholesterol to apoA-I (Fig. 5B). We also immunoprecipitated apoA-I associated sterol with an anti-apoA-I antibody. Under these conditions apoA-I mediated efflux of newly synthesized cholesterol was significantly increased at 6 and 24 h, in

contrast to the results seen in McA-RH7777 cells. Since hepatocytes also secrete lipoproteins, the immunoprecipitation accurately reflects the efflux of cholesterol to apoA-I.

In caveolin-1 deficient hepatocytes, efflux of newly synthesized cholesterol to exogenously added apoA-I decreased by 83% (Fig. 15B), in agreement with the observations of Smart et. al (131) that intracellular transport of newly synthesized cholesterol is faster in the presence of caveolin-1.

Efflux of newly synthesized cholesterol to endogenously synthesized apoA-I decreased by approximately 90% (Fig. 16B). This may also be explained by the decrease of intracellular cholesterol transport which results in a decrease of newly synthesized cholesterol at the PM.

5. Phospholipid Efflux

To further examine the possible effects of caveolin-1 on ABCA1 efflux, we looked at efflux of phospholipid to exogenously added apoA-I and endogenously synthesized apoA-I. Zheng et. al (188) had found that phospholipid efflux to exogenous apoA-I was decreased 80% in ABCA1 deficient hepatocytes. No significant defect in phospholipid metabolism was observed in caveolin-1 null mice (Fig 13A). To our surprise, phospholipid efflux to exogenously added apoA-I decreased by 68% (Fig. 15A). This was not as high as efflux of newly synthesized cholesterol to exogenously added apoA-I or HDL, but still very significant. This decrease in phospholipid efflux to apoA-I would suggest a relationship between ABCA1 and caveolin-1, for which there is no evidence in the literature.

We also found that phospholipid efflux to endogenously synthesized apoA-I decreased by over 90% in the absence of caveolin-1 (Fig. 16A). These unexpected results made us question the effectiveness of our adenoviral vector infection and expression in the caveolin-1 deficient hepatocytes, and we collected the samples of cell lysate and performed a western blot for human apoA-I (Fig. 17). ApoA-I expression was the same in all of the hepatocytes. Frank et. al (107) found that overexpression of caveolin-1 in mouse hepatocytes increased the secretion of apoA-I. When fed a western diet, caveolin-1 deficient mice on an apoE deficient background have low apoA-I secretion(4).

The dramatic changes we found in phospholipid efflux do not reflect the circulating levels of HDL repoted by Razani et. al (171) in the caveolin-1 null mice. They only observed a small decrease in plasma HDL. The small change in efflux of exogenously added cholesterol is more reflective of the influence of caveolin-1 on efflux in these cells.

ABCA1 is a primary component involved in apoA-I phospholipidation, and the importance of this step in HDL metabolism is seen in Tangiers patients (196) and the ABCA1 deficient mice (197). In ABCA1 null mice plasma apoA-I was very low and HDL was almost nonexistent (197). Since plasma levels of HDL are almost unchanged in the caveolin-1 knockout mouse (171), we conclude that caveolin-1 does not play a significant role in the phospholipidation of apoA-I by ABCA1.

6. Conclusion

My hypothesis was that caveolin-1 would influences cholesterol metabolism in hepatic cells. Effectively, whether caveolin-1 was overexpressed or deficient in the hepatic cells used, cholesterol synthesis and efflux were altered.

In agreement with other investigators, we found that overexpression of caveolin-1 resulted in caveolae formation, and an increase in intracellular cholesterol transport. This demonstrated that our cell models all were appropriate to examine the role of caveolin-1 in cholesterol homeostasis.

Cholesterol synthesis was consistently affected by caveolin-1 in the three cell models in response to intracellular cholesterol transport. The mechanism for this response should be studied in the future to understand the relationship between cholesterol synthesis and intracellular transport. Cholesterol synthesis is tightly regulated, and sensors in the ER detect changes in cholesterol concentration. Transport of cholesterol from the ER mediated by caveolin-1 would decrease the regulatory pool of cholesterol transport and increase cholesterol synthesis to maintain the cholesterol concentration in the ER. Fu et. al (105) also observed an increase in cholesterol transport in the presence of caveolin-1. An increase in cholesterol efflux would also increase requirement for cholesterol synthesis since cholesterol uptake did not increase.
The efflux of newly synthesized cholesterol in caveolin-1 deficient hepatocytes also effectively demonstrated the importance of caveolin-1 in intracellular transport, and confirmed observations by Smart et. al (131).

The efflux of cholesterol to HDL is the initial step in the reverse cholesterol transport process (9). This process is thought to be one of the key elements of the

atheroprotective nature of HDL (198). Studies with ABCA1 deficient cells demonstrate the influence of efflux on circulating levels of HDL (199;200). We found that the influence of caveolin-1 on efflux to HDL was small, which agrees with observations that HDL levels are largely unaffected by caveolin-1 deficiency (171). The efflux of cholesterol to apoA-I was also not significantly affected by caveolin-1 expression, with the exception of lipidation of endogenously synthesized apoA-I. The interpretation of this finding will require other experiments.

Caveolin-2 does not change the efflux of cholesterol inspite of its role of stabilizing caveolae (54).

In conclusion, we have found that caveolin-1 consistently increases cholesterol synthesis through its effect on cholesterol transport from ER to plasma membrane. Stable or transient transfections of caveolin-1 have little effect on cholesterol efflux, however cells derived from caveolin-1 deficient mice display decreased efflux of newly synthesized lipids. This effect remains to be elucidated. Therefore, caveolin-1 is mainly involved in cellular cholesterol transport. This narrows the focus of caveolin-1 research to a particular process in cholesterol metabolism, namely its intracellular transport between the ER and the plasma membrane.

References

1. Tracy,R.P. 2003. Thrombin, inflammation, and cardiovascular disease: an epidemiologic perspective. *Chest* 124:49S-57S.

2. Riccioni,G., De Santis,A., Cerasa,V., Menna,V., Di Ilio,C., Schiavone,C., Ballone,E., and D'Orazio,N. 2003. Atherosclerotic plaque formation and risk factors. *Int.J Immunopathol.Pharmacol.* 16:25-31.

3. Castelli,W.P. 1984. Epidemiology of coronary heart disease: the Framingham study. *Am.J Med.* 76:4-12.

4. Frank,P.G., Lee,H., Park,D.S., Tandon,N.N., Scherer,P.E., and Lisanti,M.P. 2004. Genetic ablation of caveolin-1 confers protection against atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.* 24:98-105.

5. Ross,R. 1999. Atherosclerosis is an inflammatory disease. *Am.Heart J* 138:S419-S420.

6. Lusis,A.J. 2000. Atherosclerosis. *Nature* 407:233-241.

7. Glass,C.K. and Witztum,J.L. 2001. Atherosclerosis. the road ahead. *Cell* 104:503-516.

8. Frank,P.G. and Lisanti,M.P. 2004. Caveolin-1 and caveolae in atherosclerosis: differential roles in fatty streak formation and neointimal hyperplasia. *Curr.Opin.Lipidol.* 15:523-529.

9. Barter,P., Kastelein,J., Nunn,A., and Hobbs,R. 2003. High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions. *Atherosclerosis* 168:195-211.

10. Rader,D.J. 2002. High-density lipoproteins and atherosclerosis. *Am.J Cardiol.* 90:62i-70i.

11. Rader,D.J. 2003. Regulation of reverse cholesterol transport and clinical implications. *Am.J Cardiol.* 92:42J-49J.

12. Nofer,J.R., Fobker,M., Hobbel,G., Voss,R., Wolinska,I., Tepel,M., Zidek,W., Junker,R., Seedorf,U., von Eckardstein,A. *et al.* 2000. Activation of phosphatidylinositol-specific phospholipase C by HDL-associated lysosphingolipid. Involvement in mitogenesis but not in cholesterol efflux. *Biochemistry* 39:15199-15207.

13. Kingsbury,K.J. and Bondy,G. 2003. Understanding the essentials of blood lipid metabolism. *Prog.Cardiovasc.Nurs.* 18:13-18.

14. Ginsberg,H.N. 1998. Lipoprotein physiology. *Endocrinol.Metab Clin.North Am.* 27:503-519.

15. Segrest,J.P., Garber,D.W., Brouillette,C.G., Harvey,S.C., and Anantharamaiah,G.M. 1994. The amphipathic alpha helix: a multifunctional structural motif in plasma apolipoproteins. *Adv.Protein Chem.* 45:303-369.

16. Hussain,M.M., Kancha,R.K., Zhou,Z., Luchoomun,J., Zu,H., and Bakillah,A. 1996. Chylomicron assembly and catabolism: role of apolipoproteins and receptors. *Biochim.Biophys.Acta* 1300:151-170.

17. Mahley,R.W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 240:622-630.

18. Packard,C., Caslake,M., and Shepherd,J. 2000. The role of small, dense low density lipoprotein (LDL): a new look. *Int.J Cardiol.* 74 Suppl 1:S17-S22.

19. Packard,C.J., Demant,T., Stewart,J.P., Bedford,D., Caslake,M.J., Schwertfeger,G., Bedynek,A., Shepherd,J., and Seidel,D. 2000. Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions. *J Lipid Res.* 41:305-318.

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

21. Anderson,R.G., Goldstein,J.L., and Brown,M.S. 1976. Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc.Natl.Acad.Sci.U.S.A* 73:2434-2438.

22. von Eckardstein,A., Nofer,J.R., and Assmann,G. 2001. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arterioscler.Thromb.Vasc.Biol.* 21:13-27.

23. von Eckardstein,A. and Assmann,G. 2000. Prevention of coronary heart disease by raising high-density lipoprotein cholesterol? *Curr.Opin.Lipidol.* 11:627-637.

24. Shepherd,J. 1994. Lipoprotein metabolism. An overview. *Drugs* 47 Suppl 2:1-10.

25. Fielding,P.E., Nagao,K., Hakamata,H., Chimini,G., and Fielding,C.J. 2000. A two-step mechanism for free cholesterol and phospholipid efflux from human vascular cells to apolipoprotein A-1. *Biochemistry* 39:14113-14120.

26. Tall,A.R. 1990. Plasma high density lipoproteins. Metabolism and relationship to atherogenesis. *J Clin.Invest* 86:379-384.

27. Palade, GE. Fine Structure of Blood Capillaries. J Appl Physiol 24, 1424-1436. 1953.

28. YAMADA,E. 1955. The fine structure of the gall bladder epithelium of the mouse. *J.Biophys.Biochem.Cytol.* 1:445-458.

29. Glenney,J.R., Jr. and Zokas,L. 1989. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. *J.Cell Biol.* 108:2401-2408.

30. Rothberg,K.G., Heuser,J.E., Donzell,W.C., Ying,Y.S., Glenney,J.R., and Anderson,R.G. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell* 68:673-682.

31. Kurzchalia,T.V., Dupree,P., Parton,R.G., Kellner,R., Virta,H., Lehnert,M., and Simons,K. 1992. VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. *J.Cell Biol.* 118:1003-1014.

32. Glenney,J.R., Jr. 1992. The sequence of human caveolin reveals identity with VIP21, a component of transport vesicles. *FEBS Lett.* 314:45-48.

33. Glenney,J.R., Jr. and Soppet,D. 1992. Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts. *Proc.Natl.Acad.Sci.U.S.A* 89:10517-10521.

34. Scherer,P.E., Okamoto,T., Chun,M., Nishimoto,I., Lodish,H.F., and Lisanti,M.P. 1996. Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. *Proc.Natl.Acad.Sci.U.S.A* 93:131-135.

35. Tang,Z., Scherer,P.E., Okamoto,T., Song,K., Chu,C., Kohtz,D.S., Nishimoto,I., Lodish,H.F., and Lisanti,M.P. 1996. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J.Biol.Chem.* 271:2255-2261.

36. Engelman,J.A., Zhang,X.L., and Lisanti,M.P. 1998. Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known fragile site (FRA7G) that is frequently deleted in human cancers. *FEBS Lett.* 436:403-410.

37. Engelman,J.A., Zhang,X.L., and Lisanti,M.P. 1999. Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1). Methylation of a CpG island in the 5' promoter region of the caveolin-1 gene in human breast cancer cell lines. *FEBS Lett.* 448:221-230.

38. Hurlstone,A.F., Reid,G., Reeves,J.R., Fraser,J., Strathdee,G., Rahilly,M., Parkinson,E.K., and Black,D.M. 1999. Analysis of the CAVEOLIN-1 gene at human chromosome 7q31.1 in primary tumours and tumour-derived cell lines. *Oncogene* 18:1881-1890.

39. Sotgia,F., Minetti,C., and Lisanti,M.P. 1999. Localization of the human caveolin-3 gene to the D3S18/D3S4163/D3S4539 locus (3p25), in close proximity to the human oxytocin receptor gene. Identification of the caveolin-3 gene as a candidate for deletion in 3p-syndrome. *FEBS Lett.* 452:177-180.

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

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

42. Bist,A., Fielding,P.E., and Fielding,C.J. 1997. Two sterol regulatory element-like sequences mediate up-regulation of caveolin gene transcription in response to low density lipoprotein free cholesterol. *Proc.Natl.Acad.Sci.U.S.A* 94:10693-10698.

43. Fra,A.M., Pasqualetto,E., Mancini,M., and Sitia,R. 2000. Genomic organization and transcriptional analysis of the human genes coding for caveolin-1 and caveolin-2. *Gene* 243:75-83.

44. Timme,T.L., Goltsov,A., Tahir,S., Li,L., Wang,J., Ren,C., Johnston,R.N., and Thompson,T.C. 2000. Caveolin-1 is regulated by c-myc and suppresses c-myc-induced apoptosis. *Oncogene* 19:3256-3265.

45. Razani,B., Altschuler,Y., Zhu,L., Pestell,R.G., Mostov,K.E., and Lisanti,M.P. 2000. Caveolin-1 expression is down-regulated in cells transformed by the human papilloma virus in a p53-dependent manner. Replacement of caveolin-1 expression suppresses HPV-mediated cell transformation. *Biochemistry* 39:13916-13924.

46. Galbiati,F., Volonte,D., Engelman,J.A., Watanabe,G., Burk,R., Pestell,R.G., and Lisanti,M.P. 1998. Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J.* 17:6633-6648.

47. Zenklusen,J.C., Bieche,I., Lidereau,R., and Conti,C.J. 1994. (C-A)n microsatellite repeat D7S522 is the most commonly deleted region in human primary breast cancer. *Proc.Natl.Acad.Sci.U.S.A* 91:12155-12158.

48. Zenklusen,J.C., Thompson,J.C., Troncoso,P., Kagan,J., and Conti,C.J. 1994. Loss of heterozygosity in human primary prostate carcinomas: a possible tumor suppressor gene at 7q31.1. *Cancer Res.* 54:6370-6373.

49. Jenkins,R.B., Qian,J., Lee,H.K., Huang,H., Hirasawa,K., Bostwick,D.G., Proffitt,J., Wilber,K., Lieber,M.M., Liu,W. *et al.* 1998. A molecular cytogenetic analysis of 7q31 in prostate cancer. *Cancer Res.* 58:759-766.

50. Zenklusen,J.C., Thompson,J.C., Klein-Szanto,A.J., and Conti,C.J. 1995. Frequent loss of heterozygosity in human primary squamous cell and colon carcinomas at

7q31.1: evidence for a broad range tumor suppressor gene. *Cancer Res.* 55:1347-1350.

51. Zenklusen,J.C., Weitzel,J.N., Ball,H.G., and Conti,C.J. 1995. Allelic loss at 7q31.1 in human primary ovarian carcinomas suggests the existence of a tumor suppressor gene. *Oncogene* 11:359-363.

52. Shridhar,V., Sun,Q.C., Miller,O.J., Kalemkerian,G.P., Petros,J., and Smith,D.I. 1997. Loss of heterozygosity on the long arm of human chromosome 7 in sporadic renal cell carcinomas. *Oncogene* 15:2727-2733.

53. Scherer,P.E., Tang,Z., Chun,M., Sargiacomo,M., Lodish,H.F., and Lisanti,M.P. 1995. Caveolin isoforms differ in their N-terminal protein sequence and subcellular distribution. Identification and epitope mapping of an isoform-specific monoclonal antibody probe. *J.Biol.Chem.* 270:16395-16401.

54. Fujimoto,T., Kogo,H., Nomura,R., and Une,T. 2000. Isoforms of caveolin-1 and caveolar structure. *J.Cell Sci.* 113 Pt 19:3509-3517.

55. Galbiati,F., Volonte,D., Gil,O., Zanazzi,G., Salzer,J.L., Sargiacomo,M., Scherer,P.E., Engelman,J.A., Schlegel,A., Parenti,M. *et al.* 1998. Expression of caveolin-1 and -2 in differentiating PC12 cells and dorsal root ganglion neurons: caveolin-2 is up-regulated in response to cell injury. *Proc.Natl.Acad.Sci.U.S.A* 95:10257-10262.

56. Ueda,Y., Gong,E., Royer,L., Cooper,P.N., Francone,O.L., and Rubin,E.M. 2000. Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics. *J Biol.Chem.* 275:20368-20373.

57. Scherer,P.E., Lisanti,M.P., Baldini,G., Sargiacomo,M., Mastick,C.C., and Lodish,H.F. 1994. Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J.Cell Biol.* 127:1233-1243.

58. Scherer,P.E., Lewis,R.Y., Volonte,D., Engelman,J.A., Galbiati,F., Couet,J., Kohtz,D.S., van Donselaar,E., Peters,P., and Lisanti,M.P. 1997. Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *J.Biol.Chem.* 272:29337-29346.

59. Song,K.S., Scherer,P.E., Tang,Z., Okamoto,T., Li,S., Chafel,M., Chu,C., Kohtz,D.S., and Lisanti,M.P. 1996. Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J.Biol.Chem.* 271:15160-15165.

60. Parton,R.G. 1996. Caveolae and caveolins. *Curr.Opin.Cell Biol.* 8:542-548.

61. Razani,B., Woodman,S.E., and Lisanti,M.P. 2002. Caveolae: from cell biology to animal physiology. *Pharmacol.Rev.* 54:431-467.

62. Machleidt,T., Li,W.P., Liu,P., and Anderson,R.G. 2000. Multiple domains in caveolin-1 control its intracellular traffic. *J.Cell Biol.* 148:17-28.

63. Sargiacomo,M., Sudol,M., Tang,Z., and Lisanti,M.P. 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J.Cell Biol.* 122:789-807.

64. Dupree,P., Parton,R.G., Raposo,G., Kurzchalia,T.V., and Simons,K. 1993. Caveolae and sorting in the trans-Golgi network of epithelial cells. *EMBO J.* 12:1597-1605.

65. Dietzen,D.J., Hastings,W.R., and Lublin,D.M. 1995. Caveolin is palmitoylated on multiple cysteine residues. Palmitoylation is not necessary for localization of caveolin to caveolae. *J.Biol.Chem.* 270:6838-6842.

66. Li,S., Seitz,R., and Lisanti,M.P. 1996. Phosphorylation of caveolin by src tyrosine kinases. The alpha-isoform of caveolin is selectively phosphorylated by v-Src in vivo. *J.Biol.Chem.* 271:3863-3868.

67. Kurzchalia,T.V., Dupree,P., and Monier,S. 1994. VIP21-Caveolin, a protein of the trans-Golgi network and caveolae. *FEBS Lett.* 346:88-91.

68. Lisanti,M.P., Tang,Z., Scherer,P.E., Kubler,E., Koleske,A.J., and Sargiacomo,M. 1995. Caveolae, transmembrane signalling and cellular transformation. *Mol.Membr.Biol.* 12:121-124.

69. Schlegel,A., Schwab,R.B., Scherer,P.E., and Lisanti,M.P. 1999. A role for the caveolin scaffolding domain in mediating the membrane attachment of caveolin-1. The caveolin scaffolding domain is both necessary and sufficient for membrane binding in vitro. *J.Biol.Chem.* 274:22660-22667.

70. Schlegel,A. and Lisanti,M.P. 2000. A molecular dissection of caveolin-1 membrane attachment and oligomerization. Two separate regions of the caveolin-1 C-terminal domain mediate membrane binding and oligomer/oligomer interactions in vivo. *J.Biol.Chem.* 275:21605-21617.

71. Couet,J., Li,S., Okamoto,T., Ikezu,T., and Lisanti,M.P. 1997. Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *J.Biol.Chem.* 272:6525-6533.

72. Song,K.S., Tang,Z., Li,S., and Lisanti,M.P. 1997. Mutational analysis of the properties of caveolin-1. A novel role for the C-terminal domain in mediating homo-typic caveolin-caveolin interactions. *J.Biol.Chem.* 272:4398-4403.

73. Jagannadham,M.V., Sharadadevi,A., and Nagaraj,R. 2002. Effects of deleting a tripeptide sequence observed in muscular dystrophy patients on the conformation of

synthetic peptides corresponding to the scaffolding domain of caveolin-3. *Biochem.Biophys.Res.Commun.* 298:203-206.

74. Couet,J., Sargiacomo,M., and Lisanti,M.P. 1997. Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *J.Biol.Chem.* 272:30429-30438.

75. Li,S., Couet,J., and Lisanti,M.P. 1996. Src tyrosine kinases, G alpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J.Biol.Chem.* 271:29182-29190.

76. Oka,N., Yamamoto,M., Schwencke,C., Kawabe,J., Ebina,T., Ohno,S., Couet,J., Lisanti,M.P., and Ishikawa,Y. 1997. Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. *J Biol.Chem.* 272:33416-33421.

77. Monier,S., Parton,R.G., Vogel,F., Behlke,J., Henske,A., and Kurzchalia,T.V. 1995. VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. *Mol.Biol.Cell* 6:911-927.

78. Sargiacomo,M., Scherer,P.E., Tang,Z., Kubler,E., Song,K.S., Sanders,M.C., and Lisanti,M.P. 1995. Oligomeric structure of caveolin: implications for caveolae membrane organization. *Proc.Natl.Acad.Sci.U.S.A* 92:9407-9411.

79. Monier,S., Dietzen,D.J., Hastings,W.R., Lublin,D.M., and Kurzchalia,T.V. 1996. Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol. *FEBS Lett.* 388:143-149.

80. Uittenbogaard,A. and Smart,E.J. 2000. Palmitoylation of caveolin-1 is required for cholesterol binding, chaperone complex formation, and rapid transport of cholesterol to caveolae. *J.Biol.Chem.* 275:25595-25599.

81. Cao,H., Courchesne,W.E., and Mastick,C.C. 2002. A phosphotyrosine-dependent protein interaction screen reveals a role for phosphorylation of caveolin-1 on tyrosine 14: recruitment of C-terminal Src kinase. *J Biol.Chem.* 277:8771-8774.

82. Lisanti,M.P., Tang,Z.L., and Sargiacomo,M. 1993. Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae. *J Cell Biol.* 123:595-604.

83. Mora,R., Bonilha,V.L., Marmorstein,A., Scherer,P.E., Brown,D., Lisanti,M.P., and Rodriguez-Boulan,E. 1999. Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. *J.Biol.Chem.* 274:25708-25717.

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

85. Ito,J., Nagayasu,Y., Kato,K., Sato,R., and Yokoyama,S. 2002. Apolipoprotein A-I induces translocation of cholesterol, phospholipid, and caveolin-1 to cytosol in rat astrocytes. *J Biol.Chem.* 277:7929-7935.

86. Uittenbogaard,A., Everson,W.V., Matveev,S.V., and Smart,E.J. 2002. Cholesteryl ester is transported from caveolae to internal membranes as part of a caveolin-annexin II lipid-protein complex. *J.Biol.Chem.* 277:4925-4931.

87. Liu,P., Rudick,M., and Anderson,R.G. 2002. Multiple functions of caveolin-1. *J.Biol.Chem.* 277:41295-41298.

88. Nichols,B. 2003. Caveosomes and endocytosis of lipid rafts. *J.Cell Sci.* 116:4707-4714.

89. Parton,R.G., Way,M., Zorzi,N., and Stang,E. 1997. Caveolin-3 associates with developing T-tubules during muscle differentiation. *J.Cell Biol.* 136:137-154.

90. Uehara,K. and Miyoshi,M. 1999. Tubular invaginations with caveolae and coated pits in the sinus endothelial cells of the rat spleen. *Histochem.Cell Biol.* 112:351-358.

91. Vasile,E., Qu,H., Dvorak,H.F., and Dvorak,A.M. 1999. Caveolae and vesiculo-vacuolar organelles in bovine capillary endothelial cells cultured with VPF/VEGF on floating Matrigel-collagen gels. *J Histochem.Cytochem.* 47:159-167.

92. Kandror,K.V., Stephens,J.M., and Pilch,P.F. 1995. Expression and compartmentalization of caveolin in adipose cells: coordinate regulation with and structural segregation from GLUT4. *J Cell Biol.* 129:999-1006.

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

94. Brown,D.A. and London,E. 1997. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem.Biophys.Res.Commun.* 240:1-7.

95. Brown,D.A. and London,E. 1998. Structure and origin of ordered lipid domains in biological membranes. *J Membr.Biol.* 164:103-114.

96. Anderson,R.G. 1998. The caveolae membrane system. *Annu.Rev.Biochem.* 67:199-225.

97. Shaul,P.W. and Anderson,R.G. 1998. Role of plasmalemmal caveolae in signal transduction. *Am.J.Physiol* 275:L843-L851.

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

99. Frank,P.G., Marcel,Y.L., Connelly,M.A., Lublin,D.M., Franklin,V., Williams,D.L., and Lisanti,M.P. 2002. Stabilization of caveolin-1 by cellular cholesterol and scavenger receptor class B type I. *Biochemistry* 41:11931-11940.

100. Fra,A.M., Williamson,E., Simons,K., and Parton,R.G. 1995. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc.Natl.Acad.Sci.U.S.A* 92:8655-8659.

101. Engelman,J.A., Wykoff,C.C., Yasuhara,S., Song,K.S., Okamoto,T., and Lisanti,M.P. 1997. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J.Biol.Chem.* 272:16374-16381.

102. Thiele,C., Hannah,M.J., Fahrenholz,F., and Huttner,W.B. 2000. Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nat.Cell Biol.* 2:42-49.

103. Fielding,C.J., Bist,A., and Fielding,P.E. 1999. Intracellular cholesterol transport in synchronized human skin fibroblasts. *Biochemistry* 38:2506-2513.

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

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

106. Arakawa,R., Abe-Dohmae,S., Asai,M., Ito,J.I., and Yokoyama,S. 2000. Involvement of caveolin-1 in cholesterol enrichment of high density lipoprotein during its assembly by apolipoprotein and THP-1 cells. *J Lipid Res.* 41:1952-1962.

107. Frank,P.G., Pedraza,A., Cohen,D.E., and Lisanti,M.P. 2001. Adenovirus-mediated expression of caveolin-1 in mouse liver increases plasma high-density lipoprotein levels. *Biochemistry* 40:10892-10900.

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

109. Kawano,M., Miida,T., Fielding,C.J., and Fielding,P.E. 1993. Quantitation of pre beta-HDL-dependent and nonspecific components of the total efflux of cellular cholesterol and phospholipid. *Biochemistry* 32:5025-5028.

110. Fielding,C.J. and Fielding,P.E. 2001. Caveolae and intracellular trafficking of cholesterol. *Adv.Drug Deliv.Rev.* 49:251-264.

111. Gargalovic,P. and Dory,L. 2003. Caveolins and macrophage lipid metabolism. *J.Lipid Res.* 44:11-21.

112. Gargalovic,P. and Dory,L. 2001. Caveolin-1 and caveolin-2 expression in mouse macrophages. High density lipoprotein 3-stimulated secretion and a lack of significant subcellular co-localization. *J Biol.Chem.* 276:26164-26170.

113. Takahashi,Y. and Smith,J.D. 1999. Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. *Proc.Natl.Acad.Sci.U.S.A* 96:11358-11363.

114. Acton,S., Rigotti,A., Landschulz,K.T., Xu,S., Hobbs,H.H., and Krieger,M. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 271:518-520.

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

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

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

118. Temel,R.E., Trigatti,B., DeMattos,R.B., Azhar,S., Krieger,M., and Williams,D.L. 1997. Scavenger receptor class B, type I (SR-BI) is the major route for the delivery of high density lipoprotein cholesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. *Proc.Natl.Acad.Sci.U.S.A* 94:13600-13605.

119. Rigotti,A., Trigatti,B.L., Penman,M., Rayburn,H., Herz,J., and Krieger,M. 1997. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc.Natl.Acad.Sci.U.S.A* 94:12610-12615.

120. Webb,N.R., Connell,P.M., Graf,G.A., Smart,E.J., de Villiers,W.J., de Beer,F.C., and van der Westhuyzen,D.R. 1998. SR-BII, an isoform of the scavenger receptor

BI containing an alternate cytoplasmic tail, mediates lipid transfer between high density lipoprotein and cells. *J Biol.Chem.* 273:15241-15248.

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

122. Matveev,S., Uittenbogaard,A., van Der,W.D., and Smart,E.J. 2001. Caveolin-1 negatively regulates SR-BI mediated selective uptake of high-density lipoprotein-derived cholesteryl ester. *Eur.J.Biochem.* 268:5609-5616.

123. Blair,A., Shaul,P.W., Yuhanna,I.S., Conrad,P.A., and Smart,E.J. 1999. Oxidized low density lipoprotein displaces endothelial nitric-oxide synthase (eNOS) from plasmalemmal caveolae and impairs eNOS activation. *J Biol.Chem.* 274:32512-32519.

124. Uittenbogaard,A., Shaul,P.W., Yuhanna,I.S., Blair,A., and Smart,E.J. 2000. High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae. *J.Biol.Chem.* 275:11278-11283.

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

126. Walter,M., Gerdes,U., Seedorf,U., and Assmann,G. 1994. The high density lipopro. *Biochem.Biophys.Res.Commun.* 205:850-856.

127. Aoki,T., Nomura,R., and Fujimoto,T. 1999. Tyrosine phosphorylation of caveolin-1 in the endothelium. *Exp.Cell Res.* 253:629-636.

128. Rogler,G., Trumbach,B., Klima,B., Lackner,K.J., and Schmitz,G. 1995. HDL-mediated efflux of intracellular cholesterol is impaired in fibroblasts from Tangier disease patients. *Arterioscler.Thromb.Vasc.Biol.* 15:683-690.

129. Fielding,C.J. and Fielding,P.E. 1995. Role of an N-ethylmaleimide-sensitive factor in the selective cellular uptake of low-density lipoprotein free cholesterol. *Biochemistry* 34:14237-14244.

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

131. Smart,E.J., Ying,Y., Donzell,W.C., and Anderson,R.G. 1996. A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane. *J.Biol.Chem.* 271:29427-29435.

132. Fujimoto,T., Kogo,H., Ishiguro,K., Tauchi,K., and Nomura,R. 2001. Caveolin-2 is targeted to lipid droplets, a new "membrane domain" in the cell. *J.Cell Biol.* 152:1079-1085.

133. Ostermeyer,A.G., Paci,J.M., Zeng,Y., Lublin,D.M., Munro,S., and Brown,D.A. 2001. Accumulation of caveolin-1 in the endoplasmic reticulum redirects the protein to lipid storage droplets. *J.Cell Biol.* 152:1071-1078.

134. Pol,A., Luetterforst,R., Lindsay,M., Heino,S., Ikonen,E., and Parton,R.G. 2001. A caveolin dominant negative mutant associates with lipid bodies and induces intracellular cholesterol imbalance. *J.Cell Biol.* 152:1057-1070.

135. Servetnick,D.A., Brasaemle,D.L., Gruia-Gray,J., Kimmel,A.R., Wolff,J., and Londos,C. 1995. Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and Leydig cells. *J Biol.Chem.* 270:16970-16973.

136. Londos,C., Brasaemle,D.L., Gruia-Gray,J., Servetnick,D.A., Schultz,C.J., Levin,D.M., and Kimmel,A.R. 1995. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. *Biochem.Soc.Trans.* 23:611-615.

137. Blanchette-Mackie,E.J., Dwyer,N.K., Barber,T., Coxey,R.A., Takeda,T., Rondinone,C.M., Theodorakis,J.L., Greenberg,A.S., and Londos,C. 1995. Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J Lipid Res.* 36:1211-1226.

138. Brasaemle,D.L., Barber,T., Wolins,N.E., Serrero,G., Blanchette-Mackie,E.J., and Londos,C. 1997. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res.* 38:2249-2263.

139. Ostermeyer,A.G., Ramcharan,L.T., Zeng,Y., Lublin,D.M., and Brown,D.A. 2004. Role of the hydrophobic domain in targeting caveolin-1 to lipid droplets. *J.Cell Biol.* 164:69-78.

140. Pol,A., Martin,S., Fernandez,M.A., Ferguson,C., Carozzi,A., Luetterforst,R., Enrich,C., and Parton,R.G. 2004. Dynamic and regulated association of caveolin with lipid bodies: modulation of lipid body motility and function by a dominant negative mutant. *Mol.Biol.Cell* 15:99-110.

141. Conner,S.D. and Schmid,S.L. 2003. Regulated portals of entry into the cell. *Nature* 422:37-44.

142. Fishman,P.H. 1982. Internalization and degradation of cholera toxin by cultured cells: relationship to toxin action. *J Cell Biol.* 93:860-865.

143. Tran,D., Carpentier,J.L., Sawano,F., Gorden,P., and Orci,L. 1987. Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. *Proc.Natl.Acad.Sci.U.S.A* 84:7957-7961.

144. Schnitzer,J.E., Oh,P., Pinney,E., and Allard,J. 1994. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J.Cell Biol.* 127:1217-1232.

145. Le,P.U. and Nabi,I.R. 2003. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. *J Cell Sci.* 116:1059-1071.

146. Parton,R.G., Joggerst,B., and Simons,K. 1994. Regulated internalization of caveolae. *J.Cell Biol.* 127:1199-1215.

147. McIntosh,D.P., Tan,X.Y., Oh,P., and Schnitzer,J.E. 2002. Targeting endothelium and its dynamic caveolae for tissue-specific transcytosis in vivo: a pathway to overcome cell barriers to drug and gene delivery. *Proc.Natl.Acad.Sci.U.S.A* 99:1996-2001.

148. Nichols,B.J., Kenworthy,A.K., Polishchuk,R.S., Lodge,R., Roberts,T.H., Hirschberg,K., Phair,R.D., and Lippincott-Schwartz,J. 2001. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J Cell Biol.* 153:529-541.

149. Puri,V., Watanabe,R., Singh,R.D., Dominguez,M., Brown,J.C., Wheatley,C.L., Marks,D.L., and Pagano,R.E. 2001. Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J Cell Biol.* 154:535-547.

150. Lisanti,M.P., Scherer,P.E., Vidugiriene,J., Tang,Z., Hermanowski-Vosatka,A., Tu,Y.H., Cook,R.F., and Sargiacomo,M. 1994. Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J.Cell Biol.* 126:111-126.

151. Chun,M., Liyanage,U.K., Lisanti,M.P., and Lodish,H.F. 1994. Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc.Natl.Acad.Sci.U.S.A* 91:11728-11732.

152. Rybin,V.O., Xu,X., Lisanti,M.P., and Steinberg,S.F. 2000. Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J.Biol.Chem.* 275:41447-41457.

153. Yamamoto,M., Toya,Y., Schwencke,C., Lisanti,M.P., Myers,M.G., Jr., and Ishikawa,Y. 1998. Caveolin is an activator of insulin receptor signaling. *J.Biol.Chem.* 273:26962-26968.

154. Yamamoto,M., Toya,Y., Jensen,R.A., and Ishikawa,Y. 1999. Caveolin is an inhibitor of platelet-derived growth factor receptor signaling. *Exp.Cell Res.* 247:380-388.

155. Mineo,C., Gill,G.N., and Anderson,R.G. 1999. Regulated migration of epidermal growth factor receptor from caveolae. *J.Biol.Chem.* 274:30636-30643.

156. Garcia-Cardena,G., Fan,R., Stern,D.F., Liu,J., and Sessa,W.C. 1996. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J.Biol.Chem.* 271:27237-27240.

157. Ros-Baro,A., Lopez-Iglesias,C., Peiro,S., Bellido,D., Palacin,M., Zorzano,A., and Camps,M. 2001. Lipid rafts are required for GLUT4 internalization in adipose cells. *Proc.Natl.Acad.Sci.U.S.A* 98:12050-12055.

158. Ju,H., Venema,V.J., Liang,H., Harris,M.B., Zou,R., and Venema,R.C. 2000. Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular endothelial cells: localization of JAK/STAT signalling proteins in plasmalemmal caveolae. *Biochem.J.* 351:257-264.

159. Galbiati,F., Volonte,D., Brown,A.M., Weinstein,D.E., Ben Ze'ev,A., Pestell,R.G., and Lisanti,M.P. 2000. Caveolin-1 expression inhibits Wnt/beta-catenin/Lef-1 signaling by recruiting beta-catenin to caveolae membrane domains. *J.Biol.Chem.* 275:23368-23377.

160. Okamoto,T., Schlegel,A., Scherer,P.E., and Lisanti,M.P. 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J.Biol.Chem.* 273:5419-5422.

161. Liu,J., Garcia-Cardena,G., and Sessa,W.C. 1996. Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. *Biochemistry* 35:13277-13281.

162. Michel,J.B., Feron,O., Sacks,D., and Michel,T. 1997. Reciprocal regulation of endothelial nitric-oxide synthase by Ca2+-calmodulin and caveolin. *J.Biol.Chem.* 272:15583-15586.

163. Sowa,G., Pypaert,M., and Sessa,W.C. 2001. Distinction between signaling mechanisms in lipid rafts vs. caveolae. *Proc.Natl.Acad.Sci.U.S.A* 98:14072-14077.

164. Garcia-Cardena,G., Oh,P., Liu,J., Schnitzer,J.E., and Sessa,W.C. 1996. Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc.Natl.Acad.Sci.U.S.A* 93:6448-6453.

165. Drab,M., Verkade,P., Elger,M., Kasper,M., Lohn,M., Lauterbach,B., Menne,J., Lindschau,C., Mende,F., Luft,F.C. *et al.* 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 293:2449-2452.

166. Razani,B. and Lisanti,M.P. 2001. Caveolin-deficient mice: insights into caveolar function human disease. *J.Clin.Invest* 108:1553-1561.

167. Cohen,A.W., Park,D.S., Woodman,S.E., Williams,T.M., Chandra,M., Shirani,J., Pereira,d.S., Kitsis,R.N., Russell,R.G., Weiss,L.M. *et al.* 2003. Caveolin-1 null

mice develop cardiac hypertrophy with hyperactivation of p42/44 MAP kinase in cardiac fibroblasts. *Am.J Physiol Cell Physiol* 284:C457-C474.

168. Zhao,Y.Y., Liu,Y., Stan,R.V., Fan,L., Gu,Y., Dalton,N., Chu,P.H., Peterson,K., Ross,J., Jr., and Chien,K.R. 2002. Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. *Proc.Natl.Acad.Sci.U.S.A* 99:11375-11380.

169. Park,D.S., Cohen,A.W., Frank,P.G., Razani,B., Lee,H., Williams,T.M., Chandra,M., Shirani,J., De Souza,A.P., Tang,B. *et al.* 2003. Caveolin-1 null (-/-) mice show dramatic reductions in life span. *Biochemistry* 42:15124-15131.

170. Cohen,A.W., Razani,B., Schubert,W., Williams,T.M., Wang,X.B., Iyengar,P., Brasaemle,D.L., Scherer,P.E., and Lisanti,M.P. 2004. Role of caveolin-1 in the modulation of lipolysis and lipid droplet formation. *Diabetes* 53:1261-1270.

171. Razani,B., Combs,T.P., Wang,X.B., Frank,P.G., Park,D.S., Russell,R.G., Li,M., Tang,B., Jelicks,L.A., Scherer,P.E. *et al.* 2002. Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities. *J.Biol.Chem.* 277:8635-8647.

172. Razani,B., Engelman,J.A., Wang,X.B., Schubert,W., Zhang,X.L., Marks,C.B., Macaluso,F., Russell,R.G., Li,M., Pestell,R.G. *et al.* 2001. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J.Biol.Chem.* 276:38121-38138.

173. Huo,Y. and Ley,K. 2001. Adhesion molecules and atherogenesis. *Acta Physiol Scand.* 173:35-43.

174. Razani,B., Wang,X.B., Engelman,J.A., Battista,M., Lagaud,G., Zhang,X.L., Kneitz,B., Hou,H., Jr., Christ,G.J., Edelmann,W. *et al.* 2002. Caveolin-2-deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae. *Mol.Cell Biol.* 22:2329-2344.

175. Zhang,W., Razani,B., Altschuler,Y., Bouzahzah,B., Mostov,K.E., Pestell,R.G., and Lisanti,M.P. 2000. Caveolin-1 inhibits epidermal growth factor-stimulated lamellipod extension and cell migration in metastatic mammary adenocarcinoma cells (MTLn3). Transformation suppressor effects of adenovirus-mediated gene delivery of caveolin-1. *J Biol.Chem.* 275:20717-20725.

176. Sowa,G., Pypaert,M., Fulton,D., and Sessa,W.C. 2003. The phosphorylation of caveolin-2 on serines 23 and 36 modulates caveolin-1-dependent caveolae formation. *Proc.Natl.Acad.Sci.U.S.A* 100:6511-6516.

177. McManus,D.C., Scott,B.R., Frank,P.G., Franklin,V., Schultz,J.R., and Marcel,Y.L. 2000. Distinct central amphipathic alpha-helices in apolipoprotein A-I contribute to the in vivo maturation of high density lipoprotein by either activating lecithin-cholesterol acyltransferase or binding lipids. *J Biol.Chem.* 275:5043-5051.

178. Links, P. H. Caveolin-1 Expression and its Role in Cholesterol Efflux in Rat Hepatoma Cells. 2000. Faculty of Medicine, Department of Biochemistry, Microbiology and Immunology, University of Ottawa.
Ref Type: Thesis/Dissertation

179. Fielding,P.E., Russel,J.S., Spencer,T.A., Hakamata,H., Nagao,K., and Fielding,C.J. 2002. Sterol efflux to apolipoprotein A-I originates from caveolin-rich microdomains and potentiates PDGF-dependent protein kinase activity. *Biochemistry* 41:4929-4937.

180. Brown,D.A. and London,E. 1998. Functions of lipid rafts in biological membranes. *Annu.Rev.Cell Dev.Biol.* 14:111-136.

181. Brown,D.A. and London,E. 2000. Structure and function of sphingol. *J Biol.Chem.* 275:17221-17224.

182. Brown,M.S. and Goldstein,J.L. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89:331-340.

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

184. Lange,Y. and Ramos,B.V. 1983. Analysis of the distribution of cholesterol in the intact cell. *J Biol.Chem.* 258:15130-15134.

185. Bretscher,M.S. and Munro,S. 1993. Cholesterol and the Golgi apparatus. *Science* 261:1280-1281.

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

187. Brown,D.A. 2001. Lipid droplets: proteins floating on a pool of fat. *Curr.Biol.* 11:R446-R449.

188. Zheng,H., Kiss,R.S., Franklin,V., Wang,M.D., Haidar,B., and Marcel,Y.L. 2005. ApoA-I lipidation in primary mouse hepatocytes. Separate controls for phospholipid and cholesterol transfers. *J Biol.Chem.* 280:21612-21621.

189. Malerod,L., Juvet,K., Gjoen,T., and Berg,T. 2002. The expression of scavenger receptor class B, type I (SR-BI) and caveolin-1 in parenchymal and nonparenchymal liver cells. *Cell Tissue Res.* 307:173-180.

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

191. Matveev,S., van der Westhuyzen,D.R., and Smart,E.J. 1999. Co-expression of scavenger receptor-BI and caveolin-1 is associated with enhanced selective cholesteryl ester uptake in THP-1 macrophages. *J.Lipid Res.* 40:1647-1654.

192. Wang,L., Connelly,M.A., Ostermeyer,A.G., Chen,H.H., Williams,D.L., and Brown,D.A. 2003. Caveolin-1 does not affect SR-BI-mediated cholesterol efflux or selective uptake of cholesteryl ester in two cell lines. *J.Lipid Res.* 44:807-815.

193. Shepherd,J., Patsch,J.R., Packard,C.J., Gotto,A.M., Jr., and Taunton,O.D. 1978. Dynamic properties of human high density lipoprotein apoproteins. *J Lipid Res.* 19:383-389.

194. Denis,M., Haidar,B., Marcil,M., Bouvier,M., Krimbou,L., and Genest,J. 2004. Characterization of oligomeric human ATP binding cassette transporter A1. Potential implications for determining the structure of nascent high density lipoprotein particles. *J Biol.Chem.* 279:41529-41536.

195. Denis,M., Haidar,B., Marcil,M., Bouvier,M., Krimbou,L., and Genest,J., Jr. 2004. Molecular and cellular physiology of apolipoprotein A-I lipidation by the ATP-binding cassette transporter A1 (ABCA1). *J Biol.Chem.* 279:7384-7394.

196. Bodzioch,M., Orso,E., Klucken,J., Langmann,T., Bottcher,A., Diederich,W., Drobnik,W., Barlage,S., Buchler,C., Porsch-Ozcurumez,M. *et al.* 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat.Genet.* 22:347-351.

197. Joyce,C., Freeman,L., Brewer,H.B., Jr., and Santamarina-Fojo,S. 2003. Study of ABCA1 function in transgenic mice. *Arterioscler.Thromb.Vasc.Biol.* 23:965-971.

198. Fredenrich,A. and Bayer,P. 2003. Reverse cholesterol transport, high density lipoproteins and HDL cholesterol: recent data. *Diabetes Metab* 29:201-205.

199. Marcil,M., Brooks-Wilson,A., Clee,S.M., Roomp,K., Zhang,L.H., Yu,L., Collins,J.A., van Dam,M., Molhuizen,H.O., Loubster,O. *et al.* 1999. Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet* 354:1341-1346.

200. Hobbs,H.H. and Rader,D.J. 1999. ABC1: connecting yellow tonsils, neuropathy, and very low HDL. *J Clin.Invest* 104:1015-1017.

201. Kennedy, M.A., Barrera, G.C., Nakamura, K., Baldan, A., Tarr, P., Fishbein, M.C., Frank, J., Francone, O.L., Edwards, P.A. 2005. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab.* 1:121-131.

Curriculum Vitae

Andrea G. McKenzie
University of Ottawa Heart Institute
40 Ruskin St.
Ottawa, K2Y 4W7
ON, Canada
(613) 761-4468 ag_mckenzie@hotmail.ca

Education

2001 – present	Masters of Science candidate in the Faculty of Medicine, Department of Biochemistry University of Ottawa Heart Institute Supervisor: Dr. Y.L. Marcel Thesis Title:The Effect of Caveolin-1 on the Synthesis and Efflux of Cholesterol in Hepatoma Cells and Hepatocytes
1996-2000	Bachelor of Science, Honours Biochemistry. University of Ottawa

Employment

Summer 1999	Summer Student in the lab of Dr. Y.L. Marcel University of Ottawa Heart Institute
1999-2000	Honour's Project Supervisor: Dr. Y.L. Marcel University of Ottawa Heart Institute
2000-2001	Lab Technician in the lab of Dr. Y.L. Marcel University of Ottawa Heart Institute

Presentations and Posters

- M.Sc. Graduate Students Research Presentation
  University of Ottawa (2002)

- Research Presentation, Lipoprotein Group, University of Ottawa Heart Institute (2001)

- Graduate Students' Poster Day, Department of Biochemistry, Microbiology and Immunology, University of Ottawa (2002)

- Honour Students' Poster Day, Department of Biochemistry, University of Ottawa (1999)

Publications

Kiss, R.S., McManus, D.C., Franklin, V., Tan, W.L., McKenzie, A., Chimini, G., Marcel, Y.L., The lipidation by hepatocytes of human lipoprotein A-I occurs by both ABCA1-dependent and independent pathways (2003) *J. Biol. Chem.* **278**, 10119.

B. R. Scott, D. C. McManus, V. Franklin, A. G. McKenzie, T. Neville, D. L. Sparks, and Y. L. Marcel. The N-terminal globular domain and the first class A amphipathic helix of apolipoprotein A-I are important for lecithin:cholesterol acyltransferase activation and the maturation of high density lipoprotein in vivo. (2001) *J Biol.Chem.* **276,** 48716.

Collaborators

Many people contributed to the experiments in this thesis. Philip Links trained me in the majority of the techniques required for the project, and made the stable caveolin-1 expressing McA-RH7777 cell lines. Dr. M.P. Lisanti provided the caveolin-1, GFP and tTA adenoviruses used in the HepG2 cells. Hui Zheng assisted me with the caveolin-1 deficient hepatocyte experiments involving adenovirus infection. Vivian Franklin isolated the hepatocytes from the caveolin-1 deficient mouse experiments. Peter Rippstein prepared the electron microscopy sections, taught me how to use the electron microscope.