California State University, Northridge

REGULATION OF CHOLESTEROL EFFLUX FROM CULTURED HUMAN SKIN FIBROBLASTS BY LIPOPROTEIN LIPASE AND PHOSPHOLIPIDS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry

by

David Akopian

June 2007

The thesis of David Akopian is approved:

Dr. Paula Fischhaber	Date
Dr. Joseph Hajdu	Date
Dr. Jheem D. Medh, Chair	Date

California State University, Northridge

Dedication

This work is dedicated to my parents for their love, countless sacrifices, and unwavering support and encouragement.

Acknowledgement

I am deeply grateful to my thesis advisor, Dr. Jheem D. Medh, for guiding me through the project, for always being available to answer questions, discuss my work and the related literature, for teaching me the laboratory techniques, and always being supportive and enthusiastic. I would like to thank the members of my thesis committee, Dr. Paula Fischhaber and Dr. Joseph Hajdu, for their support, advice, and proofreading the text. I would also like to thank Dr. Rheem Medh for making me feel welcome to use whatever I needed in her lab, and Dr. James Schaffer for making the thermal cycler in his lab available and spending time with me on educational discussions. I would like to thank the chemistry department stockroom personnel, especially Marcia, for always promptly providing any solvents and chemicals I needed. My gratitude extends to the professors of the chemistry department with whom I interacted and who taught me so much.

My work at CSUN would not have been as enjoyable without constant interaction with graduate and undergraduate students. I would like to thank the students with whom I have shared the laboratory, especially Ye, Anna, Shawn, Tricia, Veronica, and Sima, for being interesting conversationalists and fun coworkers.

This project was supported by an NIH Minority Biomedical Research Support (MBRS) Support of Continuous Research Excellence (SCORE) Award (S06 GM48680), NIH Academic Research Enhancement Award (AREA) grant (R15 HL083946), and intramural grants from the California State University Northridge (CSUN) Office of Research and Sponsored Projects.

TABLE OF CONTENTS

SIGNATURE PAGE ii
DEDICATION. iii
ACKNOWLEDGEMENT. iv
LIST OF FIGURES. vi
LIST OF TABLES. viii
LIST OF ABBREVIATIONS. ix
ABSTRACT. xi
INTRODUCTION. 1
MATERIALS AND METHODS. 43
RESULTS. 63
DISCUSSION. 87
REFERENCES. 106

List of Figures

Figure 1.	Main lipids of lipoprotein particles.	1
Figure 2.	Schematic diagram of an LDL particle.	4
Figure 3.	The stages of atherogenesis.	5
Figure 4.	Structure of LPL.	6
Figure 5.	The role of LPL in restructuring lipoproteins of plasma.	8
Figure 6.	Maturation of an HDL particle.	13
Figure 7.	Antiatherogenic properties of HDL.	14
Figure 8.	Reverse cholesterol transport.	18
Figure 9.	Schematic representation of ABCA1.	20
Figure 10.	Schematic representation of HDL-mediated cholesterol efflux.	39
Figure 11.	Effect of LPL on ABCA1-mediated cholesterol efflux.	40
Figure 12.	RT-PCR analysis of LPL mRNA levels in human macrophages overexpressing LPL.	63
Figure 13.	RT-PCR analysis of LPL mRNA levels in fibroblasts overexpressing LPL.	64
Figure 14.	Western blot analysis of LPL isolated from the cell culture medium of fibroblasts overexpressing LPL.	65
Figure 15.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts overexpressing LPL.	66
Figure 16.	Western blot analysis of ABCA1 protein levels in LPL-overexpressing cells in the presence of cAMP and cholesterol.	67
Figure 17.	Effect of LPL on ABCA1-mediated cholesterol efflux.	68
Figure 18.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts treated with Intralipid.	69
Figure 19.	Western blot analysis of ABCA1 protein levels in fibroblasts treated with Intralipid.	70
Figure 20.	Effect of Intralipid on ABCA1-mediated cholesterol efflux	71
Figure 21.	Effect of Intralipid and LPL on ABCA1-mediated cholesterol efflux	72

Figure 22.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts treated with adLPL and Intralipid.	73
Figure 23.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts treated with saturated and unsaturated fatty acids.	74
Figure 24.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts treated with triglycerides and phosphatidylcholine.	75
Figure 25.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts treated with different amounts of phosphatidylcholine.	76
Figure 26.	RT-PCR analysis of ABCA1 mRNA levels in fibroblasts treated with a mixture of cholesterol and triglycerides and a mixture of cholesterol and phosphatidylcholine.	77
Figure 27.	Western blot analysis of ABCA1 protein levels in fibroblasts treated with a mixture of cholesterol and triglycerides and a mixture of cholesterol and phosphatidylcholine.	78
Figure 28.	Effect of phosphatidylcholine on ABCA1-mediated cholesterol efflux.	79
Figure 29.	Effect of phosphatidylcholine on ABCA1-independent cholesterol efflux.	80
Figure 30.	Effect of phosphatidylcholine on ABCA1-independent cholesterol efflux.	81
Figure 31.	Effect of DTNB on ABCA1-mediated cholesterol efflux.	82
Figure 32.	Effect of DTNB on ABCA1-independent cholesterol efflux.	83
Figure 33.	TLC analysis of the lipids isolated from fibroblasts treated with phosphatidylcholine in the absence and presence of DTNB.	84
Figure 34.	RT-PCR analysis of LCAT mRNA levels in fibroblasts treated with phosphatidylcholine.	85
Figure 35.	RT-PCR analysis of the levels of ABCA1 mRNA isolated from fibroblasts at two different time points after treatment with phosphatidylcholine.	86
Figure 36.	Mechanism of the phospholipid-mediated decrease in ABCA1 mRNA levels and ABCA1-mediated cholesterol efflux.	102

List of Tables

Table 1.	Sequence-specific primers.	2
Table 2.	Heterogeneity of lipoprotein particles.	48

List of Abbreviations

ABCA1	ATP-binding cassette protein A1
ApoA-I	Apolipoprotein A-I
ApoE	Apolipoprotein E
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
cAMP	cyclic Adenosine Monophosphate
CETP	Cholesteryl Ester Transfer Protein
cpt-AMP	8-(4-Chlorophenylthio)-cAMP
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
CVD	Cardiovascular Disease
DMEM	Dulbecco's Modified Eagle's Medium
15d-PGJ_2	15-deoxy-12,14 Δ-prostaglandin-J_2
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
FBS	Fetal Bovine Serum
FSF	Foreskin Fibroblast
GGPP	Geranylgeranyl Pyrophosphate
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HSPG	Heparan Sulfate Proteoglycan
IDL	Intermediate Density Lipoprotein

IL-1	Interleukin-1
LCAT	Lecithin:cholesterol Acyltransferase
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
LXR	Liver X Receptor
LXRE	LXR Response Element
MCP-1	Monocyte Chemoattractant Protein-1
PC	Phosphatidylcholine
PKA	Protein Kinase A
PL	Pancreatic Lipase
PLTP	Phospholipid Transfer Protein
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	PPAR Response Element
PUFA	Polyunsaturated Fatty Acids
RCT	Reverse Cholesterol Transport
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
RXR	Retinoid X Receptor
SDS-PAGE	Sodium Dodecylsulfate – Polyacrylamide Gel Electrophoresis
SMC	Smooth Muscle Cell
THP-1	Human leukemia monocyte
TNF-1	Tumor Necrosis Factor-1
VCAM	Vascular Cell Adhesion Molecule
VLDL	Very Low Density Lipoprotein

ABSTRACT

REGULATION OF CHOLESTEROL EFFLUX FROM CULTURED HUMAN SKIN FIBROBLASTS BY LIPOPROTEIN LIPASE AND PHOSPHOLIPIDS

by

David Akopian

Master of Science in Biochemistry

Removal of excess cellular cholesterol from extrahepatic tissues to the liver for degradation is essential in maintaining proper lipid balance in cells and opposing the development of atherosclerosis. The ATP-binding cassette protein A1 (ABCA1), a cholesterol transporter, which unidirectionally translocates cholesterol and phospholipids from cells to cholesterol acceptors, plays a pivotal role in preventing the accumulation of cholesteryl esters in various tissues. Unlike ABCA1, the enzyme lipoprotein lipase (LPL) was shown to be proatherogenic in atherosclerotic lesions by facilitating the formation and cellular uptake of proatherogenic lipids. The objective of the first part of our studies was to investigate the effect of LPL on ABCA1-mediated cholesterol efflux.

We overexpressed LPL in human skin fibroblasts, using adenoviral delivery of the gene, and analyzed ABCA1 mRNA and protein levels by reverse-transcription polymerase chain reaction (RT-PCR) and western blotting, respectively. ABCA1-mediated cholesterol efflux from the LPL-overexpressing cells was measured using radioactively labeled cholesterol. We showed that overexpression of LPL causes an increase in the levels of ABCA1 mRNA and protein and augments ABCA1-mediated cholesterol efflux. Our data suggest that the enzyme may play a regulatory role in ABCA1-mediated cholesterol transport and have an antiatherogenic effect in atherosclerotic lesions.

In the second part of our studies, we investigated the effect of phospholipids on ABCA1 mRNA and protein levels and ABCA1-dependent cholesterol efflux. We found that treatment of cholesterol-loaded fibroblasts with phosphatidylcholine (PC) results in

downregulation of ABCA1 mRNA and protein levels and reduces ABCA1-mediated cholesterol efflux. Our data show that PC indirectly regulates the function of the transporter by depleting cellular cholesterol. Phospholipids are the main lipid components of high density lipoprotein (HDL), which removes cellular cholesterol from extrahepatic tissues to the liver for degradation. Our studies suggest that in vivo in addition to playing an important role in cellular cholesterol efflux phospholipids of HDL may regulate ABCA1 on a transcriptional level.

INTRODUCTION

Plasma lipoproteins

In the plasma, lipids constitute a diverse group of molecules with widely differing properties and functions. Four of the most abundant representatives of lipids, carried in the plasma as lipid-protein complexes, lipoproteins, are shown in Figure 1.

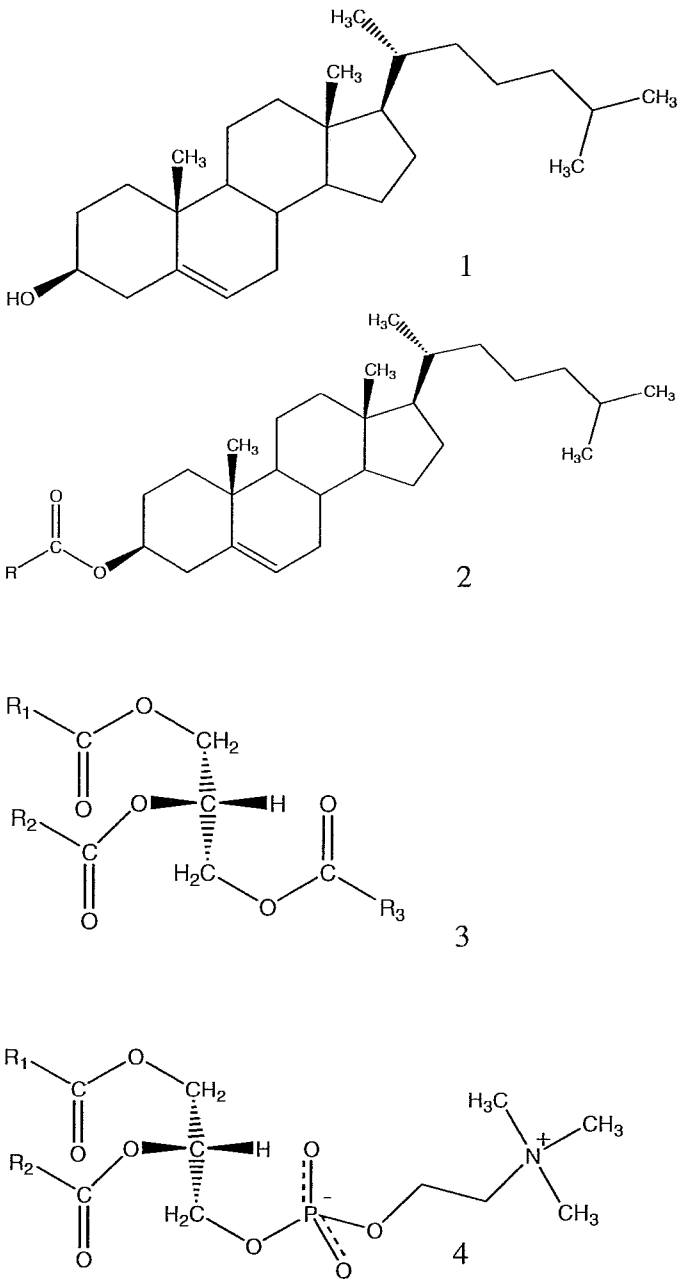


Figure 1. Main lipids of lipoprotein particles.[1] 1. Cholesterol. 2. Cholesteryl ester. R represents a hydrocarbon chain of a fatty acid. In cholesteryl palmitate R is $C_{15}H_{31}$, in cholesteryl oleate – $C_{17}H_{33}$. 3. Triacylglycerol. R_1, R_2, and R_3 are hydrocarbon chains of fatty acids, which are identical or different. 4. Phospholipid. Phospholipids are polar molecules with hydrophobic fatty acyl chains and a hydrophilic alcohol moiety. In phosphatidylcholine, shown in the figure, the alcohol group is choline, linked to the glycerol backbone via a phosphate group. Other biologically important alcohol groups of phospholipids are serine, inositiol, and ethanolamine. In lipoprotein particles, a monolayer of phospholipid associates with apolipoproteins, sequestering the hydrophobic interior of the particle containing cholesteryl ester and triglycerides from the aqueous environment of the plasma.

The hydrophobic nature of cholesteryl esters and triacylglycerols prevents their dissolution in the aqueous medium of the plasma. Specialized amphipathic proteins called apolipoproteins associate with lipids, solubilizing them and forming lipoprotein particles, whose hydrophilic exterior allows to transport lipids in the circulation. Based on the source and physicochemical properties of lipoproteins, they can be divided into five main classes, summarized in Table 1, with further subdivision within each class.

	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/cm^3)	<0.95	<1.006	1.006-1.019	1.019-1.063	1.063-1.210
Particle diameter (Å)	750-12,000	300-800	250-350	180-250	50-120
Particle mass (kD)	400,000	10,000-80,000	5,000-10,000	2300	175-360
% Protein	1.5-2.5	5-10	15-20	20-25	40-55
% Phospholipid	7-9	15-20	22	15-20	20-35
% Free cholesterol	1-3	5-10	8	7-10	3-4
% Triacyglycerols	84-89	50-65	22	7-10	3-5
% Cholesteryl esters	3-5	10-15	30	35-40	12
Major apolipoproteins	A-I, A-II, B-48, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100, C-III, E	B-100	A-I, A-II, C-I, C-II, C-III, D, E

Table 1. Heterogeneity of lipoprotein particles in the plasma.[2]

Chylomicrons assembled in the intestinal cells from dietary fat are triglyceride-rich lipoproteins, which deliver fatty acids to the tissues for storage and energy production. Enzymatic degradation of triglycerides in chylomicrons converts them to so called chylomicron remnants which are metabolized by the liver. Very low density lipoprotein (VLDL) particles assembled in the liver are the main carries of endogenous triglycerides and cholesteryl ester. Upon hydrolysis of triglycerides, they are converted to intermediate density lipoprotein (IDL), half of which is removed by the liver via LDL receptor-mediated endocytosis, with the other half catabolized to low density lipoprotein (LDL). LDL is the main carrier of cholesterol in plasma. Normal LDL is internalized by the liver and extrahepatic tissues via LDL receptor-mediated endocytosis, while oxidized or acetylated LDL particles are taken by cells via scavenger receptor-mediated endocytosis. The main function of HDL is the removal of cholesterol from extrahepatic tissues to the liver for degradation. [2,3]

The abnormal lipoprotein profile of the plasma, characterized by high levels of VLDL and LDL and low levels of HDL is one of the factors contributing to the development of atherosclerosis.

Atherogenesis

The last year's report by the American Heart Association described cardiovascular disease (CVD) as the leading cause of mortality in developed countries, claiming lives of 2500 Americans each day. Coronary artery disease (CAD), leading to myocardial infarction, is by far the most common CVD, accounting for 330,000 deaths annually in the United States.[4] Myocardial infarction, the permanent damage of the heart tissue, caused by blockage of the blood supply to myocardium, is the result of occlusion

of the lumen of a blood vessel in the region containing an atheromatous plaque. The latter forms in the process of atherogenesis.[5-8]

Although the accumulation of fatty deposits in coronary arteries plays a major role in the development of the disease, until relatively recently the mechanism of atherogenesis was viewed simplistically, with coronary arteries being thought of as inactive vessels, slowly accumulating fatty deposits on the walls that gradually occlude the lumen, blocking the passage of the blood and leading to myocardial infarction.[6] Thanks to recent advances in the area of atherosclerosis, atherogenesis is now viewed as an inflammatory process whose molecular mechanism is complex and involves multiple factors.[6-8]

LDL particles, schematically depicted in Figure 2, also known as "bad cholesterol", are the major contributor to the development of atherosclerosis. While at normal concentrations the particles enter and exit the intimal space of coronary arteries without any harm, at high levels LDL tends to accumulate in the intima where it undergoes oxidation and triggers an inflammatory response, shown in figure 3.[5-8]

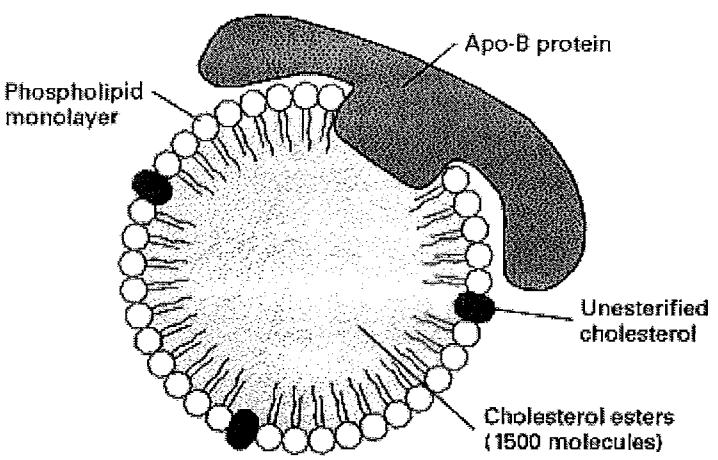


Figure 2. Schematic diagram of an LDL particle.[9] LDL is the main carrier of cholesterol in the blood. Its composition is described in Table 1. The function of LDL is to transport cholesterol to the tissues. 550 kDa apolipoprotein B-100, the largest monomeric protein known, covers approximately half of the surface of the particle, solubilizing lipids in plasma.

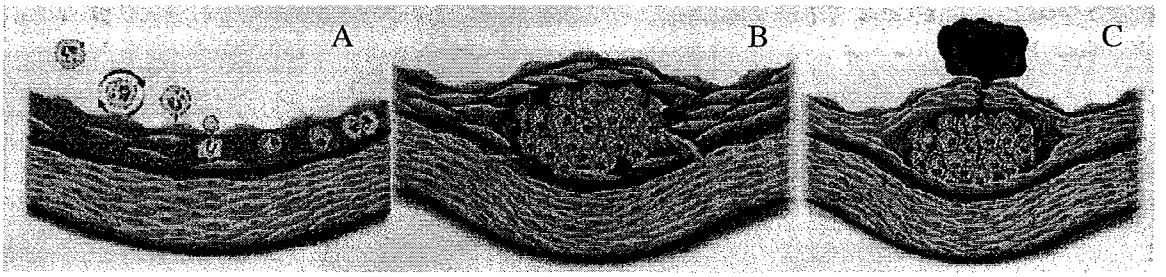


Figure 3. The stages of aterogenesis[7]. The process develops over the course of decades and can be divided into several stages. A. Recruitment of monocytes to the intima of a coronary artery by endothelial cells in response to oxidized LDL particles, which accumulate in the intima, triggering inflammatory response. Monocytes differentiate into macrophages and start engulfing LDL lipids, transforming into foam cells. B. Macrophages trigger migration and proliferation of smooth muscle cells (SMCs), which travel from the outer layers of a blood vessel toward the lumen to cover the lipid plaque, forming a fibrous cap. **C.** Secretion by macrophages of collagenases that degrade collagen produced by SMCs weakens the fibrous cap. Rupture of the atheromatous plaque leads to thrombosis and occlusion of the arterial lumen.

In response to the oxidized phospholipids in LDL, endothelial cells express vascular cell adhesion molecules (VCAMs) that attract circulating monocytes. The latter enter the intimal space of a blood vessel, differentiate into macrophages, and begin to engulf LDL particles, gradually transforming into lipid-laden foam cells, the main component of an atheromatous plaque. In addition to engulfing LDL, macrophages produce signaling molecules, such as tumor necrosis factor-β (TNF-β) and γ-interferon that trigger the migration of SMCs to the top layers of the intimal space and their proliferation to form a fibrous cap covering the fatty streak. In later stages of atherosclerosis, production by macrophages of γ-interferon inhibits collagen synthesis in SMCs. Collagenases secreted by the macrophages and foam cells degrade collagen and weaken the fibrous cap, ultimately causing its rupturing. This leads to complications in atherosclerosis. The blood in the region of the rupture is coagulated in the presence of the coagulation factor III, resulting in the formation of a thrombus. The thrombus occludes

the affected artery, blocking or dramatically reducing the supply of oxygen and nutrients to myocardium, causing myocardial infarction.[7]

Structure and function of lipoprotein lipase

One of the key factors in the development of atherosclerosis is lipoprotein lipase (LPL), which hydrolyzes triglycerides within lipoprotein particles and plays an important role in modulating the lipid profile of the plasma (Figure 4).[10,11] LPL is a secreted glycoprotein which, along with closely related hepatic lipase (HL) and pancreatic lipase (PL), belongs to the α/β-hydrolase fold family. The enzymes of this family are characterized by the catalytic fold composed of β-strands connected by α-helices.[12-14]

LPL is a homodimer composed of two identical subunits arranged head-to-tail via noncovalent interactions, with each subunit having a molecular mass of 55 kDa and consisting of two domains (Figure 4).[15-17] Molecular modeling based on the X-ray crystal structure of PL revealed a large N-terminal domain composed of the first 312 amino acid residues and a smaller C-terminal domain containing the rest of the 448 residues.[12,13] The N-terinal domain of the enzyme contains the catalytic site, while the C-terminal domain participates in determining the substrate specificity and heparin binding.[13,16]

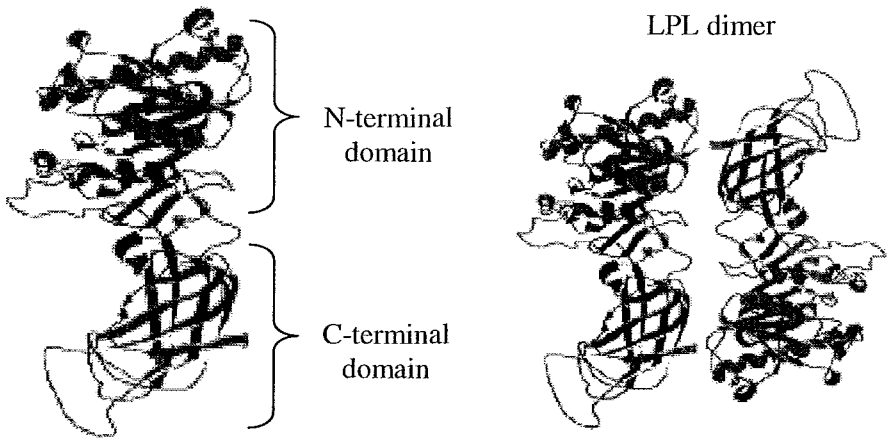


Figure 4. Subunit structure of LPL and association of the subunits head-to-tail, forming an active LPL homodimer.[17]

LPL is mainly produced by adipocytes, muscle cells, and mammary epithelial cells.[18] In the liver and the spleen, the enzyme is secreted by the scattered Kupffer cells in addition to being acquired by the liver from plasma. Production of LPL was detected in atherosclerotic lesions and lungs, where it is secreted by macrophages. Endothelial cells of blood vessels do not produce LPL but acquire it from blood and stabilize it on the lumenal surface of a blood vessel in the glycocalyx of endothelial cells via interaction with heparan sulfate proteoglycans (HSPG).[19] Here, LPL hydrolyzes triglycerides in circulating chylomicrons and VLDL particles, first converting them to cholesterol-enriched IDL and then cholesterol-rich LDL particles. The released fatty acids are oxidized in skeletal and cardiac muscle tissue to produce energy; in adipocytes they are converted to triacylglycerol and stored to be mobilized when energy is needed.[10,11,20]

Role of LPL in atherogenesis

Investigation of the physiological function of LPL has produced data that established both pro- and anti-atherogenic effects of the enzyme, depending on the site of its localization. Yla-Herttuala et al. and O'Brien et al. showed that macrophages and foam cells are the primary cells in atherosclerotic lesions that secrete LPL, suggesting its role in atherogenesis.[21,22] In murine strains, susceptibility to atherosclerosis correlates with levels of the macrophage LPL.[23,24] Thus, Babaev et al. generated mice with $LPL^{-/-}$ phenotypes for expression of the macrophage LPL and compared the size of aortic lesions in the animals after 19 weeks on an atherogenic diet to the lesions in the $LPL^{+/-}$ and $LPL^{+/+}$ animals. The $LPL^{-/-}$ mice showed a 55% reduction in the size of the aortic lesions compared to the $LPL^{+/+}$ mice.[23] Wilson et al. reported that apoE knockout mice expressing human LPL in macrophages exhibited a 51% increase in the size of aortic

lesions in the region of the aortic sinus.[24] In a more recent study, Wu et al. expressed human LPL in balloon-injured carotid arteries of rabbits with the purpose of examining its effect on production of the lipid plaque. The animals expressing LPL showed significant deposition of lipids compared to the control animals.[25]

Atherogenic properties of LPL are mediated via two main mechanisms: the lypolytic activity of LPL resulting in the formation of cholesterol-rich atherogenic lipids and LPL-mediated facilitation of the uptake of lipoproteins by cells independent of the lypolysis.[26-33]

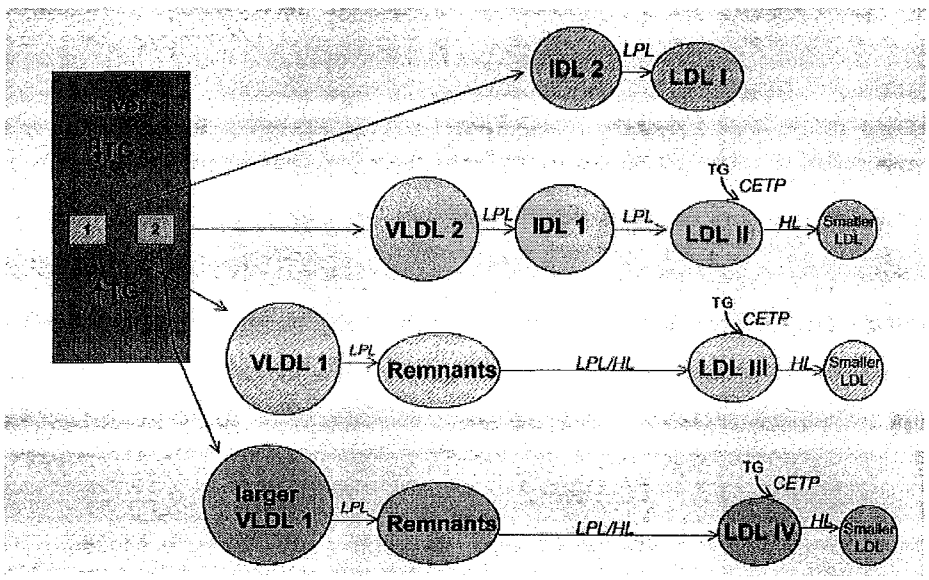


Figure 5. The role of LPL in restructuring lipoprotein particles[20]. VLDL particles packaged by the liver are the substrate for LPL. Hydrolysis of triglycerides by LPL produces cholesterol-rich lipoproteins with increased atherogenic potential. LDL is subdivided into four main classes (LDL-I – LDL-IV) based on the density. While the predominance in the plasma of both LDL-I and LDL-IV was linked to atherogenesis, small and dense LDL-IV particles have a greater atherogenic potential than large and buoyant LDL-I. The former has significantly greater capacity to enter the intimal space due to their smaller size and stronger binding to the arterial wall proteoglycans. They are also much more susceptible to oxidation than large LDL particles. Also shown, are hepatic lipase (HL), which along with LPL hydrolyzes triglycerides in lipoproteins, and cholesteryl ester transfer protein (CETP), whose function is to transport cholesteryl ester and triglycerides between lipoproteins. [20]

Zilversmit was first to propose that LPL anchored to the lumen of a blood vessel could have an atherogenic effect by hydrolyzing triglycerides in VLDL and chylomicrons and thus facilitating the formation of cholesteryl ester-enriched lipoproteins which are subsequently taken by the endothelium.[26] In support of this proposal, Lindquist et al. showed that LPL enhances the uptake of lipoproteins in mouse peritoneal macrophages[27], while Evans et al. concluded that lypolytic activity of LPL is required for the accumulation of lipids by J774 macrophages.[28] Aviram et al. investigated the effect of LPL on the uptake of LDL by SMCs, human monocyte-derived macrophages, and human skin fibroblasts. Modification of LDL particles via hydrolysis of triglycerides enhanced the accumulation of cholesterol in various cells.[29]

LPL aids in the cellular uptake of lipoprotein particles by acting as a bridge between the cell-surface receptors and the lipoprotein particles.[30-33] Beisiegel et al. reported a dramatically enhanced binding of chylomicrons to HepG2 cells and apoE-containing lipoproteins to the LDL receptor-related protein in the presence of LPL.[30] Medh et al. showed that LPL catabolizes large VLDL particles by directly interacting with the LDL receptor.[31] Takahashi et al. observed an increase in binding of VLDL particles to the VLDL receptor in the presence of LPL. The enzyme forms a bridge between the lipoprotein particles and HSPGs, thus increasing the concentration of VLDL in the vicinity of the receptor.[32] The evidence provided by Fernandez-Borja et al. from the studies with cultured human skin fibroblasts suggests that in addition to the LDL receptor-mediated lipoprotein uptake, LPL-associated lipoprotein particles are internalized via the LDL-independent HSPG pathway, with LPL anchoring the lipoprotein particles to HSPG.[33] Supporting this finding, investigation of the role of the

LDL receptor and HSPG in catabolism of VLDL particles in rat vascular smooth muscle cells showed that LPL is involved in internalization of the lipoprotein particles by interacting with both the LDL receptor and HSPG.[34]

Whether LPL is pro- or anti-atherogenic is dependent on the origin of the enzyme and its localization. While the presence of the enzyme in the endothelium of carotid arteries or its secretion by the macrophages of atherosclerotic lesions has a pro-atherogenic effect mainly through creating dense LDL particles and facilitating their uptake by the cells of the intima, the levels of circulating plasma LPL and its expression in the muscle and adipose tissue were shown to correlate with lower risk of CAD.[10,11,35-40]

Mutations in the LPL gene in humans have been associated with a proatherogenic plasma lipid profile, increased levels of plasma triglycerides, decreased levels of HDL, and progression of CAD.[35,38] Jukema and colleagues investigated the effect of Asp9Asn mutation in the LPL gene in heterozygous individuals. The carriers of the mutation had lower HDL levels and greater stenosis of coronary arteries than non-carriers.[35] Wittrup et al. examined the effect of the substitution of Asn at 291 position in the LPL gene to Ser on the plasma lipid profile and progression of CAD in 9,214 individuals.[36] The presence of the mutation was associated with higher levels of plasma triglycerides, reduced HDL levels, and a greater risk of CAD. Recently, Rip and workers reported the results of the prospective nested case-control study conducted over the course of seven years and including 25,663 individuals between the ages 45 and 79.[37] The plasma of individuals who developed CAD had a significantly lower concentration of LPL than the plasma of healthy individuals. Low concentration of LPL was associated with higher levels of plasma triglycerides and lower levels of HDL, suggesting that the atheroprotective effect

of LPL may be exerted through reducing the levels of VLDL and driving the plasma lipid profile toward higher levels of HDL.

Increased activity of LPL in animal models of atherosclerosis was also shown to be atheroprotective.[38-40] Tsutsumi et al. discovered the compound, NO-1886, which increases the activity of LPL, and investigated the long-term effect of its administration on the progression of atherosclerosis in rats. The compound increased the activity of LPL in the plasma, heart, and adipose tissue, causing an increase in the levels of HDL, and retarded the development of atherosclerosis.[38] In LDL-receptor knockout mice, overexpression of human LPL was shown to lower plasma triglyceride and cholesterol levels as well as decrease the size of aortic lesions.[39] Similarly, apoE knockout mice overexpressing human LPL displayed much smaller atherosclerotic lesions compared to the control animals.[40]

The seemingly contradictory reports concerning the role of LPL in atherogenesis stem from the diverse physiological role of the enzyme. On one hand, LPL produces atherogenic cholesterol-rich lipoprotein particles such as IDL and LDL and mediates their uptake by the macrophages and foam cells of atherosclerotic lesions. On the other hand, hydrolysis of triglycerides in chylomicrons and VLDL by LPL in muscle and adipose tissue drives the plasma lipid profile toward higher HDL levels. The source of LPL is the determinant of whether the enzyme is pro- or anti-atherogenic. While the presence of LPL in the endothelium of coronary arteries and atherosclerotic lesions is atherogenic, causing the accumulation of lipids, its production by other tissues is atheroprotective by increasing the levels of HDL.[11,39]

Atheroprotective role of HDL

Atherogenic properties of LDL are counteracted by the smallest and heaviest of lipoproteins found in the plasma, HDL particles, which remove cellular cholesterol to the liver for degradation. The main protein component of HDL, apolipoprotein A-I (apoA-I), is a 243 amino acid long polypeptide secreted into the vascular system by the liver and intestines. It folds into 10 amphipathic α-helices and acquires phospholipids and cholesterol from the liver, VLDL, chylimicrons, and extrahepatic tissues, gradually transforming from the lipid-poor apoA-I to the lipid-rich spherical HDL particle.[41-43]

Based on the electrophoretic mobility of HDL particles in agarose gel, they are divided into two main classes, pre-β-HDL and α-HDL, schematically represented in Figure 6.[41] The lipid-poor apoA-I acquires phospholipids and cholesterol from extrahepatic tissues and from the liver and lipid-rich lipoproteins, such as VLDL and chylomicrons, transforming into pre-β-HDL, which constitutes only 2-5% of plasma HDL.[42,43] These 60-70 kDa discoidal lipoproteins composed of a single lipid bilayer, with apoA-I located on the periphery, are only 5-6 nm in diameter and contain 10-40% of lipids. Depending on the content of phospholipid, mainly phosphatidylcholine, pre-β-HDL is divided into pre-β-1 HDL and pre-β-2 HDL, with the latter containing a greater proportion of phosphatidylcholine.[41]

Pre-β-HDL particles acquire cholesterol from extrahepatic tissues, storing it in the hydrophobic core as cholesteryl ester, and are transformed into spherical lipid-rich α-HDL particles. α-HDL represents a heterogeneous pool of spherical HDL particles varying in diameter from 9 to 12 nm. Based on their size and lipid content, they are subdivided into two subclasses, the smaller relatively lipid poor HDL_3 and the larger lipid-rich HDL_2.[41] During their 2-3 day presence in the plasma, HDL is continuously

remodeled by lipases and lipid transfer proteins, as it accepts additional cholesterol from the tissues before being removed from the circulation by the liver. [43-45] Cholesteryl ester transfer protein (CETP) catalyzes the exchange of cholesteryl esters and triglycerides between HDL particles and triglyceride-rich VLDL and chylomicrons. Phospholipid transfer protein (PLTP) transfers phospholipids from VLDL and chylomicrons to HDL. Hydrolysis of triglyceride-rich HDL_2 by HL produces HDL_3 and the lipid-poor apoA-I, which undergoes receptor-mediated degradation in the proximal tubule of the kidney. [43-45]

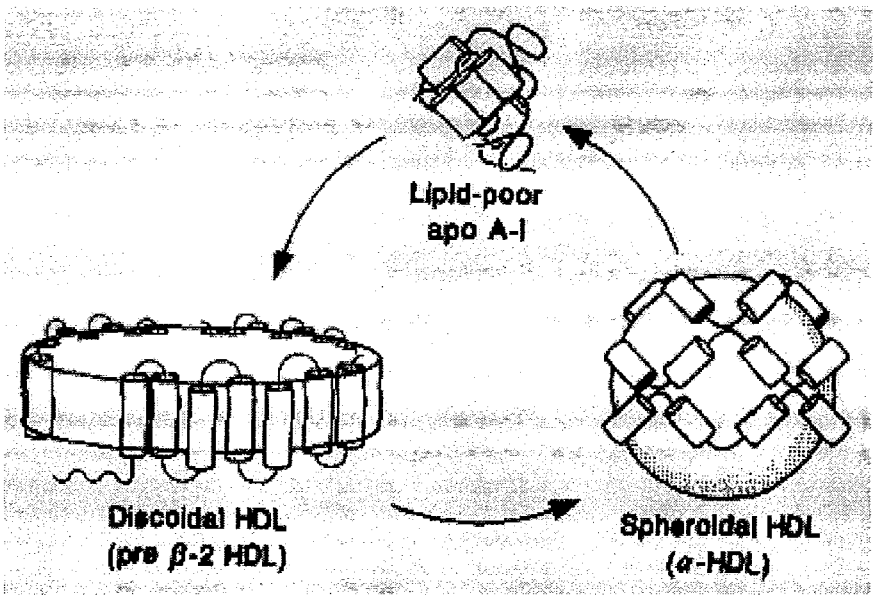


Figure 6. Maturation of an HDL particle.[41] Initial acquisition of lipids by the lipid-poor apoA-I results in the formation of discoidal pre-β HDL, which upon further lipidation matures into α-HDL. The latter undergoes remodeling by receiving cholesterol from various tissues, exchanging cholesteryl ester and triglycerides with triglyceride-rich VLDL and chylomicrons, and acquiring phospholipids from triglyceride-rich lipoproteins. Mature HDL particles are degraded by HL and apoA-I is degraded in the kidneys.

The physiological function of HDL has been extensively investigated over the course of several decades, with numerous studies demonstrating its atheroprotective role.[44,46-56] Figure 7 is a summary of the antiatherogenic properties of HDL, which

oppose the accumulation of lipids in the intima of blood vessels and counteract the pro-inflammatory effect of LDL.[44]

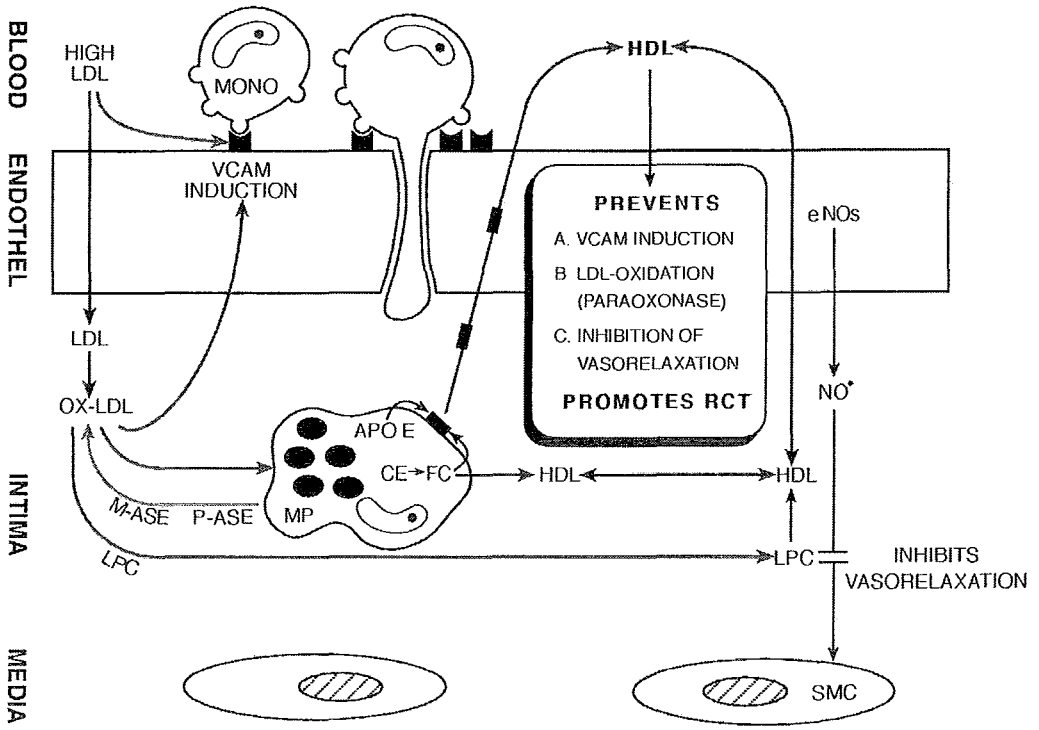


Figure 7. Antiatherogenic properties of HDL.[44] Oxidation of lipids in LDL particles, induction of VCAMs of endothelial cells by the oxidized LDL, resulting in the accumulation of monocytes in the intimal space, and the accumulation of cholesteryl esters by the monocyte-derived macrophages are the main events in atherogenesis. Macrophages (MP) secrete myeloperoxidase (M-ASE) and phospholipase (P-ASE) that oxidize LDL. HDL-associated paraoxonase catabolizes lipid peroxides, thus decreasing the amount of oxidized LDL. Independently of this effect, HDL inhibits expression of VCAMs, reducing endothelial cell-monocyte interaction, and prevents vasoconstriction, thus opposing constriction of the arterial lumen. The removal of cholesterol from the macrophages by HDL and the macrophage-secreted apoE is essential in reducing the amount of the accumulated lipids.

Expression of VCAMs by the cells of endothelium, which attract monocytes to the intima, is one of the first steps in atherogenesis. Inhibition of the experession of VCAMs by HDL protects the arterial wall from the accumulation of monocytes. Cockerill et al. showed that the cytokines, TNF-α and interleukin-1 (IL-1), inducing the

expression of VCAMs, are inhibited in the presence of HDL.[46] This effect was observed with endothelial cells and could not be reproduced with human skin fibroblasts. It was later shown that HDL classes differ in their ability to suppress the expression of VCAMs, with HDL_3 being more efficient than HDL_2.[47]

Oxidized LDL particles in the intima trigger the inflammatory cascade, which eventually results in the formation of an atheromatous plaque. The enzyme paraoxonase transported by HDL degrades oxidized phospholipids, thus exerting an atheroprotective effect. Mackness and colleagues observed a significant decrease in lipid peroxides in LDL caused by both HDL and purified paraoxonase in a concentration-dependent manner, with the effect disappearing after heat-inactivation of the enzyme.[48] Oxidized LDL particles treated with paraoxonase were much less effective than untreated LDL in inducing endothelial cell-monocyte interaction.[49]

Removal of the cellular cholesterol is yet another and undoubtedly most important atheroprotective mechanism of HDL as it opposes the accumulation of cholesterol in extrahepatic tissues. More than four decades ago Stein et al. showed that the lipid-free apoA-I is an efficient acceptor of cholesterol from the plasma membrane of Landshutz ascites cells.[50] When compared with bovine serum albumin (BSA), apoA-I was four times as efficient as the former in removing cellular cholesterol. Complexation of apoA-I with either phosphatidylcholine or sphingomyelin increased the ability of the apolipoprotein to accept cholesterol, with the rate of cholesterol release dependent on the concentration of the phospholipid. These results were reproduced by Jackson et al. who observed an increase in the removal of cellular cholesterol by various apolipoproteins in complexes with synthetic phosphatidylcholine.[51]

Oram and colleagues showed that lipoprotein particles containing ApoA-I are the main acceptors of cellular cholesterol in human plasma.[52] They incubated human skin fibroblasts in lipoprotein-deficient human serum (d>1.25) and in the medium containing the particles purified from the d>1.25 serum by anti-apoA-I affinity chromatography. No significant difference was observed between the former and the latter in the ability to remove cellular cholesterol, allowing to conclude that apoA-I-containing particles are the main acceptors of cholesterol. Incubation of fibroblasts with subfractions of HDL revealed a dramatic difference in their ability to serve as acceptors of cellular cholesterol, with cholesterol-poor HDL_3 (d=1.100-1.21) efficiently removing cholesterol and HDL_2 (d=1.063-1.100) having no cholesterol-removing activity.[52]

Castro et al. investigated efflux of ^{3}H-cholesterol from human skin fibroblasts by incubating the cells in human plasma and analyzing the lipoprotein fractions containing the radioactive label at various time points.[53] After 1 min of incubation, ^{3}H-cholesterol was associated with low-molecular weight (70 kDa) pre-β-HDL, while at 2 min of incubation, the label was found in pre-β-HDL of a higher molecular weight and α-HDL, suggesting that the lipid-poor apoA-I species serve as the primary acceptors of cellular cholesterol, transforming upon accumulating cholesterol into lipid-rich particles. These results are in agreement with the observations of Asztalos et al, who incubated firbroblasts in the medium containing different amounts of apoA-I.[54] After the incubation of the cells with [ApoA-I]<5μg/ml for 4 hours, all apoA-I was found in HDL particles with pre-α mobility. When the cells were incubated with [apoA-I]>5μg/ml, lipoproteins with pre-β mobility were observed. Thus, pre-β HDL species are intermediates in the formation of spherical HDL from the lipid-poor apoA-I.

To investigate the role of pre-β-1 HDL in the removal of cellular cholesterol, Sviridov et al. incubated human fibroblasts and THP-1 monocyte-derived macrophages in human plasma and pre-β-1 HDL-deficient plasma.[55] Interestingly, both were equally efficient in removing cholesterol from the cells. When pre-β-1 HDL was added to the pre-β-1 HDL-deficient plasma to achieve the same HDL concentration as in the whole plasma, the cholesterol-removing capacity of the reconstituted plasma exceeded that of the whole plasma. The results suggest that, while pre-β-1 HDL is an active acceptor of cholesterol, it is not essential for cholesterol-removing capacity of the plasma. In agreement with these observations, Wu and colleagues showed that both pre-β-HDL and α-HDL are capable of accepting cholesterol from human SMCs, with pre-β-HDL having a significantly greater cholesterol-removing capacity than α-HDL.[56]

Reverse cholesterol transport and ABCA1

Efflux of cellular cholesterol to HDL particles with subsequent transport of cholesterol from extrahepatic tissues to the liver for degradation is essential in maintaining the lipid balance in cells and is termed reverse cholesterol transport (RCT).[42,57,58] Removal of cholesterol from macrophages at the sites of atherogenesis via RCT plays an important role in preventing the accumulation of cholesteryl ester in cells and is crucial in protecting the arterial wall from developing atherosclerotic lesions (Figure 8).[11,43]

The ATP-binding cassette protein A1 (ABCA1), a specialized cholesterol transporter, lipidates cholesterol-free apoA-I. The newly acquired cholesterol is esterified to cholesteryl ester by the HDL-associated enzyme lecithin:cholesterol acyltransferase (LCAT). The enzyme transfers a fatty acyl chain from the second position of the glycerol

backbone of phosphatidylcholine to the hydroxyl group of cholesterol.[59-61] Cholesteryl esters travel to the core of the maturing HDL particle, which is gradually transformed under the activity of LCAT from a discoidal pre-β-migrating particle to a spherical lipid-rich HDL particle.[62] The latter interacts with the scavenger receptor class B type I (SR-BI) of hepatocytes, which mediates the uptake of the lipid-rich HDL core by cells.[63,64] The released cholesteryl esters are metabolized by the liver and excess cholesterol is excreted in the bile. In addition to the uptake by the liver, HDL particles are remodeled by HL, endothelial lipase (EL), secretory phospholipase A2, and lipid transfer proteins, with the formation of the lipid-poor apoA-I, which is degraded in the kidneys.[11]

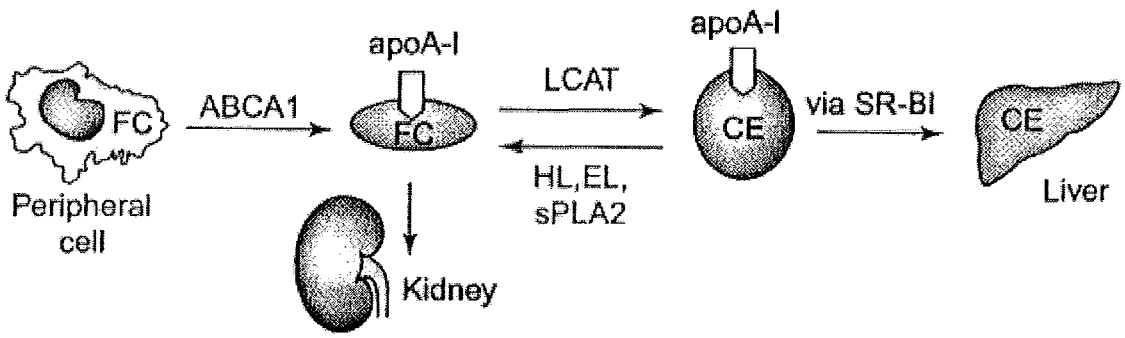


Figure 8. Reverse cholesterol transport.[11] Free cholesterol (FC) is received by cholesterol-poor ApoA-I and is converted to cholesteryl ester (CE) by LCAT, essential in HDL maturation. CE-rich HDL is removed from the circulation by the liver and excess cholesterol is excreted in the bile. HDL particles are remodeled by HL, EL, secretory phospholipase A2 in addition to lipid transfer proteins such as PLTP and CETP, resulting in the formation of lipid-poor small HDL particles, which are catabolized by the kidneys.

Function and structure of ABCA1

ABCA1 is a transmembrane protein with an essential role of transporting phospholipids, mainly phosphatidylcholine, and excess of cellular cholesterol, unidirectionally from cells to apoA-I-containing cholesterol acceptors.[42,57,58,65]

Upregulation of ABCA1-dependent cholesterol efflux upon loading the cells with cholesterol[66] and efflux of phosphatidylcholine even when the plasma membrane pool of cholesterol is depleted[67] suggest that the transport of cholesterol is an important function of ABCA1 and that phospholipids are the primary substrate of the transporter. Gillotte-Taylor and colleagues observed a 12-fold increase in ABCA1 mRNA levels, a 2-fold increase in the amount of apoA-I bound to the cell membrane, and a 6-fold increase in the rate of cholesterol efflux on loading human fibroblasts with unesterified cholesterol.[66] Wang et al. depleted cellular pools of cholesterol by incubating the cells with cyclodextrin and examined the effect of cholesterol depletion on ABCA1-mediated phospholipid efflux. Neither the binding of apoA-I nor ABCA1-dependent efflux of phosphatidylcholine was affected. Their data also suggest that phospholipid is a cofactor in ABCA1-mediated cholesterol efflux, as ABCA1 does not directly bind cholesterol and requires phospholipid for cholesterol translocation.[67]

Studies showed that ABCA1 has broad substrate specificity and can transport other small molecules and even proteins. Oram et al. observed ABCA1-mediated transport of α-tocopherol from murine fibroblasts and macrophages to apoA-I containing lipoprotein particles.[68] von Eckardstein et al. showed that secretion of apolipoprotein E from THP macrophages is mediated by ABCA1,[69] while Zhou and colleagues found that interleukin-1β secretion from macrophages is inhibited by anti-ABCA1 antisense oligonucleotides.[70]

ABCA1 is a member of the superfamily of ATP-binding cassette (ABC) proteins, which transport a wide range of substrates, ranging from inorganic ions and metals to small organic compounds and peptides, in an ATP-dependent fashion.[42,71,72] In humans

around 50 ABCs were identified, classified into subfamilies ABCA through ABCG. Mutations in these transporters result in 13 genetic disorders, such as cystic fibrosis (ABCC7), adrenoleukodystrophy (ABCD1), Stargardt disease (ABCA4), and Tangier disease (ABCA1).[72]

ABCA1 is a 2261 amino acid integral protein with twelve membrane-spanning domains, large extracellular loops, and cytoplasmic domains containing the ATP-binding folds (Figure 9).[42,71,73,74] Bungert et al. proposed a topological model of ABCA1 based on the structural studies of the closely related member of the ABCA subfamily, ABCA4, present in photoreceptors. ABCA1 consists of two structurally similar halves, with each half containing six membrane-spanning α-helices and a large extracellular loop. The 600 amino acid extracellular domain of the N-terminal half contains four glycosylation sites. The same number of glycosilation sites is present on the smaller, 275 amino acid extracellular loop of the C-terminal half.[73] Site-directed mutagenesis studies by Fitzgerald and colleagues established the importance of the large exracellular domains in ABCA1-apoA-I interaction and ABCA1-mediated cholesterol efflux, suggesting direct binding of apoA-I to the extracellular domains.[74]

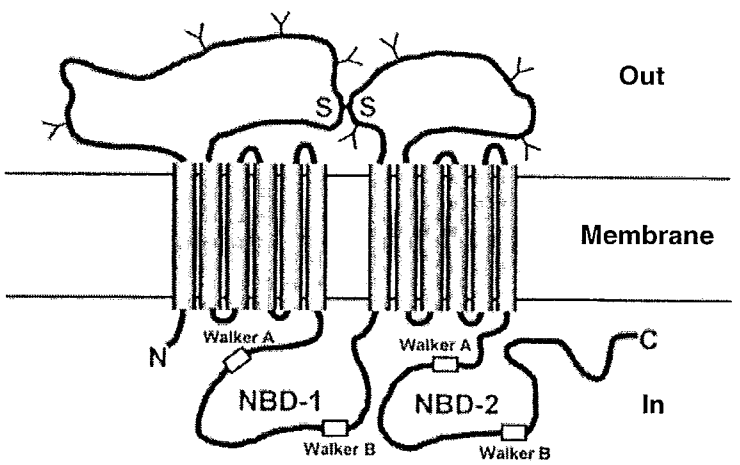


Figure 9. Schematic diagram of ABCA1.[42] NBD-1 and NBD-2 are nucleotide binding domains 1 and 2, each containing highly conserved sequences Walker A and Walker B. Motif C fourteen amino acids upstream of Walker B is not shown. Approximate glycosylation sites are represented by Y. Two large extracellular loops are linked by a disulfide bridge.

Each half of ABCA1 contains a nucleotide binding domain (NBD), which varies in length from 90 to 110 amino acids in different ABC transporters, with highly conserved sequences, Walker A, Walker B, and motif C, present in all transporters of the ABC superfamily. Binding of ATP by NBD is required for translocation of cholesterol from the cytoplasmic region of the cell to the outside.[72]

ABCA1 is widely expressed and was detected in different tissues.[75] By using in situ hybridization techniques Lawn et al. investigated the expression of ABCA1 in normal and inflamed tissues of humans and baboons and observed an increase in the expression of the protein at the sites of atherogenesis. While no ABCA1 was detected in the wall of normal arteries, atherosclerotic arteries displayed significant levels of the transporter. Among normal tissues, ABCA1 was also detected in central nervous system, kidneys, and testes.[75]

Investigation of the cellular trafficking of the transporter revealed that in addition to being localized in the plasma membrane, ABCA1 is present in intracellular vesicles. By monitoring the trafficking of a ABCA1-GFP construct by time-lapse fluorescence, Neufeld and colleagues observed a complex cellular trafficking pathway of ABCA1 between the plasma membrane, early and late endosomes, and lysosomes, the sites of ABCA1 degradation. Inhibition of the cellular trafficking of ABCA1 affected ABCA1-mediated lipid efflux, suggesting the importance of the pathway in regulating the function of the transporter in the plasma membrane.[76] In support of these findings, Zha et al. observed an increase in the vesicular trafficking between the Golgi apparatus and the plasma membrane in fibroblasts and macrophages in response to the upregulation of ABCA1-mediated cholesterol efflux by apoA-I.[77]

Mechanism of ABCA1-mediated cholesterol efflux

Despite many attempts to elucidate the mechanism of ABCA1-mediated cholesterol translocation, a clear picture of the process is still missing. It is well established that apoA-I is required for ABCA1-mediated lipid efflux, but whether the apolipoprotein directly interacts with the transporter or contacts the lipid rich plasma membrane domains in the vicinity of ABCA1 is presently unknown.[42,58]

Several studies suggest that ABCA1 creates lipid-rich domains by transporting lipids to the outer leaflet of the plasma membrane. Amphipathic helices of apoA-I then interact with the lipid-rich domains and solubilize lipids, forming nascent HDL particles.[78-81] Chambenoit and colleagues expressed functional ABCA1 and structurally intact ABCA1 lacking ATPase activity in RAW 264.7 cells and examined the binding of apoA-I to the cells. The cells expressing functional ABCA1 displayed increased binding of apoA-I. Mobility of apoA-I in the plasma membrane suggested association of the apolipoprotein with the lipid domains and argued against the interaction with ABCA1.[78] Supporting this finding, Remaley et al. observed a several-fold increase in lipid efflux from the cells expressing ABCA1 compared to the control cells in the presence of synthetic peptides with amphipathic helices. Binding of the helices to the cells correlated with their lipid affinity.[79] Gaus et al showed that apoA-I mediates cholesterol efflux by directly interacting with so called rafts, tightly packed plasma membrane domains rich in saturated phospholipids, sphingolipids, and cholesterol, and loosely packed, cholesterol-poor, non-raft domains. Inhibition of the apoA-I binding to these domains reduced cholesterol efflux.[80] Recently, Landry and colleagues reported that ATPase activity of ABCA1 disrupts raft domains of the plasma membrane, generating loosely packed lipid

domains, the preferential sites of apoA-I binding.[81] This observation suggests a model of cholesterol efflux, in which the primary role of ABCA1 is to reorganize lipids in the membrane. Formation of cholesterol-rich domains stimulates binding of ApoA-I to the membrane. Lipidation of ApoA-I upon its interaction with the plasma membrane lipids results in formation of nascent HDL, which undergoes further modifications.

Alternatively, apoA-I may directly interact with ABCA1. This proposal is supported by site-directed mutagenesis and cross-linking studies, suggesting direct association of apoA-I with the transporter.[74,82-85] Based on the sequences of naturally occurring defective ABCA1 proteins, Fitzgerald et al. generated five ABCA1 mutants with a single amino acid substitution in the extracellular domains and perfromed cross-linking studies involving apoA-I. One of the mutant proteins, W590S, retained the ability to bind apoA-I but showed no lipid efflux activity.[74] The results of this study suggested that direct binding of apoA-I to the extracellular domains is one of the requirements in ABCA1-mediated cholesterol efflux. Based on cross-linking and immunoprecipitation studies Wang et al. concluded that ABCA1 binds to apoA-I but not HDL.[82] Later Fitzgerald et al. performed competition binding assays using plasma membrane proteins and showed that cross-linking of apoA-I to ABCA1 is decreased in the presence of the competing proteins.[83] The release of apoA-I by ABCA1 was accompanied by cholesterol transfer.

Other studies support a model of ABCA1-mediated cholesterol efflux involving the direct association of the transporter with apoA-I followed by the interaction of the apolipoprotein with the lipids of the plasma membrane and the release of the lipoprotein complex.[84,85] The ability of apoA-I to interact with both the plasma membrane lipid

domains and ABCA1 suggests that the process of apoprotein lipidation may involve its simultaneous binding to the lipid bilayer and the transporter. Lipid efflux studies with apoA-I lacking various domains support this conclusion, confirming the proposal of the two-step model for ABCA1-mediated cholesterol efflux.[86] In the first step, hydrophobic α-helices of apoA-I bind to ABCA1 and the apolipoprotein is thus brought into the vicinity of the lipid domains created by the activity of ABCA1. Following the ABCA1-binding, C-terminal amphipathic α-helices of apoA-I interact with the lipid bilayer of the plasma membrane. In the next step, solubilization of the lipids by apoA-I triggers its lipidation, leading to the release of the nascent HDL particle. This model is in agreement with the wealth of research data investigating the lipidation of amphipathic α-helices of various apolipoproteins and interaction of ApoA-I with ABCA1 and the plasma membrane lipid bilayer.

Although it is well established that both phospholipids and cholesterol are the major substrates translocated by ABCA1, with the former being the primary substrate, neither the precise role of phospholipids nor the mode of lipidation of apoA-I is clear. Several lines of evidence suggest sequential lipidation of apoA-I by ABCA1, in which apoA-I first acquires phospholipids in ABCA1-dependent manner, forming a cholesterol-free nascent HDL particle with high affinity for cholesterol. Subsequently, cellular cholesterol is removed from the plasma membrane by the lipid-poor HDL independently of the ABCA1 activity. Thus Fielding and colleagues found that while cholesterol efflux from SMCs is inhibited by vanadate, phospholipid efflux is not affected, suggesting that the two processes may be uncoupled. Incubation of the aortic endothelial and umbilical vein cells, which do not express ABCA1, with naked apoA-I resulted in no cholesterol

efflux. When apoA-I was first incubated with smooth muscle cells and the conditioned medium was then incubated with the endothelial cells, cholesterol efflux was observed.[87] Wang et al. showed that depletion of the cellular pools of cholesterol by incubating HEK293 cells with β-methylcyclodextrin results in abolishment of ABCA1-mediated cholesterol efflux, while the efflux of phospholipid to apoA-I is not affected.[67]

Contrary to these findings, the results obtained by Smith et al., who measured the efflux of radioactively labeled cholesterol and phosphatidylcholine from RAW264.7 cells, suggest that ABCA1 translocates phospholipids and cholesterol simultaneously.[88]

Recently, Takahashi and colleagues purified human ABCA1, reconstituted it in synthetic liposomes, and measured its ATPase activity.[89] Liposome composed of synthetic phosphatidylcholine produced the highest ATPase activity, which was lowered in the presence of phosphatidylserine, phosphatidlyethanolamine, and phosphatidylglycerol. Synthetic liposomes containing 20% of cholesterol significantly reduced ATPase activity of ABCA1. Interestingly, while β-sitosterol had the same effect, structurally similar stigmasterol did not inhibit the activity of ABCA1, pointing to specific interaction of ABCA1 with certain sterols. Thus, while it has been demonstrated that phospholipids, primarily phosphatidylcholine, are the primary substrate of ABCA1, the mode of cholesterol translocation remains unclear.

Regulation of ABCA1-mediated cholesterol efflux

Considering the importance of ABCA1 in protecting cells from toxic levels of cholesterol, it is not surprising that this function is tightly regulated both transcriptionally and post-translationally. A significant amount of evidence of negative and positive transcriptional regulation of ABCA1 by various lipid ligands has accumulated over the

course of nearly ten years. Data pointing to the important role of post-translational regulation of ABCA1 in cellular cholesterol efflux also exist.[42,58,90]

One of the widely documented and well established observations in the regulation of ABCA1-mediated cholesterol efflux is upregulation of ABCA1 mRNA levels and dramatic increase in apoA-I-dependent cellular cholesterol efflux upon loading cells with cholesterol.[66,91-93] Thus, Mendez and colleagues observed an increase in apoA-I-mediated energy-dependent cholesterol efflux distinct from passive diffusion from cholesterol-enriched human skin firbroblasts.[91] Langmann et al. loaded macrophages with cholesterol by incubating the cells with acetylated LDL and observed an increase in ABCA1 mRNA and protein levels. This effect was erased when cellular cholesterol pools were depleted by incubating the cells with HDL_3.[92] Rong and coworkers reported transformation of SMCs into a macrophage-like state upon loading the cells with cholesterol, observing an increase in mRNA levels of ABCA1 by over 200%.[93]

The mechanism of upregulation of ABCA1 mRNA and protein levels in response to loading cells with cholesterol involves the transcription factor, liver X receptor α (LXRα), which upon binding lipid ligands, oxysterols and fatty acids, induces or represses transcription of the genes whose protein products participate in cholesterol metabolism.[94-96]

LXRα and LXRβ, members of the superfamily of nuclear receptors, are regulators of the processes involved in lipid metabolism and atherogenesis. These include intestinal cholesterol absorption, excretion of cholesterol by the liver, cellular cholesterol efflux from extrahepatic tissues, formation of VLDL by the liver, uptake of glucose and synthesis of fatty acids by the adipose tissue, inflammation, and inhibition of SMC and

macrophage proliferation. While LXRβ is ubiquitously expressed, the expression of LXRα mainly occurs in metabolically active tissues, such as liver and adipose tissue, and macrophages.[94-96]

Oxysterols, 22(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 27-hydroxycholesterol, and 24(S), 25-epoxycholesterol, natural metabolites of cholesterol, are ligands of LXRα. Upon binding an oxysterol, LXRα forms an obligate heterodimer with another nuclear receptor, retinoid X receptor (RXR), whose ligand is 9-*cis*-retinoic acid. Subsequently, the heterodimer binds to the LXR response element (LXRE) in the vicinity of the promoter of the regulated genes and induces their transcription.[94-100]

Venkateswaran and colleagues were among the first to begin to understand the mechanism of upregulation of ABCA1 mRNA by LXRα in response to cholesterol loading.[97] They overexpressed LXRα in NIH 3T3 fibroblasts and RAW264.7 macrophages and observed a dramatic increase in ABCA1 mRNA levels and apoA-I-mediated cholesterol efflux. Similar induction was achieved upon treating the cells with oxysterols. The effect could not be reproduced with the cells producing defective LXRα. Schwartz et al. transfected RAW264.7 cells with the ABCA1 promoter-luciferase gene fusion construct and observed the expression of the luciferase gene product upon stimulating the cells with 20(S)-hydroxycholesterol or 9-*cis*-retinoic acid. ApoA-I-mediated cholesterol efflux from the cells expressing ABCA1 was elevated in response to the ligands.[98] Later, Costet and colleagues reported synergistic effect of the nuclear receptor ligands. While 9-*cis*-retinoic acid or 22(R)-hydroxycholesterol alone enhanced the activity of the ABCA1 promoter only 8-fold, a 37-fold increase in the transcription

was observed when the cells were treated with both ligands simultaneously. Mutational analysis and binding assays allowed to identify the LXR/RXR binding sequence in the region located between -70 and -55 bp of the ABCA1 gene.[99]

Fu et al. observed dose-dependence of the levels of 27-hydroxycholesterol produced by the cells on the amount of cholesterol loaded into macrophages, suggesting that some of the loaded cholesterol is converted to the oxysterol. To confirm the finding that the synthesis of 27-hydroxycholesterol plays a role in activation of the ABCA1 gene, they introduced the CYP27 gene, encoding sterol 27-hydroxylase, into HEK293 cells and observed transcriptional activation of the genes regulated by LXRα. No activation of LXRα was observed in response to cholesterol loading of the fibroblasts lacking sterol 27-hydroxylase activity.[100]

Li and coworkers showed that in addition to regulating transcription of a wide array of genes, LXRα is autoregulated, with the LXRα/RXR heterodimer acting as a transcriptional activator of the LXRα gene.[101] They discovered three LXREs in the promoter region of the LXRα gene with differential binding to the LXRα/RXR and LXRβ/RXR heterodimers. Stimulation of the human macrophages with T0901317, a synthetic LXR ligand, resulted in direct transcriptional activation of the LXRα gene.

Peroxisome proliferator-activated receptors (PPARs) have also been shown to regulate LXRα and ABCA1-mediated cholesterol efflux.[96,102-105] PPARs are members of the superfamily of nuclear receptors activated by a wide range of ligands and regulating a multitude of metabolic pathways and cellular differentiation. Four subtypes of PPARs, α, β, γ, and δ, with different ligand and tissue specificities were identified. PPARα, expressed mainly in the liver, kidneys, heart, and adrenal glands, is activated by

polyunsaturated fatty acids, their oxidized products, and fibrates and regulates lipid metabolism. Widely expressed PPAR β and δ are regulators of fatty acid oxidation in response to polyunsaturated fatty acids and prostaglandins. PPARγ, expressed in adipose tissue, colon, adrenal glands, and spleen, is activated by fatty acid derivatives, prostaglandins, and insulin-sensitizing compounds and regulates adipogenesis and glucose homeostasis.[96]

Like LXRs, PPARs combine with RXRs upon ligand binding, forming PPAR/RXR heterodimers which subsequently regulate transcription of genes.[96,102] Activation of PPARα and PPARγ in response to various ligands was shown to have an atheroprotective effect.[102] Upregulation of ABCA1-mediated cholesterol efflux via induction of LXR is one of the routes through which PPARα and PPARγ produce the antiatherogenic effect.[103-105]

Chawla and colleagues reported that LXRα is under direct transcriptional regulation of PPARγ. They found that treatment of macrophages with the PPARγ-specific ligand rosiglitazone resulted in dramatic upregulation of LXRα mRNA levels. To confirm the proposal of the direct regulation of LXRα by PPARγ they performed mutational analysis of the LXRα gene, localizing the PPAR response element (PPRE) to the sequence between -722 and -710 bp of the gene.[103] Induction of LXRα in macrophages and an increase in apoA-I-mediated cholesterol efflux from cholesterol-enriched macrophages in response to diverse PPARα and PPARγ ligands were observed by Chinetti et al.. No such effect was observed in the macrophages lacking functional ABCA1.[104] Supporting these observations, Akiyama and colleagues showed that cholesterol-loaded macrophages lacking the PPARγ gene exhibit a significantly lower

apoA-I-dependent cholesterol-efflux compared to the control cells, pointing to the link between PPARγ and ABCA1.[105] These results underline the importance of the PPARγ-LXRα-ABCA1 pathway in cellular cholesterol efflux, highlighting the complexity of regulation of ABCA1-mediated cholesterol efflux by various lipid ligands.

It is important to emphasize that both PPARγ and LXRα are activated by oxidized derivatives of lipids, oxidized fatty acids and oxysterols, respectively. These derivatives are components of oxidized LDL particles engulfed by macrophages in an atherosclerotic lesion. Thus, activation of RCT by PPARγ and LXRα can be viewed as a protective mechanism evolved to counteract the accumulation cholesteryl ester derived from the oxidized LDL.[102]

Independently of LXRα, the ABCA1 gene is induced by cAMP analogues.[106-109] Prior to the discovery of ABCA1, Smith et al. reported a dramatic increase in energy-dependent cholesterol and phosphatidylcholine efflux from cells treated with 8-Br-cAMP.[106] Bortnick et al. later showed that treatment of J774 macrophages with 8-(4-chlorophenylthio)-adenosine-3`,5`-cyclic monophosphate (cpt-cAMP) caused an increase in ABCA1 mRNA levels and cholesterol efflux, which could not be observed with ABCA1-deficient cells.[107] Similar results were obtained by Oram and colleagues with RAW264 macrophages in response to treatment of the cells with 8-Br-cAMP.[108] The increase in the ABCA1 mRNA levels by the cAMP analogues suggested that the ABCA1 gene may be a direct target of cAMP. Relatively recently Le Goff and coworkers identified the cAMP response element (CRE) in the first intron of the ABCA1 gene in murine RAW264.7 macrophages and showed that the CRE binding protein (CREB) is required for cAMP-induced activation of transcription of the gene. Their data suggest that

activation of the protein kinase A (PKA) by cAMP leads to phosphorylation of CREB followed by activation of the ABCA1 transcription upon binding of the phosphorylated CREB to CRE.[109]

Gan et al. identified geranylgeranyl pyrophosphate (GGPP), a metabolite in the pathway of cholesterol biosynthesis, as a potent transcriptional deactivator of the ABCA1 gene in THP-1 and Caco-2 cells.[110] They found that GGPP exerts its inhibitory effect via two routes. It acts as an antagonist of LXR by directly associating with the nuclear receptor and thus preventing transcriptional activation of the ABCA1 gene. It also serves as a substrate for geranylgeranyl transferase, the enzyme which isoprenylates various regulatory proteins including Rho proteins. Activated Rho proteins then inhibit transcription of the ABCA1 gene possibly by interacting with LXR.

Polyunsaturated fatty acids (PUFAs) and oleic acid were also found to act as negative transcriptional regulators of ABCA1. Uehara and colleagues observed a decrease in ABCA1 mRNA and protein levels in response to PUFAs, resulting in reduction in apoA-I-mediated cholesterol efflux from human hepatocytes and murine macrophages. This effect could not be reproduced with saturated fatty acids. PUFAs were shown to combine with LXRα, thus preventing activation of the nuclear receptor by oxysterols and inhibiting transcriptional activation of the ABCA1 gene.[111]

In addition to being regulated on the level of transcription, ABCA1-mediated cholesterol efflux is affected by post-translational modifications of the transporter, such as phosphorylation. Controlled degradation is also important in regulating the activity of the transporter.[106,112-114] Haidar et al. provided evidence for the role of cAMP in regulating the activity of ABCA1.[112] They observed a dramatic increase in the levels of

phosphorylated ABCA1 augmenting apoA-I mediated cholesterol efflux upon treating human skin fibroblasts with 8-Br-cAMP. The effect was abolished when the activity of PKA was inhibited, suggesting a mechanism for activation of ABCA1 by PKA-mediated phosphorylation in response to cAMP.

Wang et al. reported that unsaturated fatty acids mediate degradation of ABCA1 in murine J774 and RAW264.7 macrophages, reducing the levels of ABCA1 in the plasma membrane and decreasing apoA-I-mediated cholesterol efflux.[113] Thus, like GGPP and cAMP, unsaturated fatty acids regulate ABCA1-mediated cholesterol efflux via more than one pathway.

Identification in the cytosolic portion of ABCA1 of the highly conserved 23 amino acid sequence (1283-1306) rich in proline, glutamic acid, serine, and threonine (PEST sequence), which mediates degradation of ABCA1 by calpain proteases, revealed yet another mode of regulation of the transporter.[113] This observation by Wang and colleagues, who also showed that in the presence of ApoA-I the calpain proteolysis of ABCA1 is inhibited, points to the intimate interaction between the cholesterol transporter and the apolipoprotein in cellular cholesterol efflux.

ABCA1 and atherosclerosis

Defects in ABCA1 in humans and animals cause abnormalities in lipid metabolism, accumulation of lipids in peripheral tissues, contribute to inflammation and progression of atherosclerosis. In humans, Tangier disease, characterized by severely low plasma HDL levels, accumulation of cholesteryl esters in peripheral tissues and macrophages, neuropathy, associated with accumulation of lipids in Schwann cells, hepatospleenomegaly, enlarged orange tonsils, and early onset of atherosclerosis, is a

recessive genetic disorder, caused by mutations in the ABCA1 gene.[57,115-120] Long before the transporter was discovered, studies showed that cells from patients with Tangier disease lack the ability to efflux cholesterol and phospholipids to apolipoprotein acceptors.[115-117] Later, genetic mapping and mutational analysis studies showed that patients with Tangier disease carry mutations in the ABCA1 gene, causing defects in the protein, thus demonstrating the pivotal role of the transporter in cholesterol efflux and lipid metabolism.[118-120]

Realization that mutations in the ABCA1 gene are the cause of Tangier disorder served as an incentive to studying the role of the transporter in lipid metabolism and atherosclerosis. In vitro studies involving cells with natural or induced ABCA1 deficiency as well as in vivo studies with ABCA1 knockout mice confirmed the importance of ABCA1 in maintaining proper lipid metabolism and protecting arteries against atherosclerosis.[121-127] Following the genetic studies which identified ABCA1 as the product of the gene mutated in patients with Tangier disease, Lawn and coworkers showed that the transporter is an important component in apolipoprotein-mediated cholesterol efflux and its expression is reduced when cellular cholesterol is depleted.[121] Aiello and colleagues investigated the effect of ABCA1 deficiency in $LDLr^{-/-}$ and $apoE^{-/-}$ mice, discovering that the absence of ABCA1 altered the plasma lipid profile and caused accumulation of cholesteryl ester-laden macrophages resulting in skin xanthomas.[122]

Since accumulation of lipids in macrophages is a hallmark of atherosclerosis, van Eck and colleagues investigated atherogenesis in $LDLr^{-/-}$ mice with leukocyte ABCA1 deficinency, discovering that the animals develop more advanced atherosclerosis than $LDLr^{-/-}$ control mice.[123] To better understand the role of ABCA1 in atherogenesis,

Albrecht et al. examined ABCA1 mRNA and protein levels in atherosclerotic tissues isolated from patients with arterial stenosis. Compared to the tissue with mild atherosclerosis, the tissue with advanced atherosclerosis had lower ABCA1 protein levels despite the fact that the mRNA levels were upregulated.[124] Thus, low expression of ABCA1 in atherosclerotic tissue may be one of the reasons contributing to the accumulation of cholesteryl esters in macrophages. Francone and colleagues assessed the lack of functional ABCA1 on the development of atherosclerosis in ABCA1-deficient mice. Macrophages isolated from the ABCA1-deficient animals had a greater content of cholesterol than normal macrophages in addition to having abnormal morphology and properties. Furthermore, the ABCA1-deficient mice displayed higher levels of inflammatory factors, such as IL-1β, the monocyte chemoattractant protein-1 (MCP-1), and the macrophage colony-stimulating factor (m-CSF), than the animals with a functional cholesterol transporter.[125] Demonstrating the atheroprotective role of ABCA1-mediated cholesterol efflux, Moore and colleagues found that deficiency of apoA in $LDLr^{-/-}$ mice results in reduced efflux of cholesterol from macrophages and decreased cholesterol transport.[126] Substantiating these results, van Eck and colleagues showed that overexpression of ABCA1 in $LDLr^{-/-}$ mice retards the development of atherosclerotic lesions.[127]

Role of LCAT in reverse cholesterol transport

Discussion of RCT would be incomplete without a more thorough description of the role of lecithin:cholesterol acyltransferase (LCAT) in this pathway. LCAT is a 416 amino acid, heavily glycosylated enzyme[128,129] produced mainly by the liver[130] and secreted in the plasma, where it circulates in association with HDL particles.[131] As

previously mentioned, LCAT transfers the fatty acyl group from the second position of the glycerol backbone of phosphatidylcholine to the hydroxyl group of cholesterol, forming cholesteryl ester and lysophosphatidylcholine[59] and reducing the content of free cholesterol in HDL. This enzymatic reaction plays an important role in the transformation of discoidal HDL into spherical, cholesteryl ester-rich particles, carrying esterified cholesterol to the liver for degradation. Reduction of free cholesterol content of the spherical HDL by LCAT favors additional efflux of free cholesterol from peripheral tissues via ABCA1-mediated pathway, thus facilitating RCT.[132,133.]

Davis and colleagues were among the first to provide evidence for the role of LCAT in removing cholesterol from extrahepatic tissues by observing that the LCAT inhibitor DNTB greatly inhibits esterification of cholesterol in the plasma of rats one hour after the removal of the liver.[134] Later Kostner et al. showed that the rate of clearance of cholesterol from the plasma correlates with the rate of its esterification by LCAT, highlighting the role of the enzyme in cholesterol metabolism.[135] Further studies involving subjects with complete or partial LCAT deficiency revealed abnormalities in the plasma lipid and apolipoprotein profiles, characterized by extremely low levels of large spherical HDL particles, the prevalence of lipid-poor discoidal HDL, and accelerated ApoA-I catabolism.[136,137] The tendency toward premature development of atherosclerosis in these subjects was also observed, although no conclusive data exist due to a small number of the cases examined.[137-139] Conversely, overexpression of human LCAT in transgenic mice enhances the rate of RCT, causing high levels of plasma HDL cholesterol, the prevalence of large spherical HDL particles, and enhanced clearance of plasma cholesterol by the liver.[132,139]

Regulation of LCAT is under intense investigation and relatively little is known in this area. Binding of LCAT to the surface of an HDL particle was found to be dependent on the concentration of phospholipids in the particle, with phosphatidylserine enhancing[140] and sphingomyelin inhibiting[141] the association of the enzyme with HDL. Activity of the enzyme was shown to be regulated by the metabolites of sphingomyelin, ceramide and ceramide phosphate, with the former activating and the latter inhibiting the enzymatic activity relative to sphingomyelin.[142] ApoA-I was found to be necessary for full activity of LCAT. Studies with synthetic peptides with sequence similarity to apoA-I and mutated apoA-I showed that several peptide sequences of ApoA-I play an important role in activating LCAT.[143,144]

Cytokines and fibrates were identified as transcriptional regulators of the LCAT gene,[145-147] while growth factors and butyrate were shown to regulate the gene on a post-transcriptional level.[146,148] The derivatives of fibric acid, fenofibrate, gemifibrozil, and clofibrate, were shown to reduce LCAT mRNA levels in hepatocytes, resulting in lowered plasma LCAT activity.[145] Treatment of human hepatoma HepG2 cells with interleukin-6 (IL-6), an inflammatory cytokine, produced an increase in LCAT mRNA levels.[146] This observation was explained later by the discovery of the IL-6 response element on the LCAT gene, spanning the sequence from -1514 to -1508 bp.[147] Transforming growth factor β (TGF-β) and sodium butyrate regulate expression of the LCAT gene on a post-transcriptional level by inducing mRNA degradation.[146,148] Regulation of the LCAT gene by inflammatory cytokines and the ligands of PPARγ points to the role of the gene in atherosclerosis.

Efflux of cholesterol via aqueous diffusion

Active unidirectional flux of cholesterol from the plasma membrane to cholesterol acceptors is an essential mechanism of cholesterol removal from peripheral tissues and thus an important component of RCT, but it is not the only mechanism responsible for cellular cholesterol efflux. Transport of cholesterol occurs via two additional pathways. SR-BI mediates bidirectional flux of cholesterol between cells and lipoproteins by interacting with a wide range of apolipoproteins. Unlike SR-BI-mediated pathway, efflux of cholesterol by passive diffusion does not require lipoprotein particles and can occur in the presence of a suitable cholesterol acceptor, such as phospholipid liposomes, phospholipid-BSA complexes, HDL particles, and cyclodextrin.[91,149-152] Efflux of cholesterol by passive diffusion is considered here.

While ABCA1-mediated efflux is a unidirectional gradient-independent process requiring ATP, passive flux of cholesterol via aqueous diffusion is bidirectional, with the net flux of cholesterol dependent on the concentration of the molecule in the donor and acceptor.[149.150] The term 'aqueous diffusion' stems from the nature of the process, which involves desorption of cholesterol molecules from the membrane, their passage through a layer of unstirred aqueous medium, such as plasma, and solubilization in the lipid substrate of the acceptor particle.[149]

Many studies have provided evidence for the exchange of cholesterol between synthetic phospholipid vesicles and between the vesicles and the cell plasma membrane. McLean and colleagues investigated the transfer of cholesterol between liposomes composed of phosphatidylcholine and cholesterol, using ^{3}H-cholesterol and ^{14}C-phosphatidylcholine.[151] Their data indicate that the rate of cholesterol transfer is six times that of phosphatidylcholine and that the exchange involves the movement of the substrate

through aqueous medium. The work by Bruckdorfer et al. supported these results, showing that the exchange of cholesterol between lipid membranes occurs even when the donor and acceptor are separated by a dialysis membrane.[152] Slotte and colleagues investigated the exchange of cholesterol between phosphatidylcholine vesicles and rat arterial SMCs, showing that the transfer occurs by aqueous diffusion and is enhanced in the presence of BSA.[153] Bartholow and colleagues examined cholesterol efflux from mouse fibroblasts into the medium containing structurally distinct phospholipids, human serum albumin (HSA), or phospholipid-HSA complexes.[154] The results of their studies indicate that the phospholipid-albumin complexes are more efficient acceptors of cholesterol than phospholipids and that the degree of unsaturation, charge, and length of fatty acyl chains of phospholipids affect the efficiency of efflux.

Like phospholipid liposomes, spherical lipid-rich HDL particles contain phospholipids at the particle-medium interface and are acceptors of cholesterol via aqueous diffusion. The flux of cholesterol is bidirectional with the concentrations of cholesterol in the HDL particle and the plasma membrane determining the direction of net transfer.[91,149,150,155-158] Mature HDL particles in the vicinity of cholesterol-rich domains of the plasma membrane are capable of accepting additional cholesterol. An important role in this process is played by LCAT, which reduces the amount of free cholesterol present in HDL and thus shifts the equilibrium toward further efflux of cellular cholesterol.[150,155-158]

A study by Mendez and colleagues clearly demonstrated the ability of HDL particles to mediate cholesterol efflux via both passive diffusion and the active energy-dependent process. They investigated cholesterol efflux from cultured human skin

fibroblasts to a series of acceptors, demonstrating that while apoA-I mediates cholesterol efflux in an energy-dependent manner, phospholipid vesicles and trypsinized HDL, lacking functional apoA-I but having an intact phospholipid monolayer, are acceptors of cholesterol via aqueous diffusion. Normal HDL was shown to accept cholesterol via both mechanisms.[91]

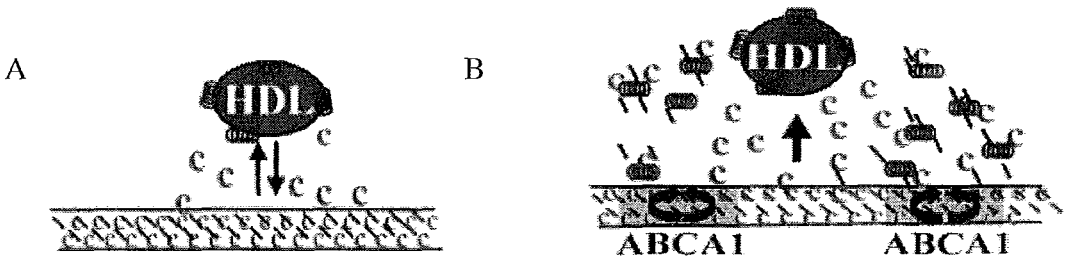


Figure 10. Schematic representation of cholesterol efflux via aqueous diffiusion (A) and the ABCA1-dependent pathway (B) to HDL particles.[149] c = cholesterol, solenoid = apoA-I, \ = phospholipid. Diffusion of cholesterol through an aqueous layer to the phospholipid monolayer of HDL is concentration-dependent. The lipidation of apoA-I in ABCA1-mediated cholesterol efflux is an energy-driven process.

Research goal 1

Effect of LPL overexpression on ABCA1-mediated cholesterol efflux in cultured human skin fibroblasts.

As described above, both LPL and ABCA1 play an essential role in atherogenesis. Whether LPL influences the activity of the transporter, however, has not been investigated. Current knowledge regarding the regulation of ABCA1 suggests that LPL may indirectly influence transcriptional regulation of the ABCA1 gene. Hydrolytic activity of LPL releases fatty acids, some of which were shown to be ligands for PPARs.[159,160] Arachidonic acid can activate PPARγ directly[159] and serves as a precursor for 15-deoxy-12,14 Δ-prostaglandin-J2 (15d-PGJ_2), which was shown to be a potent activator of PPARγ.[162] Fatty acids are also activators of PPARα.[95,101,159,160] Both PPARγ

and PPARα are transcriptional activators of the LXRα gene, whose protein product, in turn, induces transcription of the ABCA1 gene.[102-105] Products of hydrolytic activity of LPL, therefore, can act as transcriptional regulators of the ABCA1 gene (Figure 11). Fatty acids, however, were also shown to antagonize the stimulatory effect of oxysterols on LXRα,[111] suggesting that LPL may also inhibit transcription of the ABCA1 gene. Moreover, like the LXRα gene, the LPL gene contains PPRE and thus can be induced by PPARγ.[163]

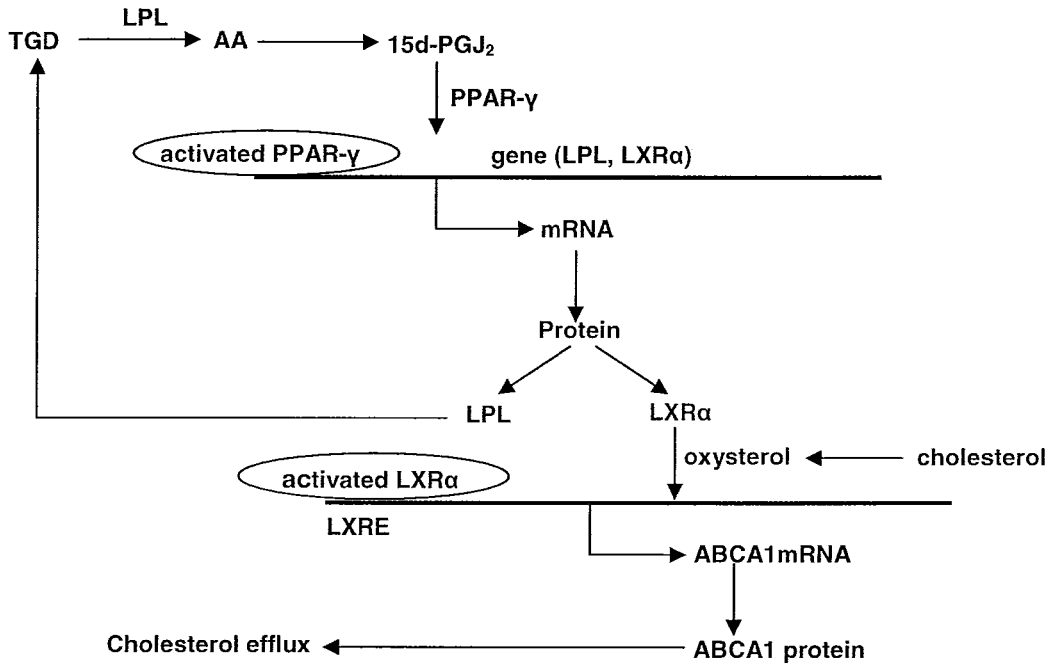


Figure 11. Diagram explaining the effect of LPL on ABCA1-mediated cholesterol efflux. Arachidonic acid (AA) generated by LPL is a precursor for 15d-PGJ_2, an activator of PPARγ. PPARγ activates transcription of LPL and LXRα. The latter stimulates transcription of the ABCA1 gene, resulting in potentiation of ABCA1-mediated cholesterol efflux.

To investigate the effect of LPL on ABCA1-dependent efflux of cholesterol we expressed the enzyme in human skin fibroblasts and examined ABCA1 mRNA and protein levels as well as efflux of cholesterol to apolipoprotein E, which is similar in

structure to apoA-I and accepts cholesterol in ABCA1-dependent cholesterol transport.[42] Overexpression of LPL produced an increase in ABCA1 mRNA and protein levels in the presence of 8-Br-cAMP and enhanced ABCA1-dependent cholesterol efflux. Our data suggest that LPL may regulate ABCA1-dependent cholesterol efflux in vivo, prompting further investigation in this area.

Research goal 2

Effect of phospholipids on ABCA1 mRNA and protein levels and ABCA1-mediated cholesterol efflux.

The role of phospholipids in cellular cholesterol efflux via both the ABCA1-mediated pathway and aqueous diffusion is well established. As discussed earlier, efflux of cellular cholesterol to phosholipid liposomes and trypsinized HDL particles is well documented.[91,152] Incubation of cells with phospholipid vesicles causes partial depletion of the cellular pools of cholesterol via aqueous diffusion.[91,150] Phospholipids are the primary substrate of ABCA1 and were shown to be essential cofactors in ABCA1-mediated transport of cellular cholesterol.[67]

Phospholipids were also shown to generate signaling molecules influencing ABCA1-mediated cholesterol efflux. Thus phosphatidic acid increased while lysophosphatidic acid decreased ABCA1-mediated cholesterol transport in normal fibroblasts and fibroblasts with partial ABCA1 deficiency, suggesting that phospholipases may be important in ABCA1-mediated cholesterol translocation.[164]

While it was shown that incubation of various cell lines with phospholipid vesicles results in enhanced efflux of cellular cholesterol, the effect of phospholipids on ABCA1 mRNA and protein levels after incubating cells with phospholipids for extended

periods of time has not been investigated. To our knowledge no published data exist as to the effect of phospholipids on ABCA1-mediated cholesterol efflux measured after incubating cells with phospholipids.

Here we show that incubation of cholesterol-loaded human skin fibroblasts with phosphatidylcholine for a period of 24 hours in the absence of apoA-I causes a decrease in ABCA1 mRNA and protein levels. Subsequent incubation of the treated cells with apoA-I for a period of 24 hours revealed that ABCA1-mediated cholesterol efflux is reduced compared to the efflux of cholesterol from the control fibroblasts. Our data suggest that phospholipids are capable of reducing ABCA1 mRNA and protein levels by serving as acceptors of cellular cholesterol and delpleting the plasma membrane cholesterol. Reduced ABCA1 mRNA levels caused by low levels of cholesterol translate into lower levels of the transporter in the plasma membrane, resulting in reduced ABCA1-mediated cholesterol efflux.

In conjunction with investigating the effect of phospholipids on ABCA1, we found that cholesterol-loading of fibroblasts and treatment of the cells with phosphatidylcholine reduces LCAT mRNA levels, with the latter more so than the former. Considering that both cholesterol and PC are substrates of LCAT, these results are intriguing and point to a possible cellular signaling pathway responding to the cellular levels of cholesterol and phospholipids and regulating LCAT on a transcriptional level.

MATERIALS AND METHODS

THP-1 cells

THP-1 cells are human monocytes derived from the blood of a one-year old male donor with acute monocytic leukemia. The cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and kept frozen in liquid nitrogen in a medium consisting of 10% dimethylsulfoxide (DMSO) and 90% fetal bovine serum (FBS) (Atlanta Biologicals).

To culture the cells, 1 ml of the cell-containing medium was thawed at 37°C, transferred into a 15-ml polystyrene conical tube, mixed with 10 ml of warm (37°C) phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4, filtered through a 0.22μm filter), and centrifuged for 5 minutes at 800 rpm to pellet the cells. The supernatant was aspirated and the cell pellet was mixed with THP-1 cell growth medium, consisting of RPMI 1640 medium (Cellgro) supplemented with 10% FBS, 2.5g/L glucose, 50 units/ml penicillin, 50μg/ml streptomycin, 10mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), 2mM glutamine, 1mM sodium pyruvate, and 50μM β-mercaptoethanol. The cells were grown in 75cm^2 polystyrene cell culture flasks in a humidified incubator at 37°C and 5% CO_2. The cells were counted under the light microscope using a hemacytometer (Reichert) and Trypan Blue dye. 400 μl of the cell-containing medium were mixed with 100 μl of Trypan Blue dye, and the number of live cells was determined by adding a small volume of the mixture onto the hemacytometer. Live cells exclude the dye, whereas dead cells acquire blue color and can be easily distinguished from the live cells. When a desired cell density was reached, the cells were centrifuged as described above, the medium was aspirated,

and the cell pellet was mixed with THP-1 cell differentiation medium similar in composition to the growth medium but containing 100ng/ml of the differentiating agent phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) and 1mg/ml BSA instead of FBS. The cells were seeded into either 6-well or 12-well tissue culture plates in the differentiation medium at the density of about 10^6 cells/ml and were allowed to differentiate into macrophages for 24 hours. THP-1 cell differentiation medium was aspirated and 1 or 2 ml of THP-1 cell growth medium was added to each well. All treatments involved differentiated cells maintained in THP-1 cell growth medium.

FSF

Normal human foreskin fibroblasts (FSF) (GM04390) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (NIGMS). They are derived from the dermis of the neonatal foreskin and are capable of dividing at least sixteen times. The cells were kept frozen under the same conditions as THP-1 cells and grown in FSF growth medium composed of Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro) supplemented with 10% FBS, 2mM glutamine, 50units/ml penicillin, 50μg/ml streptomycin, and 10mM HEPES in 75cm^2 polystyrene cell culture flasks in a humidified incubator at 37°C and 5% CO_2.

When FSF reached confluence, the medium was aspirated, the cells were washed three times with 1 ml/well of warm sterile PBS and detached from the surface by trypsinization. The cells were bathed for 10 seconds in 1 ml of 1X trypsin-EDTA reagent (0.05% trypsin, 0.53mM EDTA) (Cellgro), the solution was aspirated, and the culture flask was kept for 3 minutes at 37°C. Fresh DMEM (FBS) was added to the flask, the contents were mixed by pipetting to ensure even distribution of the cells in the medium,

and the cells were seeded into 12-well cell culture plates (1 or 2 ml/well). On reaching confluence, fibroblasts were washed twice with 1 ml/well of warm sterile PBS to remove growth factors and maintained in FSF growth arrest medium similar in composition to DMEM (FBS) but containing 1mg/ml BSA instead of FBS. All treatments involved confluent fibroblasts maintained in DMEM (BSA).

RNA isolation

TRI reagent (Sigma) affords a versatile and widely used method for fast and convenient isolation of DNA, RNA, and protein from a variety of biological samples. The reagent is a monophase solution of phenol and guanidine thiocyanate, which effectively lyses the cells and dissolves cellular components, forming a homogeneous solution. Upon addition of chloroform, protein is partitioned into the organic phase, while the aqueous phase contains RNA, with DNA present at the interphase. RNA is precipitated from the aqueous phase by adding isopropanol.

To isolate total RNA from either THP-1 cells or FSF cells, the cells were chilled on ice, the medium was aspirated, the cells were washed twice with cold (4°C) PBS, and 400 µl of TRI reagent were added to each well. The plates were kept at 4°C for 20 minutes, the wells were scraped to detach the cells from the surface, and the homogenate was transferred into 2-ml eppendorf tubes. The samples were either stored at -80°C for up to a week or used on the same day to purify RNA. 200µl of chloroform per 1 ml of TRI reagent were added to each tube, the samples were vortexed vigorously for 20 seconds and allowed to stand at room temperature for 10 minutes. The samples were centrifuged for fifteen minutes at 14,000 rpm and 4°C, and the clear aqueous phase (about 550 µl per 1 ml of TRI reagent) was removed into 1.5-ml conical eppendorf tubes. After addition of

500 µl of isopropanol per 1 ml of TRI reagent and mild vortexing, the samples were allowed to stand at room temperature for 10 minutes and were centrifuged for 10 minutes at 14,000 rpm and 4°C. The supernatant was decanted and 1 ml of 75% ethanol per 1 ml of TRI reagent was added to each pellet. The pellets were washed by brief vortexing and precipitated by centrifugation for 5 minutes at 14,000 rpm and 4°C. Ethanol was removed, the pellets were allowed to air-dry for up to 5 minutes until somewhat transparent and were dissolved in 20 to 50 µl (depending on the size of the pellet) of autoclaved double-deionized water by pipetting and incubating the samples for 10 to 15 minutes at 55-60°C. The samples were spun briefly at 13,000 rpm to precipitate the condensate and stored at 4°C.

RNA was quantitated by diluting 3 µl of the RNA solution hundred-fold with autoclaved water and measuring the absorbance of the solution at 260 and 280 nm, using the Spectronic Genesys™ 5 spectrophotometer. The concentration of RNA was calculated using the formula: [RNA] (µg/ml) = A_{260} x 40µg/ml x 100, where 40µg/ml corresponds to 1 unit of absorbance at 260 nm, and 100 is a dilution factor. Since aromatic amino acids absorb at 280 nm, the A_{260} to A_{280} ratio was used to assess the extent of contamination of the RNA preparation with protein.

RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a two-reaction method designed to amplify any segment of RNA whose partial nucleotide sequence is known, using as a starting material total RNA isolated from a biological sample. First, total RNA is converted to complimentary DNA (cDNA), using reverse transciptase and random hexamers in a process called reverse transcription. Then, a sequence of interest is

amplified by PCR, using a thermostable DNA polymerase and sequence-specific primers, to produce a desired number of copies.

Depending on the yield of RNA, 0.25 to 2 μg of RNA were mixed with 1μg of random hexamers (Promega) in a sterile 0.6-ml eppendorf tube and the final volume was adjusted to 15μl with double-deioized autoclaved water. The samples were heated to 70°C for five minutes, cooled on ice for five minutes, and spun for twenty seconds at 13,000 rpm and room temperature to precipitate the condensate. 12 μl of the solution prepared by mixing autoclaved water, 5X Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) reaction buffer (Promega), dNTP mixture (Promega), and lastly M-MLV RT (Promega), were combined with the 15 μl of the RNA-random hexamer mixture. The final concentration of the components in the 27 μl of the 1X M-MLV RT reaction buffer was the following: RNA (9.25 – 74ng/μl), random hexamers (37ng/μl), dNTPs (0.44mM), and M-MLV RT (9 u/μl). The reagents were mixed, spun briefly, and incubated at 25°C for 10 minutes followed by incubation at 42°C for 50 minutes in the Perkin Elmer DNA thermal cycler. The resulting solution of a single copy of cDNA was stored at 4°C and used as a template for amplification of the sequences of interest using PCR.

PCR mixture was prepared by mixing in a 1.5-ml sterile eppendorf tube, in the following order, double-deionized autoclaved water, 10X Thermophylic DNA polymerase buffer (supplied by Promega along with *Taq* polymerase), $MgCl_2$ buffer (Promega), dNTP mixture, sense and anti-sense primers (shown in the table below) (supplied by Invitrogen in lyophylised form and diluted to 50μM concentration with Tris-EDTA buffer (5:1 (v/v) 1M Tris-HCl:1M EDTA), and *Taq* polymerase. The mixture was

mixed by pipetting, spun briefly, and 20μl of the mixture were combined with 5μl of the cDNA solution in a sterile 0.6-ml eppendorf tube. The final concentration of the components in 1X Thermophylic DNA polymerase buffer was as follows: sense and anti-sense sequence-specific primers (1.9 μM each), dNTPs (380μM), $MgCl_2$ (1.9mM), and *Taq* polymerase (0.19 u/μl). The solution was covered with 2 drops of mineral oil to prevent evaporation of the reaction mixture during the reaction. The samples were first incubated at 94°C for 10 minutes and then subjected to amplification with a variable number of cylces (5 to 40, depending on the sequence being amplified and the amount of RNA used in RT), with each cycle involving the incubation at 94°C for 1 minute, followed by the incubation at the annealing temperature (primer-dependent) for 1 minute, and at 72°C for 1 minute. The solutions of amplified sequences were stored at 4°C.

Amplicon and its size (bp)	Primer type	Tm (°C)	Ta (°C)	Primer sequence
LPL 742	Sense	61	48	5'GGTTTATCAACTGGATGGAGGAGG3'
	Anti-sense	64		5'GGAAACTTCAGGCAGAGTGAATGGG3'
LPL 515	Sense	45	48	5'ATGGAGAGCAAAGCC3'
	Anti-sense	56		5'GTCAGACTTCCTGCAATGCC3'
ABCA1 377	Sense	62	52	5'CCCGAATGTCATCAACAATGCC3'
	Anti-sense	53		5'GAGGACACATAGGACTTCTGC3'
β-actin 285	Sense	64	60	5'TCATGAAGTGTGACGTTGACATCCGT3'
	Anti-sense	67		5'CTTAGAAGCATTTGCGGTGCACGATG3'
LXR-α 677	Sense	57	56	5'GAAGAAACTGAAGCGGCAAG3'
	Anti-sense	58		5'TAGGATCCGTGGGAACATCA3'
PPAR-γ	Sense	63	52	5'CCACTATGGACTTCATGCTTGTGAAGG3'
	Anti-sense	67		5'TGCAGGGGGGTGATATGTTTGAACTTG3'
LCAT 375	Sense	55	50	5'CTTGGCCTCAGGTGACAAC3'
	Anti-sense	54		5'CGTGTCATCACCATCCTCAT3'

Table 1. Sequence-specific primers.

Some of the sequences were amplified using 2X PCR Master Mix (GoTaq Green Master Mix, Promega) containing all necessary components for PCR except cDNA and the primers. 5 µl of the cDNA solution, 5.5µl of nuclease free water (Promega), 25µM stock solution of the sense and antisense primers (1µl each), and 12.5 µl of the GoTaq Master mix were mixed in a 0.6-ml eppendorf tube. 2 drops of mineral oil were added to each tube and the amplification was performed as described above. PCR products were stored at 4°C.

PCR products were resolved using agarose gel electrophoresis in TAE buffer (400mM Tris-Acetate, 1mM EDTA) at 80 volts. 2% agarose gel containing ethidium bromide was prepared by dissolving agarose (molecular biology grade) (Fisher Scientific) in TAE buffer and adding 1.6 µg/ml of ethidium bromide (Invitrogen). The dye intercalates between the bases of the double-stranded nucleic acids and fluoresces with bright yellow to orange glow (depending on the amount bound) upon illumination with ultraviolet light, allowing for visualization of the bands. The size of the DNA in the bands was confirmed by comparing them to the standards of the 100 bp DNA ladder (Promega) run next to the sample lanes. The gel was placed onto the UV Transilluminator (UVP, model M – 26) and photographed with the photo-documentation camera (FB-PDC-34) (Fisher Scientific). The photographs were scanned and the relative amount of DNA in each band was determined by quantitating the intensity of the bands, using Adobe Photoshop 7.0 and ImageJ software. In some cases, where the intensity of the bands was low, the intensity contributed by the background was taken into account by subtracting the intensity due to the background from the intensity of each band. To confirm that the observed differences in the amounts of selected transcripts were gene

specific, a segment of the housekeeping gene, β-actin, was amplified via RT-PCR and resolved using agarose gel electrophoresis. The intensity of the β-actin bands was measured and the intenisity of the bands of the sequence of interest was divided by that of the β-actin bands. The ratio from the experimental control was expressed as 100% and other ratios were compared to the control.

Western blot analysis of the LPL protein levels

Secretion of LPL into the cell culture medium by LPL-overexpressing fibroblasts was confirmed by immunoprecipitating LPL from the medium using sepharose derivitized with anti-LPL immunoglobulin and analyzing its levels by western blotting.

The medium was collected into 15-ml polystyrene conical tubes, mixed with glycerol (20%) and Triton X100 (0.1%) and either stored at -20°C or used on the same day to isolate LPL. Sepharose derivatized with the anti-LPL antibody (Anti-LPL IgY Sepharose, Chicken # 1, Pool 2, in PBS with 0.02% azide, produced in Dr. Medh's lab) was used to immunoprecipitate LPL from 2.5 ml of the medium. 250μl of the sepharose-IgY suspension were mixed with 1 ml of immunoprecipitation buffer (10mM Na_2HP0_4, 10mM NaH_2PO_4, 20% glycerol, 0.5% Triton X100, 0.5M NaCl), centrifuged at 13,000 rpm for 20 seconds, and the supernatant was aspirated. The washing of the sepharose-IgY was repeated twice, and 300μl of immunoprecipitation buffer were added to the pellet. After thorough mixing of the suspension, its volume was estimated and it was distributed equally among the tubes containing the medium of the LPL-overxpressing fibroblasts and that of the control cells. The samples were incubated at room temperature for 3 hours with rocking and centrifuged at 1000 rpm for 2 minutes at room temperature. The supernatant was aspirated, and 5 ml of immunoprecipitation buffer were added to the

pellet in each tube. The samples were vortexed briefly, centrifuged at 1000 rpm for 2 minutes, and the supernatant was aspirated. The washing was repeated four times, after which 200 µl of 2X reducing buffer (200mM DL-dithiothreitol in 2X sample buffer (0.04 mg/ml SDS, 125mM Tris (pH 6.8), 0.2 ml/ml of glycerol, and 3 mg of bromophenol blue)) were mixed with the pellet. The samples were stored at -20°C or resolved using sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) on the same day.

The samples were centrifuged at 1000 rpm for 2 minutes to precipitate the LPL bound to the sepharose-IgY, and the supernatant was removed into 1.5-ml eppendorf tubes. The protein contained in 35µl of the solution was resolved under denaturing conditions along with 7 to 10 µl of low and high range pre-stained SDS-PAGE standards (Bio-Rad), using SDS-PAGE in electrode buffer (25mM Tris, 192mM glycine, 0.1% SDS) at 200 volts. 7.5% resolving polyacrylamide gel was prepared by mixing 4.5 ml of double-deionized water (ddH_20), 2.5 ml of 1.5M Tris-HCl, 2.5 ml of 29.2% acrylamide (Acros Organics) – 0.8% N,N'-methylenebisacrylamide (Eastman) solution, 100 µl of 10% (w/v) SDS, 80 µl of 10% ammonium persulfate (free radical polymerization intitiator), and 10 µl of N,N,N',N'-tetramethylenediamine (TEMED) (catalyst of free radical formation from ammonium persulfate). 3.8 % stacking gel, designed to ensure stacking of the protein and its simultaneous entry into the resolving gel, was prepared by mixing 6.1 ml of ddH_2O, 2.5 ml of 0.5M Tris-HCl, 1.33 ml of 29.2% acrylamide – 0.8% N,N'-methylenebisacrylamide, 100 µl of 10% (w/v) SDS, 10 µl of TEMED, and 80 µl of 10% ammonium persulfate, and poured onto the polymerized resolving gel. After electrophoresis, the stacking gel was separated from the resolving gel and the latter was

washed in transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol, pH 8.6) to remove pieces of polyacrylamide. The resolved protein was transferred onto the polyvinylidine fluoride membrane (Immobilon^{TM}-P, cat. no. IPVH00010) in transfer buffer at 30 volts overnight or at 100 volts for 1 hour. The blot was incubated in blocking buffer (20mM Tris, 150mM NaCl, 2mM $CaCl_2$, 1% gelatin, 0.05% TWEEN-20, pH 7.4) overnight at room temperature to block nonspecific binding sites on the membrane and washed twice for 5 minutes each time in 1X blot wash buffer (20mM Tris, 150mM NaCl, 2mM $CaCl_2$, pH 7.4) It was then incubated in blocking buffer containing the primary antibody (Anti-bovine LpL IgY Chicken #2, 2nd pool (5μg/ml), produced in Dr. Medh's lab) for 2 hours at room temperature. After washing the blot 4 times for 5 minutes each time in 1X blot wash buffer, it was incubated in blocking buffer containing the secondary antibody prepared by diluting the stock antibody solution (Peroxidase-conjugated AffiniPure Rabbit Anti-Chicken IgY (IgG) (H+L), Jackson ImmunoResearch Laboratories, Inc.) 25,000-fold in blocking buffer. The blot was washed 5 times in 1X blot wash buffer, incubated for 8 minutes in 10 ml of 1:1 peroxide solution:enhancer solution from SuperSignal West Pico Mouse IgG Detection Kit (Pierce), and exposed to the X-ray film (CL-X PosureTM Film, 20 x 25 cm, Pierce) for 1, 5, 30 seconds, and 2 minutes. The X-ray film was developed using the Kodak, RP X-OMAT Processor, and the bands were scanned and analyzed using Adobe Photoshop 7.0 and ImageJ software.

Quantitation of cellular protein and western blot analysis of ABCA1 protein levels

Confluent fibroblasts cultured in DMEM (BSA) in 12-well cell culture plates were chilled on ice, the medium was aspirated, and the cells were washed twice with cold (4°C) PBS. 0.5 ml of PBS was added to each well, the cells were scraped into the buffer,

and the solution from six wells subjected to equal treatment was transferred into a 15-ml polystyrene conical tubes. The procedure was repeated to completely remove the cells, and the combined cell extract was centrifuged at 800 rpm for 5 minutes at room temperature. The supernatant was aspirated and 50 µl of the cell lysis buffer (50mM Tris-HCl, 2mM $CaCl_2$, 0.1% Triton X-100) containing the serine protease inhibitors, aprotinin (19 µg/ml), benzamidine (1mM), and PMSF (1mM), was added to the cell pellet. The content of each conical tube was mixed by pipetting, transferred into 0.6-ml eppendorf tubes, and either stored at -20°C for up to a week or used on the same day to quantitate the protein.

The samples were centrifuged at 13,000 rpm for 1 minute at room temperature to precipitate cellular debris, the supernatant was transferred into 0.6-ml eppendorf tubes, and the pellets were stored at -20°C. 6 to 10 µl of the supernatant (depending on the size of the pellet) were used for protein quantitation by the method of Lowry.

The method involves the formation of coordination complexes between peptides and Cu^{2+}, which binds amide nitrogen atoms, forming a reducing complex. Upon addition of the Folin & Ciocalteu's phenol reagent, phosphotungstic acid and phosphomolybdic acid contained in the reagent are reduced by the Cu^{2+}-peptide complex, forming chromogens, and the solution acquires blue color with the absorbance peak at 660 nm. The intensity of the color is directly related to the amount of the protein present in the sample as Cu^{2+} is present in excess and the protein is the limiting reagent. The absorbance of a series of standards each containing a known amount of BSA is measured with the purpose of generating a standard curve relating the absorbance to the protein concentration. The absorbance of the sample solution is measured and the amount of

protein in the sample is determined based on the equation of the line obtained from the standard curve.

The BSA standard curve was generated by preparing a series of dilutions of a 0.50 mg/ml solution of BSA in water. 5, 10, 20, 40, 80, 120, 160, and 200 µl of the BSA solution and 6 to 10 µl of the sample solution were diluted to 200µl using autoclaved water. 2 ml of the Lowry reagent, prepared by mixing 25 ml of the reagent A (2% Na_2CO_3 in 0.1M NaOH), 0.25 ml of 1% $CuSO_4$, and 0.25 ml of 2% sodium-potassium tartarate, were added to the BSA and sample dilutions. The contents of each test tube were mixed and kept at room temperature for ten minutes. 100 µl of the Folin & Ciocalteu's phenol reagent (Sigma) were mixed with the protein solutions, the contents of each tube were briefly vortexed and incubated at 50°C for ten minutes. The solutions were allowed to cool to room temperature and the absorbance was measured at 660 nm. All measurements were performed in duplicates. The amount of protein in the samples was determined from the equation of the best fit line with the y-intercept set to 0, obtained by plotting average absorbance values obtained from duplicates versus the amount of BSA.

Volumes of the sample solutions containing equal amounts of protein were mixed with ¼ the volume of 5X sample buffer (0.1 mg/ml SDS, 312mM Tris (pH 6.8), 0.5 ml/ml of glycerol, and 3 mg of bromophenol blue) and centrifuged briefly. The protein was resolved under denaturing conditions in 7.0% polyacrylamide gel along with low and high range pre-stained SDS-PAGE standards. Western blot analysis of ABCA1 protein levels was performed according to the protocol outlined above with the following changes. The anti-ABCA1 primary antibody (anti-ABCA1 NB 400-105 Lot K1 rabbit

sera Novus Biologicals (5μg/ml)) and goat-anti-rabbit secondary antibody (Peroxidase-conjugated ImmunoPure Goat Anti-Rabbit IgG (H+L), Jackson ImmunoResearch Laboratories, Inc.) were used. The X-ray film was exposed to the blot for 5, 3, 1, and 0.5 minutes. The bands were analyzed as described earlier.

After the exposure to the X-ray film, the membrane was incubated in 40 ml of stripping buffer (0.2M glycine, 0.5M NaCl, pH 2.8) for 5 minutes to remove the antibodies. 4 ml of 1M Tris-HCl (pH 8.0) were added to the stripping buffer, the membrane was incubated in the solution for several minutes to neutralize the acid, and the blot was washed twice in ddH_2O. Western blot analysis of β-actin protein levels was performed as described above starting at the blocking step. The blot was incubated with the anti-actin primary antibody (actin (C-11)-R sc-1615-R Lot #A0506 rabit polyclonal IgG (1.5μg/ml), Santa Cruz Biotechnology) and the goat-anti-rabbit secondary antibody used in the analysis of ABCA1 protein levels.

Transfection of the LPL gene into THP-1 cells

THP-1 cells were seeded into 6-well cell culture plates in THP-1 cell differentiation medium at the density of about 10^6 cells/ml. After incubating the cells for 24 hours, the medium was aspirated, the cells were washed twice with 1 ml of warm PBS, and THP-1 cell growth medium containing either the LPL gene-carrying adenovirus (Ad5RSVLpLwt6115) or the LacZ gene-carrying adenovirus Ad5RSVntLacZ was added to each well. Stock solutions of adLPL (2.9×10^{12} viral particles/ml) and adLacZ (1.4×10^{12} viral particles/ml) were stored at -80°C and added either directly to the cell culture medium in individual wells or first mixed with the medium to prepare a common solution containing 7×10^9 viral particles/ml, which was then transferred to the wells. The volume

of the viral stock solution used per ml of the medium was determined by assuming that approximately 70% of seeded monocytes differentiate into macrophages and only one in every one hundred viral particles is infectious. Multiplicity of infection (MOI), the ratio of the infecting viral particles to the number of cells, was set to 100, and the volume of the viral stock was calculated according to the formula: $V_{viral\ stock}$ = MOI x ($N_{cells/well}/N_{pfu/ml}$), where $N_{cells/well}$ is the number of differentiated cells in each well and $N_{pfu/ml}$ is the number of the plaque forming units (infectious viral particles) in the viral stock solution. The cells were incubated with the adenovirus for twenty four hours, total RNA was extracted using the TRI reagent-chloroform method, and the levels of LPL mRNA were analysed by RT-PCR.

Transfection of the LPL gene into FSF

Fibroblasts were seeded into 12-well plates in DMEM (FBS) and grown to confluence. The medium was aspirated, the cells were washed twice with 1ml of warm PBS, and 1 ml of DMEM (BSA) containing the adenovirus (7 x 10^9 viral particles/ml) was added to each well. The cells were incubated for 24 hours, total RNA was extracted, and a segment of the LPL RNA was amplified using RT-PCR.

To investigate the effect of the LPL overexpression on ABCA1 mRNA and protein levels, confluent fibroblasts were treated with the adenovirus in DMEM (BSA) in the presence or absence of 8-Br-cAMP (0.5mM), and mRNA and protein levels of ABCA1 were anlalyzed by RT-PCR and western blotting, respectively. A 50mM stock solution of 8-Br-cAMP was prepared by dissolving the solid (8-bromoadenosine 3', 5'-cyclic monophosphate sodium salt, 97% purity, Sigma) in autoclaved water and stored at -20°C. The stock solutions of 8-Br-cAMP, adLPL, and adLacZ were either added directly

to the wells or first dissolved in the medium, which was then transferred to the wells. The medium was collected to immunoprecipitate LPL, and the cells were harvested in TRI reagent to purify RNA. A segment of the ABCA1 mRNA was amplified using RT-PCR. Alternatively, fibroblasts were scraped into PBS and the cells were lysed to isolate protein for western blot analysis of ABCA1 protein levels.

Cholesterol efflux assays involving the LPL-overexpressing FSF

FSF were seeded into 12-well plates and cultured in DMEM (FBS) to 50% confluence. Upon reaching 50% confluence, DMEM (FBS) was aspirated and 1ml of DMEM (BSA) containing 1μCi/ml of radioactively labeled cholesterol ([1α,2α(n)-^{3}H]cholesterol in toluene solution, 1mCi, Amersham Biosciences) was added to each well. When the cells reached confluence in the radioactive growth medium, the medium was aspirated, the cells were washed twice with warm PBS, and 1 ml of DMEM (BSA) containing unlabeled cholesterol (20μg/ml), 8-Br-cAMP (0.5mM) and either AdLPL or AdLacZ (7×10^9 viral particles/ml) was added to each well. A 20 mg/ml stock solution of cholesterol prepared by dissolving cholesterol (Sigma, grade: 99+%) in absolute ethanol with sonication was stored at -20°C and sonicated before use to ensure complete dissolution of the solid. The stock solution was diluted in DMEM (BSA) to obtain the required concentration of cholesterol. The cells were incubated for 24 hours, the medium was aspirated, and the cells were washed 4 times with 1ml/well of warm (37°C) HEPES-saline BSA (0.03mM BSA, 137mM NaCl, 5.4mM KCl , 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, and 3.58mM HEPES, pH 7.4) to remove adherent lipids. 0.5 ml of DMEM (BSA) containing 0.5mM 8-Br-cAMP with or without ApoE (12.5μg/ml) was added to each well. 50mM 8-Br-cAMP solution was diluted in the medium to obtain 0.5mM 8-Br-

cAMP solution, half of which was used to dilute a 1.555mg/ml stock solution of apoE. The cells were incubated for twenty 24 hours on a rocker in a humidified 37°C and 5% CO_2 incubator. The cells were chilled on ice, the medium was collected into scintillation vials, and the cells were washed 4 times with cold (4°C) HEPES-saline BSA to remove adherent lipids. 3:2 hexanes:isopropanol (v/v) solvent was used to extract cellular lipids. 0.5 ml of the extraction solvent was added to each well and the plate was kept on ice for thirteen minutes with occasional swirling. The extract was transferred to scintillation vials, and the cells were extracted with a fresh 0.5 ml portion of the solvent for thirteen minutes at room temperature with occasional swirling. The second lipid extract was combined with the first one in the scintillation vials. 4 ml of the complete counting cocktail (3a70B, Research Products International Corp.) was added to each vial, and the radioactivity was measured using the Beckman, LS 6500 scintillation counter. The amount of the effluxed cholesterol was calculated from the formula: cholesterol efflux (%) = [radioactivity in the medium / (radioactivity in the medium + radioactivity in the lipid extract)] x 100.

Cholesterol efflux assays in the presence of Intralipid, triglycerides, and fatty acids

Intralipid is a 10% I.V. Fat Emulsion, consisting of soybean oil, phospholipid, and glycerin (Pharmacia & Upjohn Company, Clayton, NC 27520 USA). Protein-free lipid particles with S_f 100-400 were previously obtained from Intralipid by ultracentrifugal flotation, and the triglyceride concentration of the fraction was determined by GPO-Trinder colorimetric assay (Sigma). The fractions varying in triglyceride concentration from 14mg/ml to 24mg/ml were kept at 4°C. To conserve space, the Intralipid-derived fractions will be referred to as "Intralipid"in the rest of the text.

The volume of the stock solution of Intralipid required to produce the final concentration of triglycerides of 100μg/ml was dissolved in DMEM (BSA). The effect of Intralipid on cholesterol efflux from control fibroblasts and the fibroblasts over-expressing LPL was investigated according to the above protocol with the exception of incubating the cells with Inralipid along with cholesterol. The medium was added to the wells along with unlabeled cholesterol and 8-Br-cAMP, with or without the adenovirus. After incubating the cells with the lipids for 24 hours, the cells were either harvested in TRI reagent for RNA isolation and RT-PCR analysis of ABCA1 mRNA levels, or efflux of cholesterol was measured as described above, starting at the step of washing the cells with HEPES-saline BSA.

Glyceryl trioleate (≥ 99%) was purchased from Sigma and stored at -20°C. It was diluted in DMEM (BSA) to 100μg/ml. Triglycerides previously isolated from nutmeg seed were dissolved in chloroform to prepare a 100mg/ml stock solution. A stock solution of L-α-phosphatidylcholine dipalmitoyl (Sigma, synthetic, 99+ %) was prepared by dissolving 100 mg of the solid in 1 ml of chloroform. A stock solution of sodium palmitate in 50mg/ml of fatty acid-free BSA (ICN Biomedicals Inc.) was prepared as follows. 20 mg of palmitic acid (Sigma, approx. 99%) were dissolved in 1 ml of chloroform, and 390 μl of 0.2M aqueous sodium hydroxide were added to neutralize the acid. The solution was dried under nitrogen and the solid was mixed with 8 ml of 50mg/ml fatty acid-free BSA solution. Prolonged sonication afforded a silvery white homogenous mixture. A stock solution of sodium oleate was prepared by first dissolving 20 mg of oleic acid in 1ml of chloroform and neutralizing the acid with 354 μl of 0.2M aqueous sodium hydroxide. The solid was dried under nitrogen and dissolved in 2 ml of

water affording a cloudy homogeous solution. Stock solutions of palmitic acid and stearic acid (Sigma, approx. 99%) were prepared by dissolving 10 mg of the solid in 2 ml of 50mg/ml solution of fatty acid-free BSA with sonication. The resulting mixtures were homogeneous milky suspensions stored at -20°C.

All stock solutions were diluted in DMEM (BSA) to the final concentration of 100μg/ml for treating fibroblasts over a period of 24 hours. Sterile-filtered solutions of oleic and linoleic acids in Dulbecco's phosphate buffered saline (DPBS) were purchased from Sigma as complexes with BSA, with 2 moles of acid per mole of BSA. The solution of each acid contained 100mg/ml of BSA.

Cholesterol efflux assays in the presence of PC and DTNB

Fibroblasts were seeded into 12-well cell culture plates and grown to 50% confluence in DMEM (FBS). Upon reaching half-confluence, the medium was aspirated and 1 ml of DMEM (FBS) containing either 1μCi/ml or 0.5μCi/ml of radioactively labeled cholesterol was added to each well. The cells were allowed to reach confluence, the radioactive medium was aspirated, the cells were washed twice with 1ml per well of warm (37°C) PBS, and 1 ml of DMEM (BSA) containing 20μg/ml of cholesterol was added to each well. Fibroblasts in select wells were treated with 100μg/ml of PC prepared by diluting a stock solution of PC in ethanol (Sigma, L-α-phosphatidylcholine from frozen egg yolk, type XIII-E, 100mg/ml in ethanol) in DMEM (BSA) containing cholesterol. The cells were incubated in the lipid solutions for 24 hours. The medium was aspirated, the cells were harvested in TRI reagent, and total RNA was isolated for the analysis of ABCA1 mRNA levels. Alternatively, the cells were scraped into PBS for the analysis of ABCA1 protein levels by western blotting, or incubated in 1ml/well of

DMEM (BSA) containing 0.5mM 8-Br-cAMP with or without 12.5μg/ml of apoE to measure the efflux of cholesterol.

The cholesterol efflux assay was completed as previously described. Delipidated fibroblasts were washed once with HEPES-Saline BSA and harvested in TRI reagent for RNA isolation.

In a series of similar cholesterol efflux experiments, the cells were incubated with cholesterol or a mixture of cholesterol and PC in DMEM (BSA) for 24 hours, and the medium, was collected for radioactivity measurements. The cells were washed and the lipids extracted as described earlier.

Cholesterol efflux assays involving PC were also performed in the presence 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). A 100mg/ml stock solution of DTNB was prepared by dissolving the solid (Aldrich, 99%) in absolute ethanol. The stock solution was diluted in DMEM (BSA) to obtain 1mM solution of DTNB. The stock solutions of cholesterol and PC were then added to the DTNB-containing medium. After incubating the cells for 24 hours, the cells were either harvested in TRI reagent for RNA isolation or incubated in the efflux medium. Throughout the incubation period, the cells were observed under the light microscope for any morphological changes (detached cells and cellular debris) to assess the effect of DTNB, cholesterol, and PC on cellular integrity.

In a final set of cholesterol efflux experiments involving PC, only fibroblasts in select wells were incubated with cholesterol. At the end of the 24-hour incubation period, the medium was aspirated and the control and cholesterol-loaded cells were incubated in 1 ml of DMEM (BSA) for 24 hours to allow for equlibration of cellular pools of cholesterol. DMEM (BSA) was aspirated, and DMEM (BSA) containing PC (100μg/ml)

was added to the control and cholesterol-treated cells. The cells were incubated for 24 hours, the medium was collected for radioactivity measurements, and the lipids were extracted as described earlier.

TLC

Cellular lipids were analyzed using thin layer chromatography (TLC), a separation technique in which a mixture of interest is applied onto an adsorbent material (stationary phase) and is resolved using a suitable solvent system (mobile phase). Since components of the mixture have different affinity for the stationary phase and differential solubility in the mobile phase, they can be separated on the adsorbent material and identified by comparison with standards.

Confluent fibroblasts cultured in DMEM (BSA) in 12-well cell culture plates were chilled on ice, the medium was aspirated, the cells were washed and lipids extracted as previously described. A combined lipid extract was dried under nitrogen, dissolved in 1 ml of dichloromethane and concentrated by evaporating the solvent to 300 µl. 40 µl of the lipid solution were spotted onto the Silica Gel 60 F254 TLC plate (Merck, Darmstadt, Germany) and resolved using a solvent consisting of 3:7 ethyl acetate:petroleum ether (v/v). The spots were visualized by immersing the plates into a mixture of crushed iodine and silica (grade 633, 200:45 mesh) (Fisher Scientific). Cholesterol and cholesteryl ester spots were identified by comparing the plates to the standards obtained by spotting 25 µl of a 2% solution of cholesterol and cholesteryl palmitate in dichloromethane onto control plates and developing using the same solvent. The plates were photographed using VisionWorks™ Imaging Software.

RESULTS

Overexpression of LPL in FSF

Considering the role of LPL in atherogenesis and its possible effect on ABCA1 through the PPAR-LXRα regulatory pathway, we asked if the enzyme has any effect on transcriptional regulation of ABCA1 and ABCA1-mediated cholesterol efflux. To address this question, we attempted to overexpress LPL in cultured human macrophages by transfecting the LPL gene into the cells, using a recombinant replication-deficient adenovirus. However, high levels of endogenous LPL produced by THP-1 cells obscured any increase in the levels of LPL mRNA expected upon incubation of the cells with adLPL. No difference was observed in LPL mRNA levels between the cells treated with adLPL and those infected with the control adenovirus carrying the LacZ gene (adLacZ). Any attempts to detect the difference in the levels of LPL mRNA isolated from the macrophages treated with adLPL and those from the adLacZ-treated cells were unsuccessful. We extended the duration of incubation of the cells with the virus and varied the number of PCR cycles to amplify a segment of the LPL gene. No significant and consistent difference was observed despite these attempts, suggesting that THP-1 cells are not suited for this study (Figure 12).

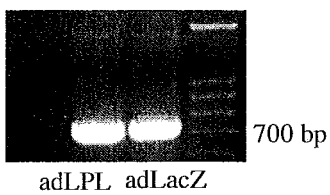


Figure 12. RT-PCR analysis of the levels of LPL mRNA from macrophages treated with adLPL. Human monocytes, THP-1, were seeded into 6-well plates in THP-1 cell differentiation medium at the density of 10^6 cells/ml. After 24 hours of incubation at 37 °C in a 5% CO_2 incubator, the differentiation medium was aspirated, and THP-1 cell growth medium containing either adLPL or adLacZ (7×10^9 viral particles/ml) was added to each well. The cells were incubated for 24 hours, total RNA was extracted and a 742 nt segment of LPL mRNA was amplified by RT-PCR (40 cycles), using LPL-specific primers, and resolved on 2% agarose gel.

To circumvent the problem posed by high levels of the macrophage LPL, we chose to work with human foreskin fibroblasts (FSF), which, unlike macrophages, do not produce LPL and thus offer a convenient system for investigating the effect of overexpression of LPL on the synthesis and function of ABCA1. Incubation of the cells with adLPL for a period of 24 hours resulted in the transfection of the LPL gene into the cells and the production of LPL mRNA as confirmed by RT-PCR (Figure 13).

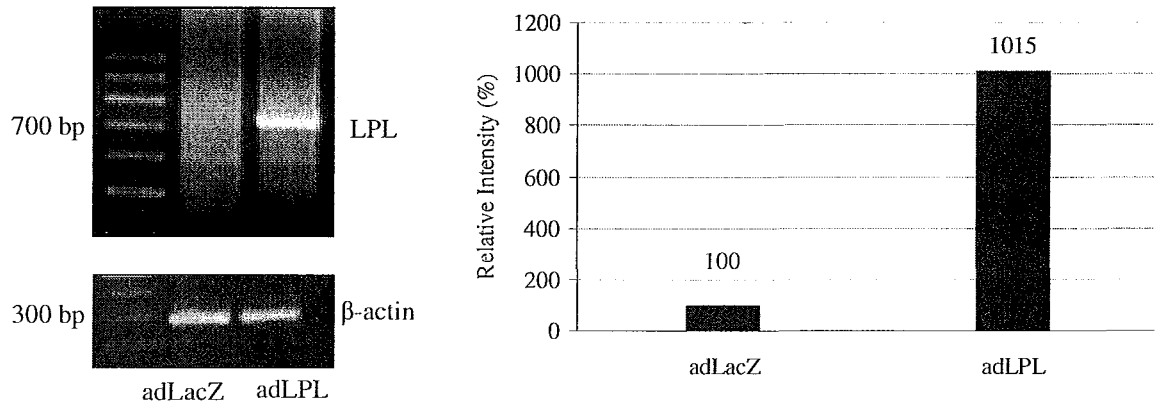


Figure 13. RT-PCR analysis of the levels of LPL mRNA isolated from fibroblasts treated with adLPL. Fibroblasts were cultured in 12-well plates in DMEM (FBS) to confluence. When the cells reached confluence, DMEM (FBS) was aspirated, the cells were washed twice with 1 ml of PBS, and 1 ml of DMEM (BSA) containing either adLPL or adLacZ (7×10^9 particles/ml) was added to each well. The cells were incubated with the virus for 24 hours. Total RNA was extracted, and a 742 nt segment of LPL mRNA was amplified by RT-PCR (40 cycles), using LPL-specific primers. A 285 nt segment of β-actin mRNA was amplified by RT-PCR (25 cycles), using β-actin-specific primers. PCR products were resolved on 2% agarose gel. The intensity of the bands was measured and the LPL to β-actin ratios were calculated. The LPL/β-actin from the cells treated with adLacZ was set to 100%. The data are representative of three experiments with similar results.

Since in vivo LPL is a secreted enzyme, we immunoprecipitated LPL from the cell culture medium derived from LPL-overexpressing fibroblasts using anti-LPL chicken IgY and analyzed its protein levels by western blotting. In agreement with extracellular

localization of the enzyme, we detected LPL in the cell culture medium as shown in Figure 14.

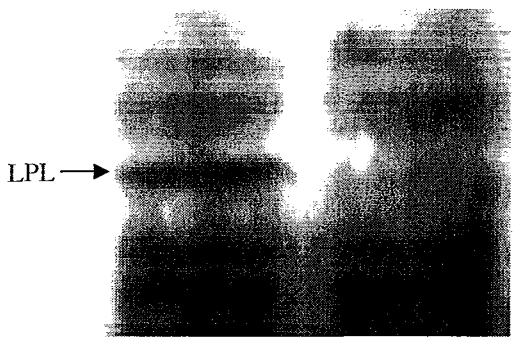


Figure 14. Western blot analysis of the levels of LPL protein isolated from the medium of the LPL-overexpressing fibroblasts. Confluent fibroblasts in DMEM (BSA) were incubated for 24 hours in the presence of adLPL or adLacZ (7 x 10^9 particles/ml). The medium was collected and LPL was immunoprecipitated with sepharose derivitized with an anti-LPL antibody. The protein was resolved in 7.5% SDS-PAGE and analyzed by western blotting. Nonspecific protein bands show that protein was loaded into both wells. The data are representative of two experiments with similar results.

Effect of overexpession of LPL on ABCA1 mRNA and protein levels

Having established a system for investigating the effect of LPL on ABCA1, we first asked if the overexpression of LPL in fibroblasts affects mRNA levels of ABCA1. Whereas incubation of fibroblasts with adLPL alone did not affect ABCA1 mRNA levels, treatment of the cells with 8-Br-cAMP, known to induce ABCA1, followed by the incubation with the virus caused an increase in ABCA1 mRNA levels (Figure 15).

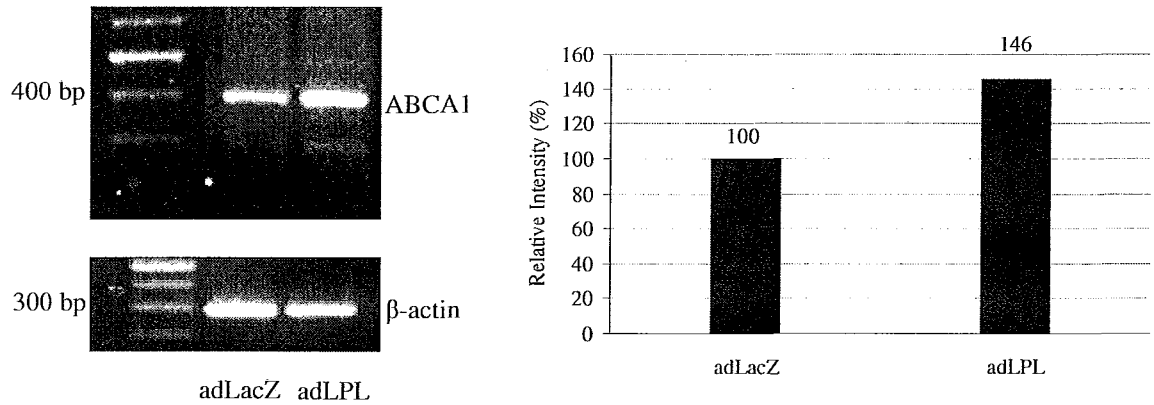
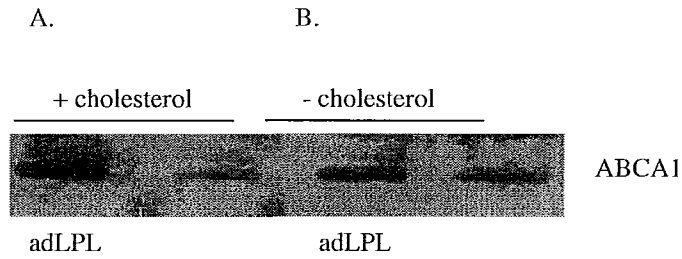


Figure 15. RT-PCR analysis of the levels of ABCA1 mRNA isolated from the LPL-overexpressing fibroblasts. Confluent fibroblasts were incubated in DMEM (BSA) for 24 hours in the presence or absence of 8-Br-cAMP (0.5 mM). Stock adLPL or adLacZ solutions were added directly to each well at 7 x 10^9 particles/ml, and the cells were incubated for 48 hours. Total RNA was extracted, and a 377 nt segment of ABCA1 mRNA was amplified using RT-PCR (25 cycles) and ABCA1-specific primers. A 285 nt segment of β-actin mRNA was amplified by RT-PCR (25 cycles), using β-actin-specific primers. PCR products were resolved on 2% agarose gel. The intensity of the bands was measured, and the ABCA1 to β-actin ratios were calculated. The ABCA1 to β-actin ratio from the cells treated with adLacZ was set to 100%. The data are representative of three experiments with similar results.

Western blot analysis of ABCA1 protein levels showed that fibroblasts overexpressing LPL have higher levels of ABCA1 protein compared to the cells infected with adLacZ. In two different experiments, fibroblasts were treated with either 8-Br-cAMP alone or 8-Br-cAMP and cholesterol (20μg/ml) followed by the incubation with adLPL. The incubation of the fibroblasts with both cholesterol and 8-Br-cAMP produced a greater difference between the intensity of the ABCA1 protein bands from the control cells and those treated with adLPL compared to the cells treated with 8-Br-cAMP alone (Figure 16).



Treatment	Relative intensity (%)	Treatment	Relative intensity (%)
cAMP/cholesterol	100	cAMP	100
cAMP/cholesterol/adLPL	146	cAMP/adLPL	120

Figure 16. Western blot analysis of the levels of ABCA1 protein isolated from the LPL-overexpressing fibroblasts. A. Confluent fibroblasts in DMEM (BSA) were treated with 8-Br-cAMP (0.5mM) and cholesterol (20μg/ml) in the presence or absence of adLPL (7 x 10^9 particles/ml) for 24 hours. Cells were lysed, and total protein was isolated and quantitated by the method of Lowry. Equal amounts of protein were resolved in 7.0% SDS-PAGE and probed with a rabbit anti-ABCA1 primary antibody and a goat peroxidase-conjugated secondary antibody. The intensity of the bands was measured and the intensity of the ABCA1 band obtained from the cells not treated with adLPL was set to 100%. B. Confluent fibroblasts were incubated in DMEM (BSA) containing 8-Br-cAMP for 24 hours. A stock solution of adLPL was added directly to select wells at 7 x 10^9 particles/ml and incubated for 48 hours. Lysis of the cells and western blot analysis of ABCA1 protein were performed as in A.

Effect of Overexpression of LPL on Cholesterol Efflux

We next asked whether the upregulation of ABCA1 protein levels translates into an increased ABCA1-mediated transport of cholesterol from the cells. To answer this question, the cells were first incubated with tritiated cholesterol and then loaded with unlabeled cholesterol to ensure the uptake of the label by the cells. The efflux of cholesterol into the medium was measured in the presence of 8-Br-cAMP with and without apoE, a cholesterol acceptor. Fibroblasts overexpressing LPL showed a

significant increase in ABCA1-mediated cholesterol efflux compared to the cells treated with adLacZ (Figure 17).

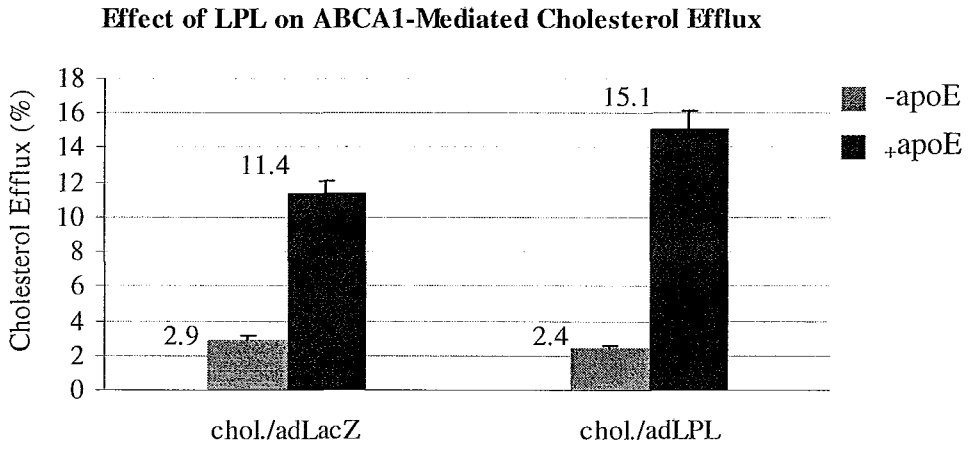


Figure 17. At 50% confluence, radioactively labeled cholesterol in DMEM (FBS) (1μCi/ml) was introduced into each well and fibroblasts were allowed to reach confluence. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated in DMEM (BSA) containing 8-Br-cAMP (0.5 mM), unlabeled cholesterol (20 μg/ml), and either adLPL or adLacZ (7 x 10^9 particles/ml) for 24 hours. The cells were washed 4 times with 1 ml of HEPES/Saline/BSA. 0.5 ml of DMEM (BSA) containing 0.5 mM 8-Br-cAMP with or without apoE (12.5μg/ml) was added to each well. The cells were incubated in a 5% CO_2 incubator at 37 °C with rocking for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA, and lipids were extracted. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of the labeled cholesterol released was calculated from the formula: % Efflux = ^3H in the medium / (^3H in the medium + ^3H in the lipid extract) x 100. Each value is an average of three measurements ± standard deviation. Student t-test was applied, assuming unequal variances, to analyze statistical significance of the results. P (11.4, 15.1) = 0.016; P (2.9, 2.4) = 0.023.

Effect of Intralipid on mRNA and protein levels of ABCA1 and ABCA1-mediated cholesterol efflux

Since LPL hydrolyzes triglycerides, we hypothesized that its effect on ABCA1 may be enhanced in the presence of the triglycerides. We decided to repeat the experiments in the presence of triglyceride-rich lipid particles obtained from Intralipid, a

10% fat emulsion, whose main components are soybean oil and phospholipids (described in Methods). We first examined the effect of Intralipid alone on ABCA1 mRNA levels in normal fibroblasts. Incubation of the cells with Intralipid for 24 hours dramatically reduced mRNA levels of ABCA1. A similar effect was observed when the cells were simultaneously incubated with both Intralipid and cholesterol as shown in Figure 18.

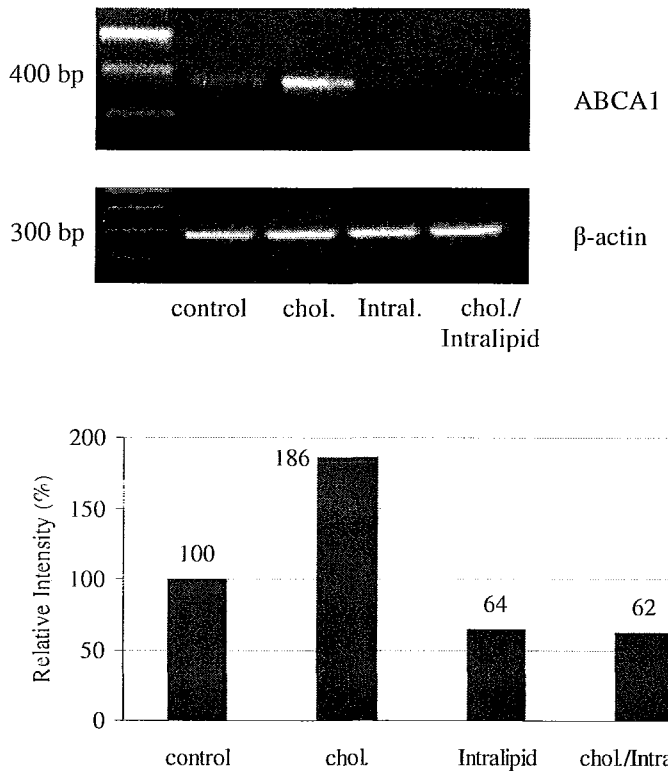


Figure 18. RT-PCR analysis of the levels of ABCA1 mRNA from fibroblasts treated with Intralipid. Fully confluent fibroblasts were incubated for 24 hours at 37 °C in a 5% CO_2 incubator in DMEM (BSA) in the presence of either cholesterol (20 μg/ml), Intralipid (100 μg/ml triglycerides), or a mixture of Intralipid and cholesterol. Total RNA was extracted, and a 377 nt segment of ABCA1 mRNA was amplified by RT-PCR (25 cycles), using ABCA1-specific primers. A 285 nt segment of β-actin mRNA was amplified by RT-PCR (25 cycles), using β-actin-specific primers. PCR products were resolved on 2% agarose gel. The intensity of the bands was measured and the background intensity was subtracted from each band. The ABCA1 to β-actin ratios were calculated and the ABCA1 to β-actin ratio from the control cells was set to 100%. The data are representative of at least three experiments with similar results.

Western blot analysis of ABCA1 protein levels from the fibroblasts incubated with Intralipid for 24 hours showed that the incubation of the cells with cholesterol and Intralipid counteracts the effect of cholesterol on ABCA1 protein levels. Cholesterol alone increased ABCA1 protein levels by more than 40%, while the incubation of the cells with cholesterol and Intralipid resulted in an increase of less than 20% (Figure 19).

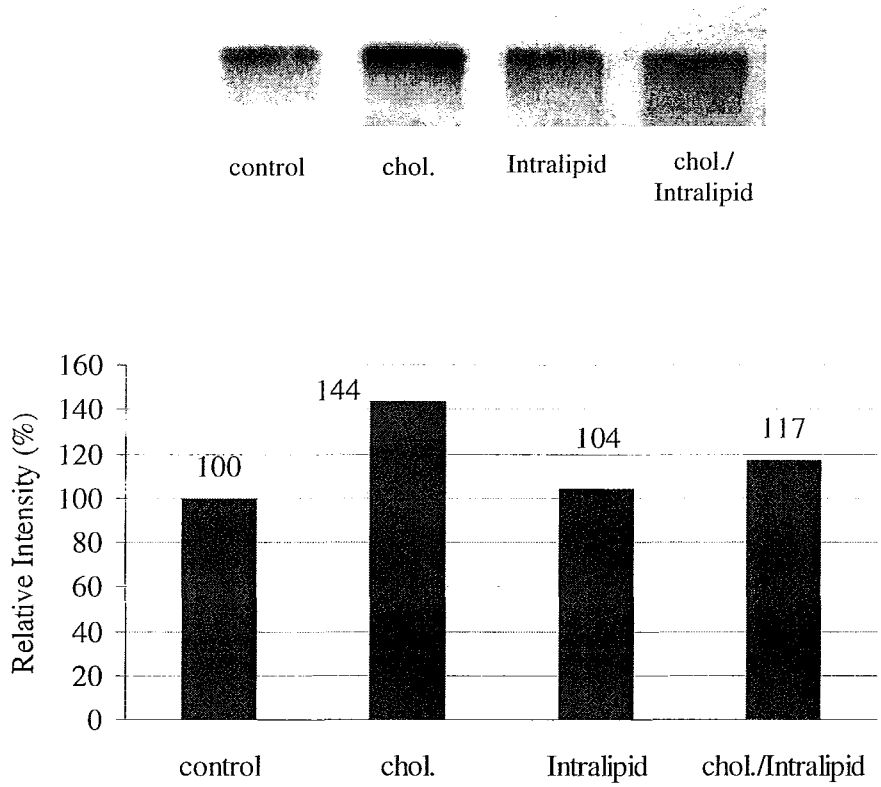


Figure 19. Western blot analysis of the levels of ABCA1 protein from fibroblasts treated with Intralipid. Fully confluent fibroblasts were incubated for 24 hours at 37 °C in a 5% CO_2 incubator in DMEM (BSA) in the presence of either cholesterol (20 µg/ml), Intralipid (100 µg/ml triglycerides), or a mixture of Intralipid and cholesterol. The cells were lysed, cellular protein was quantitated by the method of Lowry and resolved in 7.0% SDS-PAGE. ABCA1 protein levels were analyzed by western blotting using a rabbit anti-ABCA1 primary antibody and a goat peroxidase-conjugated secondary antibody.

We then performed a cholesterol efflux assay to examine the effect of Intralipid on ABCA1-mediated efflux of cholesterol. As shown on Figure 20, Intralipid caused a decrease in the amount of the effluxed cholesterol.

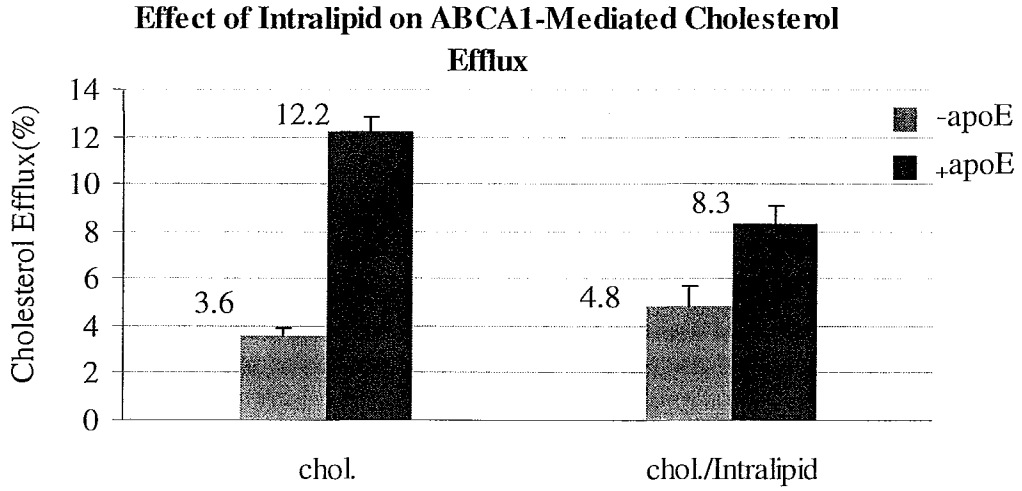


Figure 20. In order to examine the effect of Intralipid on ABCA1-mediated cholesterol efflux, we measured the efflux of cholesterol from fibroblasts after incubating the cells for 24 hours with Intralipid and compared it to the cholesterol efflux from untreated cells. FSF were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated with and without Intralipid in DMEM (BSA) containing 8-Br-cAMP (0.5mM) and unlabeled cholesterol (20μg/ml) for 24 hours. The cells were washed 4 times with 1 ml of HEPES-Saline BSA. 0.5 ml DMEM (BSA) containing 0.5mM 8-Br-cAMP with or without apoE (12.5μg/ml) was added to each well. The cells were incubated in a 5% CO_2 incubator at 37 °C with rocking for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA, and lipids were extracted. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = ^3H in the medium / (^3H in the medium + ^3H in the lipid extract) x 100. Each value is an average of three measurements ± standard deviation. *P* (12.2, 8.3) = 0.0020; *P* (3.6, 4.8) = 0.14

Overexpression of LPL in fibroblasts did not alter the effect of Intralipid on ABCA1 mRNA levels and cholesterol efflux. Figure 21 shows that the efflux from the

LPL-overexpressing cells was higher both in the absence and presence of Intralipid compared to the efflux from untreated cells. Intralipid reduced ABCA1-mediated cholesterol efflux (Figure 21) and ABCA1 mRNA levels (Figure 22), examined by RT-PCR after the cholesterol efflux assay.

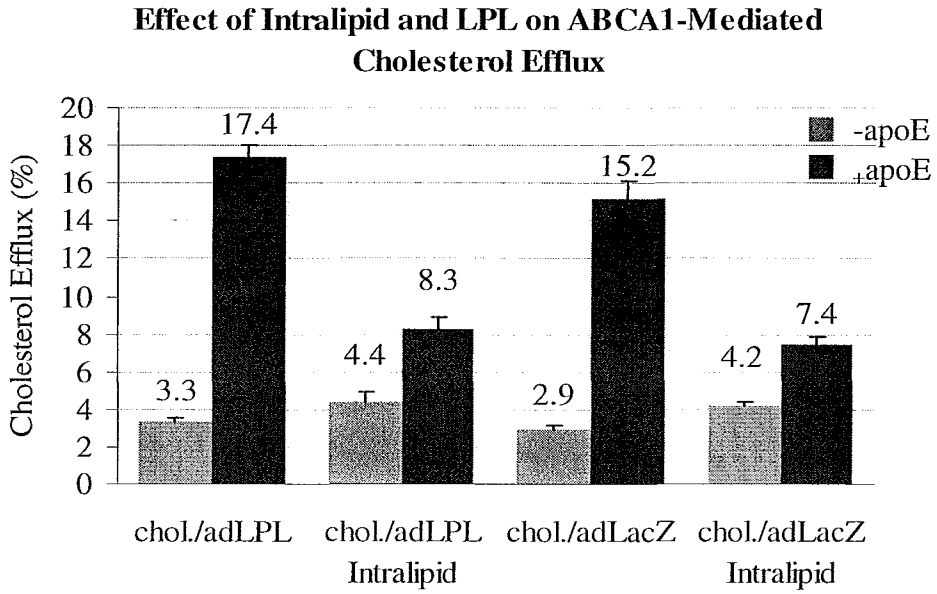


Figure 21. FSF were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated in DMEM (BSA) containing 8-Br-cAMP (0.5mM), unlabeled cholesterol (20μg/ml), and either adLPL or adLacZ (7 x 10^9 particles/ml) in the presence or absence of Intralipid for 24 hours. The cells were washed 4 times with 1 ml of HEPES-Saline BSA. 0.5 ml of DMEM (BSA) containing 0.5mM 8-Br-cAMP with or without apoE (12.5μg/ml) was added to each well. The cells were incubated in a 5% CO_2 incubator at 37 °C with rocking for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA, and lipids were extracted. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = ^3H in the medium / (^3H in the medium + ^3H in the lipid extract) x 100. Each value is an average of three measurements ± standard deviation. *P* (17.4, 15.2) = 0.037; *P* (17.4, 8.3) = 4.0×10^{-5}; *P* (8.3, 7.4) = 0.11.

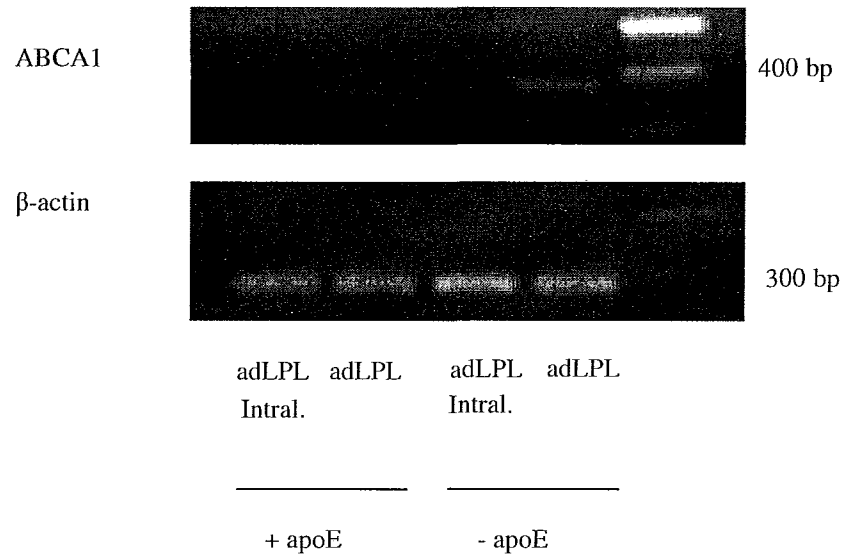


Figure 22. RT-PCR analysis of the levels of ABCA1 mRNA isolated from fibroblasts treated with Intralipid and adLPL. After completing the cholesterol efflux assay with the cells overexpressing LPL in the presence and absence of Intralipid, the delipidated cells overexpressing LPL were harvested in TRI reagent, and total RNA was isolated. A 377 nt segment of ABCA1 mRNA was amplified using RT-PCR (25 cylcels) and ABCA1-specific primers. A 285 nt segment of β-actin mRNA was amplified by RT-PCR (25 cycles), using β-actin specific primers. PCR products were resolved on 2% agarose gel.

Effect of phosphatidylcholine on ABCA1-mediated cholesterol efflux

Our next objective was to identify the major component of Intralipid responsible for the observed effects. Since Intralipid is a complex mixture of lipids consisting primarily of soybean oil and phospholipids, we chose to investigate the effect of the main classes of lipids, fatty acids, triglycerides, and phospholipids, on ABCA1 mRNA levels and ABCA1-mediated cholesterol efflux. Treatment of fibroblasts with sodium oleate, sodium palmitate, palmitic, stearic, oleic, and linoleic acids did not produce consistent results (Figure 23).

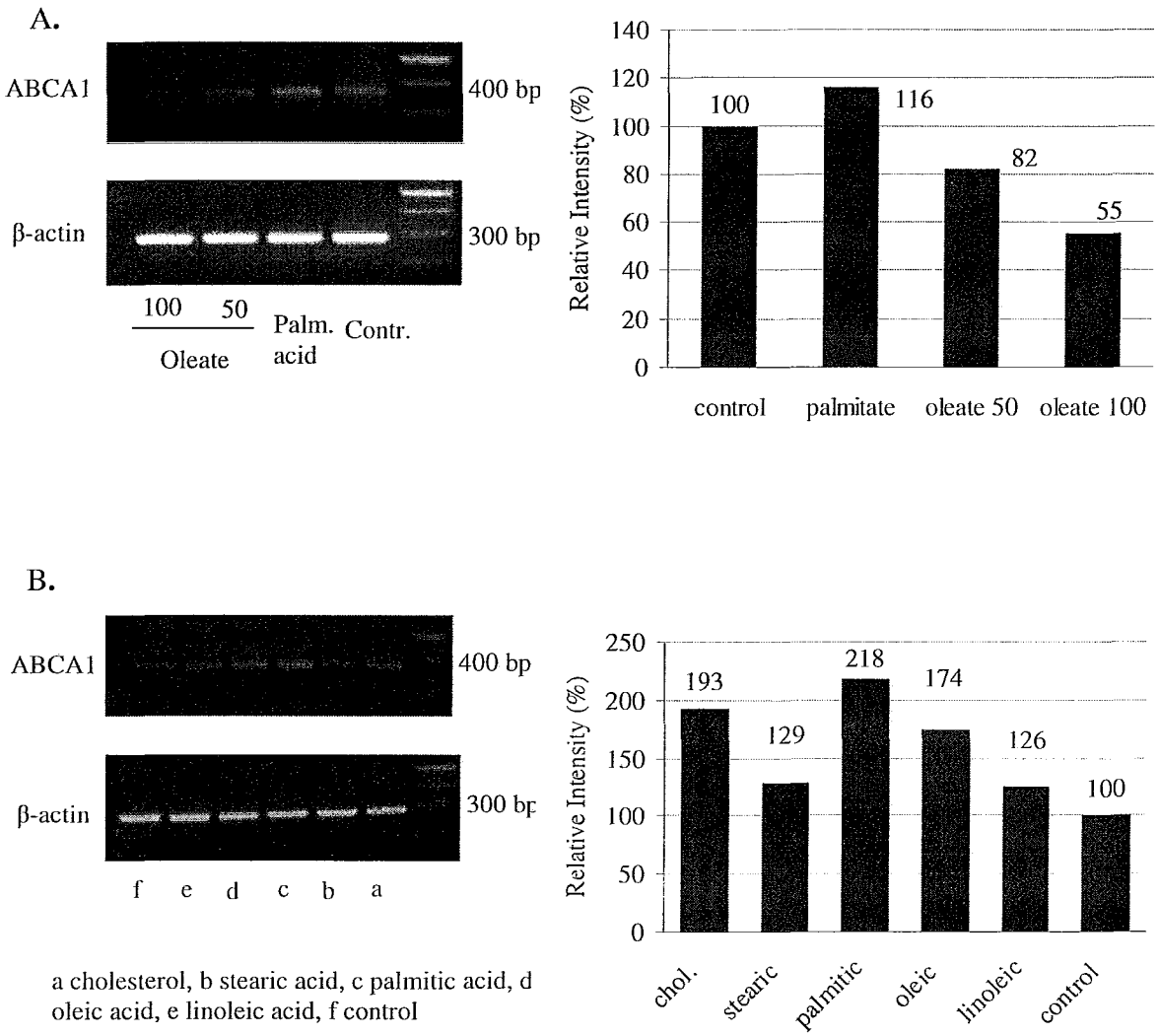
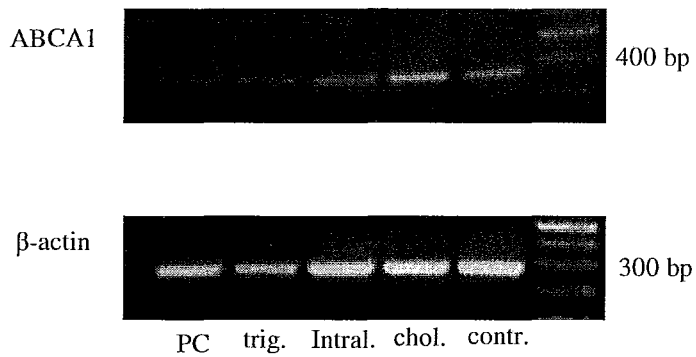


Figure 23. RT-PCR analysis of the levels of ABCA1 mRNA isolated from the cells treated with fatty acids. Panels A and B represent two independent experiments. Fully confluent fibroblasts were treated with the fatty acids for 24 hours in DMEM (BSA). Total RNA was extracted, a 377 nt segment of ABCA1 mRNA and a 285 segment of β-actin mRNA were amplified by RT-PCR, using 40 and 25 cycles, respectively. PCR products were resolved on 2% agarose gel. The ABCA1 to β-actin ratio of the band intensity obtained from control cells was set to 100%.

Incubation of the cells in DMEM (BSA) containing either triglycerides from the nutmeg seed or dipalmitoylphoshpatidylcholine revealed that while the former has some

downregulating effect on ABCA1 mRNA levels, the latter considerably deactivates the transcription of the ABCA1 gene (Figure 24).



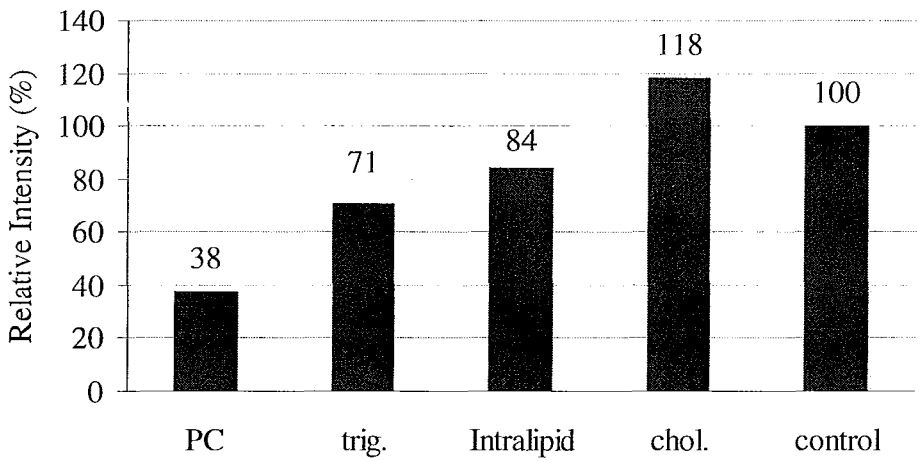
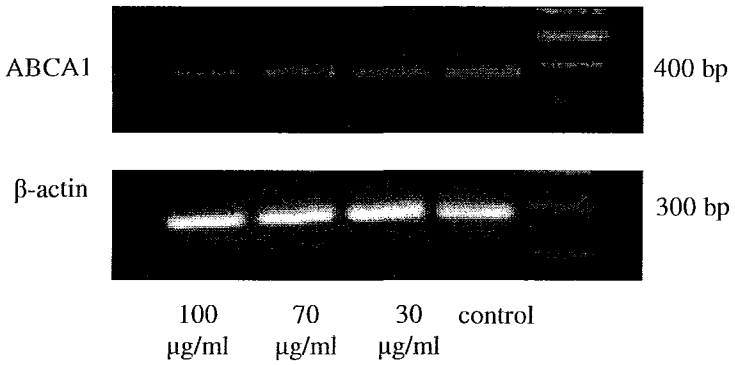


Figure 24. RT-PCR analysis of the levels of ABCA1 mRNA isolated from fibroblasts treated with the lipids. Fully confluent fibroblasts were incubated for 24 hours in DMEM (BSA) containing specified lipids. Stock solutions of the nutmeg seed triglycerides and PC in chloroform were prepared as described in Methods. Concentration of all reagents in the cell culture medium was 100μg/ml. Total RNA was purified, a 377 nt segment of ABCA1 mRNA and a 285 nt segment of β-actin mRNA were amplified by RT-PCR, using 40 and 25 cycles, respectively. PCR products were resolved on 2% agarose gel. The ABCA1 to β-actin ratio of the band intensity obtained from control cells was set to 100%.

To see if PC lowers ABCA1 mRNA levels in a concentration-dependent manner, we incubated the cells with various amounts of PC for 24 hours and analyzed ABCA1 mRNA levels using RT-PCR. PC dose-dependently reduced the levels of ABCA1 mRNA (Figure 25).



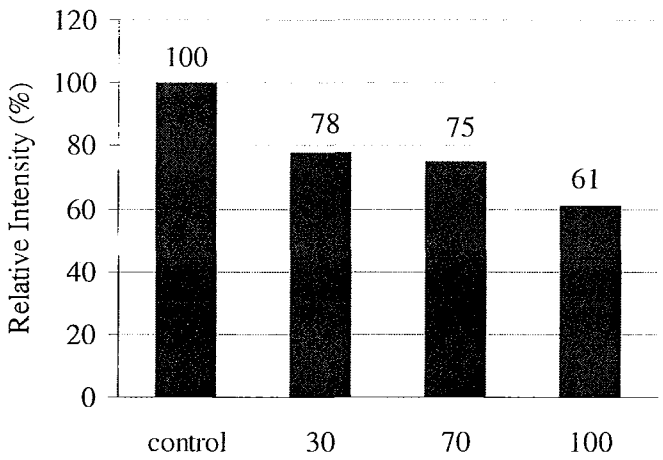
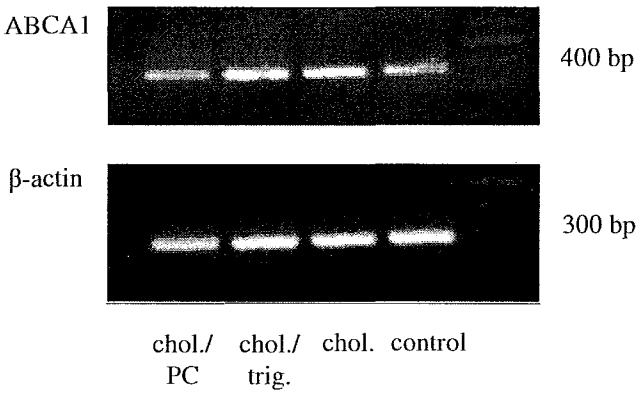


Figure 25. RT-PCR analysis of the levels of ABCA1 mRNA isolated from fibroblasts treated with various amounts of PC. The cells were incubated in DMEM (BSA) containing different amounts of dipalmitoylphosphatidylcholine for 24 hours. A stock solution of PC in ethanol (100mg/ml) was diluted in DMEM (BSA) to 100μg/ml. A 377 nt segment of ABCA1 mRNA and a 285 nt segment of β-actin mRNA were amplified by RT-PCR, using 40 and 25 cycles, respectively. PCR products were resolved on 2% agarose gel. Quantitation of the band intensity was performed as described earlier. The ABCA1 to β-actin ratio of the band intensity obtained from control cells was set to 100%.

Incubation of fibroblasts with both PC and cholesterol caused a reduction in ABCA1 mRNA levels compared to the levels from the cells treated with cholesterol alone. Thus, the effect of upregulation of ABCA1 mRNA levels by cholesterol was partially erased by PC (Figure 26).



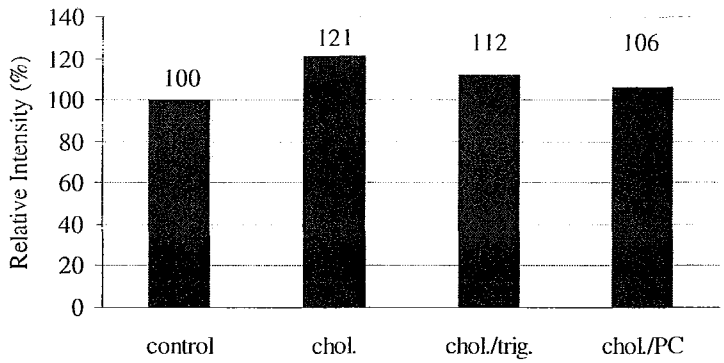


Figure 26. RT-PCR analysis of the levels of ABCA1 mRNA isolated from the cells treated with specified lipids. Fibroblasts were incubated with cholesterol, a mixture of cholesterol and triglycerides, or a mixture of cholesterol and PC for 24 hours. Stock solutions of triglycerides from the nutmeg seed (100mg/ml) and PC (100mg/ml) were prepared in chloroform and diluted in the cell culture medium to 100μg/ml. Total RNA was extracted, a 377 nt segment of ABCA1 mRNA and a 285 nt segment of β-actin mRNA were amplified by RT-PCR, using 40 and 25 cycles, respectively. PCR products were resolved on 2% agarose gel. The intensity of the bands was quantitated as described earlier. The ABCA1 to β-actin ratio of the band intensity obtained from the control cells was set to 100%.

PC had a similar effect on ABCA1 protein levels. Incubation of fibroblasts with cholesterol alone caused an increase in ABCA1 protein levels. When fibroblasts were treated simultaneously with cholesterol and PC, the upregulating effect of cholesterol on ABCA1 was dramatically reduced. No effect on ABCA1 protein levels was observed when fibroblasts were incubated with both cholesterol and triglycerides (Figure 27).

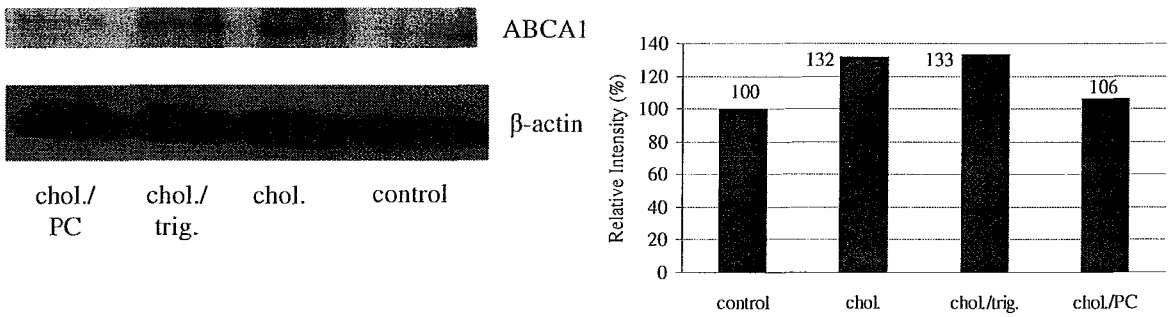


Figure 27. Western blot analysis of ABCA1 protein isolated from fibroblasts treated with specified lipids. Fully confluent fibroblasts in 12-well plates were incubated with cholesterol (20μg/ml), the nutmeg seed triglycerides (100μg/ml) and cholesterol. or PC (100μg/ml) and cholesterol for 24 hours. Stock solutions of the lipids, triglycerides in chloroform (100mg/ml), PC in chloroform (100mg/ml), and cholesterol in ethanol (20mg/ml), were dissolved in DMEM (BSA) prior to transferring the medium to the wells. The final concentrations of PC and triglycerides were 100μg/ml each, while that of cholesterol 20μg/ml. The cells were collected and lysed as described in Methods. Cellular protein was quantitated by the method of Lowry and resolved under denaturing conditions using 7.0% SDS-PAGE. Western blot analysis of ABCA1 protein levels was performed using a rabbit anti-ABCA1 primary antibody and a goat peroxidase-conjugated secondary antibody. The blot was stripped and reprobed with a rabbit anti-β-actin primary antibody and a goat peroxidase-conjugated secondary antibody. The relative intensity of the bands was determined as described earlier. The ABCA1 to β-actin ratio of the band intensity obtained from the control was set to 100%.

We then asked whether the decrease in mRNA and protein levels of ABCA1 observed upon treating fibroblasts with PC causes a reduction in ABCA1-mediated cholesterol efflux. Incubation of the fibroblasts with cholesterol and dipalmitoylphosphatidylcholine for 24 hours caused a reduction in ABCA1-mediated

cholesterol efflux. Unlike PC, triglycerides had no effect on ABCA1-dependent cholesterol efflux. No significant difference was observed in cholesterol efflux via aqueous diffusion between control cells and the cells treated with the lipids (Figure 28).

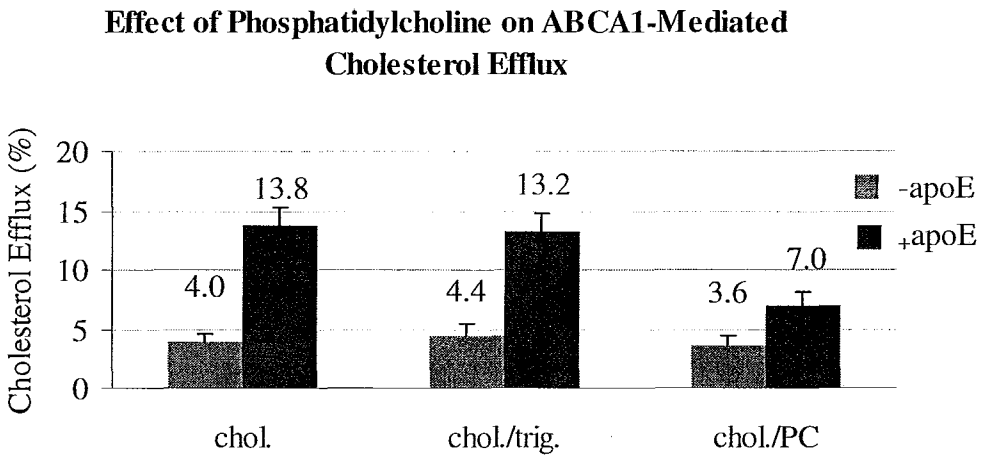


Figure 28. FSF were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES/Saline/BSA, and incubated in DMEM (BSA) containining unlabeled cholesterol (20μg/ml) or a mixture of cholesterol (20μg/ml) with either the nutmeg triglycerides (100μg/ml) or dipalmitoylphosphatidylcholine (100μg/ml) for 24 hours. The cells were washed 4 times with 1 ml of HEPES-Saline BSA. 1 ml of DMEM (BSA) containing 0.5mM 8-Br-cAMP with or without apoE (12.5μg/ml) was added to each well. The cells were incubated in a 5% CO_2 incubator at 37 °C for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA, and lipids were extracted. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = ^3H in the medium / (^3H in the medium + ^3H in the lipid extract) x 100. Each value is an average of four measurements ± standard deviation. *P* (13.8, 13.2) = 0.47; *P* (13.8, 7) = 0.0012.

To explain the observed effect of PC on ABCA1-mediated cholesterol efflux, we hypothesized that during the 24-hour incubation period extracellular pools of free cholesterol are depleted in the presence of PC, which acts as an acceptor of cholesterol by forming PC-BSA complexes and mixed micelles with cholesterol. The decrease in the amount of free cholesterol diminishes the upregulating effect of cholesterol on ABCA1

mRNA levels, resulting in downregualtion of ABCA1 mRNA and protein levels, which, in turn, cause a decrease in ABCA1-mediated cholesterol efflux. To test our proposal that depletion of cellular cholesterol occurs by desorption of plasma membrane cholesterol and its transfer to phospholipid-containing acceptor complexes, we decided to measure efflux of cholesterol during the 24-hour incubation period with phospholipid. PC alone in the absence of apoprotein E dramatically increased cholesterol efflux. (Figure 29).

Effect of Phosphatidylcholine on ABCA1-Independent Cholesterol Efflux

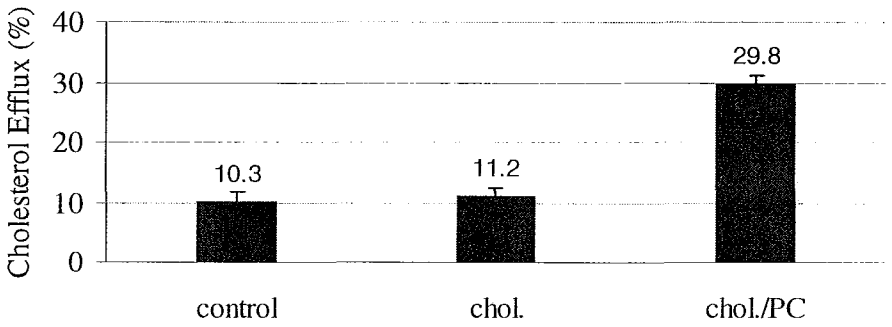


Figure 29. FSF were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated in DMEM (BSA) containing unlabeled cholesterol (20μg/ml) or a mixture of cholesterol (20μg/ml) and dipalmitoylphosphatidylcholine (100μg/ml) for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA, and lipids were extracted as described earlier. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = ^{3}H in the medium / (^{3}H in the medium + ^{3}H in the lipid extract) x 100. Each value is an average of three measurements ± standard deviation. *P* (10.3, 11.2) = 0.38; *P* (10.3, 29.8) = 9.1×10^{-7}.

Since in our experiments we incubated fibroblasts simultaneously with PC and cholesterol, we decided to examine the effect of phosphatidylcholine on cholesterol efflux when the cell culture medium is free of cholesterol. We allowed the equilibration of free pools of cellular cholesterol for 24 hours in cholesterol free-medium. This was

done by removing the cholesterol-containing medium and incubating firbroblasts in DMEM (BSA) devoid of cholesterol for 24 hours. After the equilibration period, the medium was aspirated and the efflux was performed using fresh medium for 24 hours. PC significantly increased the amount of the released cholesterol both from the cells loaded with cholesterol and control cells (Figure 30).

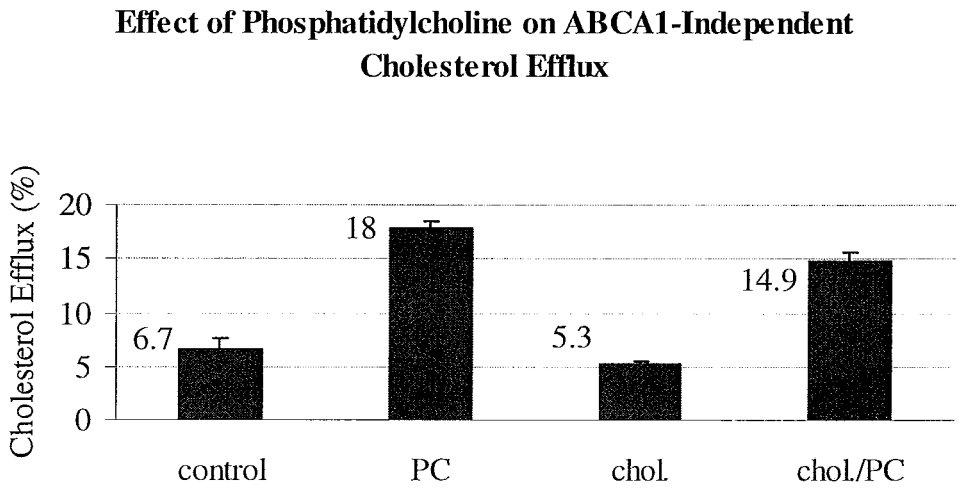


Figure 30. FSF were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated in DMEM (BSA) containining unlabeled cholesterol (20μg/ml) or a mixture of cholesterol (20μg/ml) and dipalmitoylphosphatidylcholine (100μg/ml) for 24 hours. The medium was aspirated and 1 ml of DMEM (BSA) was added to each well. The cells were incubated for 24 hours. The medium was aspirated, and efflux was performed in DMEM (BSA) in the absence or presence of dipalmitoylphosphatidylcholine for 24 hours. The medium was collected for scintillation counting, and lipids were extracted as described previously. Percentage of labeled cholesterol released was calculated from the formula: % Efflux = ^{3}H in the medium / (^{3}H in the medium + ^{3}H in the lipid extract) x 100. Each value is an average of three measurements ± standard deviation. *P* (6.7, 18.0) = 0.00029; *P* (6.7, 5.3) = 0.13; *P* (18.0, 14.9) = 0.0066.

In order to eliminate the possibility of esterification of free cholesterol in the medium in the presence of PC by LCAT and test the proposal that depletion of cellular

cholesterol by PC occurs by the transfer of the molecule onto phospholipid-containing acceptors without esterification of the effluxed cholesterol by the enzyme, we treated the cells with the LCAT inhibitor DTNB for 24 hours while incubating them in the presence of cholesterol and PC. The treatment of the cells with the inhibitor reduced ABCA1-mediated cholesterol efflux without altering the effect of PC (Figure 31).

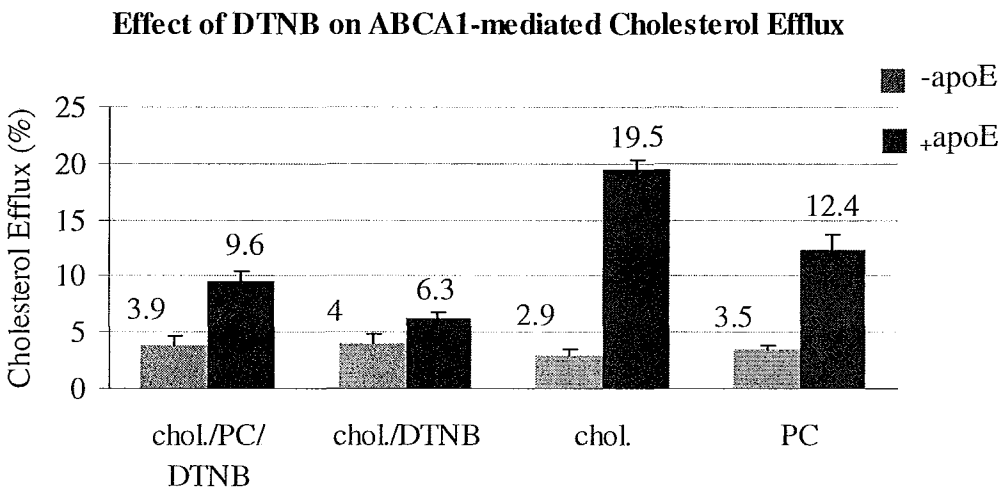


Figure 31. FSF were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated in DMEM (BSA) containining unlabeled cholesterol (20μg/ml) or a mixture of cholesterol (20 μg/ml) and dipalmitoylphosphatidylcholine (100μg/ml) for 24 hours. A stock solution of PC in ethanol (100mg/ml) was diluted in DMEM (BSA) to 100μg/ml. The cells were treated with 1mM DTNB. The cells were washed 4 times with 1 ml of HEPES-Saline BSA. 1 ml of DMEM (BSA) containing 0.5mM 8-Br-cAMP with or without apoE (12.5μg/ml) was added to each well. Fibroblasts were incubated in a 5% CO_2 incubator at 37 °C for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA, and lipids were extracted. Radioactivity in the medium and the lipid extract was measured using scintillation counting. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = ^3H in the medium / (^3H in the medium + ^3H in the lipid extract) x 100. Each value is an average of three measurements ± standard deviation. P (9.6, 6.3) = 0.0080; P (9.6, 19.5) = 9.7×10^{-5}; P (19.5, 12.4) = 0.0037.

Since DTNB reduced ABCA1-mediated cholesterol efflux from cholesterol-loaded fibroblasts in the presence and absence of PC, we asked whether the inhibitor also affects ABCA1-independent cholesterol efflux. To answer this question we measured ABCA1-independent cholesterol efflux in the presence of DTNB. The inhibitor did not alter the effect of PC on ABCA1-independent cholesterol efflux (Figure 32).

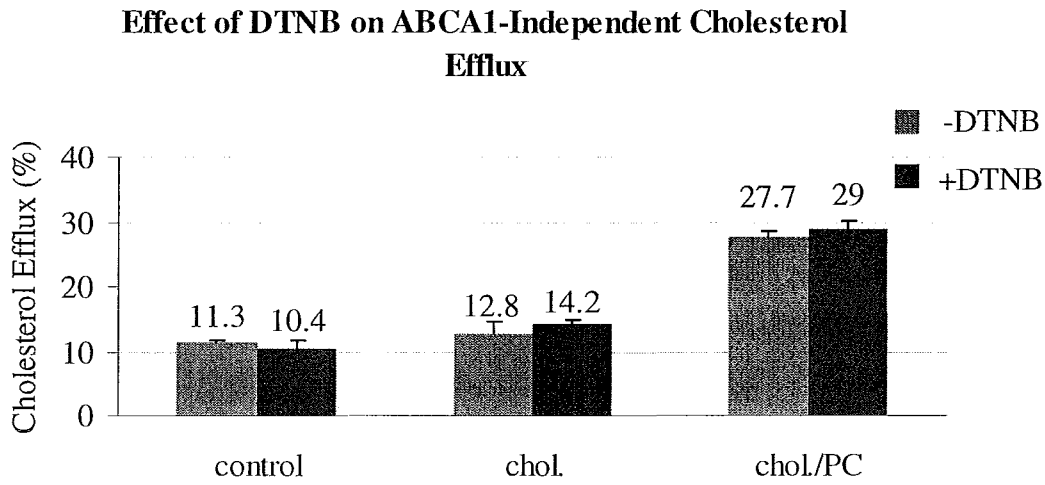


Figure 32. Fibroblasts were allowed to reach confluence in DMEM (FBS) containing tritiated cholesterol. The medium was aspirated, the cells were washed once with 1 ml of HEPES-Saline BSA, and incubated in DMEM (BSA) containining unlabeled cholesterol (20μg/ml) or a mixture of cholesterol (20μg/ml) and dipalmitoylphosphatidylcholine (100μg/ml) in the presence and absence of 1 mM DTNB for 24 hours. The medium was collected for scintillation counting, and lipids were extracted. Percentage of radioactive cholesterol released was calculated from the formula: % Efflux = ^{3}H in the medium / (^{3}H in the medium + ^{3}H in the lipid extract) x 100. Each value is an average of four measurements ± standard deviation. *P* (11.3, 10.4) = 0.27; *P* (12.8, 14.2) = 0.21; *P* (27.7, 29.0) = 0.15.

In agreement with the cholesterol efflux data, TLC of cellular lipids confirmed our proposal that phosphatidylcholine depletes cellular cholesterol. The lipid extract from the fibroblasts treated with both cholesterol and PC was deficient in free cholesterol

compared to the lipid extract from the cells treated only with cholesterol. DTNB did not have any pronounced effect on the lipid profile of the cells (Figure 33).

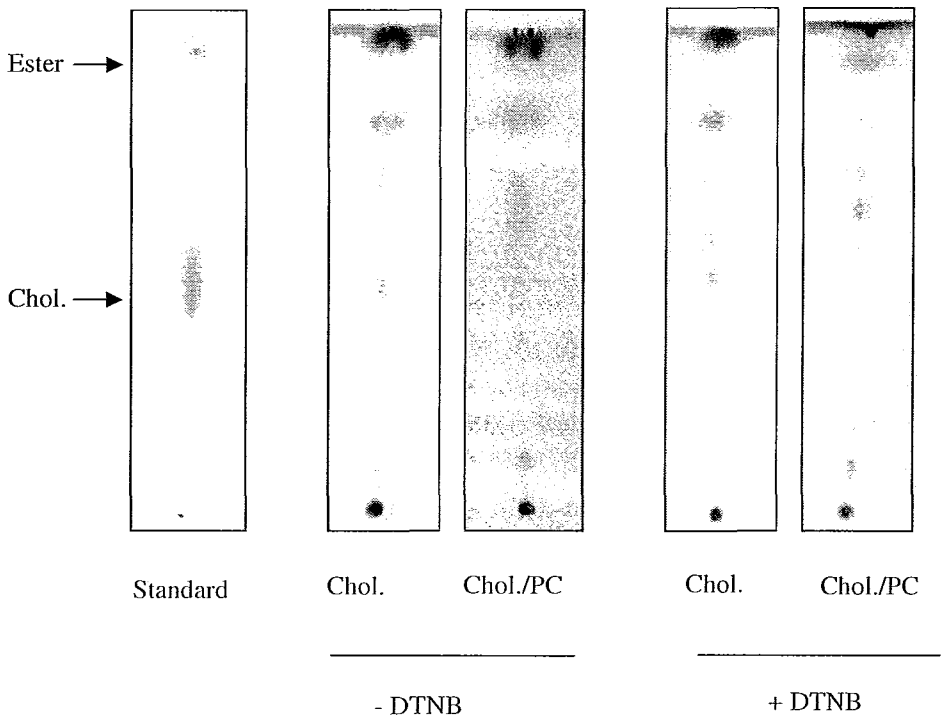


Figure 33. TLC analysis of the lipids extracted from fibroblasts treated with PC in the presence and absence of DTNB. Fully confluent fibroblasts were incubated in DMEM (BSA) with either cholesterol (20μg/ml) or a mixture of cholesterol and PC (100μg/ml) in the presence and absence of DTNB (1mM). The cells were washed 4 times with HEPES-Saline BSA, and lipids were extracted with a 3:2 hexanes:isopropanol mixture as described earlier. The lipid solution was dried under nitrogen, the solid was dissolved in dichloromethane, and its volume was reduced to 300μl. 40μl of the solution were spotted onto a silica plate and resolved in a 30:70 ethyl acetate:petroleum ether solvent. A 2% solution of a 1:1 cholesterol:cholesteryl palmitate mixture was used .

Since LCAT plays an important role in ABCA1-mediated cholesterol efflux, we asked whether PC has any effect on mRNA levels of LCAT. Both cholesterol and PC

caused a decrease in mRNA levels of LCAT, with the effect of PC being more pronounced than that of cholesterol (Figure 34).

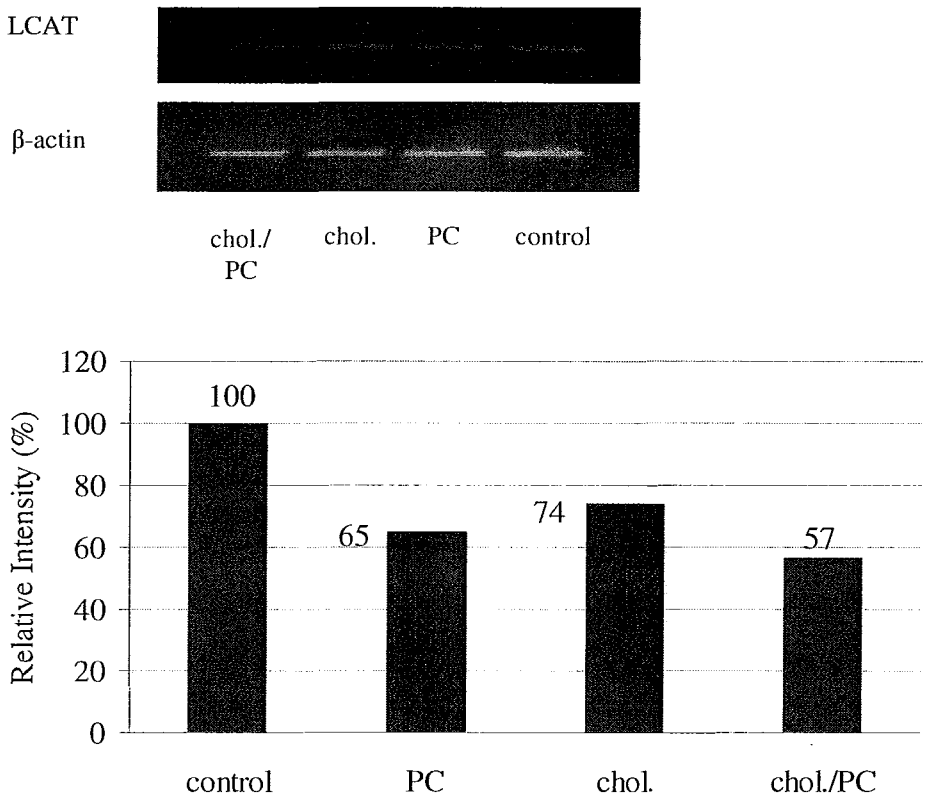


Figure 34. RT-PCR analysis of the levels of LCAT mRNA isolated from fibroblasts treated with phosphatidylcholine. Fully confluent fibroblasts were incubated in DMEM (BSA) containing either dipalmitoylphosphatidylcholine (100μg/ml), cholesterol (20μg/ml), or a mixture of PC and cholesterol for 24 hours. Total RNA was isolated, and 2 μg of total RNA was reverse-transcribed to cDNA. A 375 bp fragment of LCAT cDNA and a 285 bp fragment of β-actin cDNA were amplified, using 35 and 30 cycles, respectively. Amplification was performed using GoTaq polymerase as described in Methods. PCR products were resolved on 2% agarose gel. Quantitation of the intensity of the bands was performed as described earlier.

Lastly, we asked whether the effect of PC on ABCA1 mRNA levels is reversible. While the incubation of fibroblasts with PC for 24 hours significantly lowered ABCA1 mRNA levels compared to the control, the difference was dramatically reduced when the

phospholipid-containing medium was removed and the cells were incubated for an additional 24 hours in lipid-free medium (Figure 35).

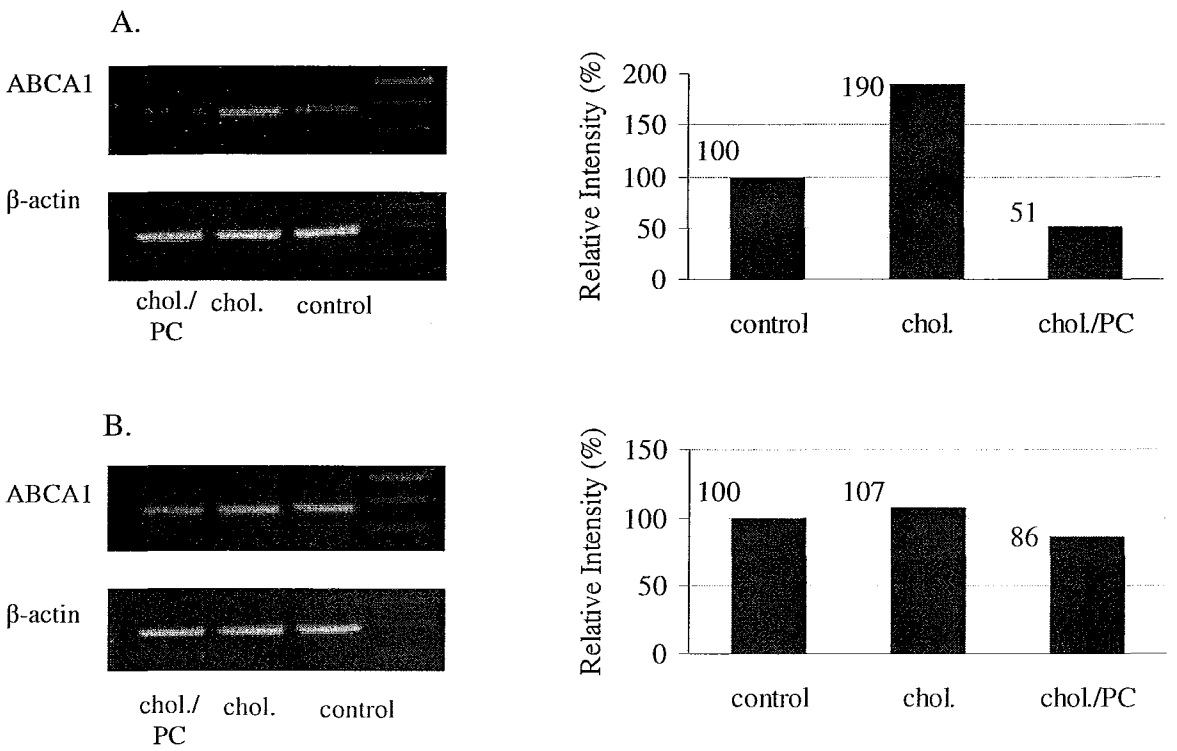


Figure 35. RT-PCR analysis of the levels of ABCA1 mRNA isolated from fibroblasts at two different time points after the treatement with PC. A. Fully confluent fibroblasts were incubated with specified lipids for 24 hours in DMEM (BSA). The medium was collected for scintillation counting and cellular lipids were extracted as described earlier. Delipidated cells were collected in TRI reagent, and total RNA was purified. A 377 nt segment of ABCA1 mRNA and a 285 nt segment of β-actin mRNA were amplified by RT-PCR, using 40 and 25 cycles, respectively. PCR products were resolved on 2% agarose gel. The intensity of the bands was quantitated as previously described. B. Fully confluent fibroblasts were incubated with specified lipids for 24 hours in DMEM (BSA). The medium was aspirated, and DMEM (BSA) containing apo A-I (12.5μg/ml) and 0.5mM 8-Br-cAMP was added to each well. The cells were incubated in a 5% CO_2 incubator at 37 °C for 24 hours. The medium was collected, the cells were washed 4 times with 1 ml of HEPES-Saline BSA and lipids were extracted. Delipidated cells were lysed in TRI reagent, total RNA was purified, and ABCA1 mRNA levels were analyzed using RT-PCR as described in A.

DISCUSSION

In this study we investigated the regulation of ABCA1-mediated cholesterol efflux with the focus on two independent, though related, areas. In the first part we investigated the effect of LPL on transcriptional regulation of the ABCA1 gene and ABCA1-dependent cholesterol efflux. While pursuing this goal, we observed a dramatic decrease in ABCA1 mRNA levels in human skin fibroblasts in the presence of Intralipid and were prompted to examine this phenomenon further. This line of research led us to examine the effect of phospholipids on ABCA1 mRNA levels and ABCA1-mediated cholesterol efflux.

Effect of LPL on ABCA1-dependent cholesterol efflux

The role of LPL in atherogenesis is well documented, and numerous reports exist, detailing diverse physiological effects of this enzyme with a pivotal function in lipid metabolism. Depending on the source and type of tissue in which it is present, LPL can exert either atherogenic or atheroprotective effect. LPL secreted by macrophages of atherosclerotic lesions is proatherogenic by participating in the formation of small dense LDL particles and facilitating the uptake of oxidized LDL by macrophages. Proatherogenic effect of LPL in a macrophage is partly offset by the function of the cholesterol transporter, ABCA1, which rids the cells of excess of cellular cholesterol and thus opposes the formation of the macrophage-derived foam cells.

Here we asked whether the enzyme has any regulatory effect on ABCA1. Based on the current knowledge of transcriptional regulation of the ABCA1 gene, we hypothesized that LPL may upregulate ABCA1 mRNA levels and augment ABCA1-mediated cholesterol efflux by producing some of the lipid ligands known to activate

transcription of the ABCA1 gene. To begin to test this hypothesis we attempted to overexpress LPL in THP-1 macrophages. Macrophages are relevant to this study since they have active lipid metabolism and are abundant in atherosclerotic lesions. However, our attempts to overexpress LPL in macrophages using adenoviral delivery of the gene were unsuccessful due to high levels of endogenously produced LPL, which rendered any difference in the amounts of the LPL transcript produced by the cells transfected with the control adenovirus carrying the LacZ gene and those transfected with the adenovirus expressing LPL unnoticeable (Figure 12).

We were aware of the possibility that equal intensity of the LPL bands may be due to the reasons other than the high endogenous expression of LPL in macrophages. Limited sensitivity of the conventional PCR must be taken into account. In order to accurately quantitate specific mRNA present in the sample, PCR products must be analyzed while the reaction is in the exponential phase. This phase spans only about five amplification cycles before reaching a plateau phase, at which saturation occurs and any distinction between the DNA contents of the samples is impossible. Sensitivity of PCR before the linear phase is also limited. Low levels of double stranded products present at early phases of the reaction bind small amounts of ethidium bromide. The resulting fluorescence is too low to accurately reflect the amount of amplified DNA and cannot be used for quantitative measurements.

To ensure that the analyzed samples were the products of PCR in the exponential phase, we compared the amount of the LPL PCR products in the control and transfected cells using a variable number of amplification cycles. A 742 bp segment of the LPL cDNA was amplified using 5, 10, 19, 20, 21, 26, 27, 28, 33, and 40 cycles, but no

significant or consistent difference in the intensity of the control and the sample bands was observed. We also varied duration of incubation of the cells with the adenovirus to exclude the possibility of poor efficiency of transfection, but could detect no significant difference in the amount of the LPL transcript produced by the control and transfected cells. It is important to stress here that even if some difference was detected it would probably be insufficient to study the effect of LPL in macrophages as any significant interference by the endogenously expressed LPL would partly mask the effect of the overexpressed LPL.

We therefore abandoned macrophages and chose to work with human skin fibroblasts. Fibroblasts have been extensively used to investigate the genes participating in lipid metabolism and atherosclerosis. They proved to be a convenient system in our case, as they produce very low levels of the LPL transcript. Incubation of confluent fibroblasts with adLPL for 24 hours resulted in a 10-fold increase in the amount of the LPL transcript compared to the cells incubated with adLacZ (Figure 13).

Overproduction of the LPL transcript led to the synthesis of the protein and its secretion into cell culture medium. Using sepharose derivitized with anti-LPL chicken antibody we were able to immunoprecipitate LPL from the medium of the LPL-overexpressing fibroblasts as revealed by subsequent western blot analysis of the protein levels in the immunoprecipitate (Figure 14). This is in agreement with extracellular localization of LPL, which is secreted by the cells in vivo, circulates in plasma, and is anchored to the cells of the endothelium by interacting with cell-surface proteoglycans. No LPL was detected in the immunoprecipitate from the medium of the cells treated with adLacZ (Figure 14).

Having established a system for investigation of the effect of LPL, we first asked if overexpression of the enzyme has any effect on ABCA1 mRNA levels. Incubation of the cells with adLPL alone for twenty four hours did not have any detectable effect on mRNA levels of ABCA1. However, when fibroblasts were first treated with 8-Br-cAMP, a stable cAMP analogue, for 24 hours and then incubated with the adenovirus for 48 hours, a 40% increase in ABCA1 mRNA levels was observed in the cells overexpressing LPL (Figure 15).

cAMP analogues, such as 8-Br-cAMP and cpt-cAMP, are known to act as transcriptional activators of the ABCA1 gene by activating PKA and triggering phosphorylation of CREB, which subsequently induces transcription by binding to CRE of the ABCA1 gene.[106-109] Such effect was observed in murine macrophages, such as RAW264.7 cells, and accounts for a dramactic increase in ABCA1-mediated cholesterol efflux. [107] Comparison of the sequences of the murine and human ABCA1 genes revealed that in the latter CRE is not conserved,[109] explaining why cAMP analogues have only a minor effect on ABCA1 mRNA levels in human skin fibroblasts. Earlier studies failed to detect upregulation of ABCA1 mRNA levels in response to 8-Br-cAMP in immortalized skin fibroblasts or reported induction of the ABCA1 gene only after extended periods of incubation.[107] This is in agreement with our data, which show lack of induction of the ABCA1 gene in fibroblasts treated with 8-Br-cAMP.

Overexpression of LPL in fibroblasts in the absence of 8-Br-cAMP did not have any effect on ABCA1 mRNA levels, suggesting that LPL alone is not sufficient to cause significant induction of the ABCA1 gene. It is possible, however, that small differences in the amount of the ABCA1 transcript in the control and adLPL-treated cells were not

detected due to limitations imposed by PCR. It would be informative to compare ABCA1 mRNA levels in the LPL-overexpressing fibroblasts and the cells treated with adLacZ using real time quantitative RT-PCR.

Performed in the presence of the high-affinity DNA dye, CYBR Green, real-time PCR allows to continuously monitor the levels of double-stranded products throughout the reaction. This makes the techniques much more quantitative in nature than conventional PCR and useful in observing small effects.

Considering the results discussed above and the limitations of conventional PCR, a 40% increase in ABCA1 mRNA levels upon incubating the cells with both 8-Br-cAMP and adLPL is significant and suggests that LPL and 8-Br-cAMP may act synergistically to increase ABCA1 mRNA levels in human skin fibroblasts. A synergistic effect of two ligands on transcription of the ABCA1 gene has been previously observed.[99] Thus, transcription of the ABCA1 gene in RAW264.7 macrophages can be activated by either 9-*cis*-retinoic acid or 22(R)-hydroxycholesterol and results in 8-fold induction of ABCA1 mRNA levels in each case. When both ligands were used simultaneously, 37-fold induction of the transcription was observed.[99] 9-cis-retinoic acid and 22(R)-hydroxycholesterol are both required in the formation of the LXRα/RXR heterodimer, which subsequently binds to LRXE and activates transcription of the ABCA1 gene. In our case, the synergistic effect may be achieved by transcriptional activation of the ABCA1 promoter via two mechanisms involving different response elements on the gene. Some of the products of hydrolytic activity of LPL are precursors of the ligands of PPARγ. Arachidonic acid, for example, can be converted to 15d-PGJ_2, which upon binding to PPARγ activates it and stimulates transcription of the LXRα gene. The latter

acts as a transcriptional activator of the ABCA1 gene (Figure 11).[102-105,169] On the other hand, cAMP may exert its effect via CRE. Although 8-Br-cAMP is not a transcriptional regulator of the human ABCA1 gene, the observation that prolonged treatment of fibroblasts with 8-Br-cAMP induces the gene suggests that cAMP has some effect on transcription of the gene.[107] While separately these pathways have a negligible effect on transcription of the ABCA1 gene, the combined effect may be significant.

Considering that CRE is weak in the human ABCA1 gene and that fibroblasts do not have active lipid metabolism, an alternative mechanism may account for the observed effect of LPL and cAMP on mRNA levels of ABCA1. In human skin fibroblasts, 8-Br-cAMP was shown to trigger phosphorylation of ABCA1 by activating PKA.[112] It is not farfetched to suggest that activation of ABCA1 by 8-Br-cAMP combined with possible stabilization of the transporter in the plasma membrane by LPL triggers a signal transduction pathway resulting in transcriptional activation of the ABCA1 gene. In analogy with the synergistic effect on a transcriptional level, simultaneous activation and stabilization of ABCA1 by 8-Br-cAMP and LPL, respectively, may produce a sufficiently strong signal to trigger transcriptional activation of the gene. Interaction of ABCA1 with apolipoproteins and α-helices of synthetic peptide has been reported and was shown to decrease the rate of degradation of ABCA1 by calpain proteases and play a role in ABCA1-mediated cholesterol and phospholipid efflux.[83,113] It is conceivable that LPL secreted into the medium interacts with extracellular loops of ABCA1 stabilizing the transporter and thus decreasing the rate of its catabolism.

Western blot analysis of the ABCA1 protein levels showed that the LPL-overexpressing cells in the presence of 8-Br-cAMP produce a greater amount of the

ABCA1 protein compared to the amount detected in the control cells (Figure 16). This increase is comparable to the extent of the upregualtion of ABCA1 mRNA levels and suggests that the transcript is translated. A greater increase in the levels of the ABCA1 protein in the cells treated with cholesterol in addition to 8-Br-cAMP lends support to the idea of the synergistic transcriptional activation of the ABCA1 gene. The mechanism of induction of the ABCA1 gene by LXRα in the presence of oxysterols formed from cholesterol (Figure 11) may combine with the effect of LPL and 8-Br-cAMP to increase the levels of ABCA1 mRNA.

Along with the increase in ABCA1 mRNA and protein levels, we observed an increase in ABCA1-mediated cholesterol efflux from the LPL-overexpressing fibroblasts loaded with cholesterol. It is noteworthy that a 40% increase in the amount of the effluxed cholesterol is similar to the increase in ABCA1 mRNA and protein levels, suggesting that the potentiation of ABCA1-mediated cholesterol efflux is caused by the rise in the levels of ABCA1 mRNA. No significant changes were observed in cholesterol efflux in the absence of apoE, suggesting that overexpression of LPL does not affect ABCA1-independent cholesterol efflux (Figure 17).

In summary, the evidence provided here shows that LPL may play a regulatory role in ABCA1-mediated cholesterol efflux. Our data suggest that in addition to exerting a proatherogenic effect in atherosclerotic lesions, LPL may also be antiatherogenic by augmenting ABCA1-mediated cholesterol efflux.

Effect of Intralipid on ABCA1-mediated cholesterol efflux

Considering that LPL is a hydrolytic enzyme converting triglycerides to glycerol and fatty acids, we hypothesized that it may exert its effect on ABCA1 primarily through

generating lipid ligands. Thus, in the presence of the substrate the effect of LPL is expected to be more pronounced. To test this possibility, we decided to incubate the LPL-overxpressing fibroblasts with triglyceride-rich lipid particles isolated from Intralipid as a way of providing the substrate for LPL. We first incubated the cells with Intralipid alone and found that after treating the cells with Intralipid for 24 hours ABCA1 mRNA levels were dramatically reduced relative to the control (Figure 18). As expected, cholesterol, stimulating the LXRα pathway, increased ABCA1 mRNA levels, while incubation of the cells with both cholesterol and Inralipid erased the upregulating effect of cholesterol. Similar effects were observed on the level of ABCA1 protein (Figure 19) and ABCA1-mediated cholesterol efflux (Figure 20).

LPL did not alter the observed effect of Intralipid. Both the levels of ABCA1 mRNA and ABCA1-mediated cholesterol efflux were reduced in the LPL-overxpressing cells (Figures 21 and 22). It is noteworthy that ABCA1-mediated cholesterol efflux was greater from the LPL-overxpressing cells than the cells treated with adLacZ both in the presence and absence of Inralipid (Figure 21), suggesting that LPL and Intralipid exert independent and antagonistic effects on ABCA1-dependent cholesterol efflux. These data, however, do not invalidate the proposed mechanism of upregualtion of ABCA1 mRNA levels by LPL. It is possible that with the amounts of lipid particles used in our experiments any effect of LPL in the presence of the substrate was masked by the downregulating effect of Intralipid.

It would be informative to investigate whether the effect of Intralipid on ABCA1 in the LPL-overexpressing cells is dose-dependent. If the mechanism through which LPL affects ABCA1 mRNA levels involves its catalytic activity and formation of lipid

ligands, then at low concentrations of Intralipid, when its downregulating effect is masked by the activity of LPL, ABCA1-mediated cholesterol efflux from the LPL-overexpressing cells treated with Intralipid should be greater than the efflux from the control cells. These studies can be conducted in the future with the purpose of elucidating the mechanism via which LPL elevates ABCA1 mRNA levels. It would be informative to infect fibroblasts with the adenovirus expressing LPL deficient in enzymatic activity as a way to test the importance of the LPL's catalytic activity in regulating ABCA1. Inhibition of PPAR and LXR in the LPL-overexpressing fibroblasts and analysis of ABCA1 mRNA levels may contribute toward understanding the pathway linking LPL and ABCA1. Specific silencing of the LPL gene in macrophages using RNA interference and analysis of ABCA1 mRNA levels would also be informative in elucidating the effect of LPL on ABCA1.

Intralipid is a phospholipid-stabilized soybean oil emulsion. It is a complex mixture containing representatives of different classes of lipids, such as fatty acids, triglycerides, and phospholipids. The role of Intralipid in cholesterol efflux has previously been investigated. Whitfield et al. investigated the effect of Intralipid on cholesterol efflux from HepG2 cells, showing that the emulsion inhibited the uptake of cholesterol by the cells and enhanced efflux of cellular cholesterol. Neither the component of the Intralipid nor the mechanism by which it produced the effect was identified.[165] In their studies, the efflux was measured in the presence of Intralipid. Considering the data by Whitfield et al., we were surprised to observe a dramatic decrease in ABCA1 mRNA levels and a significant reduction in ABCA1-mediated cholesterol efflux measured after the cells were incubated with Intralipid for 24 hours.

We decided to investigate this phenomenon further and identify the component responsible for the effect.

Unsaturated fatty acids were shown to act as negative regulators of ABCA1-mediated cholesterol efflux by acting as transcriptional deactivators of the ABCA1 gene and promoting proteolysis of the transporter.[111] Phospholipids are the primary substrate for ABCA1 and were shown to be essential in translocation of cholesterol by ABCA1.[67] Several in vitro studies investigated the role of triglycerides in cellular cholesterol efflux, but no data exist as to the effect of triglycerides on ABCA1 mRNA and protein levels and ABCA1-mediated cholesterol efflux.[166-168] We decided to examine the effect of each class of the above lipids on ABCA1 mRNA levels and ABCA1-mediated cholesterol efflux.

Effect of fatty acids on ABCA1 mRNA levels

Whereas oleate dose-dependently decreased ABCA1 mRNA levels, oleic and linoleic acids had an upregulating effect (Figure 23). This could be explained by the presence of structurally different complexes formed in the BSA-containing medium when fatty acids and their salts are introduced into the solution. Oleate containing a charged carboxylate is expected to form micelles in addition to complexation with BSA, and these micelles may act as cholesterol acceptors similarly to phospholipid vesicles, secluding the hydrophobic portion of the cholesterol molecule in the apolar core of the particle. The depletion of cholesterol may cause the decrease in ABCA1 mRNA levels. This possibility, however, is contradicted by the observation that palmitate upregulates ABCA1 mRNA levels, pointing to the influence of structural differences in the hydrocarbon chain of fatty acids. In fact, Bartholow and colleagues showed that the

degree of unsaturation and the length of fatty acyl chains of various phospholipids influence cholesterol efflux to phospholipid vesicles.[154] Similar factors may be at work with fatty acids. Moreover, unsaturated and polyunsaturated fatty acids were shown to act as transcriptional downregulators of the ABCA1 gene, while saturated fatty acids had some or no upregulating effect.[111] This is in agreement with our data regarding the effect of oleate and saturated acids, but contradicts the results obtained with oleic and linoleic acids. The discrepancy may be explained by the metabolic state of the cells during the treatments, the duration of the treatments, and the amounts of the fatty acids used.

Effect of triglycerides on ABCA1-mediated cholesterol efflux

In vitro studies involving various cell lines showed that triglycerides influence cellular cholesterol efflux. Lada et al. depleted the stores of triglycerides in the THP-1-derived macrophages and examined the effect of the depletion on cellular cholesterol efflux.[166] The removal of triglycerides altered the physical state of the droplets of cholesteryl ester in the cells and caused a decrease in the amount of the effluxed cholesterol. HDL rich in triglycerides was shown to facilitate cholesterol efflux from J774 macrophages loaded with cholesterol.[167] Palmer and coworkers incubated macrophage foam cells with various concentrations of triglycerides for up to 24 hours and showed that the treatement lowered apoA-I-mediated cholesterol efflux.[168] Here, we showed that triglycerides are not likely to be the component of Intralipid responsible for the effect produced by the emulstion in cultrured human skin fibroblasts, since incubation of the fibroblasts with triglycerides for 24 hours did not subsequently affect apoA-I-mediated cholesterol efflux (Figure 28). Considering that triglycerides constitute only a portion of lipids in Intralipid, the observed effect of triglycerides on ABCA1 mRNA and

protein levels (Figures 24 and 27, respectively) does not explain the effect observed with the emulsion.

Effect of phospholipids on ABCA1 mRNA and protein levels and ABCA1-mediated cholesterol efflux

In our studies phospholipids were the only substrate that consistently reduced ABCA1 mRNA and protein levels (Figures 24 and 27, respectively) as well as ABCA1-mediated cholesterol efflux (Figure 28) upon incubation with fibroblasts. These effects paralleled those observed with Intralipid, allowing us to conclude that phospholipids are the major constituent of Intralipid responsible for its effect.

The most likely mechanism by which phospholipids downregulate ABCA1-mediated cholesterol efflux is the depletion of cellular pools of cholesterol, leading to downregulation of ABCA1 mRNA and protein levels. It was previously shown that phospholipid vesicles are efficient acceptors of cellular cholesterol and that the cholesterol-removing capacity of phospholipids increases upon its complexation with BSA or HSA.[153,154] Here, we showed that incubation of fibroblasts with phospholipids for 24 hours subsequently reduces apoA-I-mediated cholesterol efflux. The cholesterol-accepting capacity of phospholipids explains this effect. In our experiments, we first introduced the radioactively labeled cholesterol into fibroblasts and then incubated the cells with both cholesterol and phospholipids for 24 hours in the BSA-containing medium. Considering that the molar ratio of PC to BSA in the medium was 9 to 1 (100μg/ml PC and 1mg/ml BSA) and that phospholipids form complexes with BSA with the molar ratio of 1 to 1,[169] it is reasonable to assume that all BSA in the medium was complexed with PC. Complexation of phospholipids with BSA creates efficient acceptors

of cholesterol, which scavenge cholesterol from the medium as well as remove it from the plasma membrane.

Since the molar ratio of PC to cholesterol in the medium was 1 to 4 (100μg/ml PC and 20μg/ml cholesterol) and only a portion of PC complexed with BSA, it is safe to assume that the remaining PC formed multilamelar vesicles or micelles in association with free cholesterol. Considering that the molar ratio of phospholipids to cholesterol in phospholipid vesicles is close to 1:1,[153] the remaining free cholesterol was available for the uptake by the plasma membrane.

The above discussion suggests that the cells incubated with both cholesterol and phospholipids are relatively cholesterol-poor compared to the cells incubated only with cholesterol. Since cholesterol-loading of the cells was shown to elevate ABCA1 mRNA and protein levels, causing an increase in ABCA1-dependent cholesterol efflux, the removal of cholesterol available to the cells from the medium as well as depletion of the plasma membrane cholesterol is expected to produce the opposite effect. In fact, depletion of the cellular pools of cholesterol by HDL and cyclodextrin was shown to reduce ABCA1-mediated cholesterol efflux.[92,149,150] Here we showed that phospholipids are capable of depleting cellular cholesterol, causing a decrease in ABCA1 mRNA and protein levels, lowering apoE-dependent efflux of cholesterol by 50% compared to the control cells (Figure 28).

The mechanism by which phospholipids exert this effect was investigated by measuring the amount of radioactive cholesterol released into the medium while the cells were incubated with phospholipids for 24 hours. Incubation of the cells with phospholipids resulted in a 3-fold increase in the amount of the effluxed cholesterol,

compatible with our proposal of the phospholipid-mediated depletion of cellular cholesterol pools (Figure 29).

As was mentioned earlier, incubation of the cells with a mixture of cholesterol and phospholipids makes the accurate determination of the plasma membrane cholesterol-removing capacity of phospholipids impossible, since phospholipids form complexes with free cholesterol in the medium and thus partly lose the ability to accept cellular cholesterol. To address this question, we first incubated the cells with either cholesterol, phospholipids, or a mixture of cholesterol and phospholipids. We then removed the lipid-containing medium, and incubated the cells for 24 hours in fresh medium free of lipids. Lastly, we performed cholesterol efflux in the absence or presence of phospholipids for 24 hours. Phospholipids increased the amount of radioactive cholesterol released into the medium 3-fold, similar to the increase observed when the efflux was performed in the presence of cholesterol in the medium (Figure 30). It is interesting to note that the amount of the label released is lower than that released during the first 24-hour incubation of the cells with phospholipids as shown in Figures 29 and 30. This decrease is accounted for by the removal of the label by BSA during the second 24-hour incubation period.

Also noteworthy is the difference in the amount of the label released from the cells incubated with phospholipids alone and those incubated with a mixture of cholesterol and phospholipids. This can be explained by dilution of the label in the plasma membrane of the cells incubated with both cholesterol and phospholipids. Unlabeled cholesterol is incorporated into the plasma membrane of the cells and is removed along with the label upon incubation with phospholipids. Thus the proportion of

radioactive cholesterol removed is lower compared to amount of the labeled cholesterol removed from the cell not loaded with cholesterol.

TLC analysis of the lipids extracted from the cells incubated in the presence of cholesterol alone or a mixture of cholesterol and phospholipids supports the proposal of the phospholipid-mediated depletion of cellular cholesterol (Figure 33). The absence of the cholesterol spot on the TLC plate spotted with the lipids from the cells treated with cholesterol and phospholipids suggests that a significant amount of cholesterol is removed from the cells in the presence of phospholipids.

Our data suggest that the depletion of cholesterol occurs by passive diffusion of the molecule to phospholipid vesicles and phospholipid-BSA complexes without esterification of cholesterol in the medium by LCAT. LCAT is a heavily glycosylated enzyme secreted into plasma. To eliminate the possibility of it being released into the medium by fibroblasts and esterifying cholesterol in the presence of phospholipids, thus reducing the amount of free cholesterol available to the cells, we incubated the cells with 1mM DTNB, an LCAT inhibitor. The release of cholesterol via the ABCA1-independent pathway was not affected and phospholipids still increased the amount of radioactive cholesterol released 3-fold compared to the label released from the control cells (Figure 32).

Although DTNB did not affect ABCA1-independent cholesterol efflux, it reduced ABCA1-mediated efflux of cholesterol without altering the effect of phospholipids (Figure 31). These data are compatible with our proposal of the diffusion-mediated efflux of cholesterol to BSA-phospholipid complexes or phospholipid vesicles and eliminates the involvement of LCAT in the phospholipid-mediated depletion of cellular cholesterol.

The observation that DTNB affects ABCA1-mediated cholesterol efflux can be explained by its reactivity. DNTB contains a disulfide bridge and is capable of oxidizing thiols. Therefore it can have an effect on proteins containing disulfide bridges or free thiols. ABCA1 contains a disulfide bridge linking two large extracellular loops of the transporter. It is conceivable that the inhibitor affects the structural organization of the extracellular loops of ABCA1 and thus reduces its activity. It is also possible that DTNB affects intracellular trafficking of ABCA1 and reduces the amount of the transporter in the plasma membrane.

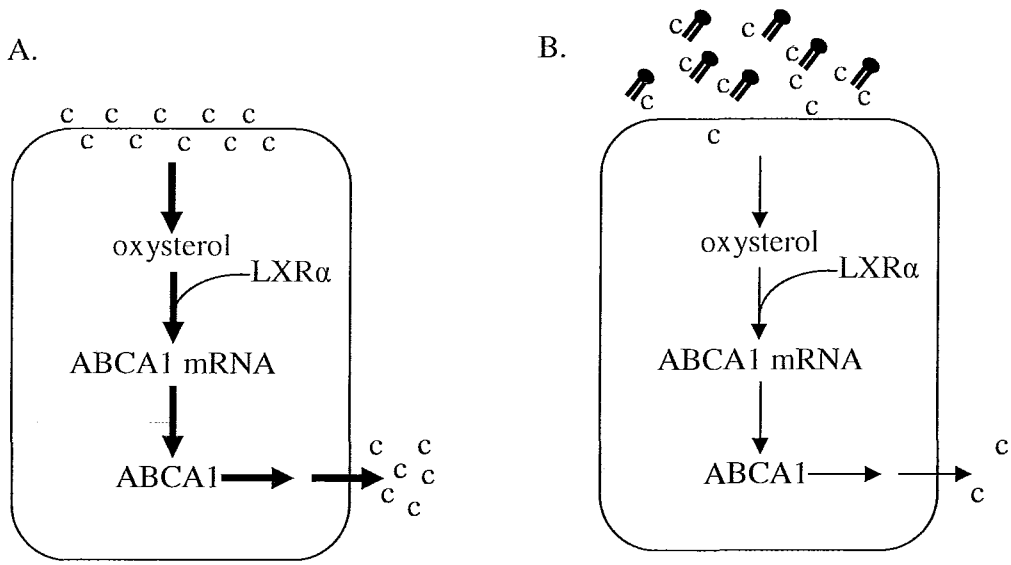


Figure 36. Mechanism of the phospholipid-mediated decrease in ABCA1mRNA levels and ABCA1-mediated cholesterol efflux. A. High levels of cellular cholesterol (c) lead to overproduction of oxysterols, which activate LXRα, a transcriptional activator of the ABCA1 gene. High levels of ABCA1 mRNA result in high concentration of ABCA1 in the plasma membrane and upregulation of ABCA1-mediated cholesterol efflux. B. In the presence of phospholipids, cellular cholesterol is depleted and the oxysterol-LXRα pathway is deactivated, causing a decrease in ABCA1 mRNA levels and ABCA1-mediated cholesterol efflux.

The results of our studies indicate that the effect produced by phospholipids on ABCA1 mRNA levels is transient. Thus, while phospholipids causes a significant decrease in ABCA1 mRNA levels measured after incubating the cells for 24 hours with phospholipids, the effect is partly erased when the phospholipid-containing medium is removed and the cells are maintained in phospholipid-free medium for 24 hours as shown in Figure 35. This is in agreement with our proposal of the decrease in ABCA1 mRNA levels via phospholipid-mediated cholesterol depletion depicted in Figure 36.

Interestingly, we observed a decrease in LCAT mRNA levels upon incubation of the cells with phospholipids and cholesterol, with the former having a more pronounced effect than the latter. Considering the importance of LCAT in ABCA1-mediated cholesterol efflux in vivo, these results are significant and suggest that cholesterol and phospholipids or their metabolites may regulate the LCAT gene on a transcriptional level. The finding that both phospholipids and cholesterol lower LCAT mRNA levels is surprising considering that both are substrates of LCAT and their presence, therefore, would be expected to enhance transcription of the gene. No data exist in the literature regarding the transcriptional regulation of the LCAT gene by cholesterol and phospholipids, and additional experiments are required to investigate this effect. Since the liver is the primary source of LCAT and the amount of the LCAT transcript produced by human fibroblasts is low, in the future we will use cultured hepatocytes to investigate the effect of phospholipids and cholesterol on the transcriptional regulation of the LCAT gene.

In conclusion, we identified phospholipids as the component of Intralipid that reduces ABCA1 mRNA and protein levels, decreasing ABCA1-mediated cholesterol

efflux. We showed that depletion of cellular cholesterol via passive efflux of cholesterol into the phospholipid-rich medium over a period of 24 hours causes transcriptional deactivation of the ABCA1 gene, reducing ABCA1 mRNA and protein levels. In light of the knowledge that phospholipid is the major substrate of ABCA1, the observed effect of phospholipids on the levels of ABCA1 mRNA is interesting since it decreases the amount of the transporter in the plasma membrane and therefore reduces the efflux of both cholesterol and phospholipid. In future studies we will label fibroblasts with radioactive choline to investigate the effect of phospholipids on ABCA1-mediated phospholipid efflux. We will also examine the mechanism of ABCA1-mediated cholesterol efflux by measuring efflux of cholesterol and phospholipid at different time points. It would be informative to repeat the experiments with THP-1-derived macrophages as they have active lipid metabolism and are more relevant to the study of atherogenesis than fibroblasts.

Our data suggest a role for HDL in regulation of ABCA1-mediated cholesterol efflux in vivo. As described earlier, phospholipid- and apolipoprotein-rich HDL particles accept cellular cholesterol via both active lipidation of apoA-I by ABCA1 and passive desorption of cholesterol into the phospholipid monolayer of the particle. The results of our studies show that phospholipids of HDL may indirectly deactivate transcription of the ABCA1 gene via depleting cellular cholesterol in the absence of apoA-I or LCAT. In vitro studies with cultured cells incubated with trypsinized HDL particles are required to investigate this further. It would also be informative to conduct in vivo studies, involving animals infused with phospholipid liposomes or phospholipid-BSA complexes. Quantitation of ABCA1 mRNA levels in different tissues following the treatment with

phospholipids would show if our proposal is valid. Similar studies with animal models of atherosclerosis would yield information as to the effect of phospholipids on ABCA1 mRNA levels in vivo and the development of atherosclerosis.

REFERENCES

1. Berg, J.M., Tymoczko, J.L., Stryer, L. 2002. Phospholipids are the major class of membrane lipids, pp. 322-323; squalene (C_{30}) is synthesized from six molecules of isopentenyl pyrophosphate (C_5), pp. 725-726. Biochemistry 5th Edition. W.H. Freeman and Company, 41 Madison Avenue, New York, New York 10010.
2. Voet, D., Voet, J.G. 1995. Lipoprotein Structure. Biochemistry, 2nd Eddition, pp. 316-325, John Willey & Sons, Inc.
3. Nelson, D.L., Cox, M.M. 2000. Cholesterol and Other Lipids Are Carried on Plasma Lipoproteins. Lehninger Principles of Biochemistry, 3rd Eddition, pp. 804-810, Worth Publishers, New York, NY 10010.
4. Thom, T., Haase, N., Rosamond, W., Howard, V.J., Rumsfeld, J., Manolio, T., Zheng, Z., Flegal, K., O'Donnell, C., Kittner, S., Lloyd-Jones, D., Goff, Jr.D.C., Hong, Y., Adams, R., Friday, G., Furie, K., Gorelick, P., Kissela, B., Marler, J., Meigs, J., Roger, V., Sidney, S., Sorlie, P., Steinberger, J., Wasserthiel-Smoller, S., Wilson, M., Wolf, P. 2006. Heart Disease and Stroke Statistics – 2006 Update: A Report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **113**:85-151.
5. Libby, P. 1995. Molecular Bases of the Acute Coronary Syndromes. *Circulation* **91**:2844-2850.
6. Libby, P. 2001. Current Concepts of the Pathogenesis of the Acute Coronary Syndromes. *Circulation* **104**:365-372.
7. Libby, P., Ridker, P.M., Maseri, A. 2002. Inflammation and Atherosclerosis. *Circulation* **105**:1135-1143.

8. Holvoet, P., Vanhaecke, J., Janssens, S., Van de Werf, F., Collen, D. 1998. Oxidized LDL and Malondialdehyde-Modified LDL in Patients with Acute Coronary Syndromes and Stable Coronary Artery Disease. *Circulation* **98**:1487-1494.

9. Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P., Darnell, J. Structure of an LDL particle. Molecular Cell Biology, 3rd Eddition, p. 725, Scientific American Books, Inc.

10. Mead, J.R., Cryer, A., Ramji, D. 1999. Lipoprotein lipase, a key role in atherosclerosis. *FEBS Letters* **462**:1-6.

11. Jin, W., Marchadier, D., Rader, D.J. 2002. Lipases and HDL metabolism. *TRENDS in Endocrinology & Metabolism* **13**:174-178.

12. Hide, W., Chan, L., Li, W. 1992. Structure and evolution of the lipase family. *Journal of Lipid Research* **33**:167-178.

13. Wong, H., Davis, R.C., Thuren, T., Goers, J.W., Nikazy, J., Waite, M., Schotz, M.C. 1994. Lipoprotein Lipase Domain Function. *Journal of Biological Chemistry* **269**:10319-10323.

14. Derewenda, Z.S., Derewenda, U. 1991. Relationships among serine hydrolases: evidence for a common structural motif in triacylglyceride lipases and esterases. *Biochemistry and Cell Biology* **69**:842-851.

15. van Tilbeurgh, H., Roussel, A., Lalouel, J.M., Cambillau, C. 1994. Lipoprotein lipase. Molecular model based on the pancreatic lipase x-ray structure: consequences for heparin binding and catalysis. *Journal of Biological Chemistry* **269**:4626-4633.

16. Kobayashi, Y., Nakajima, T., Inoue, I. 2002. Molecular modeling of the dimeric structure of human lipoprotein lipase and functional studies of the carboxy-terminal domain. *European Journal of Biochemistry* **269**:4701-4710.

17. Zhang, L., Lookene, A., Wu, G., Olivecrona, G. Calcium Triggers Folding of Lipoprotein Lipase into Active Dimers. *Journal of Biological Chemistry* **280**:42580-42591.

18. Camps, L., Reina, M., Llobera, M., Vilaro, S., Olivecrona, T. 1990. Lipoprotein lipase: cellular origin and functional distribution. *American Journal of Physiology* **258**:673-681.

19. Camps, L., Reina, M., Llobera, M., Bengtsson-Olivecrona, G., Olivecrona, T., Vilaro, S. 1991. Lipoprotein lipase in lungs, spleen, and liver: synthesis and distribution. *Journal of Lipid Research* **32**:1877-1888.

20. Berneis, K., Krauss, R.M. 2002. Metabolic origins and clinical significance of LDL heterogeneity. *Journal of Lipid Research* **43**:1363-1379.

21. Yla-Herttuala, S., Lipton, B.A., Rosenfeld, M.E., Goldberg, I.J., Steinberg, D., Witztum, J.L. 1991. Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions. *Proceedings of the National Academy of Sciences of the U.S.A.* **88**:10143-10147.

22. O'Brien, K.D., Gordon, D., Deeb, S., Ferguson, M., Chait, A. 1992. Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *Journal of Clinical Investigation* **89**:1544-1550.

23. Babaev, V.R., Fazio, S., Gleaves, L.A., Carter, K.J., Semenkovich, C.F., Linton, M.F. 1999. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *Journal of Clinical Investigation* **103**:1697-1705.

24. Wilson, K., Fry, G.L., Chappell, D.A., Sigmund, C.D., Medh, J.D. 2001. Macrophage-Specific Expression of Human Lipoprotein Lipase Accelerates Atherosclerosis in Transgenic Apolipoprotein E Knockout Mice but Not in C57BL/6 Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **21**:1809-1815.

25. Wu, X., Wang, J., Fan, J., Chen, M., Chen, L., Huang, W., Liu, G. 2006. Localized vessel expression of lipoprotein lipase in rabbits leads to rapid lipid deposition in the balloon-injured wall. *Atherosclerosis* **187**:65-73.

26. Zilversmit, D.B. 1973. A proposal linking atherogenesis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circulation Research* **33**:633-638.

27. Lindqvist, P., Ostlund-Lindqvist, A., Witztum, J.L., Steinberg, D., Little, J.A. 1983. The Role of Lipoprotein Lipase in the Metabolism of Triglyceride-rich Lipoproteins by Macrophages. *Journal of Biological Chemistry* **258**:9086-9092.

28. Evans, A.J., Sawyez, C.G., Wolfe, B.M., Connelly, P.W., Maguire, G.F., Huff, M.W. 1996. Evidence that cholesteryl ester and triglyceride accumulation in J774 macrophages induced by very low density lipoprotein subfractions occurs by different mechanisms. *Journal of Lipid Research* **34**:703-717.

29. Aviram, M., Bierman, E.L., Chait, A. 1988. Modification of low density lipoproteins by lipoprotein lipase induces enhanced uptake and cholesterol accumulation in cells. *Journal of Biological Chemistry* **263**:15416-15422.

30. Beisiegel, U., Weber, W., Bengtsson-Olivecrona, G. 1991. Lipoprotein Lipase Enhances the Binding of Chylomicrons to Low Density Lipoprotein Receptor-Related Protein. *Proceedings of the National Academy of Sciences of the U.S.A.* **88**:8342-8346.

31. Medh, J.D., Bowen, S.L., Fry, G.L., Ruben, S., Andrack, M., Inoue, I., Lalouel, J., Strickland, D.K., Chappell, D.A. 1996. Lipoprotein Lipase Binds to Low Density Lipoprotein Receptors and Induces Receptor-mediated Catabolism of Very Low Density Lipoproteins in Vitro. *Journal of Biological Chemistry* **271**:17073-17080.

32. Takahashi, S., Suzuki, J., Kohno, M., Oida, K., Tamai, T., Miyabo, S., Yamamoto, T., Nakai, T. 1995. Enhancement of the Binding of Triglyceride-rich Lipoproteins to the Very Low Density Lipoprotein Receptor by Apolipoprotein E and Lipoprotein Lipase. *Journal of Biological Chemistry* **270**:15747-15754.

33. Fernandez-Borja, M., Bellido, D., Vilella, E., Olivecrona, G. 1996. Lipoprotein lipase-mediated uptake of lipoprotein in human fibroblasts: evidence for an LDL receptor-independent internalization pathway. *Journal of Lipid Research* **37**:464-481.

34. Weaver, A.M., Lysiak, J.J., Gonias, S.L. 1997. LDL receptor family-dependent and -independent pathways for the internalization and digestion of lipoprotein lipase-associated beta-VLDL by rat vascular smooth muscle cells. *Journal of Lipid Research* **38**:1841-1850.

35. Jukema, J.W., van Boven, J., Groenemeijer, B., Zwinderman, A.H., Reiber, J.H.C, Bruschke, A.V.G., Henneman, J.A., Molhoek, G.P., Bruin, T., Jansen, H., Gagne, E., Hayden, M.R., Kastelein, J.P. 1996. The Asp_9Asn Mutation in the Lipoprotein Lipase Gene is Associated with Increased Progression of Coronary Atherosclerosis. *Circulation* **94**:1913-1918.

36. Wittrup, H.H., Tybjaerg-Hansen, A., Abildgaard, S., Steffensen, R., Schnohr, P., Nordestgaard, B.C. 1997. A Common Substitution (Asn291Ser) in Lipoprotein Lipase Is Associated with Increased Risk of Ischemic Heart Disease. *Journal of Clinical Investigation* **99**:1606-1613.

37. Rip, J., Nierman, M.C., Wareham, N.J., Luben, R., Bingham, S.A., Day, N.E., van Miert, J.N.I., Hutten, A.B., Kastelein, J.J.P., Kuivenhoven, J.A., Khaw, K., Boekholdt, S.M. 2006. Serum Lipoprotein Lipase Concentration and Risk for Future Coronary Artery Disease: The EPIC-Norfolk Prospective Population Study. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**:637-642.

38. Tsutsumi, K., Inoue, Y., Shima, A., Iwasaki, K., Kawamura, M., Murase, T. 1993, The Novel Compound NO-1886 Increases Lipoprotein Lipase Activity with Resulting Elevation of High Density Lipoprotein Cholesterol, and Long-term Administration Inhibits Atherogenesis in the Coronary Arteries of Rats with Experimental Atherosclerosis. *Journal of Clinical Investigation* **92**:411-417.

39. Shimada, M., Ishibashi, S., Inaba, T., Yagyu, H., Harada, K., Osuga, J., Ohashi, K., Yazaki, Y., Yamada, N. 1996. Suppression of diet-induced atherosclerosis in low density lipoprotein receptor knockout mice overexpressing lipoprotein lipase. *Proceedings of the National Academy of Sciences of the U.S.A.* **93**:7242-7246.

40. Yagyu, H., Ishibashi, S., Chen, Z., Osuga, J., Okazaki, M., Perrey, S., Kitamine, T., Shimada, M., Ohashi, K., Harada, K., Shionoiri, F., Yahagi, N., Gotoda, T., Yazaki, Y., Yamada, N. 1999. Overexpressed lipoprotein lipase protects against atherosclerosis in apolipoprotein E knockout mice. *Journal of Lipid Research* **40**:1677-1685.

41. Fielding, C.J., Fielding, P.E. 1995. Molecular physiology of reverse cholesterol transport. *Journal of Lipid Research* **36**:211-228.

42. Oram, J.F. 2003. HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23**:720-727.

43. Lewis, G.F., Rader, D.J. 2005. New Insight into the Regulation of HDL Metabolism and Reverse Cholesterol Transport. *Circulation Research* **96**:1221-1232.

44. Stein, O., Stein, Y. 1999. Atheroprotective mechanisms of HDL. *Atherosclerosis* **144**:285-301.

45. Akopian, D., Medh, J.D. 2006. Genetics and molecular biology: phospholipid transfer protein in atherosclerosis. *Current Opinion in Lipidology* **17**:695-698.

46. Cockerill, G.W., Rye, K., Gamble, J.R., Vadas, M.A., Barter, P.J. 1995. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arteriosclerosis, Thrombosis, and Vascular Bio*logy **15**:1987-1994.

47. Ashby, D.T., Rye, K., Clay, M.A., Vadas, M.A., Gamble, J.R., Barter, P.J. 1998. Factors Influencing the Ability of HDL to Inhibit Expression of Vascular Cell

Adhesion Molecule-1 in Endothelial Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* **18**: 1450-1455.

48. Mackness, M.I., Arrol, S., Abbott, C., Durrington, P.N. 1993. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis* **104**:129-135.

49. Watson, A.D., Berliner, J.A., Hama, S.Y., La Du, B.N., Faull, K.F., Fogelman, A.M., Navab, M. 1995. Protective Effect of High Density Lipoprotein Associated Paraoxonase. Inhibition of the Biological Activity of Minimally Oxidized Low Density Lipoprotein. *Journal of Clinical Investigation* **96**:2882-2891.

50. Stein, O., Stein, Y. 1973. The removal of cholesterol from landschutz ascites cells by high-density lipoprotein. *Biochimica et Biophysica Acta* **326**:232-244.

51. Jackson, R., Gotto, A.M., Stein, O., Stein, Y. 1975. A Comparative Study on the Removal of Cellular Lipids from Landschutz Ascites Cells by Human Plasma Apolipoproteins. *Journal of Biological Chemistry* **250**:7204-7209.

52. Oram, J.F., Albers, J.J., Cheung, M.C., Bierman, E.L. 1981. The Effect of Subfractions of High Density Lipoprotein on Cholesterol Efflux from Cultured Fibroblasts. *Journal of Biological Chemistry* **256**:8348-8356.

53. Castro, G.R., Fielding, C.J. 1988. Early Incorporation of Cell-Derived Cholesterol into Pre-β-Migrating High-Density Lipoprotein. *Biochemistry* **27**:25-29.

54. Asztalos, B., Zhang, W., Roheim, P.S., Wong, P. 1997. Role of Free Apolipoprotein A-I in Cholesterol Efflux. Formation of Pre-α-Migrating High-Density Lipoprotein Particles. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17**:1630-1636.

55. Sviridov. D., Miyazaki, O., Theodore, K., Hoang, A., Fukamachi, I., Nestel, P. 2002. Delineation of the role of pre-beta 1-HDL in cholesterol efflux using isolated pre-beta 1-HDL. *Arteriosclerosis, Thrombosis, and Vascular Biology* **22**:1482-1488.

56. Wu, X.W., Yang, L.C., Xu, Y.H., Fu, Q., Fu, M.D. 2004. Effect of purified pre-beta 1 HDL on efflux of ^{3}H-cholesterol loaded in human smooth muscle cells. *Sichuan Da Xue Xue Bao Yi Xue Ban*. **35**:18-20.

57. Oram, J.F., 2000. Tangier disease and ABCA1. *Biochimica et Biophysica Acta* **1529**:321-330.

58. Knight, B.L. 2004. ATP-binding cassette transporter A1: regulation of cholesterol efflux. *Biochemical Society Transactions* **32**:124-127.

59. Glomset, J.A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *Journal of Lipid Research* **9**:155-167.

60. Aron, L., Jones, S., Fielding, C.J. 1978. Human Plasma Lecithin-Cholesterol Acyltransferase. Characterization of Cofactor-Dependent Phospholipase Activity. *Journal of Biological Chemistry* **253**:7220-7226.

61. Nakamura, Y., Kotite, L., Gan, Y., Spencer, T.A., Fielding, C.J., Fielding, P.E. 2004. Molecular mechanism of reverse cholesterol transport: reaction of pre-beta-migrating high-density lipoprotein with plasma lecithin/cholesterol acyltransferase. *Biochemistry* **43**:14811-14820.

62. Gong, E.L., Nichols, A.V., Forte, T.M., Blanche, P.J., Shore, V.G. 1988. Transformation of large discoidal complexes of apolipoprotein A-I and

phosphatidylcholine by lecithin-cholesterol acyltransferase. *Biochimica et Biophysica Acta* **961**:72-85.

63. Greene, D.J., Skeggs, J.W., Morton, R.E. 2001. Elevated triglyceride content diminishes the capacity of high density lipoprotein to deliver cholesteryl esters via the scavenger receptor class B type I (SR-BI). *Journal of Biological Chemistry* **276**:4804-4811.
64. Harder, C.J., Vassiliou, G., McBride, H.M., McPherson, R. 2006. Hepatic SR-BI-mediated cholesteryl ester selective uptake occurs with unaltered efficiency in the absence of cellular energy. *Journal of Lipid Research* **47**:492-503.
65. Wang, N., Tall, A.R. 2003. Regulation and Mechanisms of ATP-Binding Cassette Transporter A1-Mediated Cellular Cholesterol Efflux. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23**:1178-1184.
66. Gillotte-Taylor, K., Nicker, M., Johnson, W.J., Francone, O.L., Holvoet, P., Lund-Katz, S., Rothblat, G.H., Phillips, M.C. 2002. Effects of Enrichment of Fibroblasts with Unesterified Cholesterol on the Efflux of Cellular Lipids to Apolipoprotein A-I. *Journal of Biological Chemistry* **277**:11811-11820.
67. Wang, N., Silver, D.L., Thiele, C., Tall, A.R. 2001. ATP-binding Cassette Transporter A1 (ABCA1) Functions as a Cholesterol Efflux Regulatory Protein. 2001. *Journal of Biological Chemistry* **276**:23742-23747.
68. Oram, J.F., Vaughan, A.M., Stocker, R. 2001. ATP-binding transporter A1 mediates cellular secretion of alpha-tocopherol. *Journal of Biological Chemistry* **276**:39898-39902.

69. Von Eckardstein, A., Langer, C., Engel, T., Schaukal, I., Cignarella, A., Reinhardt, J., Lorkowski, S., Li, Z., Zhou, X., Cullen, P., Assmann, G. 2001. ATP binding cassette transporter ABCA1 modulates the secretion of apolipoprotein E from human monocyte-derived macrophages. *FASEB Journal* **15**: 1555-1561.

70. Zhou, X., Engel, T., Goepfert, C., Erren, M., Assmann, G, von Eckardstein, A. 2002. The ATP binding cassette transporter A1 contributes to the secretion of interleukin 1 beta from macrophages but not from monocytes. *Biochemical and Biophysical Research Communications* **291**:598-604.

71. Dean, M., Hamon, Y., Chimini, G. 2001. The human ATP-binding cassette (ABC) transporter superfamily. *Journal of Lipid Research* **42**:1007-1017.

72. Stefkova, J., Poledne, R., Hubacek, J.A. 2004. ATP-binding Transporters in Human Metabolism and Diseases. *Physiological Res*earch **53**:235-243.

73. Bungert, S., Molday, L.L., Molday, R.S. 2001. Membrane topology of the ATP binding cassette transporter ABCR and its relationship to ABC1 and related transporters: identification of N-linked glycosylation sites. *Journal of Biological Chemistry* **276**:23539-23546.

74. Fitzgerald, M.L., Morris, A.L., Rhee, J.S., Andersson, L.P., Mendez, A.J., Freeman, M.W. 2002. Naturally occurring mutations in the largest extracellular loops of ABCA1 can disrupt its direct interaction with apolipoprotein A-I. *Journal of Biological Chemistry* **277**:33178-33187.

75. Lawn, R.M., Wade, D.P., Couse, T.L., Wilcox, J.N., 2001. Localization of human ATP-binding cassette transporter 1 (ABC1) in normal and atherosclerotic tissues. *Arteriosclerosis, Thrombosis, and Vascular Biology* **21**:378-385.

76. Neufeld, E.B., Remaley, A.T., Demosky, S.J., Stonik, J.A., Cooney, A.M., Comly, M., Dwyer, N.K., Zhang, M., Blanchette-Mackie, J., Santamarina-Fojo, S., Brewer, Jr.H.B. 2001. Cellular Localization and Trafficking of the Human ABCA1 Transporter. *Journal of Biological Chemistry* **276**:27584-27590.

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

78. Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., Chimini, G. 2001. Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. *Journal of Biological Chemistry* **276**:9955-9960.

79. Remaley, A.T., Thomas, F., Stonik, J.A., Demosky, S.J., Bark, S.E., Neufeld, E.B., Bocharov, A.V., Vishnyakova, T.G., Patterson, A.P., Eggerman, T.L., Santamarina-Fojo, S., Brewer, H.B. 2003. Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and ABCA1-independent pathway. *Journal of Lipid Research* **44**:828-836.

80. Gaus, K., Kritharides, L., Schmitz, G., Boettcher, A., Drobnik, W., Langmann, T., Quinn, C.M., Death, A. 2004. Apolipoprotein A-I interaction with plasma membrane lipid rafts controls cholesterol export from macrophages. *FASEB Journal* **18**:574-576.

81. Landry, Y.D., Denis, M., Nandi, S., Bell, S., Vaughan, A.M., Zha, X. ATP-binding cassette transporter A1 expression disrupts raft membrane microdomains

through its ATPase-related functions. *Journal of Biological Chemistry* **281**:36091-36101.

82. Wang, N., Silver, D.L., Costet, P., Tall, A.R. 2000. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *Journal of Biological Chemistry* **275**:33053-33058.

83. Fitzgerald, M.L., Morris, A.L., Chroni, A., Mendez, A.J., Zannis, V.I., Freeman, M.W. 2004. ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *Journal of Lipid Research* **45**:287-294.

84. Chroni, A., Liu, T., Fitzgerald, M.L., Freeman, M.W., Zannis, V.I. 2004. Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry* **24**:2126-2139.

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

86. Vehachalam, C., Liu, L., Nickel, M., Dhanasekaran, P., Anantharamaiah, G.M., Lund-Katz, S., Rothblat, G.H., Phillips, M.C. 2004. Influence of ApoA-I structure on the ABCA1-mediated efflux of cellular lipids. *Journal of Biological Chemistry* **279**:49931-49939.

87. Fielding, P.E., Nagao, K., Hakamata, H., Chimini, G., 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.

88. Smith, J.D., Le Goff, W., Settle, M., Brubaker, G., Waelde, G., Horwitz, A., Oda, M.N. 2004. ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *Journal of Lipid Research* **45**:635-644.

89. Takahashi, K., Kimura, Y., Kioka, N., Matsuo, M., Ueda, K. 2006. Purification and ATPase activity of human ABCA1. *Journal of Biological Chemistry* **281**:10760-10768.

90. Tall, A.R., Costet, P., Wang, N. 2002. Regulation and mechanisms of macrophage cholesterol efflux. *Journal of Clinical Investigation* **110**:899-903.

91. Mendez, A.J. 1997. Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion. *Journal of Lipid Research* **38**:1807-1821.

92. Langmann, T., Klucken, J., Reil, M., Liebisch, G., Luciani, M., Chimini, G., Kaminski, W.E., Schmitz, G. 1999. Molecular Cloning of the Human ATP-binding Cassette Transporter 1 (hABC1): Evidence for Sterol-Dependent Regulation in macrophages. *Biochemical and Biophysical Research Communications* **257**:29-33.

93. Rong, J.X., Shapiro, M., Trogan, E., Fisher, E.A. 2003. Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proceedings of the National Academy of Sciences of the U.S.A.* **100**:13531-13536.

94. Ulven, S.M., Dalen, K.T., Gustafsson, J.A., Nebb, H.I. 2005. LXR is crucial in lipid metabolism. *Prostaglandins, Leukotrienes, & Essential Fatty Acids* **73**:59-63.

95. Bruemmer, D., Law, R.E. 2005. Liver x receptors: potential novel targets in cardiovascular diseases. *Current Drug Targets: Cardiovascular & Haematological Disorders* **5**:533-540.

96. Li, A.C., Glass, C.K. 2004. PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. *Journal of Lipid Research* **45**:2161-73.

97. Venkateswaran, A., Laffitte, B.A., Joseph, S.B., Mak, P.A., Wilpitz, D.C., Edwards, P.A., Tontonoz, P. 2000. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXRα. *Proceedings of the National Academy of Sciences of the U.S.A.* **97**:12097-12102.

98. Schwartz, K., Lawn, R.M., Wade, D.P. 2000. ABC1 Gene Expression and Apo-I-Mediated Cholesterol Efflux Are Regulated by LXR. *Biochemical and Biophysical Research Communications* **274**:794-802.

99. Costet, P., Luo, Y., Wang, N., Tall, A.R. 2000. Sterol-dependent Transactivation of the *ABC1* Promoter by the Liver X Receptor/Retinoid X Receptor. *Journal of Biological Chemistry* **275**:28240-28245.

100. Fu, X., Menke, J.G., Chen, Y., Zhou, G., MacNaul, K.L., Wright, S.D., Sparrow, C.P., Lund, E.G. 2001. 27-Hydroxycholesterol Is an Endogenous Ligand for Liver X Receptor in Cholesterol-loaded Cells. *Journal of Biological Chemistry* **276**:38378-38387.

101. Li, Y., Bolten, C., Bhat, B.G., Woodring, J., Li, S., Prayaga, S.K., Xia, C., Lala, D.S. 2002. Induction of Human Liver X Receptor α Gene Expression Via an Autoregulatory Loop Mechanism. *Molecular Endocrinology* **16**:506-514.

102. Marx, N., Duez, H., Fruchart, J.C., Staels, B. 2004. Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells. *Circulation Research* **94**:1168-1178.

103. Chawla, A., Boisvert, W.A., Lee, C., Laffitte, B.A., Barak, Y., Joseph, S.B., Liao, D., Nagy, L., Edwards, P.A., Curtiss, L.K., Evans, R.M., Tontonoz, P. 2001. A PPARγ-LXR-ABCA1 Pathway in Macrophages Is Involved in Cholesterol Efflux and Atherogenesis. *Molecular Cell* **7**:161-171.

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

105. Akiyama, T.E., Sakai, S., Lambert, G., Nicol, C.J., Matsusue, K., Pimprale, S., Lee, Y., Ricote, M., Glass, C.K., Brewer, Jr.H.B., Gonzalez, F.J. 2002. Conditional Disruption of the Peroxisome Proliferator-Activated Receptor γ Gene in Mice Results in Lowered Expression of ABCA1, ABCG1, and apoE in Macrophages and Reduced Cholesterol Efflux. *Molecular and Cellular Biology* **22**:2607-2619.

106. Smith, J.D., Miyata, M., Ginsberg, M., Grigaux, C., Shmookler, E., Plump, A.S. 1996. Cyclic AMP Induces Apolipoprotein E Binding Activity and Promotes Cholesterol Efflux from a Macrophage Cell Line to Apolipoprotein Acceptors. *Journal of Biological Chemistry* **271**:30647-30655.

107. Bortnick, A.E., Rothblat, G.H., Stoudt, G., Hoppe, K.L., Royer, L.J., McNeish, J., Francone, O.L. 2000. The Correlation of ATP-binding Cassette 1 mRNA Levels with Cholesterol Efflux from Various Cell Lines. *Journal of Biological Chemistry* **275**:28634-28640.

108. Oram, J.F., Lawn, R.M., Garvin, M.R., Wade, D.P. 2000. ABCA1 Is the cAMP-inducible Apolipoprotein Receptor That Mediates Cholesterol Secretion from Macrophages. *Journal of Biological Chemistry* **275**:34508-34511.

109. Le Goff, W., Zheng, P., Brubaker, G., Smith, J.D. 2006. Identification of the cAMP-Responsive Enhancer of the Murine ABCA1 Gene: Requirement for CREB1 and STAT3/4 Elements. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**:527-533.

110. Gan, X., Kaplan, R., Menke, J.G., MacNaul, K., Chen, Y., Sparrow, C.P., Zhou, G., Wright, S.D., Cai, T. 2001. Dual Mechanisms of ABCA1 Regulation by Geranylgeranyl Pyrophosphate. *Journal of Biological Chemistry* **276**:48702-48708.

111. Uehara, Y., Engel, T., Li, Z., Goepfert, C., Rust, S., Zhou, X., Langer, C., Schachtrup, C., Wiekowski, J., Lorkowski, S., Assmann, G., von Eckardstein, A. 2002. Polyunsaturated Fatty Acids and Acetoacetate Downregulated the Expression of the ATP-Binding Cassette Transporter A1. *Diabetes* **51**:2922-2928.

112. Haidar, B., Denis, M., Krimbou, L., Marcil, M., Genest, Jr.J. 2002. cAMP induces ABCA1 phosphorylation activity and promotes cholesterol efflux from fibroblasts. *Journal of Lipid Research* **43**:2087-2094.

113. Wang, Y., Oram, J.F. 2002. Unsaturated Fatty Acids Inhibit Cholesterol Efflux from Macrophages by Increasing Degradation of ATP-binding Cassette Transporter A1. *Journal of Biological Chemistry* **277**:5692-5697.

114. Wang, N., Chen, W., Linsel-Nitschke, P., Martinez, L.O., Agerholm-Larsen, B., Silver, D.L., Tall, A.R. 2003. A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I. *Journal of Clinical Investigation* **111**:99-107.

115. Freeman, M.W. 1999. Effluxed lipids: Tangier Island's latest export. *Proceedings of the National Academy of Sciences of the U.S.A.* **96**:10950-10952.

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

117. Remaley, A.T., Schumacher, U.K., Stonik, J.A., Farsi, B.D., Nazih, H., Brewer, Jr.H.B. 1997. Decreased Reverse Cholesterol Transport from Tangier Disease Fibroblasts. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17**:1813-1821.

118. Oram, J.F., Mendez, A.J., Lymp, J., Kavanagh, T.J., Halbert, C.L. 1999. Reduction in apolipoprotein-mediated removal of cellular lipids by immortalization of human fibroblasts and its reversion by cAMP: lack of effect with Tangier disease cells. *Journal of Lipid Research* **40**:1769-1781.

119. Brooks-Wilson, A., Marcil, M., Clee, S.M., Zhang, L., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J.A., Molhuizen, H.O.F., Loubser, O., Ouelette, B.F.F., Fichter, K., Ashbourne-Excoffon, K.J.D., Sensen, C.W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S.,

Kastelein, J.J.P., Genest, Jr.J., Hayden, M.R. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nature Genetics* **22**:336-345.

120. Bodzioch, M. Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., Kaminski, W.E., Hahmann, H.W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K.J., Schmitz, G. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nature Genetics* **22**:347-351.

120. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J., Deleuze, J., Brewer, H.B., Duverger, N., Denefle, P., Assmann, G. 1999. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nature Genetics* **22**:352-355.

121. Lawn, R.M., Wade, D.P., Garvin, M.R., Wang, X., Schwartz, K., Porter, J.G., Seilhamer, J.J., Vaughan, A.M., Oram, J.F. 1999. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *Journal of Clinical Investigation* **0**:08119-R31.

122. Aiello, R.J., Brees, D., Bourassa, P., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., Francone, O.L. 2002. Increased Atherosclerosis in Hyperlipidemic Mice With Inactivation of ABCA1 in Macrophages. *Arteriosclerosis, Thrombosis, and Vascular Biology* **22**:630-637.

123. Van Eck, M., Bos, S.T., Kaminski, W.E., Orso, E., Rothe, G., Twisk, J., Bottcher, A., Van Amersfoort, E.S., Christiansen-Weber, T.A., Fung-Leung, W., Van Berkel, J.C., Schmitz, G. 2002. Leukocyte ABCA1 controls susceptibility to

atherosclerosis and macrophage recruitment into tissues. *Proceedings of the National Academy of Sciencses of the U.S.A.* **99**:6298-6303.

124. Albrecht, C., Soumian, S., Amey, J.S., Sardini, A., Higgins, C.F., Davies, A.H., Gibbs, R.G.J. 2004. ABCA1 Expression in Carotid Atherosclerotic Plaques. *Stroke* **35**:2801-2806.

125. Francone, O.L., Royer, L., Boucher, G., Haghpassand, M., Freeman, A., Brees, D., Aiello, R.J. 2005. Increased Cholesterol Deposition, Expression of Scavenger Receptors, and Response to Chemotactic Factors in Abca1-Deficient Macrophages. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25**:1198-1205.

126. Moore, R.E., Navab, M., Millar, J.S., Zimetti, F., Hama, S., Rothblat, G.H., Rader, D.J. 2005. Increased Atherosclerosis in Mice Lacking Apolipoprotein A-I Attributable to Both Impaired Reverse Cholesterol Transport and Increased Inflammation. *Circulation Research* **97**:763-771.

127. Van Eck, M., Singaraja, R.R., Ye, D., Hildebrand, R.B., James, E.R., Hayden, M.R., Van Berkel, T.J. 2006. Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**: 929-934.

128. Yang, C.Y., Manoogian, G., Pao, Q., Lee, F.S., Knapp, R.D., Gotto, Jr.A.M., Pownall, H.J. 1987. Lecithin:cholesterol acyltransferase. Functional regions and a structural model of the enzyme. *Journal of Biological Chemistry* **262**:3086-3091.

129. Harada, Y., Inoue, I., Takahashi, K. 1994. Lecithin:cholesterol acyltransferase. *Nippon Rinsho* **52**:3158-3163.

130. Warden, C.H., Langner, C.A., Gordon, J.I., Taylor, B.A., McLean, J.W., Lusis, A.J. 1989. Tissue-specific expression, developmental regulation, and chromosomal mapping of the lecithin:cholesterol acyltransferase gene. Evidence for expression in brain and testes as well as liver. *Journal of Biological Chemistry* **264**:21573-211581.

131. Park, M.S., Kudchodkar, B.J., Frohlich, J., Pritchard, H., Lacko, A.G. 1987. Study of the components or reverse cholesterol transport in lecithin:cholesterol acyltransferase deficiency. *Archives of Biochemistry and Biophysics* **258**:545-554.

132. Francone, O.L., Haghpassand, M., Bennet, J.A., Royer, L., McNeish, J. 1997. Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport. *Journal of Lipid Research* **38**:813-822.

133. Nakamura, Y., Kotite, L., Gan, Y., Spencer, T.A., Fielding, C.J., Fielding, P.E. 2004. Molecular mechanism of reverse cholesterol transport: reaction of pre-beta-migrating high-density lipoprotein with plasma lecithin/cholesterol acyltransferase. *Biochemistry* **43**:14811-14820.

134. Davis, R.A., Helgerud, P., Dueland, S., Drevon, C.A. 1982. Evidence that reverse cholesterol transport occurs in vivo and requires lecithin-cholesterol acyltransferase. *Biochimica et Biophysica Acta* **689**:410-414.

135. Kostner, G.M., Knipping, G., Groener, J.E., Zechner, R., Dieplinger, H. 1987. The role of LCAT and cholesteryl ester transfer proteins for the HDL and LDL structure and metabolism. *Advances in Experimental Medicine and Biology* **210**:79-86.

136. Elkahlil, L., Majd, Z., Bakir, R., Perez-Mendez, O., Castro, G., Poulain, P., Lacroix, B., Duhal, N., Fruchart, J.C., Luc, G. 1997. Fish-eye disease: structural and in vivo metabolic abnormalities of high-density lipoproteins. *Metabolism* **46**:474-483.

137. Asztalos, B.F., Schaefer, E.J., Horvatch, K.V., Yamashita, S., Miller, M., Franceschini, G., Calabresi, L. 2007. Role of LCAT in HDL remodeling: investigation of LCAT deficiency states. *Journal of Lipid Research* **48**:592-599.

138. Kuivenhoven, J.A., Pritchard, H., Hill, J., Frohlich, J., Assmann, G., Kastelein, J. 1997. The molecular pathology of lecithin:cholesterol acyltransferase (LCAT) deficiency syndromes. *Journal of Lipid Research* **38**:191-205.

139. Vaisman, B.L., Klein, H.G., Rouis, M., Berard, A.M., Kindt, M.R., Talley, G.D., Meyn, S.M., Hoyt, Jr.R.F., Marcovina, S.M., Albers, J.J. 1995. Overexpression of human lecithin:cholesterol acyltransferase leads to hyperalphalipoproteinemia in transgenic mice. *Journal of Biological Chemistry* **270**:12269-12275.

140. Bolin, D.J., Jonas, A. 1994. Binding of lecithin:cholesterol acyltransferase to reconstituted high density lipoproteins is affected by their lipid but not apolipoprotein composition. *Journal of Biological Chemistry* **269**:7429-7434.

141. Bolin, D.J., Jonas, A. 1996. Sphingomyelin inhibits the lecithin-cholesterol acyltransferase reaction with reconstituted high density lipoproteins by decreasing enzyme binding. *Journal of Biological Chemistry* **271**:19152-19158.

142. Subbaiah, P.V., Horvath, P., Achar, S.B. 2006. Regulation of the activity and fatty acid specificity of lecithin-cholesterol acyltransferase by sphingomyelin and its metabolites, ceramide and ceramide phosphate. *Biochemistry* **45**:5029-5038.

143. Banka, C.L., Bonnet, D.J., Black, A.S., Smith, R.S., Curtis, L.K. 1991. Localization of an apolipoprotein A-I epitope critical for activation of lecithin-cholesterol acyltransferase. *Journal of Biological Chemistry* **266**:23886-23892.

144. Lindholm, E.M., Bielicki, J.K., Curtiss, L.K., Rubin, E.M., Forte, T.M. 1998. Deletion of amino acids Glu146-->Arg160 in human apolipoprotein A-I (ApoA-I Seattle) alters lecithin:cholesterol acyltransferase activity and recruitment of cell phospholipid. *Biochemistry* **37**:4863-4868.

145. Staels, B., van Tol, A., Skretting, G., Auwerx, J. 1992. Lecithin:cholesterol acyltransferase gene expression is regulated in a tissue-selective manner by fibrates. *Journal of Lipid Research* **33**:727-735.

146. Skretting, G., Gjernes, E., Prydz, H. 1995. Regualtion of lecithin:cholesterol acyltransferase by TGF-beta and interleukin-6. *Biochimica et Biophysica Acta* **1255**:267-272.

147. Feister, H.A., Auerbach, B.J., Cole, L.A., Krause, B.R., Karathanasis, S.K. 2002. Identification of an IL-6 response element in the human LCAT promoter. *Journal of Lipid Research* **43**:960-970.

148. Skretting, G., Giernes, E., Prydz, H. 1997. Sodium butyrate inhibits the expression of the human lecithin:cholesterol acyltransferase gene in HepG2 cells by a post-transcriptional mechanism. *FEBS Letters* **404**:105-110.

149. Yancey, P.G., Bortnick, A.E., Kellner-Weibel, G., de la Llera-Moya, M., Phillips, M.C., Rothblat, G.H. 2003. Importance of Different Pathways of Cellular Cholesterol Efflux. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23**:712-719.

150. Phillips, M.C., Gillotte, K.L., Haynes, M.P., Johnson, W.J., Lund-Katz, S., Rothblat, G.H. 1998. Mechanisms of high density lipoprotein-mediated efflux of cholesterol from cell plasma membranes. *Atherosclerosis* **137**:S13-S17.

151. McLean, L.R., Phillips, M.C. 1981. Mechanism of cholesterol and phosphatidylcholine exchange or transfer between unilamelar vesicles. *Biochemistry* **20**:2893-2900.

152. Bruckdorfer, K.R., Crowe, J., Sherry, M.K. 1984. Evidence for a water-soluble intermediate in exchange of cholesterol between membranes. *Biochimica et Biophysica Acta* **778**:489-496.

153. Slotte, J.P., Lundberg, B. 1983. Transfer of [3H]cholesterol between lipid vesicles and rat arterial smooth muscle cells in vitro. *Biochimica et Biophysica Acta* **750**:434-439.

154. Bartholow, L.C., Geyer, R.P. 1982. Sterol efflux from mammalian cells induced by human serum albumin-phospholipid complexes. Dependence on phospholipid acyl chain length, degree of saturation, and net charge. *Journal of Biological Chemistry* **257**:3126-3130.

155. Rothblat, G.H., Mahlberg, F.H., Johnson, W.J., Phillips, M.C. 1992. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. *Journal of Lipid Research* **33**:1091-1097.

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

157. Phillips, M.C., Johnson, W.J., Rothblat, G.H. 1987. Mechanisms and consequences of cellular cholesterol exchange and transfer. *Biochimica et Biophysica Acta* **906**:223-276.

158. Johnson, W.J., Mahlberg, F.H., Rothblat, G.H., Phillips, M.C. 1991. Cholesterol transport between cells and high-density lipoproteins. *Biochimica et Biophysica Acta* **1085**:273-298.

159. Michaud, S.E., Renier, G. 2001. Direct regulatory effect of fatty acids on a macrophage lipoprotein lipase: potential role of PPARs. *Diabetes* **50**:660-666.

160. Ziouzenkova, O., Rerrey, S., Asatryan, L., Hwang, J., MacNaul, K.L., Moller, D.E., Rader, D.J., Sevanian, A., Zechner, R., Hoefler, G., Plutzky, J. 2003. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an anti-inflammatory role for lipoprotein lipase. *Proceedings of the National Academy of Sciences of the U.S.A* **100**:2730-2735.

161. Fang, X, Dillon, J.S., Hu, S., Harmon, S.D., Yao, J., Anjaiah, S., Falck, J.R., Spector, A.A. 2007. 20-carboxy-arachidonic acid is a dual activator of peroxisome proliferator-activated receptor alpha and gamma. *Prostaglandins & Other Lipid Mediators* **82**:175-184.

162. Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., Evans, R.M. 1995. 15-Deoxy-delta 12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**:803-812.

163. Schoonjans, K. Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B., Auwerx, J. 1996. PPARalpha and PPARgamma activators

direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO Journal* **15**:5336-5348.

164. Haidar, B., Mott, S., Boucher, B., Yin Lee, C., Marcil, M., Genest, Jr.J. 2001. Cellular cholesterol efflux is modulated by phospholipid-derived signaling molecules in familial HDL deficiency/Tangier disease fibroblasts. *Journal of Lipid Research* **42**:249-257.

165. Whitfield, P.D., Clayton, P.T., Muller, D.P.R. 2000. Effect of Intravenous Emulsions on Hepatic Cholesterol Metabolism. *Journal of Pediatric Gastroenterology and Nutrition* **30**:538-546.

166. Lada, A.T., Willingham, M.C., St. Clair, R.W. 2002. Triglyceride depletion in THP-1 cells alters cholesteryl ester physical state and cholesterol efflux. *Journal of Lipid Research* **43**:618-628.

167. Skeggs, J.W., Morton, R.E. 2002. LDL and HDL enriched in triglyceride promote abnormal cholesterol transport. *Journal of Lipid Research* **43**:1264-1274.

168. Palmer, A.M., Murphy, N., Graham, A. 2004. Triglyceride-rich lipoproteins inhibit cholesterol efflux to apolipoprotein (apo) A1 from human macrophage foam cells. *Atherosclerosis* **173**:27-38.

169. Zborowski, J., Roerdink, F., Scherphof, G. 1977. Leakage of sucrose from phosphatidylcholine liposomes induced by interaction with serum albumin. *Biochimica et Biophysica Acta* **497**:183-191.