uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

André Gauthier
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Biochemistry)
GRADE / DEGREE

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

Regulation of Low Density Lipoprotein Receptor-related Protein Gene Expression & Cholesteryl Ester Transfer Protein-mediated HDL Selective Uptake in the Liver

TITRE DE LA THÈSE / TITLE OF THESIS

Ruth McPherson
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

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

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Katherine Cianflone

Balwant Tuana

James Van Huysse

Zemin Yao

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

Regulation of Low Density Lipoprotein Receptor-related Protein Gene Expression
&
Cholesteryl Ester Transfer Protein-mediated HDL Selective Uptake in the Liver

by

André Gauthier

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Ph.D.)

Department of Biochemistry, Microbiology & Immunology
Faculty of Medicine, University of Ottawa
January 2006

Your file Votre référence
ISBN: 978-0-494-15021-4
Our file Notre référence
ISBN: 978-0-494-15021-4

For Karine, Benjamin & Dominic

ABSTRACT

Regulation of Low Density Lipoprotein Receptor-related Protein Gene Expression
&
Cholesteryl Ester Transfer Protein-mediated Selective Uptake in the Liver

by André Gauthier

Disordered lipoprotein metabolism is a major risk factor for coronary artery disease (CAD). This thesis focuses on two proteins that may exert protective effects against CAD: (1) the regulation of LDL receptor-related protein (LRP) gene expression and (2) hepatic cholesteryl ester transfer protein (CETP)-mediated selective uptake.

LRP is a large multifunctional receptor, which is implicated in numerous biologically important processes including lipoprotein metabolism, cell signal transduction and atherosclerosis. Despite this, regulation of LRP gene expression remains largely unknown. We have identified a novel transcriptional regulatory element, known as a peroxisome proliferator response element (PPRE), at position -1185 to -1173 in the 5'-flanking region of the human LRP gene. The PPRE is transactivated by peroxisome proliferator activated receptors (PPAR) α and γ when activated by ligands such as long chain fatty acids, rosiglitazone (PPARγ-specific) and fenofibric acid (PPARα-specific). This results in upregulation of LRP expression and function in several cell types including adipocytes (PPARγ-expressing) and hepatocytes (PPARα-expressing). These data suggest that LRP gene expression could be preferentially modulated by the use of PPAR-selective agonists and may have important implications for the regulation of human preadipocyte differentiation and triglyceride-rich lipoprotein metabolism in adipocytes and hepatocytes.

CETP is a hydrophobic glycoprotein that plays a central role in human high-density lipoprotein (HDL) metabolism. We have examined the role of CETP in HDL-cholesteryl ester (CE) selective uptake in primary mouse hepatocytes expressing CETP via adenovirus-mediated gene transfer (ad-CETP) or incubated in CETP-containing media. These studies demonstrate that CETP mediates hepatocyte selective uptake of HDL-derived CE independently of other known lipoprotein receptors. Furthermore, CETP-mediated selective uptake occurs by two pathways, only one of which requires CETP transfer activity. Hepatic expression of ad-CETP *in vivo* resulted in an acute drop in plasma cholesterol in particles in the HDL density range. Finally, CETP-mediated selective uptake *in vivo* was only partially inhibited by concomitant administration of torcetrapib, a compound which inhibits CETP neutral lipid transfer activity. Taken together, these studies demonstrate a novel and important role for CETP in the selective uptake of HDL-derived CE by hepatocytes, findings which are of fundamental importance to human lipoprotein metabolism.

ACKNOWLEDGEMENTS

I would first like to thank my thesis supervisor, Dr Ruth McPherson. I owe her a great deal of gratitude for the support she has given me over the course of my graduate studies. Her confidence in my abilities from the very start of my graduate career has helped to instill a feeling of confidence in my work, which I have carried forward into my medical training. She has been especially supportive of my own ideas and always encouraged me to develop independently. I am most thankful for her support and help in achieving my own personal career goals.

I would like to thank the other members of the Lipoprotein & Atherosclerosis Research Group. Drs Yves Marcel, Heidi McBride, Ross Milne, Daniel Sparks and Zemin Yao were always willing to discuss new research ideas and critique manuscripts and continually offered valuable advice. I am also thankful for the effort Dr Milne spent critiquing this thesis in addition to participating as a member of my thesis advisory committee along with Drs Lynn Megeney and Mary-Ellen Harper. I am grateful to all the members of the research group, past and present. The camaraderie amongst labs was incredible and certainly helped to make the Heart Institute a wonderful place to work. I also greatly appreciated the friendship and support from all of the graduate students who have passed through our research group over the years.

I would also like to extend my gratitude to all the individuals in the McPherson lab over my tenure as a graduate student. Technical assistance from Thet Naing, Nihan

Kavaslar and Andrew Meng was greatly appreciated. I would especially like to thank Paulina Lau for her help in the laboratory. Her experience in the lab was extremely helpful, especially in the beginning, and she continued to offer invaluable support and advice throughout my time in the lab. I would also like to thank Drs Xiaohui Zha and Gerard Vassiliou; without their guidance and support, many of the experiments would not have been possible. I would like single out two individuals form Dr Marcel's lab: Dan McManus for his teaching me adenovirus and mouse tail vein injection techniques and Vivian Franklin for teaching me the art of primary hepatocyte isolation. Without these techniques, the CETP experiments would not have been possible.

Finally, the love and support of my family was certainly an important factor in my success as a graduate student. Without the support and encouragement from my wife, Karine, I would not have found as much personal and professional success has I have. I would especially thank her for her patience while I was a graduate student working all hours of the day and weekends and for her continued patience and support while I pursue my medical training. This thesis is dedicated to her and our children, Benjamin and Dominic.

TABLE OF CONTENTS

ABSTRACT iii
ACKNOWLEDGEMENTS v
LIST OF FIGURES ix
LIST OF TABLES x
ABBREVIATIONS (in alphabetical order) xi

CHAPTER 1 – INTRODUCTION 1
1.1 – Atherosclerosis and Cardiovascular Disease 1
1.1.1 – Cardiovascular Disease *1*
1.1.2 – Atherosclerosis and Risk Factors *1*
1.1.3 – Structure of a Normal Artery *2*
1.1.4 – Natural Progression of Atherosclerosis *3*
1.2 - Lipoproteins 8
1.2.1 – Lipoprotein Classification *8*
1.2.2 – Overview of Lipoprotein Metabolism *10*
1.2.2A – Metabolism of apolipoprotein B-containing Lipoproteins 10
1.2.2B – Metabolism of HDL and Reverse Cholesterol Transport 15
1.2.3 – Lipoproteins and the Pathogenesis of Atherosclerosis *18*
1.2.4 – Strategies Aimed at Raising HDL *20*

CHAPTER 2 - REGULATION OF LRP GENE EXPRESSION 22
2.1 – Background 22
2.1.1 – LRP and the LDL Receptor Gene Family *22*
2.1.2 – Protein Structure of LRP *23*
2.1.3 – Ligands of LRP *23*
2.1.4 – LRP Functions *25*
2.1.4A – LRP and Adipocyte Lipoprotein Metabolism 26
2.1.4B – LRP and Hepatic Lipoprotein Metabolism 27
2.1.4C – LRP in Signalling 28
2.1.5 – Human LRP Gene Structure and Regulation *30*
2.1.5A – Organization of the Human LRP Gene 30
2.1.5B – Organization of the Human LRP Promoter 30
2.1.5C – Factors Affecting LRP Expression 31
2.1.6 – Adipogenesis *33*
2.1.7 – Peroxisome Proliferator-Activated Receptors (PPARs) *34*
2.1.8 – Ligand Activation of PPARs *35*
2.2 – Rationale 36
2.3 – Materials & Methods 38
2.4 – Results 50
2.4.1 – The Human LRP Promoter Contains a Conserved Peroxisome Proliferator Response Element (PPRE) *50*
2.4.2 – Endogenous LRP mRNA Levels are Increased Upon Exposure to PPARγ Ligands *50*

2.4.3 – PPARγ Ligands Act at the Transcriptional Level to Increase LRP mRNA... 53
2.4.4 – LRP PPRE Luciferase Constructs are Responsive to Rosiglitazone in Dual Luciferase Assay ... 55
2.4.5 – Mutagenesis of the PPRE Results in the Loss of Enhancer Activity ... 57
2.4.6 – PPARγ:RXRα Heterodimers Selectively Bind the PPRE Identified in the LRP Promoter Region ... 57
2.4.7 – Functional Cell Surface LRP is Increased Upon Exposure to PPARγ Ligands ... 61
2.4.8 – ^{125}I-α2M Binding at 0°C is Increased in Primary Human Hepatocytes Treated with the PPARα Agonist Fenofibric Acid ... 64*
2.4.9 – Mouse LRP mRNA Levels are not Increased Upon Stimulation by PPAR Agonists ... 67
2.4.10 – LRP Promoter PPRE not Conserved Between Human and Mouse ... 67
2.4.11 – Nuclear Extracts Containing PPARγ do not Bind to Mouse PPRE Sequence in vitro ... 69
2.4.12 – LRP Function is not Stimulated by PPAR Agonists in Murine Derived Cells ... 69
2.5 – Discussion ... 73

CHAPTER 3 – CETP-MEDIATED HDL SELECTIVE UPTAKE IN THE LIVER ... 79
3.1 – Background ... 79
3.1.1 – Cholesteryl Ester Transfer Protein ... 79
3.1.2 – CETP Gene Structure and Regulation ... 80
3.1.3 – CETP Function ... 84
3.1.4 – CETP and Atherosclerosis ... 85
3.1.5 – Pharmacological Inhibition of CETP ... 88
3.1.6 – Selective Uptake ... 90
3.1.7 – CETP and Selective Uptake ... 90
3.2 – Rationale ... 91
3.3 – Materials & Methods ... 92
3.4 – Results ... 102
3.4.1 – Expression of CETP Using Adenovirus-mediated Gene Transfer ... 102
3.4.2 – CETP Mediates Selective Uptake of HDL-derived CE in Primary Mouse Hepatocytes ... 104
3.4.3 – CETP-mediated Selective Uptake Does Not Depend on CETP Secretion into the Media ... 106
3.4.4 – CETP-mediated Selective Uptake is Independent of Other Lipoprotein Receptors ... 106
3.4.5 – CETP-mediated Selective Uptake is Independent of CETP Transfer Activity ... 108
3.4.6 – Exogenously Added Recombinant CETP Mediates HDL-derived ^{3}H-CE Selective Uptake, an Effect Which is Partially Inhibitable by Torcetrapib ... 110
3.4.7 – Exogenously added rCETP can Associate with Primary Hepatocytes in Culture and Mediate Uptake of HDL-derived ^{3}H-CE ... 110
3.4.8 – ^{3}H-CE Uptake, Mediated by Exogenous rCETP, is Independent of Other Classical Selective Uptake Pathways ... 114

3.4.9 – Effects of Torcetrapib on Reversible and Irreversible Components of CETP Selective Uptake *116*
3.4.10 – Ad-CETP$_{w/t}$ and ad-CETP$_{KD}$ Expression in vivo *116*
3.4.11 – CETP$_{KD}$ Expression in vivo Decreases Total Plasma Cholesterol and Total Cholesterol of Lipoprotein Fractions Corresponding to HDL *119*
3.4.12 – In vivo CETP$_{KD}$ Expression Mediates Selective Uptake of HDL-derived ^{3}H-CE in vitro *121*
3.4.13 – CETP$_{w/t}$ Mediates Hepatic Selective Uptake of HDL-derived CE in vivo *121*
3.5 – Discussion 125

CHAPTER 4 – CONCLUSIONS & FUTURE DIRECTIONS 133

REFERENCES 136
CONTRIBUTIONS OF COLLABORATORS 167
APPENDIX I 168
CURRICULUM VITAE 170

LIST OF FIGURES

Figure 1.1. Schematic diagram of hypothetical sequence of cellular interactions in atherosclerosis. 5
Figure 1.2. The exogenous and endogenous lipoprotein metabolic pathways. 13
Figure 1.3. HDL metabolism and reverse cholesterol transport. 16

Figure 2.1. RT-PCR of selected adipocyte genes during differentiation. 37
Figure 2.2. Nucleotide sequence of the human LRP promoter. 51
Figure 2.3. Endogenous LRP mRNA levels in SW872 cells are modulated by PPARγ ligands at the transcriptional level. 52
Figure 2.4. Endogenous LRP mRNA levels in differentiated primary human adipocytes are modulated by rosiglitazone. 54
Figure 2.5. LRP promoter confers regulation of Luciferase activity by rosiglitazone. ... 56
Figure 2.6. Site-directed mutagenesis of the PPRE abolishes the response to rosiglitazone. 58
Figure 2.7. PPARγ:RXRα heterodimers selectively bind PPRE in the LRP promoter... 59
Figure 2.8. LRP protein levels are increased in SW872 cells upon exposure to PPARγ ligands. 62
Figure 2.9. Increased cellular degradation of ^{125}I-α2M* by cells treated with PPARγ ligands. 63
Figure 2.10. Increased cell surface binding of ^{125}I-α2M* by cells treated with a PPARγ ligand 65

Figure 2.11. ^{125}I-α2M binding is increased by PPARα ligand fenofibric acid in cultured primary human hepatocytes. 66
Figure 2.12. Endogenous LRP mRNA levels in murine derived cell lines are not affected by PPAR agonists. 68
Figure 2.13. Comparison of mouse and human LRP promoter sequences and evaluation of PPARγ binding by EMSA. 70
Figure 2.14. ^{125}I-α2M* binding and degradation are not affected by PPAR agonists in murine derived cells. 71

Figure 3.1. Expression of CETP transgenes in Cos7 cells following adenovirus-mediated gene transfer. 103
Figure 3.2. $CETP_{w/t}$ transgene expression mediates selective uptake of HDL-derived ^{3}H-CE in primary mouse hepatocytes. 105
Figure 3.3. CETP-mediated selective uptake is independent of secretion, SR-B1 or LDLR family members. 107
Figure 3.4. Selective uptake, mediated by adenovirally expressed CETP, does not require transfer activity. 109
Figure 3.5. Exogenously added CETP mediates selective uptake in primary mouse hepatocytes by 2 distinct mechanisms. 111
Figure 3.6. CETP added to media can associate with primary mouse hepatocytes and mediate selective uptake. 113
Figure 3.7. Selective uptake mediated by exogenously added CETP is independent of classical lipoprotein receptors. 115
Figure 3.8. Effects of torcetrapib on reversible and irreversible components of selective uptake. 117
Figure 3.9. CETPw/t and $CETP_{KD}$ are secreted into plasma *in vivo* 118
Figure 3.10. Effects of hepatic ad-$CETP_{KD}$ expression on selective uptake in vivo. 120
Figure 3.11. Effects of *in vivo* hepatic expression of ad-$CETP_{KD}$ on selective uptake *in vitro*. 122
Figure 3.12. Effects of hepatic expression of ad-CETPw/t on selective uptake *in vivo*. 123

LIST OF TABLES

Table 1.1 American Heart Association classification of human atherosclerotic lesions... 4
Table 1.2 Classification and physical properties of plasma lipoproteins 9
Table 1.3 Major apolipoprotein source & function. 11

ABBREVIATIONS (in alphabetical order)

α2M:	alpha 2 macroglobulin
α2M*:	activated alpha 2 macroglobulin
ABCA1:	ATP-binding cassette transporter A1
ABCG1:	ATP-binding cassette transporter G1
ABCG4:	ATP-binding cassette transporter G4
ABCG5:	ATP-binding cassette transporter G5
ABCG8:	ATP-binding cassette transporter G8
ACAT-2:	acyl coenzyme A:cholesterol acyltransferase-2
ad-CETP:	CETP-expressing adenovirus
ad-Luc:	luciferase-expressing adenovirus
aP2:	adipocyte fatty acid binding protein
apoB:	apolipoprotein B
apoC:	apolipoprotein C
apoE:	apolipoprotein E
BPI:	bactericidal/permeability-increasing protein
CAD:	coronary artery disease
CE:	esterified cholesterol (cholesteryl esters)
C/EBP:	CAAT enhancer binding protein
CETP:	cholesteryl ester transfer protein
$CETP_{KD}$:	human CETP with K233D and K239D point mutations
$CETP_{w/t}$:	wild-type human CETP
CHD:	coronary heart disease
CR:	chylomicron remnants
CVD:	cardiovascular disease
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	dimethyl sulfoxide
DR-1:	direct repeat spaced by 1 nucleotide
DR-2:	direct repeat spaced by 2 nucleotides
DR-4:	direct repeat spaced by 4 nucleotides
eCFP:	enhanced cyan fluorescent protein
ECM:	extracellular matrix
EGF:	epidermal growth factor
ER:	endoplasmic reticulum
FATP:	fatty acid transport protein
FC:	free cholesterol (unesterified cholesterol)
FFA:	free fatty acids
hACOX:	human fatty acyl coA oxidase
HDL:	high density lipoprotein
HDL-C:	HDL cholesterol
HDL-CE:	HDL-derived cholesteryl esters
HL:	hepatic lipase
HSPGs:	heparan sulphate proteoglycans
IDL:	intermediate density lipoprotein

kb:	kilo base pairs
LBP:	lipopolysaccharide binding protein
LCAT:	lecithin-cholesterol acyltransferase
LDL:	low density lipoprotein
LDL-C:	LDL cholesterol
LDLR:	low density lipoprotein receptor
Lp(a):	lipoprotein A
LPDS:	lipoprotein depleted serum
LPL:	lipoprotein lipase
LRP:	low density lipoprotein receptor-related protein
LTP/LBP:	lipid transfer protein/lipopolysaccharide binding protein
MTP:	microsomal triglyceride transfer protein
PDGF:	platelet-derived growth factor
PDGF-BB:	platelet-derived growth factor isoform BB
PDGFR:	platelet-derived growth factor receptor
PFU:	plaque forming units
PL:	phospholipids
PLTP:	phospholipid transfer protein
PPAR:	peroxisome proliferator-activated receptor
PPRE:	peroxisome proliferator response element
RAP:	receptor associated protein
rCETP:	partially purified recombinant human CETP
RCT:	reverse cholesterol transport
RT-PCR:	reverse-transcriptase polymerase chain reaction
RQ-RT-PCR:	relative-quantitative reverse-transcripase polymerase chain reaction
RXRα:	retinoid X receptor alpha
SR-B1:	scavenger receptor B1
SMCs:	smooth muscle cells
TG:	triglyceride
TRLs:	triglyceride-rich lipoproteins
TZD:	thiazolidinedione
VLDL:	very low density lipoprotein
VLDLR:	very low density lipoprotein receptor
VSMCs:	vascular smooth muscle cells

CHAPTER 1 – INTRODUCTION

1.1 – Atherosclerosis and Cardiovascular Disease

1.1.1 – Cardiovascular Disease

The World Health Organization estimated that nearly 16.6 million deaths in 2001 were attributable to cardiovascular disease (CVD) accounting for one third of global deaths [1]. The most common forms of CVD are coronary artery disease (CAD) (coronary atherosclerosis) and stroke, accounting for 43% (7.2 million) and 33% (5.5 million) of CVD deaths, respectively. In Canada, 36% of deaths in 1999 were due to CVD, the main presentations being CAD and stroke [2]. Over the past several decades CVD death and hospitalization rates have decreased largely due to improved treatments and drug therapies, as evidenced by the increase in prescriptions for cardiovascular diseases. However, the total number of deaths and hospitalizations related to CVD has remained constant and is predicted to rise because of the aging population [2]. The total cost of CVD to the Canadian economy was estimated to be approximately 18.5 billion dollars in 1998 [3], making it the most costly disease in Canada and the largest burden on our national health care system.

1.1.2 – Atherosclerosis and Risk Factors

Atherosclerosis of coronary arteries, or CAD, is the major cause of CVD. It is a disease characterized by the gradual accumulation of lipids and fibrous elements in the walls of arteries [4]. Although lesions can grow sufficiently large to gradually block blood flow, most lesions do not encroach on the vessel lumen to a large extent. Rather, the

lesions are generally small and stable due to a fibrous cap consisting of vascular smooth muscle cells (VSMCs) and extracellular matrix that enclose a lipid-rich necrotic core. Lesions pose a major risk when physical trauma and inflammation result in lesion rupture or erosion of the fibrous cap. This may ultimately result in thrombus formation and hence acute occlusion of the vessel causing a heart attack or stroke.

There are numerous risk factors that can individually, or in combination, lead to CAD (reviewed in [4,5]). These include age, male sex, diabetes mellitus, dyslipidemia, cigarette smoking, hypertension, a family history of heart disease, physical inactivity and abdominal obesity. "Emerging" risk factors include elevated blood levels of homocysteine [6] and inflammatory markers such as C-reactive protein [7] and pro-coagulant factors including plasminogen activator inhibitor-1 (PAI-1) [8]. Some of these risk factors are not modifiable such as age, ethnicity, sex and genetics but others are modifiable by lifestyle changes (diet, obesity, cigarette smoking and sedentary lifestyle) or medications (dyslipidemia and hypertension). Of the modifiable risk factors, most converge to promote atherosclerosis, a process that involves an evolution from fatty streaks (due to lipid deposition within the vessel walls) to complex plaques and clinical disease [5].

1.1.3 – Structure of a Normal Artery

In order to better understand the pathological and cellular basis of atherosclerosis, a brief overview of the structure of an artery will be considered first. An artery generally consists of three concentric layers known as the intima, media and adventitia. The inner most endothelial layer and subendothelial connective tissue comprise the intima. The vascular endothelium is a continuous layer of endothelial cells connected by tight junctions that line the entire cardiovascular system. Aside from its role as a selectively

permeable barrier between blood and tissue, it is a multifunctional tissue having numerous synthetic and metabolic roles including the regulation of coagulation and fibrinolytic pathways, extracellular matrix production, regulation of inflammation and immunity and regulation of cell growth. The intima is separated from the media by the internal elastic lamina which is not continuous; rather, it is full of pores to allow diffusion of nutrients and oxygen into the media. The media consists of many layers of smooth muscle cells (SMCs) and in highly elastic arteries like the aorta, contains alternating layers of SMCs and elastic fibers. The outer limit of the media is defined by the external elastic lamina which also separates media from the adventitia. The adventitia contains connective tissue, nerve fibers and the vasa vasorum (blood vessels responsible for feeding the external layers of the arteries) [9].

1.1.4 – Natural Progression of Atherosclerosis

The histological classification of atherosclerosis, as recently summarized by the American Heart Association [10], divides atherosclerotic lesions into six types, beginning with isolated foam cells, developing into fatty streaks, atheromas and fibroatheromas, and culminating in the complicated lesion (**Table 1.1**). A model of atherosclerotic lesion development is shown in **Figure 1.1**.

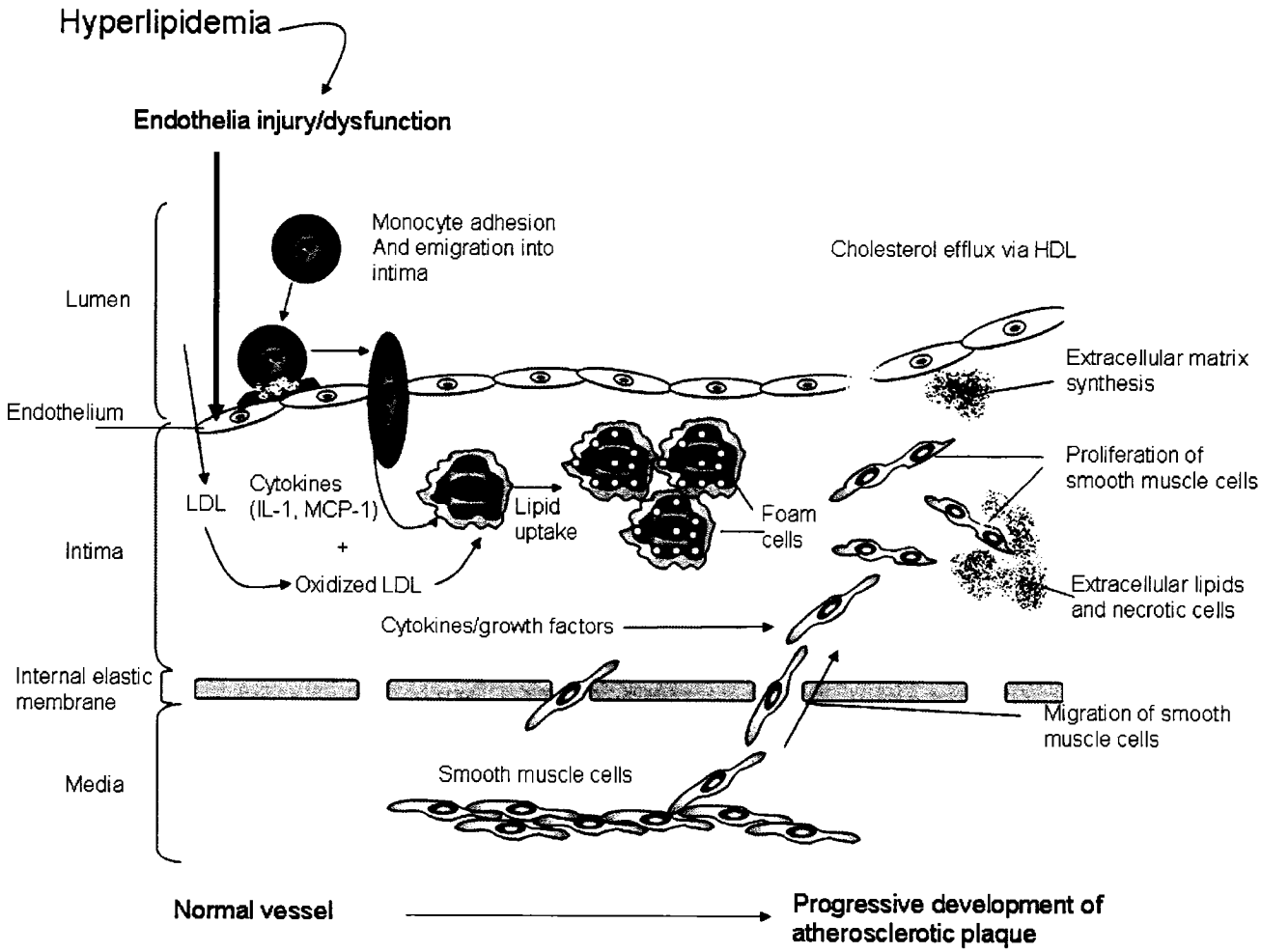
The earliest lesions of atherosclerosis, also called fatty streaks, consist of an aggregation of lipid-rich macrophages, also called foam cells, and T lymphocytes within the intima of the artery wall [11]. These lesions are not significantly raised and do not cause any disturbances in blood flow. The key processes in the development of early atherosclerotic lesions (type I & II) are intimal thickening and lipid accumulation. The

Lesions Type and Histology	Main Growth Mechanism	Typical Age of Onset	Clinical Correlation
Type I (initial) Isolated macrophage foam cells	Growth mainly by lipid accumulation	< 10 years old	Clinically Silent
Type II (fatty streak) Mainly localized intracellular lipid accumulation			
Type III (intermediate) Type II + small extracellular pools of lipid		> 20 years old	
Type IV (atheroma) Type II + large intracellular pools forming a lipid core			
Type V (fibroatheroma) Lipid core and fibrotic layer; can contain calcifications	Accelerated smooth muscle cell migration and proliferation and collagen increase	> 30 years old	Clinically overt disease
Type VI (complicated) Surface defect, hematoma-hemorrhage, thrombus	Thrombosis, hematoma		

Table 1.1 American Heart Association classification of human atherosclerotic lesions. From type I to type III, changes in lesion morphology occur primarily because of increasing accumulation of lipid within foam cells. Type IV lesions have increased intracellular lipid deposition in addition to increased foam cell lipid. Type V lesions involve further derangement of intima but through proliferative and reparative mechanisms in addition to increased lipid deposition of type IV. In complicated lesions, thrombotic deposits may form repeatedly over varied time spans in the same location and may be the principal mechanism for gradual occlusion of medium-sized arteries. [Adapted from (10)]

Figure 1.1. Schematic diagram of hypothetical sequence of cellular interactions in atherosclerosis.

Hyperlipidemia is thought to cause endothelial injury, resulting in adhesion of platelets and monocytes and release of growth factors, including platelet-derived growth factor (PDGF), which lead to smooth muscle cell migration and proliferation. Foam cells of atheromatous plaques are derived from both macrophages and smooth muscle. The macrophages take up oxidized LDL via scavenger receptors. Extracellular lipid is derived from LDL that has crossed into the intima from the vessel lumen, particularly in the presence of hypercholesteremia, and also from degenerating foam cells. The accumulation of foam cells, extracellular lipid and necrotic cells and tissue constitute the necrotic lipid core of the lesion. Cholesterol accumulation in the plaque reflects an imbalance between influx and efflux, and HDL plays a key role in helping to clear cholesterol from these accumulations. Smooth muscle cell migration into the intima, results in proliferation and excess production of extracellular matrix and ultimately the thickening of the intima and encroachment into the lumen. [Adapted from (9)]


Hyperlipidemia
Endothelia injury/dysfunction
Monocyte adhesion
And emigration into
intima
Cholesterol efflux via HDL
Lumen
Endothelium
Extracellular matrix
synthesis
Proliferation of
smooth muscle cells
LDL
Cytokines
(IL-1, MCP-1)
+
Oxidized LDL
Lipid
uptake
Foam
cells
Intima
Extracellular lipids
and necrotic cells
Cytokines/growth factors
Internal elastic
membrane
Migration of smooth
muscle cells
Media
Smooth muscle cells
Normal vessel
Progressive development of
atherosclerotic plaque

intimal thickening, which is intimately related to the presence of fatty streaks, is the result of smooth muscle cell migration and proliferation within the intima of the vessel wall [9]. Type I & II lesions are present in areas of intimal thickening even in infants but generally disappear shortly after and only return after puberty [12]. Fatty streaks are often found at bifurcations of blood vessels. These areas are extremely susceptible to atherosclerosis because the disturbed flow around the endothelial cells results in decreased cellular organization and allows the passage of lipoproteins through the normally selectively permeable endothelium [13]. The lipid that infiltrates the extracellular space of the intima is trapped there by interactions between apolipoprotein-B (apoB) and matrix proteoglycans [14]. Once trapped, apoB-containing lipoproteins may be readily oxidized by free radicals and it is these oxidized lipoproteins that are easily taken up by macrophages to form foam cells (discussed below). Intermediate lesions (type III) are the result of increased deposition of lipids within the extracellular matrix (ECM) in areas of already increased intima thickness and these form the bridge between early and advanced lesions. These lesions are the result of the increased permeability of the endothelium to macromolecules such as lipoproteins [13].

Atheromatous plaques (type IV) are the beginning of intima disruption and are made up of three principle components: (i) cells, including SMCs, macrophages and other leukocytes; (ii) ECM, including collagen, elastic fibers and proteoglycans; and (iii) abundant intracellular and extracellular lipid. Structurally, the atheromatous plaque appears as a raised focal lesion initiating within the intima, having a soft, yellow necrotic core of lipid (mainly cholesterol and cholesteryl esters), covered by a firm, white fibrous cap [9]. These lesions are larger and begin to slightly encroach into the lumen of the artery

but are generally clinically silent. In type V lesions (fibroatheromatous), the intima is thickened by substantial reparative fibrous (mainly collagenous) tissue layers. Type V lesions display further encroachment of the artery lumen and may or may not be clinically overt depending on the size of the artery and blood flow demands. The presence of fibrous connective tissue layers in addition to one or more lipid cores may be labelled type Va (fibroatheroma). The sides of the fibrous cap, referred to as the shoulder, are enriched in macrophages, SMCs and leukocytes and represent the weakest portion of the lesion. These areas are also the most common sites for the formation of thrombus because of the presence of newly formed blood vessels [9]. Furthermore, fibroatheromatous plaques often undergo calcification which increases the risk for coronary events [10].

Advanced lesions (type VI) generally contain all of the characteristics of the previous lesions: lipid-laden macrophages and SMCs, deep necrotic core of extracellular lipids and layers of newly formed fibrous connective tissue [10]. In addition, they may also have surface defects, hematomas and thrombotic deposits which further damage, deform and thicken the intima of the artery quickly converting this lesion from clinically silent to overt disease. The strength of the fibrous cap is very important in this type of lesion since they are at the highest risk for developing acute obstructions caused by thrombus formation following focal rupture, ulceration or erosion of the luminal surfaces of the plaques. The occlusion of an artery feeding the heart muscle results in a myocardial infarction whereas the occlusion of an artery feeding the brain results in a stroke.

1.2 - Lipoproteins

1.2.1 – Lipoprotein Classification

Plasma concentrations of individual lipoproteins are very important predictors of atherosclerosis risk. As will be discussed below, low density lipoprotein (LDL) plays a crucial role in the formation of fatty streaks and the progression to a full blown atherosclerotic lesion. Furthermore, high density lipoprotein (HDL) has several anti-atherogenic properties including anti-oxidant and anti-inflammatory activities and importantly, its ability to participate in the removal of cholesterol from macrophages within the artery wall [15].

Lipoproteins are complexes of proteins and lipids that transport insoluble lipids (triglycerides and cholesteryl esters) and fat-soluble vitamins throughout the body (plasma, interstitial fluid and lymph). The general structure of all lipoproteins is that of an amphipathic phospholipid (PL) monolayer stabilized by interactions of the apolipoproteins and free cholestesterol (FC) (or unesterified cholesterol) surrounding a core of neutral lipids composed of triglycerides (TG) and cholesteryl esters (CE), and the chemical composition of each of these differs between classes. There are five major classes of lipoproteins that can be identified by buoyancy: high density lipoproteins (HDL - ρ=1.063-1.21 g/ml), low density lipoproteins (LDL - ρ=1.019-1.063 g/ml), intermediate density lipoproteins (IDL - ρ=1.006-1.019 g/ml), and very low density lipoproteins (VLDL - ρ=0.93-1.006 g/ml) [16]. Chylomicrons (ρ=0.93) are triglyceride-rich lipoproteins that are synthesized by the intestine [16]. Particles from each of the lipoprotein classes vary slightly in density, size, migration during gel electrophoresis, and protein and lipid content. These differences and others are summarized in **Table 1.2.**

Lipoprotein	Density (g/ml)	Size (nm)	Electrophoretic mobility	Major Lipid Component(s)	Apolipoproteins		Other constituents
					Major	Other	
Chylomicrons	0.930	75-1200	Origin	TG	apoB-48	A-1, A-IV, C-1, C-II, C-III	Retinyl esters
Chylomicron remnants	0.930-1.006	30-80	Slow pre-β	CE, TG	apoB-48	E, A-1, A-IV, C-1, C-II, C-III	Retinyl esters
VLDL	0.930-1.006	30-80	Pre-β	TG	apoB-100	E, A-1, A-II, A-V, C-1, C-II, C-III	Vitamin E
IDL	1.006-1.019	25-35	Slow pre-β	CE	apoB-100	E, C-1, C-II, C-III	Vitamin E
LDL	1.019-1.063	18-25	β	CE	apoB-100		Vitamin E
HDL							
HDL_2	1.063-1.125	5-12	α	CE, PL	apoA-1	A-II, A-IV, E, C-I, C-II, C-III, D	LCAT, CETP, paraoxonase
HDL_3	1.125-1.210	5-12	α	PL	apoA-1		
Lp(a)	1.050-1.120	25	Pre-β	CE	apoB-100	Apo(a)	

Table 1.2 Classification and physical properties of plasma lipoproteins. Lipoproteins can be distinguished by density (determined by ultracentrifugation), size (determined using gel electrophoresis), electrophoretic mobility (reflects the size and surface charge of the particle, with β being the position of LDL and α the position of HDL), major lipid components and associated apolipoproteins. All of the lipoprotein classes contain phospholipids (PL), esterified (CE) and unesterified cholesterol (FC), and triglycerides (TG) to varying degrees. VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; Lp(a), lipoprotein (a); LCAT, lecithin-cholesterol acyltransferase; CETP, cholesteryl ester transfer protein. [Adapted from (25)]

Lipoproteins also differ in their apolipoprotein content, specialized proteins that are required for the assembly and structure of lipoproteins. They also serve to mediate lipoprotein binding to cell surface receptors and to activate enzymes involved in lipoprotein metabolism [17] (summarized in **Table 1.3**).

1.2.2 – Overview of Lipoprotein Metabolism

Cholesterol is a crucial component of all cells and plays several extremely important roles. It is an essential component of the plasma membrane [18] and an important regulator of membrane fluidity and function [19,20]. Furthermore, cholesterol is the sole precursor of bile acids, steroid hormones and certain vitamins [21]. Excess cholesterol is toxic to cells as demonstrated by disruption of the normal plasma membrane and impaired function of membrane-associated enzymes, channels and receptors [22,23]. In addition, excess cholesterol is one factor responsible for the formation of gallstones [24]. For these reasons, cholesterol and lipid movement and storage is tightly regulated.

1.2.2A – Metabolism of apolipoprotein B-containing Lipoproteins

ApoB-containing lipoprotein metabolism within the body can be divided into two pathways: exogenous lipoprotein metabolism and endogenous lipoprotein metabolism (reviewed in [25]). Humans do not have dietary cholesterol requirements because the body is fully capable of synthesizing all the cholesterol that it needs [26]. In the typical Western diet however, 1/3 of the cholesterol entering the intestinal lumen is of dietary origin [26] and any excess cholesterol consumption is excreted in the feces [27]. Lipoproteins play an essential role in the absorption of dietary cholesterol, long-chain fatty acids and fat-soluble vitamins. The transport of dietary lipids is mediated by the exogenous pathway

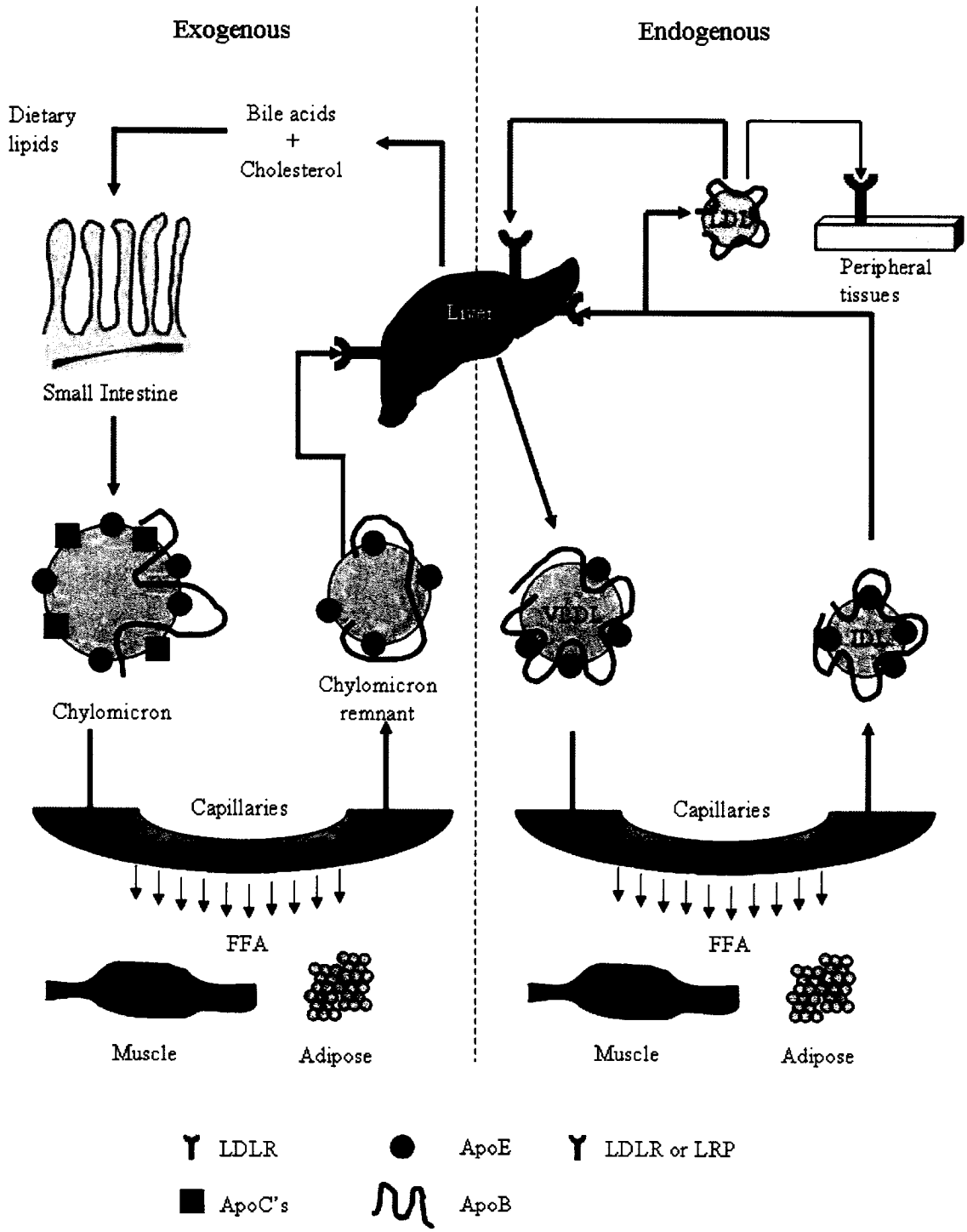
Apolipoprotein	Primary Source	Function
ApoA-1	Intestine, liver	Structural protein for HDL; activates LCAT
ApoA-II	Liver	Structural protein for HDL
ApoB-48	Intestine	Structural protein for chylomicrons and CR
ApoB-100	Liver	Structural protein for VLDL, LDL, IDL, Lp(a); ligand for binding to LDL receptor
ApoC-II	Liver	Cofactor for LPL
ApoC-III	Liver	Inhibits lipoprotein binding to receptors
ApoE	Liver	Ligand for binding to LDL receptor family (LDLR, LRP, VLDLR)

Table 1.3 Major apolipoprotein source & function. Apolipoproteins shown have well described functions. Other apolipoproteins such as apoC-1, C-IV and A-V shown in Table 1.2 have unclear functions. HDL, high density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; CR, chylomicron remnants; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein A; LPL, lipoprotein lipase; LDLR, LDL receptor; LRP, LDL receptor-related protein; VLDLR, VLDL receptor. [Adapted from (25)]

of lipoprotein metabolism (**Figure 1.2**). Dietary triglycerides are hydrolyzed by pancreatic lipases within the intestinal lumen which are further emulsified with dietary cholesterol and bile acids to form micelles [27]. Intestinal cholesterol absorption represents a major route for the entry of exogenous cholesterol potentially impacting serum cholesterol levels. The cholesterol-enriched micelles must first move through a diffusion barrier overlying the surface of the absorptive intestinal cells (enterocytes) after which the cholesterol within the micelles is taken up by the enterocytes at the brush border surface by Niemann-Pick C1-Like 1 (NPC1L1) [28], the molecular target of the intestinal cholesterol aborption inhibitor, ezetimibe [29]. Cholesterol taken up by the enterocytes can then be excreted back into the lumen of the intestine or into the lymph by a process that is primarily regulated by ABCG5 and G8 [26,30]. SR-B1 has also been implicated in the uptake of micelle-derived cholesterol although it is not absolutely required since knockout mice still absorb dietary cholesterol [31]. Furthermore, its role in cholesterol uptake appears to be indirectly associated with cholesterol efflux to micelles rather than direct uptake of cholesterol [32]. Once the cholesterol is absorbed, it is esterified by the action of acyl-coenzyme A:cholesterol acyltransferase-2 (ACAT-2), by the addition of a fatty acid, to form cholesteryl esters [33]. Long-chain fatty acids are incorporated into triglycerides and packaged with apoB-48 (generated solely in the intestine via mRNA editing of the full-length apoB transcript), CE, retinyl esters, PL and FC to form chylomicrons, a process that requires microsomal transfer protein (MTP) [34]. Nascent chylomicrons are secreted into the intestinal lymph by the intestinal epithelial cells and delivered to the systemic circulation. ApoC-II, which is transferred to circulating chylomicrons, acts as a cofactor for lipoprotein lipase (LPL) in a hydrolysis reaction in

Figure 1.2. The exogenous and endogenous lipoprotein metabolic pathways.

The exogenous pathway transports dietary lipids to the periphery and the liver. The exogenous pathway transports hepatic lipids to the periphery. LPL, lipoprotein lipase; FFA, free fatty acids; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; LDLR, LDL receptor; LRP LDL receptor-related protein. [Adapted from (25)]


Exogenous
Endogenous
Dietary lipids
Bile acids + Cholesterol
Small Intestine
Liver
LDL
Peripheral tissues
Chylomicron
Chylomicron remnant
VLDL
IDL
Capillaries
Capillaries
FFA
FFA
Muscle
Adipose
Muscle
Adipose
LDLR
ApoE
LDLR or LRP
ApoC's
ApoB

which the triglycerides of the circulating chylomicrons are hydrolysed releasing free fatty acids (FFA) [35]. The released FFA are taken up by other cells and tissues, including muscle and adipose tissue, and either oxidized to generate energy or re-esterified and stored as TG. The chylomicrons are progressively metabolized in the circulation producing chylomicron remnants (TG-depleted, cholesterol-enriched particles). These remnants are rapidly removed from the circulation by the liver in an apoE-dependent mechanism involving particle binding to the LDLR or LRP followed by endocytosis [36].

The endogenous pathway of lipoprotein metabolism involves the hepatic secretion and metabolism of VLDL to IDL and LDL and represents the transport of *de novo* synthesized cholesterol (**Figure 1.2**). VLDL particles resemble chylomicrons in protein composition (Table 1.2) except for apoB; intestinal cells produce only apoB-48 whereas hepatocytes produce full length apoB (apoB-100). The triglycerides of VLDL are derived primarily from the re-esterification of long-chain fatty acids generated from the hydrolysis of the TG storage pool within hepatocytes [37]. The majority of TG transported in the plasma in the post-absorptive phase is via VLDL [38]. In hepatocytes, the packaging of VLDL particles requires the activity of MTP [38]. Once secreted into the plasma, VLDL acquires its other apolipoproteins, namely apoE and apoCs, which are largely responsible for its metabolism. VLDL are hydrolyzed by LPL especially in muscle and adipose tissue, since the FFA released from this reaction are used for energy or re-esterified and stored as TG. VLDL remnants undergo further hydrolysis in the circulation until they are reduced in size and composition to IDL. VLDL remnants and IDL can be cleared from the circulation via the interaction of the apoE and the members of the LDLR family (LDLR, LRP and VLDLR) and subsequent endocytosis. In mice, approximately 50% of

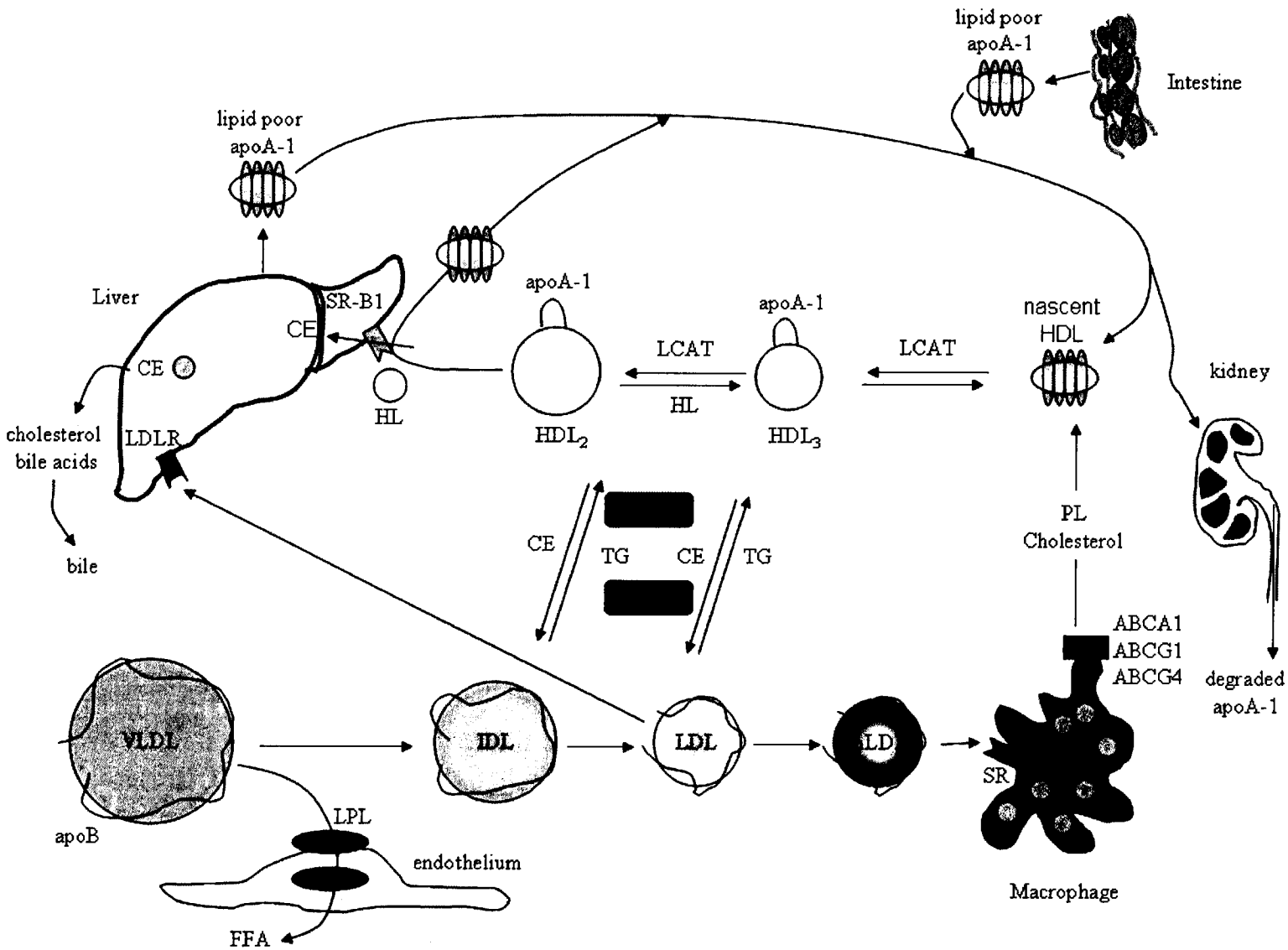
cholesterol and 30% of TG is removed from the circulation by hepatic LDLR-mediated clearance of remnant lipoproteins [39]. The remainder of IDL in the circulation is remodeled by hepatic lipase (HL) and cholesteryl ester transfer protein (CETP) to form LDL, during which most of the TG is hydrolyzed and all of the apolipoproteins except apoB-100 are transferred to other lipoproteins [25]. The cholesterol in LDL accounts for approximately 70% of the plasma cholesterol in normal individuals and 2/3 of these particles are cleared by LDL receptor-mediated endocytosis in the liver [25]. When LDL receptors are down-regulated or dysfunctional, LDL accumulates in the plasma with potentially harmful consequences (discussed below).

1.2.2B – Metabolism of HDL and Reverse Cholesterol Transport

A schematic diagram of HDL metabolism and reverse cholesterol transport is given in **Figure 1.3**. The mechanism of HDL synthesis, secretion, lipidation and its role in reverse cholesterol transport (RCT) as been extensively reviewed in the literature [15,40-46]. Briefly, poorly-lipidated apoA-1 particles are secreted by both the liver and the intestine. These poorly-lipidated apoA1 particles readily acquire cholesterol and phospholipids from the liver and peripheral tissues through ABCA1-mediated efflux forming nascent HDL. ABCG1 and ABCG4 can then efflux cholesterol to HDL from peripheral tissues, particularly lipid-laden macrophages [47,48]. FC that has been transferred to HDL is subsequently esterified to CE by LCAT, which is associated with HDL. As the particle grows and acquires more PL and CE, the CE is sequestered in the hydrophobic core of the mature HDL particle. Additional apolipoproteins (A-II, A-IV, E and Cs) are transferred to the surface of HDL from chylomicrons and VLDL during their lipolysis. ApoA-1 is also secreted into the plasma on chylomicrons from the intestine, after which

Figure 1.3. HDL metabolism and reverse cholesterol transport.

Lipid-poor apoA-1 is secreted from the liver and intestine and quickly gains cholesterol and phospholipids effluxed from macrophages and peripheral cells via ABCA1-mediated efflux. The cholesterol transferred to this nascent HDL particle is esterifed by LCAT and stored in the core of the lipoprotein particle to generate mature HDL_2 particles. ABCG1 and ABCG4 are also responsible for the cholesterol efflux to HDL from peripheral tissues, particularly macrophages. Following CETP-mediated exchange of CE for TG, the TG within HDL is hydrolyzed by HL. In the direct route of reverse cholesterol transport, the CE that remains with the HDL particle can be selectively taken up by SR-B1 in the liver, resulting in small lipid-poor HDL particles that are susceptible to clearance by the kidney. In the indirect route of reverse cholesterol transport, a portion of the CE transferred to apoB-containing lipoproteins (VLDL, IDL and LDL) is transported to the liver for endocytic particle uptake by the LDL receptor, or an LDLR family member such as LRP and VLDLR. LCAT, lecithin cholesterol acyltransferase; HL, hepatic lipase; LPL, lipoprotein lipase; FFA, free fatty acids; LDLR, LDL receptor; CE, cholesterol esters; mLDL, modified LDL; SR, scavenger receptor. [Adapted from (41)]


lipid poor
apoA-1
Intestine
lipid poor
apoA-1
Liver
SR-B1
CE
CE
HL
apoA-1
apoA-1
LCAT
HL
LCAT
nascent
HDL
kidney
cholesterol
bile acids
LDLR
bile
HDL2
HDL3
CE
TG
CE
TG
PL
Cholesterol
ABCA1
ABCG1
ABCG4
degraded
apoA-1
VLDL
IDL
LDL
LD
SR
LPL
apoB
endothelium
FFA
Macrophage

it can be exchanged onto HDL particles in the circulation. In the circulation, CETP mediates the exchange of CE for TG between HDL and apoB-containing lipoproteins, respectively, and the TG is later hydrolyzed by HL generating smaller HDL particles. HDL cholesterol is transported back to hepatocytes by both direct and indirect pathways of reverse cholesterol transport (RCT). In the direct pathway, the CE still associated with the mature HDL particles can be selectively removed from the particles by SR-B1 in the liver by a process termed selective uptake (discussed further in section 3.1.6) which constitutes the final step in RCT. Selective uptake results in small, lipid-depleted apoA-1 particles that are returned to the circulation to acquire more CE and PL. Unfortunately, these small lipid-depleted particles are also susceptible to clearance by the kidney. In the indirect pathway, a portion of the CE transferred to apoB-containing particles (VLDL, IDL and LDL) via the action of CETP is transported back to liver for uptake by LDLR family members (LDLR, LRP and VLDLR).

Additional HDL receptors, other than SR-B1, have been identified. HDL particles highly enriched in apoE can be cleared by holoparticle uptake via members of the LDLR family. In addition, LRP can mediate the selective uptake of HDL-derived CE in adipocytes [49,50]. HL and LPL have also been shown to mediate selective uptake of HDL-derived CE dependent on heparan sulphate proteoglycans (HSPGs) in culture and *in vivo* [51,52]. These were both independent of SR-B1 but the role of other endocytic receptors was not excluded. Finally, CETP has also been shown to mediate the selective uptake of HDL-derived cholesteryl esters in adipocytes [53,54], which is further discussed in chapter 3.

1.2.3 – Lipoproteins and the Pathogenesis of Atherosclerosis

There are several lines of major evidence implicating elevated plasma concentrations of LDL cholesterol in the pathogenesis of atherosclerosis. For example, the major lipids in atheromatous plaques are plasma-derived cholesterol and cholesteryl esters, and oxidized LDL is observed in macrophages in arteries at sites of fatty streaks [9]. Genetic defects in lipoprotein metabolism causing hyperlipoproteinemia, such as familial hypercholesteremia due to complete (homozygous FH) or partial (heterozygous FH) absence of functional LDL receptors, are associated with accelerated atherosclerosis [55]. Epidemiological data demonstrate that for both men and women, there is a positive and curvilinear relationship between plasma LDL cholesterol concentrations and age-specific risk of CAD. Conversely, there is a strong inverse correlation between plasma concentrations of HDL cholesterol levels and risk for both CAD and stroke [56]. Overall, a 1% decrease in HDL cholesterol (HDL-C) results in a 1% to 2% increased risk for CAD [57]. Finally, numerous studies demonstrate that lowering levels of serum cholesterol, particularly LDL cholesterol via statin treatment, slows the rate of progression of atherosclerosis and dramatically reduces the risk of clinical CAD events [58].

The mechanisms whereby LDL cholesterol contributes to atherogenesis are complex. With chronic hyperlipidemia, lipoproteins accumulate within the intima at sites of increased endothelial permeability. Increased lipid deposition within the intima results in increased production of oxidized lipoproteins induced by the free radicals generated in macrophages or endothelial cells in the arterial wall. Oxidized LDL plays several important roles [9]. It is readily ingested by macrophages through scavenger receptors forming foam cells. Oxidized LDL also acts as a chemoattractant increasing monocyte

accumulation in lesions. It stimulates the release of growth factors and cytokines resulting in the migration and proliferation of smooth muscle cells within the intima. Oxidized LDL may directly impair endothelial cell function through decreased mRNA expression of endothelial nitric oxide synthase (eNOS) and increased degradation of nitric oxide (NO), the major endothelial-relaxing factor. Finally, oxidized LDL is cytotoxic to endothelial cells and smooth muscle cells resulting in further endothelial and intimal damage.

Whereas LDL functions in the delivery of cholesterol to many tissues, HDL plays an important role as a carrier of excess cholesterol in the reverse cholesterol transport pathway (as discussed above) and is a negative risk factor for CAD. Aside from this very important role, HDL is also believed to have at least two other antiatherogenic mechanisms. HDL is also atheroprotective via its anti-oxidative effects. Oxidized (or modified) LDL, as discussed above, is readily taken up by macrophages in the vessel wall via scavenger receptors resulting in foam cell formation. HDL has been shown in mice to reduce the oxidation of LDL and this resulted in a reduction in atherosclerosis [59] and it is the HDL-associated paraoxonase that is believed to be responsible for these effects [60]. HDL may also slow lesion progression by selectively decreasing the production of vascular cell adhesion molecules such as E-selectin, P-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 that are responsible for the uptake of cells into the vessel wall [61-63], a key component in the development of atherosclerotic lesions. HDL also preserves endothelial function through maintaining normal vasoreactivity by stimulating nitric oxide and prostacyclin production [64,65].

1.2.4 – Strategies Aimed at Raising HDL

As discussed above, one of the major atherosclerosis risk factors modifiable by medications is dyslipidemia. The negative impact of dyslipidemia on overall health has prompted the development of very effective drugs aimed at reducing plasma LDL-cholesterol and TG levels including fibric acid derivatives, nicotinic acid (niacin), bile-acid binding resins, intestinal sterol absorption inhibitors, and statins (reviewed in [66,67]). Although these numerous classes of medications are extremely successful at reducing plasma concentrations of triglyceride-rich lipoproteins and LDL, statins, fibrates and niacin have the added benefit of increasing HDL cholesterol but, with the exception of niacin, their HDL effects are modest at best and in some cases, the side-effects preclude widespread use (niacin) [68].

Several other approaches have been used to target HDL and increase HDL cholesterol. One short-term approach used to investigate the atheroprotective effects of HDL involved five weekly infusions of poorly-lipidated apoA-1 Milano into patients with known cardiovascular disease [69]. ApoA-1 Milano is a naturally occurring variant of apoA-1 that contains a single point mutation resulting in a cysteine substitution. About 70% of ApoA-1 Milano is expressed as a dimer, linked by the sulfhydryl groups. This restricts HDL size and growth, but allows the remaining 30% of monomeric ApoA-1 Milano to act as an antioxidant, protecting lipids from free radical oxidation. This resulted in a significant reduction in the volume of coronary atheroma after only six weeks. Additional short term therapies under development include the infusion of synthetic peptides based on the amphipathic structure of apoA-1 and the reinfusion of

autologous delipidated HDL [70]. Long-term approaches aimed at raising HDL cholesterol levels involve the inhibition of CETP, which is discussed further in chapter 3.

CHAPTER 2 - REGULATION OF LRP GENE EXPRESSION

2.1 – Background

2.1.1 – LRP and the LDL Receptor Gene Family

The LDL receptor (LDLR) gene family consists of a class of structurally related cell surface receptors that are involved in diverse biological functions with varied organ, tissue and cell type distribution. Members of this family include LDLR, for which the class is named, very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (apoE2R), LDL receptor-related protein (LRP) which is also known as the α2 macroglobulin (α2M) receptor, LRP5 and LRP6, multiple epidermal growth factor-containing protein 7 (MEGF7), LRP1B and megalin.

Although LDLR appears to be involved solely in lipoprotein metabolism, LRP and other members of this family have other distinct biological functions [71]. These distinct biological functions are made possible through the varied arrangement of their common structural units. Members of the LDLR receptor gene family are made up of five common structural units: ligand-binding-type (or complement-type) cysteine-rich repeats, epidermal growth factor (EGF)-like cysteine-rich repeats, YWTD domains, a single membrane-spanning segment, and a cytoplasmic tail that can contain multiple NPxY motifs (reviewed in [72]). The difference between the various members is the arrangement of these constituent modules and thus the ligands, that they can bind.

2.1.2 – Protein Structure of LRP

LRP is synthesized as a 600 kDa polypeptide and undergoes multiple co- and post-translational modifications including N-linked glycosylation and proteolytic cleavage [73]. It is this cleavage that takes place in the late-Golgi [74] that is responsible for generating the 515 kDa α-subunit and 85 kDa β-subunit. These two subunits associate non-covalently at the cell-surface as a heterodimeric protein to generate the mature LRP. The β-subunit includes a cytoplasmic tail and a membrane spanning region, which is non-covalently associated with the extracellular α-subunit [73,75]. The α-subunit is composed primarily of multiple copies of three different types of repeats. These include the four clusters of 2,8,10, and 11 ligand-binding-type cysteine-rich repeats similar to the cluster of 7 such motifs that comprise the LDL-receptor ligand binding domains. In addition, the LRP α-subunit contains EGF-type repeats separated from each other by YWTD repeats. The α–chain of LRP contains several distinct but overlapping sequences that bind to several classes of biologically diverse ligands. Additionally, the intracellular portion of the □-subunit binds many adapter and scaffold proteins.

2.1.3 – Ligands of LRP

In contrast to the LDL receptor, LRP recognizes more than 30 ligands that can be sub-classified in several families that are structurally diverse with extremely varied functions. This diverse ligand recognition is made possible by the 31 ligand-binding-type repeats, each with its own unique surface contour and charge distribution [76]. Ligands associate with the receptor through these repeats within the clusters sometimes via multiple interactions with two or more clusters. LRP ligands include lipoproteins

and proteins involved in lipoprotein metabolism, proteinases, proteinase-inhibitor complexes, extracellular matrix proteins, bacterial toxins, viruses and other intracellular proteins such as adapter and scaffold proteins, many of which are involved in intracellular signaling (reviewed in [72,77-79]).

The largest group of LRP ligands are proteinases and proteinase-inhibitor complexes which include proteinases of the fibrinolytic pathway [80], serpin-enzyme complexes [81], □2-macroglobulin [75,82] and complement C3 [83] and more recently, proteins involved in blood coagulation such as factors IXa, VIIIa and VIIa [84,85], antithrombin III [81] and tissue factor plasminogen inhibitor [86].

Another important group of LRP ligands are those involved in lipoprotein metabolism and include lipoprotein lipase (LPL) [87], hepatic lipase (HL) [88] and apoE-rich lipoproteins [89,90].

Enormous progress has been made on the identification of adapter and scaffold proteins that bind to the intracellular domain of LRP solidifying its role in cellular communication and signal transduction pathways [91]. These include proteins such as Disabled-1 [92], Shc [93,94], FE65 [92] and Jun N-terminal kinase (JNK) interacting protein 1 (JIP-1) and 2[95] which interact with the NPxY motifs of LRP through either a phosphotyrosine binding (PTB) domain or PDZ domain.

Other documented LRP ligands include pathogens such as rhinovirus [96] and pseudomonas exotoxin A [97] as well as beta-amyloid precursor protein (APP) [98].

A 39kD receptor-associated protein (RAP) is an endoplasmic reticulum (ER) resident protein which functions intracellularly as a molecular chaperone for LRP and

other members of the LDLR family [99] and regulates its ligand-binding activity. RAP is required for proper folding and export of LRP from the ER [100] as demonstrated by the severely impaired cell-surface presentation of LRP in RAP-deficient mice [101]. It is believed that RAP functions by preventing the premature binding of co-expressed ligands, such as apoE [100]. RAP binds LRP directly via adjacent complement-type repeats, both containing a conserved acidic residue [102], and thus stearically interferes with binding of other LRP ligands including α2M* and remnant lipoproteins. For this very reason, RAP is often used as a control when studying LRP function. Interestingly, RAP is the only LRP ligand confirmed to bind within cluster III of the ligand-binding-type-repeats [78].

2.1.4 – LRP Functions

LRP is expressed in mammals, most notably humans [103] and mice [104], as well as chickens [105] and the nematode Caenorhabditis elegans [106] suggesting a common ancestry and common functions. Although LRP is essential for normal development as demonstrated by the embryonic lethality of the LRP knockout mouse [107,108], its exact role is unclear, however, its function in regulating tissue remodeling pathways might play a role. Tissue distribution in mammals is extremely varied with high expression in neurons and astrocytes within the central nervous system, hepatocytes, smooth muscle cells, fibroblasts, testes and ovaries, macrophages, kidney, lung and intestinal cells as well as adipocytes [109-111]. Because of the large variety of ligands, LRP is a multifunctional scavenger receptor whose numerous biological functions are likely tissue- and cell-dependent. These processes include: homeostasis of proteinases and proteinase inhibitors, some of which are involved in coagulation and fibrinolysis; cellular entry of

viruses and toxins; activation of lysosomal enzymes; neurotransmission; and metabolism of lipoproteins and β amyloid in the central nervous system (reviewed in [72,77-79]). For the purpose of this thesis, discussion below will be limited to the functions of LRP important in adipocytes and atherosclerosis, specifically adipocyte and hepatocyte lipoprotein metabolism and intracellular signalling.

2.1.4A – LRP and Adipocyte Lipoprotein Metabolism

This laboratory has demonstrated that LRP is absent from human preadipocytes and is expressed during differentiation concomitant with PPARγ and C/EBPα (Benoist & McPherson, unpublished data), consistent with a functional role in human adipocyte metabolism. Since cholesterol synthesis is limited in human adipocytes, the majority of cholesterol is provided by lipoproteins via specific receptors or by passive diffusion [112,113]. Both *in vivo* and *in vitro* studies suggest that LRP promotes adipocyte acquisition of lipoprotein-derived fatty acids and cholesterol. This effect is markedly enhanced in the presence of insulin, which elicits a rapid translocation of LRP to the cell surface [114]. Herz's group [111] infused ^{3}H cholesterol-labelled chylomicron remnants (CR) into rats and demonstrated that the uptake of labelled CR cholesterol by epididymal fat pads was increased 24-fold in the postprandial hyperinsulinemic state as compared to the fasting state. Similarly, when human adipose tissue was maintained in organ culture, incubation with CR in the presence of insulin resulted in marked accumulation of chylomicron cholesterol (McPherson *et al.*, unpublished results). Overall, these studies suggest that adipocyte LRP enhances the clearance of CR cholesterol and that adipose tissue may account for over 20% of CR cholesterol clearance in the rat, a species with low adipose tissue stores as compared to human. LRP also functions in adipocyte HDL metabolism.

This laboratory has demonstrated that adipocyte LRP mediates the selective uptake of HDL-derived CE through a novel efflux-recapture mechanism which requires apo E and heparan sulphate proteoglycans (HSPG) [49,50]. Thus, CR cholesterol clearance could be due in part to transfer of CR cholesterol to HDL and selective uptake of HDL cholesterol by adipocytes, an effect which may be mediated by LRP.

2.1.4B – LRP and Hepatic Lipoprotein Metabolism

Since hepatocytes are highly enriched in both LDLR and LRP [115], when LRP was first identified based on its structural homology with the LDL receptor, its primary function in the liver was estimated to be in lipoprotein metabolism and cholesterol homeostasis. Although the LDL receptor (LDLR) plays a significant role in the hepatic clearance of remnant lipoproteins, evidence that the LDL receptor-related protein (LRP) is an important alternate receptor for chylomicron remnant (CR) clearance has been provided by several groups including those of Mahley [36,116,117], Herz [101,118-122], Redgrave [123-125], and others [126-129]. The space of Disse is the space between the endothelial cells and hepatocytes in the liver, enabling intercellular exchange of metabolites. It is rich in HSPG, apoE and hepatic lipase. ApoE can mediate the trapping of remnants by binding to HSPG, followed by the transfer of remnants from the HSPG to LRP for endocytosis or by formation of a ternary complex which is internalized. LRP not only binds the remnant lipoproteins via its interaction with apoE, but also HL and LPL which are responsible for the formation of these remnants from the triglyceride-rich chylomicrons [130]. Following heparinase treatment, LRP-mediated remnant uptake by the hepatocyte is markedly impaired, suggesting an important role for the HSPG matrix. Orlando has recently proposed a model whereby dissociation of HSPG from LRP on the cell surface allows

binding and internalization of VLDL independently of LRP endocytic activity [131]. In rat hepatocytes *in vivo*, endocytosis of hepatic lipase is partially mediated by LRP and that of lipoprotein lipase almost exclusively mediated by LRP [5]. Functional impairment of the LRP/HSPG pathway by over-expression of RAP via an adenoviral construct had little effect on plasma lipids in normal mice but resulted in a 10-fold increase in plasma cholesterol and triglycerides in LDLR null mice [118]. In RAP-knockout mice, in which LRP expression is decreased by 75%, there was little change in plasma lipids in LDLR-expressing mice but marked remnant accumulation in double knockout mice lacking both LDLR and RAP [101]. Similarly, inducible inactivation of the hepatic LRP gene by Cre-mediated recombination in LDLR-deficient mice resulted in accumulation of cholesterol-rich remnants confirming the role of LRP in CR clearance [122]. Finally, human subjects with homozygous familial hypercholesterolemia do not exhibit marked accumulation of CR demonstrating that LRP is an effective backup receptor for remnant clearance when the LDLR is absent or impaired [132].

2.1.4C – LRP in Signalling

The members of the LDL receptor family, including LRP, play a well-established role in signal transduction [133]. LRP interacts with numerous cytosolic adaptor and scaffold proteins including Disabled-1, JIP-2, PSD-95, Shc and FE65, originally identified as a transcriptional activator [91,94,134,135]. The intracellular domain of LRP has been proposed to be part of a pre-assembled signalling complex that is released into the cell once cleaved by γ-secretase-like activity [136]. The cytoplasmic adaptor proteins bound to the cytosolic tail of LRP, controlled in part by its phosphorylation state, appear to regulate the subcellular localization and signalling functions of the intracellular domain

of LRP thus regulating its numerous intracellular effects. For example, LRP negatively regulates transcriptional activity of the amyloid precursor protein, Fe65 and Tip60 complex in the nucleus [137]. LRP has also been identified as a co-receptor for calreticulin and has a signalling function in the thrombospondin cell adhesion regulatory pathway [138]. By interacting with active tissue-type plasminogen activator (tPA) at the cell surface, LRP also appears to control permeability of the blood-brain barrier [139], vascular tone [140], and the expression of matrix metalloproteinases (MMPs) [141] through intracellular signals which remain unclear. An additional mechanism whereby LRP regulates cell signalling involves the sequestration of activated JNK in the plasma membrane thus inhibiting nuclear activation of the JNK-dependent transcription factors, Elk-1 and cJun [142].

The most clinically important signalling pathway involving LRP is that of the platelet-derived growth factor isoform BB (PDGF-BB) and platelet-derived growth factor receptor (PDGFR). PDGF induces the proliferation and migration of vascular smooth muscle cells (VSMC), a critical step in the formation of atherosclerotic lesions [4]. The mechanism is proposed to be PDGF-BB induced phosphorylation of the second NPxY motif within the intracellular domain of LRP when LRP is complexed with PDGFR-β, Src tyrosine kinase and PI3 kinase [143,144]. ApoE inhibits both PDGF-induced VSMC proliferation and migration [145,146] and LRP phosphorylation [144] leading Boucher et al. [147] to determine the effects of PDGF signalling by LRP on atherosclerosis *in vivo*. Mice with VSMC-specific deletion of LRP displayed severe hyperplasia of the aorta and increased susceptibility to atherosclerotic lesions as well as increased phosphorylated PDGFR expression indicating an important function of LRP in vascular wall integrity and resistance to atherosclerosis.

2.1.5 – Human LRP Gene Structure and Regulation

Considering the overall importance of LRP function, very few details surrounding the regulation of its gene expression are available. Several specific consensus sequences have been identified in the promoter but very few have actually been confirmed as functional discrete elements. Furthermore, the specific mechanisms or discrete promoter elements responsible for many of the observed changes in LRP mRNA and protein expression by some of the molecules discussed below are still unknown.

2.1.5A – Organization of the Human LRP Gene

The human LRP gene consists of 89 exons spanning 92 kb[148] on chromosome 12q13-q14 and encodes an mRNA of 15 kb that is translated into a peptide of 4525 amino acids[103].

2.1.5B – Organization of the Human LRP Promoter

A portion of the 5'-flanking region of the LRP gene was described by Stanley's group [149,150]. Despite the sequence homology between LRP and the LDL receptor in the coding region, there is little apparent similarity in the promoter region. In CHO cells, the minimal promoter driving expression of the LRP gene was contained within a 1.6 kb fragment. This region is GC-rich and does not contain a classical TATA box. However, an SP1 sequence at -80 and two clusters of SP1 sequences between -620 and -752 were characterized and shown to be critical for expression of the gene. In addition, the promoter region also contains an NRF-1 element located at -152 which may account for the regulation of LRP gene expression by cAMP and IFNγ. Although a consensus sterol response element, similar to that found in the LDL receptor promoter, exists at +233 in

the 5'-untranslated region of LRP[150], cholesterol treatment of macrophages actually increased LRP mRNA expression unlike the decrease observed with the LDL receptor [149]. The significance of this element in either regulation of gene transcription or mRNA stability remains obscure.

2.1.5C – Factors Affecting LRP Expression

In rats, LRP is very low in fetal cells but is induced shortly before birth [151] and there is a particularly high level of LRP mRNA expression in rat brain in the perinatal period [152]. In a rat model of nephritic syndrome, hepatic LRP mRNA and protein levels were increased and this could be reversed with statin treatment [153]. In contrast, a model of chronic renal failure showed significantly reduced hepatic LRP mRNA and protein [154]. In differentiated placental trophoblasts, LRP protein levels are down-regulated by cAMP [155]. Impaired expression of LRP has been reported in a number of neoplastic cells [156] although LRP expression is increased in malignant astrocytes [157]. Epidermal growth factor (EGF) has been shown to decrease LRP mRNA expression in neoplastic astrocytes but not normal human astrocytes [158]. In HepG2 cells, dexamethasone increased LRP mRNA and LRP receptor activity by two-fold and nuclear run-on experiments demonstrated that this effect was at the post-transcriptional level [159]. Other studies using HepG2 cells found that the HIV-1 protease inhibitor, Nelfinavir, reduced LDL receptor and LRP mRNA and protein levels by reducing active levels of SREBP-1 in the nucleus [160]. In macrophages, LRP expression is decreased by lipopolysaccharide and interferon γ and the effect of IFγ on LRP expression is at the transcriptional level and is inhibited by TGFβ [161]. On the other hand, IFγ has been reported to increase LRP gene expression in astrocytes [162]. Insulin was shown to have a biphasic effect on LRP mRNA expression in

macrophages [163]. *In vivo* studies demonstrated that insulin infusion had a small effect on skeletal muscle LRP mRNA abundance in normal subjects but had no effect in either overweight or diabetic subjects [164].

LRP gene expression is upregulated during monocyte differentiation into macrophages [165], especially in the presence of VLDL-1 [166]. Another study found that VLDL significantly increased VLDL receptor mRNA but only slightly increased LRP mRNA in macrophages, suggesting that foam cell formation more likely involves the VLDL receptor rather than LRP [167]. Several studies have shown that expression of LRP is increased in atherosclerotic lesions, particularly in intimal SMC and lipid-rich foam cells [168,169], two key cell types in the development of atherosclerotic lesions. Badimon and colleagues [170] reported upregulation of LRP expression in porcine vascular smooth muscle cells by LDL and attributed this to a decrease in nuclear SREBP-2. However, no effort was made to identify and characterize cis-acting element(s) and their cognate transcription factors within the LRP promoter. More recently, they reported increased LRP mRNA levels in plaque-derived vascular smooth muscle cells from human coronaries compared to non-plaque [171] correlating increased lipid deposition to LRP expression levels. Dietary cholesterol increased LRP mRNA levels in circulating monocytes [172] similar to previous *in vitro* observations [149], however in more recent studies patients suffering from myocardial infarction had reduced LRP protein levels in circulating monocytes compared to controls, despite significantly increased LRP mRNA levels [173].

2.1.6 – Adipogenesis

Although LRP is highly expressed in adipocytes, few data are available on factors involved in its activation, regulation, and function during adipogenesis. Following hormonal induction, confluent preadipocytes undergo mitosis and clonal expansion and coordinately express C/EBPβ and δ. C/EBPβ induces the expression of PPARγ, which initiates the adipogenic program [174]. Specific adipocyte proteins important in triglyceride accumulation such as LPL, fatty acid transport protein-1 (FATP-1), acetyl coA synthetase (ACS), CD36, and adipocyte fatty acid binding protein (aP2) [175-178], all contain at least one peroxisome proliferator response element (PPRE) in their 5'-flanking sequences and are all regulated by PPARγ. The free cholesterol concentration in the membrane of the adipocyte core lipid droplet is tightly regulated at approximately 2 mg per gram of TG suggesting a structural role for cholesterol in the formation of the lipid droplet [179,180]. However, adipocytes have a very low capacity for cholesterol synthesis and studies in this laboratory using ^{14}C-glycerol incorporation into TG demonstrate that lipoprotein cholesterol availability is rate-limiting for adipocyte TG synthesis (Benoit and McPherson, unpublished data). Adipocyte LRP functions in chylomicron remnant cholesterol clearance by an unknown mechanism both *in vitro* and *in vivo* [111], and in HDL-CE acquisition and thus may play a role in the maturation of the pre-adipocytes into fat cells. This laboratory has demonstrated that LRP can also play a role in the selective uptake of HDL-CE by human adipocytes [49,50,181]. Thus, co-ordinate regulation of LRP and fatty acid transporters may be a mechanism by which adipocytes can regulate cholesterol uptake to match TG synthesis during differentiation.

2.1.7 – Peroxisome Proliferator-Activated Receptors (PPARs)

Three PPAR subtypes (α, γ and δ or β) have been identified and they are members of the nuclear hormone receptor superfamily. PPARγ is further divided into PPARγ1 and PPARγ2 isoforms, which are identical except, due to different first exons, PPARγ2 contains an additional 30 amino acids at its N-terminus. Nuclear hormone receptors are ligand-dependent transcription factors that stimulate transcription of specific genes via dimerization with their obligate partner RXRα, recognition of and binding to specific DNA sequences known as a peroxisome proliferator response element (PPRE), and activation by ligand [182,183]. The PPRE consists of a hexameric nucleotide repeat of the recognition motif (TGACCT) spaced by one nucleotide (DR-1) [182,184]. Within a given species, the DNA binding domains of the three PPARs are 80% identical (slightly higher between PPARγ2 and PPARα [185]), thus PPARα and PPARγ recognize the same basic DNA sequence although the flanking sequence can confer some specificity for PPAR subtype [185,186]. Despite the homology in the DNA binding domains, their ligand-binding domains only share approximately 65% homology [187]. PPARδ is ubiquitously expressed and until recently, its function was unclear. PPARδ is an important regulator of embryonic and tissue development and it also plays a regulatory role in fatty acid catabolism in several tissues, including skeletal muscle and adipose tissue (reviewed in [188]). The tissue distribution of the PPAR α and γ subtypes however, is very distinct. PPARα is predominantly expressed in liver, heart, kidney, intestinal mucosa and brown adipose tissue [182]. These are all sites with high fatty acid catabolism and peroxisomal metabolism. PPARγ is expressed mainly in adipose tissue, where it plays a major role in adipocyte differentiation, but is also found in skeletal muscle, heart, brain, vascular

smooth muscle cells and monocyte/macrophages [182,183,189]. PPARγ2 is relatively adipose specific, although in animal models of obesity, hepatic expression of PPARγ2 has been documented [190].

2.1.8 – Ligand Activation of PPARs

The transcriptional activity of each of the PPAR subtypes is enhanced by a multitude of distinct compounds. Long chain polyunsaturated fatty acids are less specific ligands recognizing all PPAR subtypes [191] however their actual effectiveness *in vivo* is questioned by the supra-physiological concentrations required for efficient activation [192]. The arachidonic acid metabolite, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 is a physiological ligand for PPARγ [193] and leukotriene B4 [194] for PPARα. Members of the thiazolidinedione (TZD) class of insulin-sensitizing drugs, rosiglitazone for example, are specific ligands for PPARγ [195,196] and do not activate PPARα whereas members of the fibrate class of drugs, for example fenofibric acid, are selective PPARα activators [182,191].

TZDs are routinely used to improve peripheral insulin sensitivity in type 2 diabetic patients without affecting pancreatic β-cell insulin secretion [197]. TZD-treated patients also respond with a reduction in fasting and postprandial serum glucose and free fatty acids. These effects have been attributed to the primary effect of enhanced lipid storage in subcutaneous adipocytes and improved adipocyte function [198,199]. We propose that part of the beneficial effect of TZD administration on adipocyte lipid metabolism is through the upregulation of LRP and subsequent increase in free fatty acid uptake and storage.

Fibrates substantially decrease plasma triglyceride levels and this effect has been primarily attributed to an increase in LPL activity and decreased expression of apoCIII

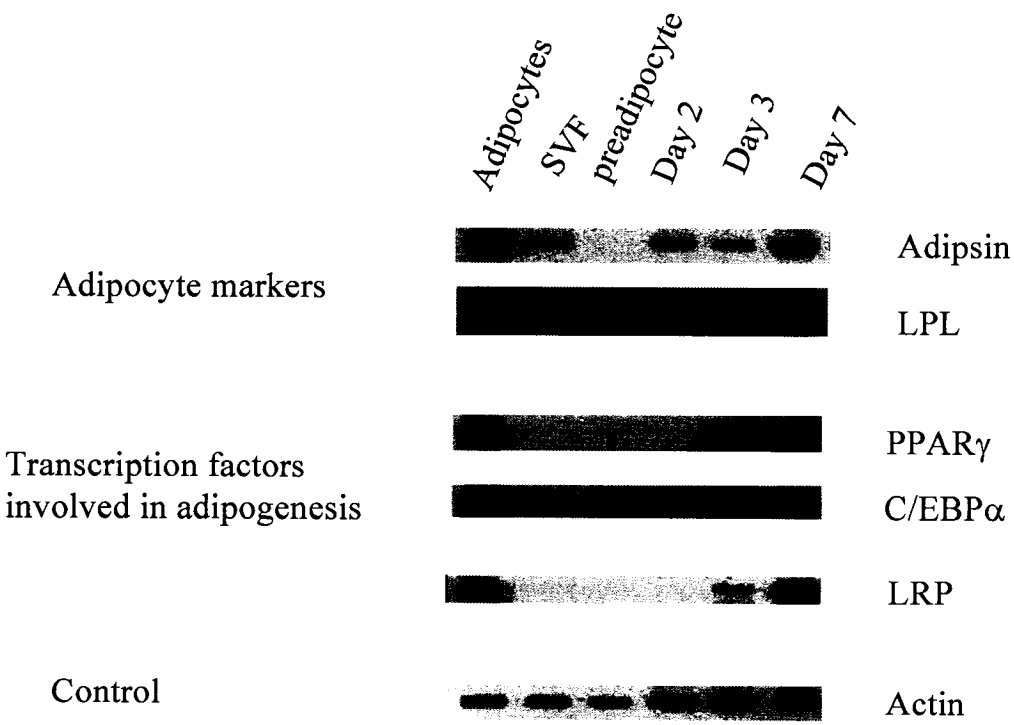
[182,189,191]. Fenofibric acid treatment results in a dramatic improvement in the lipoprotein phenotype in type III dysbetalipoproteinemia due to homozygosity for apoE2. Most [90,156,200], but not all [123] reports indicate that in contrast to the LDLR, LRP binds apoE2 efficiently. Thus we propose that a PPARα-mediated increase in hepatic LRP expression may explain in part the triglyceride-lowering effects of fibric acid derivatives.

2.2 – Rationale

This laboratory has examined LRP gene expression during human preadipocyte differentiation and in response to free fatty acid (FFA) availability. LRP mRNA was absent in human preadipocytes and the appearance of LRP mRNA during differentiation coincided with that of the peroxisome proliferator activated receptor gamma (PPARγ) (Benoist and McPherson, unpublished results; **Figure 2.1**). These data suggested that LRP is regulated by PPARγ in the adipocyte. The primary objective of these studies was the elucidation of the mechanism by which PPARγ regulates LRP gene expression in adipocytes. The specific activation by various distinct activators or ligands and distinct tissue-specific expression of the different PPAR subtypes suggests a mechanism for highly tissue-specific regulation of genes with a PPRE, including LRP therefore in additional studies, it was hypothesized that regulation of hepatic LRP could be achieved with PPARα agonists. The specific functions of LRP in each cell type may differ and an understanding of the regulation of LRP gene expression by the TZD or fibrate classes of drugs could have considerable clinical importance in light of LRP's important function in lipoprotein metabolism and atherosclerosis.

Figure 2.1. RT-PCR of selected adipocyte genes during differentiation.

LRP mRNA is absent in primary human preadipocytes and appears several days following the induction of adipocyte differentiation. mRNA transcripts for indicated genes were measured by RT-PCR and their products were resolved on 1% agarose gel and viewed by ethidium bromide staining. The key transcription factors involved in adipogenesis, C/EBPα and PPARγ, are absent in the preadipocytes and appear only once differentiation has been induced. The adipocyte markers adipsin and lipoprotein lipase (LPL) confirm that the differentiation has occurred. LRP mRNA appears several days following the induction of adipocyte differentiation, concomitant with the appearance of other adipocyte markers, suggesting that it might be regulated by PPARγ. These experiments were performed by Dr Benoit Gauthier. SVF, stromal vascular fraction.


Adipocytes
SVF
preadipocyte
Day 2
Day 3
Day 7
Adipocyte markers
Adipsin
LPL
Transcription factors involved in adipogenesis
PPARγ
C/EBPα
LRP
Control
Actin

2.3 – Materials & Methods

Reagents

Cell culture medium and reagents were purchased from Life Technologies and cell culture plasticware was purchased from Falcon. Chemical reagents were from Fisher or Sigma chemicals. Restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs. Taq DNA polymerase was purchased from Invitrogen. Maxiprep, miniprep and gel purification kits were purchased from Qiagen. Iodobeads and Super Signal chemiluminescence reagents were purchased from Pierce. Horseradish peroxidase-conjugated secondary antibodies and radioisotopes were purchased from Amersham. LRP antibodies were kindly provided by Dr. G Bu. Rosiglitazone (BRL 49653) was kindly provided by Dr. Steven Smith of Smith Kline Beecham. Dr. Michael Saunders of Glaxo Wellcome Inc provided the plasmid for RXRα and Dr. Bruce Spiegelman provided the plasmid for PPARγ2.

Human Preadipocyte Isolation and Culture

Subcutaneous adipose tissue was collected from healthy normolipemic subjects undergoing reduction mammoplasty procedures. Preadipocytes were isolated and cultured from adipose tissue through collagenase digestion, centrifugation and filtration as previously described [53,201-203]. The preadipocytes were cultured in differentiation media for 10 to 14 days. The cells were then insulin starved in the presence or absence of varying concentrations of the PPARγ ligand, rosiglitazone, for 24 h prior to assays. The control cells, which were also insulin starved, were treated with vehicle only (DMSO).

Cell Culture

The human liposarcoma cell line SW872 (American Type Culture Collection, Rockville, MD) was previously characterized and has been shown to be a good cell model for adipocyte gene expression [204-206]. Cells were cultured in Dulbecco's modified Eagle's/HAM F12 (3:1) (DMEM/F12) (Life Technologies, Burlington, ON) supplemented with 5% fetal bovine serum and 1% L-glutamine (Life Technologies, Burlington, ON), 10 mM $NaCO_3$, and 50 μg/ml gentamycin (NovoPharm, Toronto, ON) in the presence of 5% CO_2 at 37°C. Lipoprotein deficient fetal bovine serum (LPDS) was prepared as described previously [207] and dialyzed against PBS for 24 h. The effect of fatty acids and TZDs on LRP mRNA and protein levels was determined by incubating cells for 24 h in medium containing either LPDS or fetal calf serum in the presence or absence of oleic acid (18:1) or arachidonic acid (20:4) (Sigma Chemical Co., St. Louis, MO) or rosiglitazone (Smith Kline Beecham Pharmaceuticals, King of Prussia, PA). All conditions were studied in triplicate.

3T3-L1 mouse pre-adipocyte cell line was obtained from ATCC and maintained at low passage number. 3T3-L1 preadipocytes were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Two days post-confluence, differentiation was induced by the addition of 100 nM insulin, 0.25 μM dexamethasone and 0.5 mM isobutylmethylxanthine (IBMX) in DMEM containing 10% fetal bovine serum. Cells were incubated with this differentiation medium for 2 days. The medium was then changed to DMEM with 10% fetal bovine serum containing 100 nM insulin only and this medium was changed every 2 days until the cells were terminally

differentiated (approximately 8 days following addition of differentiation media). Cells were incubated with either vehicle (DMSO) or rosiglitazone at the indicated concentrations for 24 hours prior to RT-PCR or ^{125}I-α2M* binding and degradation assays as described below.

Primary mouse hepatocytes were isolated from retired breeders of the C57Bl6J (wild type) mouse strain purchased from Charles River (Wilmington, MA) using established protocols [208,209]. Briefly, mice were sedated and livers perfused with collagenase solution. The cells were seeded on fibronectin-coated (4 μg/well) 24-well plates at an initial density of 2.9 X 10^5 cells per well in William's medium containing penicillin (100 units/ml), streptomycin sulfate (100 units/ml), Fungizone (250 ng/ml; Invitrogen) and 10% fetal bovine serum (Sigma). Six hours following the initial plating, the cells were washed in William's medium without fetal bovine serum and then fresh media (as above) was added with either vehicle (DMSO) or fenofibric acid for 24 hours prior to ^{125}I-α2M* binding and degradation assays as described below.

Primary human hepatocytes isolated from a recently deceased individual who was otherwise in good health were obtained commercially (Gentest, BD Biosciences). The cells were seeded on BIOCOAT MATRIGEL matrix cellware and maintained in Hepato-STIM media containing 10 ng/ml epidermal growth factor (EGF) and 2 mM L-glutamine. Cells were treated with 250 μM fenofibric acid or vehicle control (DMSO) for 24 hours prior to the ^{125}I-α2M* degradation assay, as described below.

RNA Extraction, Northern Blot and RT-PCR

Total cellular RNA was isolated from both differentiated primary human adipocytes and SW872 cells with Tri-Reagent (Bio/Can, Mississauga, ON) according to

the manufacturer's instructions. RNA samples from differentiated primary adipocytes that were to be used in RT-PCR reactions were treated with amplification grade DNAse1 to deplete the samples of any DNA contamination according to the manufacturer's instructions (Life Technologies, Burlington, ON). RNA concentration was determined spectrophotometrically using $OD_{260/280}$.

Total RNA (5 μg) was separated by agarose gel electrophoresis using the NorthernMax-Gly kit and transferred to BrightStar-Plus nylon membrane according to the manufacturer's instructions (Ambion, Austin, TX). DNA probes were synthesized by RT-PCR; 1st strand DNA was synthesized as described below and PCR was performed using the following primers: LRPf 5'-GAGTACCAGGTCCTGTACATCGCTG-3' and LRPr 5'-CTCGTCAATCATGCCCGAGATGAGC-3' and β-actin-f 5'-GCCCCTCCATCGTCCACCGC-3' and β-actin-r 5'-GGGCACGAAGGCTCATCATT-3'. The PCR products were gel purified using the QiaexII kit (Qiagen) and the purified DNA was subsequently labeled using the Rediprime II random prime labeling kit according the manufacturer's instructions (Amersham). The probes were cleaned up with NICK columns (Amersham) and the specific activity was determined by scintillation counting. Hybridizations and washes were performed according the NorthernMax-Gly kit instructions (Ambion).

RT-PCR was performed using a two-step approach. First strand cDNA was synthesized using 2.5 μg of total RNA, 10 μM random decamer primers (Ambion, Austin, TX) and 200 units of MMLV reverse transcriptase (Life Technologies, Burlington, ON) and incubated at 42°C for 1 hour. Consecutive PCR reactions were then performed on the 1st strand cDNA using the primers shown below and the SYBR green

"taq-start" polymerase and the LightCycler Apparatus according the manufacturer's instructions (Roche, Montreal, PQ). Some of the data from the LightCycler was repeated using relative-quantitative RT-PCR as described below. For the RT-PCR experiments performed on murine-derived cells, the following primers were designed using the Light Cycler Primer Design Software from Roche: mouse LRP forward 5'-CCCAAGCATTTCGTGT-3' and reverse 5'-CGCGTTCGTCTGAACC-3'; GAPDH forward 5'-GTCGGAGTCAACGGAT-3' and reverse 5'-CCACGACGTACTCAGC-3'; β-actin forward 5'-CTACGAGCTGCCTGAC-3' and reverse 5'-GGGTACATGGTGGTGC-3'. These primers were used along with Taq DNA polymerase to amplify their respective sequences from an RT reaction on RNA isolated from primary mouse hepatocytes. The amplicons were subsequently cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmids containing each of these genes were purified from bacterial culture using the Maxi Prep DNA isolation kits (Qiagen). Serial dilutions of these plasmids were generated and used as templates in real-time PCR reactions to generate a standard curve that can be used for future runs on the Light Cycler to measure mRNA abundance in experimental samples.

For certain SW 872 samples, relative-quantitative RT-PCR (RQ-RT-PCR) was performed using the Quantum RNA 18s Internal Standards kit from Ambion (Austin, TX). This kit has been shown previously to allow the accurate determination of relative changes in gene expression between samples [210]. Briefly, first strand cDNA was synthesized by reverse transcription at 42 °C for 1 hour using 2.5 μg of total RNA, 10 μM random decamer primers (Ambion, Austin, TX) and 200 units of MMLV reverse transcriptase (Life Technologies, Burlington, ON). LRP forward primer (5'-

GAGTACCAGGTCCTGTACATCGCTG-3') and reverse primer (5'-CTCGTCAATCATGCCCGAGATGAGC-3') were designed to amplify a region of the LRP mRNA that is approximately 400 bp in size whereas the primers provided in the 18s Internal Standards kit produced a band that is approximately 500 bp. A cycle number of 23 was determined to be within the linear range of PCR and was used for all subsequent PCR reactions. The 18s primer:competimer ratio of 3:7 was experimentally determined so that the LRP and 18s PCR products were amplified to give similar yields so that they could be compared between samples. PCR was performed on 1 μl of the RT reaction using 20 pmol of each LRP primer and 4 μl of 18s primer/competimer mix with the following PCR conditions; 1 cycle X 95°C, 3 min., 23 cycles X 95 °C, 30 sec., 66 °C, 30 sec., 72 °C, 30 sec. Cocktails containing all shared components were used to reduce variation between samples. PCR products were subjected to electrophoresis through a 1.5% agarose gel and visualized with ethidium bromide staining. Band intensities were measured using the ChemiDoc apparatus and Quantity One software (Bio-Rad, Mississauga, ON). Relative intensity was calculated by dividing the 400 bp band corresponding to the LRP message by the 488 bp band corresponding to the 18s message.

To determine if the effect of rosiglitazone was at the transcriptional level, 500 nM of this ligand was added to cells cultured as described above in the presence or absence of 10 μg/ml α-amanitin (Sigma Chemical Co., St. Louis, MO), a potent inhibitor of RNA polymerase II [211]. Total RNA was isolated and subject to relative-quantitative RT-PCR as described above.

LRP Reporter Gene Constructs

Complementary oligomers with flanking Nhe1 and Xho1 restriction sites (5' – CTAGCCTCCT*TGAACTCTGACAT*GCAGACC – 3') were annealed and subcloned into the luciferase reporter vector, pGL3-Promoter (Promega Corp.) (a vector map is provided in Appendix I). The pGL3-promoter vector contains only an SV40 promoter and is suitable for experimentally testing the function of enhancer elements such as the PPRE in the LRP promoter. This new vector contains a single copy of the putative PPRE upstream of the SV40 promoter and is designated pGL3-PPRE. For pGL3-PPRE3, oligomers consisting of 3 tandem copies of the LRP PPRE (5'-CTAGCCTCCTTGAACTCTGACATGCTCCTGAACTCTGACATGCTCCTGAACTCTGACATGCAGAC-3') were synthesized and hybridized prior to cloning into the pGL3-promoter vector.

A 1.9 kb fragment of the 5'-flanking region of LRP was also prepared by PCR amplification from the LRP-BAC construct prepared by Dr. Jan Boren [212], which contains the entire 92 kb gene of human LRP, using the primers 5'-GCAACGAGCTCCGTAAAAGGGGGAAG-3' and 5'-GCAGCAGATCTTTCCCCGGACTGAAG-3'. This fragment was subcloned into the SacI and BglII sites of the luciferase reporter vector, pGL3-Basic (Promega Corp.)(a vector map is provided in Appendix I) and designated pGL3-LRP. The Mutagenesis of the PPRE was performed by PCR using PFUTurbo (Stratagene, La Jolla, CA) according to their Quikchange site-directed mutagenesis protocol. The complementary primers (5'-CCCGCTCCTTGAACTCAACGATGCAGACACC-3') were designed to mutate a single half-site of the PPRE so that PPARγ would no longer bind the response element (mutated

nucleotides are underlined). This construct was designated pGL3-$LRP_{mutant\ PPRE}$. Both pGL3-LRP and pGL3-$LRP_{mutant\ PPRE}$ were sequenced to confirm that the promoter sequence was correct (compared to GenBank Accession Y18524) and to verify the mutagenesis.

Transient Transfection and Luciferase Activity Promoter Assays

Confluent SW872 cells were trypsinized and seeded at a density of 1.25 X 10^5 cells/well in 12 well plates 48 h prior to transfection. The cells were approximately 70-80% confluent at the time of transfection. Fresh media containing fetal bovine serum was added 12 h preceding transfections. The cells were co-transfected with 4 μg of the Firefly luciferase reporter vector (either pGL3-basic, pGL3-LRP, PGL3-PPRE or pGL3-$LRP_{mutant\ PPRE}$) and 0.25 μg of the Renilla luciferase-bearing reporter vector, pRL-CMV (Promega Corp.) using the calcium phosphate-DNA precipitate method [213]. Four hours after the transfection, the cells were subjected to glycerol (15%) for 2 min, and washed 3 times with PBS before the addition of media. Cells were treated 12 h later with DMSO alone (vehicle control) or varying concentrations of rosiglitazone. After 24 h (36 hours total post-transfection) the cells were scraped in 250 μl of reporter lysis buffer (Promega Corp.) and kept on ice until assayed.

Luciferase activities derived from both Firefly (LRP constructs) and Renilla (pRL-CMV) proteins were measured using the Dual-Luciferase Reporter Assay System (Promega Corp.) and recorded using a Monolight 2010c luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Renilla luciferase activity was then used to standardize for transfection efficiency.

Preparation of Nuclear Protein Extracts

Nuclear proteins were extracted from SW872 cells[214] or from primary human adipocytes[215] as described. Protein concentration was determined using BCA protein reagent (BioLynx, Brockville, ON) according the manufacturer's instructions.

Electrophoretic Mobility Shift Assays (EMSA)

Double stranded oligonucleotides (oligomers) corresponding to the PPRE of human LRP (5'-CCCCGCTCCT*TGAACTCTGACAT*CGAGACACCTA-3') were radioactively end-labeled with [γ^{32}P]-dATP (Amersham Pharmacia Biotech, Baie D'Urfé, PQ) using T4 polynucleotide kinase (Life Technologies, Burlington, ON) and purified from unincorporated nucleotides by gel filtration through G-50 spin columns (Amersham Pharmacia Biotech, Baie D'Urfé, PQ). The same procedure was used for oligomers corresponding to the PPRE of the human fatty acyl coA oxidase gene (hACOX), a gene containing a known PPRE in its promoter [216] (5'-TCCGAACG*TGACAGCTGACCT*GGTCCCCTTT-3') and oligomers corresponding to the mutated form of the human LRP PPRE (the mutated half-site is underlined) (5'-CCCCGCTCCT*TGAACTC*<u>*AACGAT*</u>CGAGACACCTA-3'). The specific activities of the oligomers were approximately 250 cpm/fmol. These were diluted to 60 fmol/μl for use in the assay. PPARγ (from Dr. Bruce Spiegelman) was subcloned into the MluI and NotI sites of pSPORT1 (Life Technologies, Burlington, ON) using PCR based methods. RXRα in pSG5 was provided by Dr. Michael Saunders. Both constructs are driven by the T7 RNA polymerase promoter for use in the TnT T7 Coupled Reticulocyte Lysate System (Promega Corp.) for *in-vitro* transcription/translation. All EMSA reactions were carried out on ice in 20 μl binding buffer (12.5 mM HEPES-KOH (pH7.6), 6 mM $MgCl_2$,

5.5 mM EDTA and 50 mM KCl) supplemented with 5 mM DTT, 0.25 μg low fat milk, 0.05 μg poly dI:dC and 10% glycerol. For EMSA reactions with TnT purified proteins, 2 μl of the TNT reaction was added to the reaction mix along with 1 μl (60 fmol) of labeled oligomers. For EMSA reactions with nuclear protein extracts, 6 μl of nuclear extracts were added to the reaction mix with 1 μl (60 fmol) of labeled oligomers. These reactions were left on ice for 20 minutes. Following the 20 min incubation, 2 μl of 20% ficoll was added to the samples. DNA/protein complexes were then resolved by electrophoresis through 6% polyacrylamide gels in 0.25X Tris-borate running buffer (20 mM Tris-borate pH 7.2, 0.5 mM EDTA). Supershift assays were performed using the PPARγ NuShift kit following the manufacturer's protocol (Active Motif). The non-specific antibody used was mouse monoclonal anti-β-actin (Santa-Cruz).

For experiments comparing human and mouse PPRE sequences EMSA and supershift assays were done using the PPARγ NuShift kit (Active Motif). Oligomers corresponding to the human LRP PPRE sequence were the same as those above. The oligomers corresponding to the mouse LRP PPRE sequence were the following: (5'-TGAGCTTTCC*AGAACTTTCACTT*CAGGTCACAGA-3'). Radioactive end-labeling was as described above.

Cell Surface Fluorescent Detection of LRP

Cells were cultured on 35 mm cover glass bottom dishes (MatTek, Ashland, MA) as described above and supplemented with 160 μM arachidonic acid, 500 nM rosiglitazone, or control for 24 hours prior to experiment. α2M was activated by incubating purified α2M with 400 mM methylamine for 16 hours at room temperature. Activated α2M (α2M*) was fluorescently labeled using Cy3 Monofunctional reactive

dye (Amersham Pharmacia Biotech, Baie D'Urfé, PQ) to a dye:protein ratio of 1.3 according to the manufacturer's instructions. Cells were placed on ice for 1 h in 3:1 DMEM/F12 supplement with 2 mg/ml BSA buffered with 10 mM HEPES. Labeled α2M* was diluted in the same media to a concentration of 1 μg/ml and was added to the cells at 0 °C for 45 min. The cells were washed with ice cold PBS 3 times prior to being fixed with 4% paraformaldehyde for 10 min at 0 °C. Cells were rinsed with PBS and kept in 2 ml of PBS at room temperature for fluorescence microscopy. Binding was competed with 30-fold excess of unlabelled α2M*. Cells were viewed with an Olympus IX50 fluorescent microscope and images were taken using a coded CCD camera (MicroMax) and WinView software from Princeton Instruments (Princeton, NJ).

Western Blotting of LRP

Total cellular protein (5 μg) from SW872 cells incubated in the presence or absence of various PPARγ ligands, was subjected to SDS-polacrylamide gel electrophoresis and transferred to nitrocellulose [217]. The LRP was detected [217] by chemiluminescent detection (Pierce Biochemicals) using a polyclonal rabbit antisera (from Dr. G. Bu) followed with a secondary antibody conjugated to horseradish peroxidase. The blot was developed and the bands quantified using the ChemiDoc apparatus and Quantity One software (Bio-Rad, Mississauga, ON). Triplicate cell samples were processed and are summarized in the graph.

Molecular biology techniques were essentially as described by Sambrook *et al.* [218].

^{125}I-α2M Degradation and Binding Assays*

SW872 cells were cultured for 24 h with either 160 μM arachidonic acid or 500 nM rosiglitazone in media containing CS prior to measuring their ability to degrade ^{125}I-labelled activated alpha 2 macroglobulin (^{125}I-α2M*) as previously described [219]. Differentiated primary human adipocytes cultured in differentiating media, were starved of insulin in the presence or absence of increasing concentrations of rosiglitazone prior to measuring their ability to degrade or bind ^{125}I-α2M* [219]. Control cells were treated with vehicle only (DMSO). As a further control, cells were treated in the presence or absence of RAP.

Analyses of data (B_{max} and K_d) were done using GraphPad Prism v.4.02 software (GraphPad Software Inc.).

Statistical Analysis

Results are expressed as the mean ± SEM. Where indicated, the statistical significance of the differences between groups was determined using Student's *t* test or ANOVA using GraphPad InStat v.3.06 statistical analysis software (GraphPad Software Inc.).

2.4 – Results

2.4.1 – The Human LRP Promoter Contains a Conserved Peroxisome Proliferator Response Element (PPRE)

Analysis of the human LRP promoter, accession Y18524, has identified a novel sequence (**TGAACTcTGACAT**) in the 5'-flanking sequence (-1185 to –1173) with homology to a consensus PPRE (**Figure 2.2**). The consensus PPRE is a direct repeat spaced by a single nucleotide (DR-1) with the sequence TGACCTnTGACCT. This newly identified element contains a single mismatch per half-site of the DR-1 for a total homology of 83%.

2.4.2 – Endogenous LRP mRNA Levels are Increased Upon Exposure to PPARγ Ligands

The effect of PPARγ ligands on levels of LRP mRNA was determined in the adipocytic cell line, SW872, by relative-quantitative RT-PCR. The relative intensity was determined by the ratio of the LRP band intensity compared to the 18s band intensity and these values were normalized to the control samples to give values of fold increase. The fold increases of LRP mRNA in cells upon treatment with oleic acid, arachidonic acid and rosiglitazone are summarized in **figure 2.3A, B & C** (respectively). PPARγ ligands, rosiglitazone (500 nM), arachidonic acid (160 μM) and oleic acid (0.8 mM) increased LRP mRNA levels by 1.5 fold, 1.6 fold and 1.3 fold respectively. The maximum effect of the ligands was observed after 24 h treatment. In SW 872 cells, increases in LRP mRNA levels were not observed for concentrations of rosiglitazone above 1 μM (data not shown). When cells were cultured in media containingLPDS instead of complete fetal

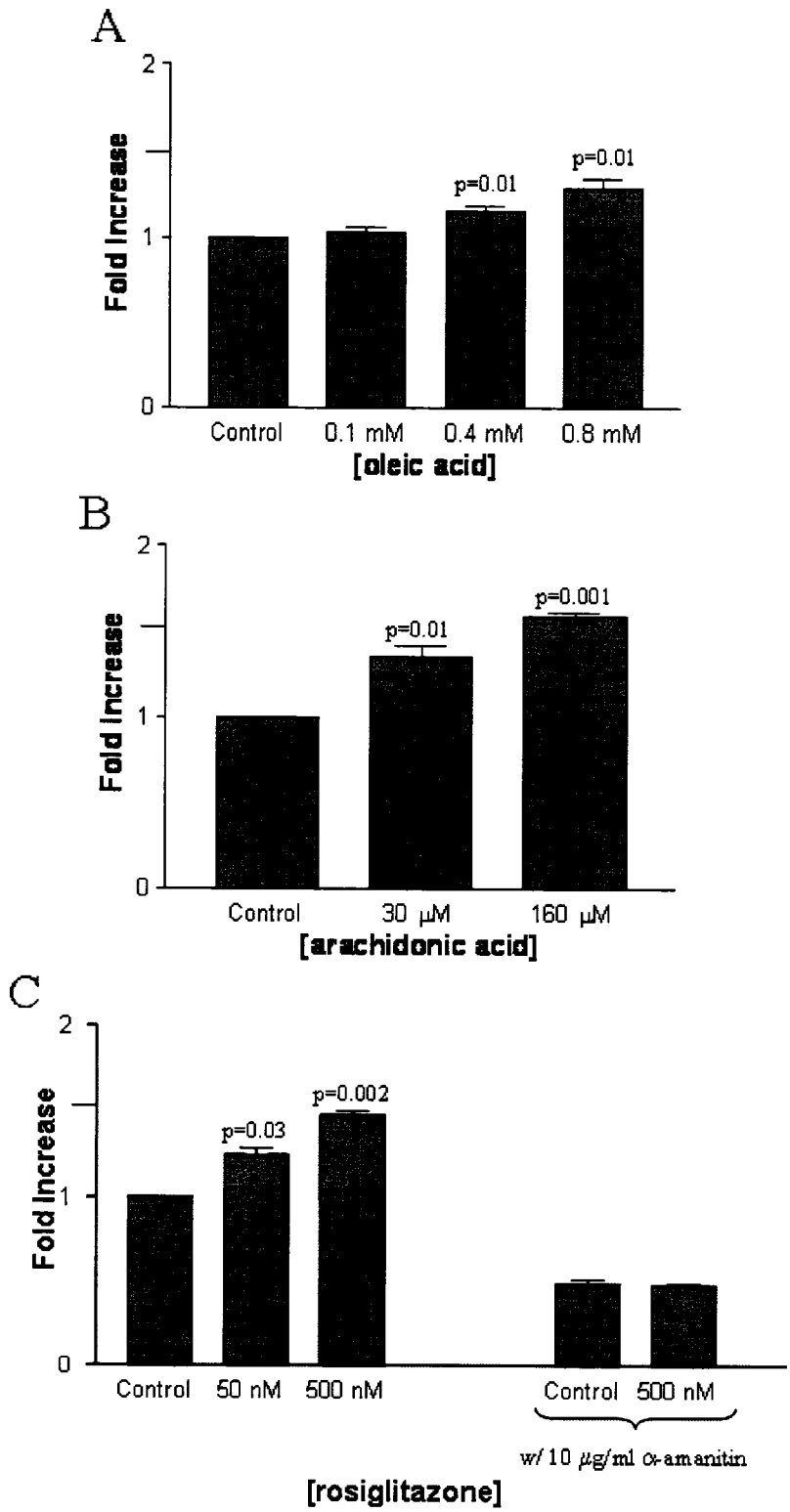
Figure 2.2. Nucleotide sequence of the human LRP promoter.

The sequence is numbered with reference to the putative transcription start site which is situated 466 bp upstream of the LRP open reading frame. Boxes show previously identified DNase protected footprint regions using rat liver nuclear extract [149]. Previously identified putative response elements are underlined [150]. The arrows indicate the direction and type of site (forward arrow = sense strand; reverse arrow = antisense strand). By sequence scanning, we have found that the human LRP promoter contains a novel peroxisome proliferator response element at -1185 to -1173 with 83% homology to the consensus PPRE sequence (TGACCTnTGACCT). The promoter also contains two CCAAT-boxes to which C/EBPα may bind but this has not been confirmed experimentally. There are other numerous DR-1 and DR-2 sites with slightly less homology to the consensus but since their significance is unknown they have not been indicated.


-1254 CGTA AAANGGGGAA CAGTCCCTCC TCTCCAGCGT CAATTTTTGG CAGCCGACGT CGCTGCCCCG CTCCTTGAAC
-PPRE→
-1180 TCTGACATGC AGACACCTAG AAAGTCAGAC ACTAAGGTAA CAGCCATAAA ACNCCGCCCA GAAGGGGGCA GTGACCAAAA
-1100 GCACGTTCAC TGGCCCCTGG GAACCGCCTG GCGCCTGCCT TCTGCAAAGT ATCATTCCCG TGTGGGCTGA GCCTGGGGAC
-1020 AAAGGTCCGG CGCTCAGCAG ACACCTCCTT GGAACCCGAG GCCTGGAAAC GAAGGTCCAG AGTCCCTCAT CTTATTCCAA
-940 ATCCTGGCGC TCGGAATCTA AAACCCTAAC TTGATGGCAA GCCAGTCAGA AGGTATTAAA AAAAAAAAAA AAAGAAAAAA
-860 CAGAAAAATA AATGAGCCCC GACTTCTTGG GCGAAAGGGG GTGGCCTTTC CTCAACCCCA CCCGCTGGTG ACTCACCTCC
-Sp 1→ -Sp 1→
-780 CTCCAAACCA GGGCCTGCCC CTCCCGCGCC TAGGGCTGGG CGAGCAGGGC GGGCGGGTAC TAAGGTGGGC TCCATCCCCG
-Sp 1→ -Sp 1→
-700 AGCCCCACGC GGGCGGACAA GCTCCGGCGT GTCCCCTCGG GTGTCCCTGT TTACTCCGAG CCCGGGAGCG AGGTGGGGGC
←Sp 1- -CCAAT-box→
-620 GGGTCCTCGC GGTCCCTCCC CAACCCCGCC CCCTCCTTCG CAGGCCCCAA TCCCAGTCCG GTCTTCCAAA GTCTCCAAAT
-540 CAAAGCTCAG CCTTTCCCTG CACCTTCCCC GCGGACTGGC GGTCCCTGTC CCCACCCCGG GCAGAGGAGG CACCTTCAGG
-460 GTTCCCCTAG AAAATCGAGC CTCGGCCCAG ACCACTGAGT CCCGGACGCC CCCGGAGGAG CCTGAAATCC TAGAGTATGA
←Sp 1- -CCAAT-box→
-380 CACTGAGTTT CAAAGGGGAG CCGCTCAGCT CTCCGCCCAC TGCCCAATCC CACCCTCGAC CCCTTCTGCT CTCTGGAGCA
-300 CCAGGGAGGA GGGCCCAGCC AGGGGAGAGG GCGCCCCAGG CCCCAAACTA GCCAGCGAGT CCCCGGGACC CTCACTCTTG
-NRF-1→
-220 GACCGCTTTC CAGAACTCCG AACCCTGTCG CGGGTCCGCG TCGCCCTCCC CCGGGCAGCG CGTCAAATCG GCGCATGCGC
←Sp 1-
-140 ACTCACTGGA TTGCCGCGTA GCTCTTTCTC CCCCCACCAA CCAACCTTTT TTCTTTCCCC GCCCCTTCCC TCCCTCCCTC
transcription start site
-60 CTCAACCCGT CCCCTCCCTC TCCCCCATCA GCCCCCCCCT CGGCACTTCA GTCCGGGGAA CAGCGGTGCG AGCTCCAGGC
21 CCATGCACTG AGGAGGCGGA AACAAGGGGA GCCCCCAGAG CTCCATCAAG CCCCCTCCAA AGGCTCCCCT ACCCGGTCCA
101 CGCCCCCCAC CCCCCCTCCC CGCCTCCTCC CAATTGTGCA TTTTTGCAGC CGGAGGCGGC GCCGAGATGG GGCTGTGAGC
←SRE-1-
181 TTCGCCCGGG GAGGGGGAAA GAGCAGCGAG GAGTGAAGCG GGGGGGTGGG GTGAAGGGTT TGGATTTCGG GGCAGGGGGC

Figure 2.3. Endogenous LRP mRNA levels in SW872 cells are modulated by PPARγ ligands at the transcriptional level.

The effect of PPARγ ligands on levels of LRP mRNA was determined by incubating SW872 cells for 24 h in DMEM/HAMF12 (3:1) supplemented with complete serum in the presence or absence of the PPARγ ligands: **A.** oleic acid; **B.** arachidonic acid; **C.** rosiglitazone. Also a potent inhibitor of RNA polymerase II, α-amanitin, was added to SW872 cells in the presence or absence of 500 nM rosiglitazone to inhibit transcription (**C**). Reverse transcription was performed on total RNA isolated from these cells and multiplex PCR was performed using LRP and 18s gene specific primers (RQ-RT-PCR). PCR products were visualized by EtBr staining on the ChemiDoc and band intensities were determined using Quantity One software. The intensity of the LRP product was divided by the intensity of the 18s product to obtain a value termed relative intensity. These results were normalized to the control and shown as fold increases. The bars in each graph represent the mean of triplicate experiments and the error bars represent the standard error of the mean. One tailed *p* values from paired *t* test are shown above bars to indicate significant differences.


A
Fold Increase
2
1
0
p=0.01
p=0.01
Control
0.1 mM
0.4 mM
0.8 mM
[oleic acid]
B
Fold Increase
2
1
0
p=0.01
p=0.001
Control
30 µM
160 µM
[arachidonic acid]
C
Fold Increase
2
1
0
p=0.03
p=0.002
Control
50 nM
500 nM
Control
500 nM
w/ 10 µg/ml α-amanitin
[rosiglitazone]

bovine serum, the increases in LRP mRNA levels upon stimulation with 160 μM arachidonic acid or 500 nM rosiglitazone were similar to those shown in figure 2.3 B & C (data not shown). These data support the reports demonstrating that the previously identified sterol response element in the 5' untranslated region of the LRP promoter is inactive [149,150].

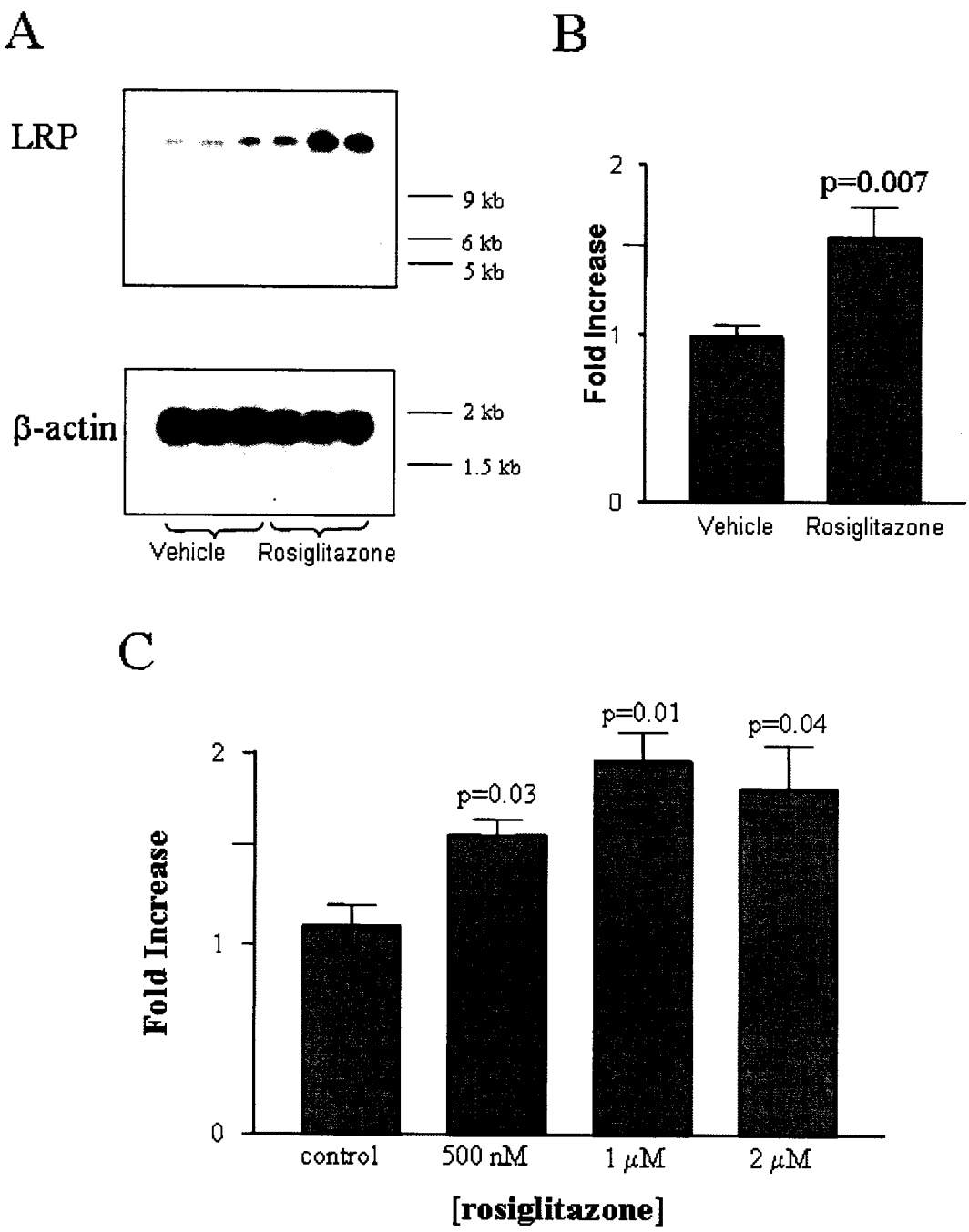
The effect of rosiglitazone on LRP mRNA levels was also determined in differentiated primary human adipocytes, as measured by Northern blot and real time PCR, and these results are summarized in **Figure 2.4**. According to the Northern blot analysis, LRP mRNA is increased by rosiglitazone treatment by approximately 1.6 fold. Using real-time RT-PCR, there was a dose dependent increase in LRP mRNA levels (ranging from 1.4 to 1.8 fold) after 24 h ligand treatment. The levels of LRP mRNA were also verified using relative-quantitative RT-PCR and the fold increases in LRP expression were similar to those shown in Figure 2.4 (data not shown).

2.4.3 – PPARγ Ligands Act at the Transcriptional Level to Increase LRP mRNA

The increase in LRP mRNA could be due to an mRNA stabilization effect or to increased transcription of the mRNA. To distinguish between these possibilities, a potent inhibitor of RNA polymerase II activity, α-amanitin, was used to inhibit new transcriptional activity. If the level of mRNA were increased by a stabilization effect of rosiglitazone or other PPARγ ligands, then the increase in mRNA levels would still be observed when both α-amanitin and rosiglitazone were added to cells concomitantly. We did not observe an increase in LRP mRNA in cells cultured with both rosiglitazone and α-amanitin (**Figure 2.3C**). Although there was a small decrease in LRP mRNA levels

Figure 2.4. Endogenous LRP mRNA levels in differentiated primary human adipocytes are modulated by rosiglitazone.

A. Northern blot analysis of total RNA from differentiated primary human adipocytes. Differentiated primary human adipocytes were cultured for 24 h in differentiating media without insulin in the presence or absence of 1 μM rosiglitazone. Total RNA was subsequently isolated and separated by agarose gel electrophoresis and transferred to BrightStar-Plus nylon membrane. The membrane was probed with either an LRP or β-actin random labelled probe. A representative blot is shown here (n=3). **B.** Quantification of Northern blots. Northern blots were scanned using the ChemiDoc and densitometry was performed using Quantity One software. Data shown are the average of three independent Northern blots from the RNA of three individual tissue samples (each done in triplicate) and the error bars represent the standard error of the mean. **C.** Quantification of mRNA changes using real-time RT-PCR. Differentiated primary human adipocytes were cultured for 24 h in differentiating media without insulin in the presence or absence of various concentrations of rosiglitazone. Reverse transcription was performed on total RNA isolated from these cells and independent real-time PCR reactions were performed using LRP and 18s gene specific primers on the LightCycler. These results were normalized to the control and shown as fold increases. The bars in each graph represent the mean of triplicate experiments and the error bars represent the standard error of the mean. The same RT reaction was subjected to RQ-RT-PCR and the results were similar to those shown here. Two-tailed p-values from student's *t* test are shown above bars to indicate significant differences. A p-value of 0.02 for all values is obtained using ANOVA.


A
LRP
9 kb
6 kb
5 kb
β-actin
2 kb
1.5 kb
Vehicle
Rosiglitazone
B
Fold Increase
2
1
0
p=0.007
Vehicle
Rosiglitazone
C
Fold Increase
2
1
0
p=0.03
p=0.01
p=0.04
control
500 nM
1 μM
2 μM
[rosiglitazone]

with the addition of α-amanitin, this decrease was identical in both the vehicle treated and rosiglitazone treated cells suggesting this is due to normal turnover of the mRNA. These results support a role for these ligands as transcriptional up-regulators of LRP gene expression.

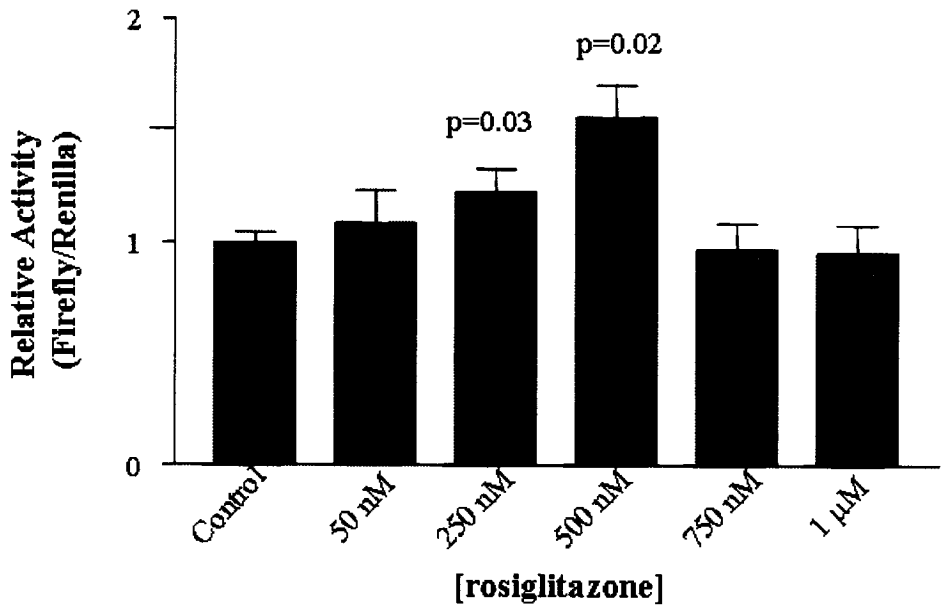
2.4.4 – LRP PPRE Luciferase Constructs are Responsive to Rosiglitazone in Dual Luciferase Assay

Although the basal transcriptional activity of the pGL3-PPRE and pGL3-PPRE3 constructs were approximately 2 fold higher than the pGL3-LRP construct due to the context of the SV40 ideal promoter (data not shown), the promoter context of the endogenous LRP promoter, which is present in pGL3-LRP, is the more physiologically relevant of the three constructs. This portion of the promoter drove the basal activity of the reporter gene as determined by the Dual Luciferase Reporter assay system (**Figure 2.5A**). The values were normalized to the cells treated with DMSO only (vehicle control). The empty pGL3-basic vector had no basal activity above the background measurements of the instrument (data not shown). For pGL3-LRP (**Figure 2.5A**), as well as pGL3-PPRE and pGL3-PPRE3 (**Figure 2.5B and C**), there was a dose dependent response of the luciferase activity upon treatment with rosiglitazone that corresponded to the increases in LRP mRNA shown in figure 2.3. The ratio of firefly to renilla luciferase activities are shown in the figures; it is important to note that the luciferase activity increases with rosiglitazone treatment and the renilla does not decrease.

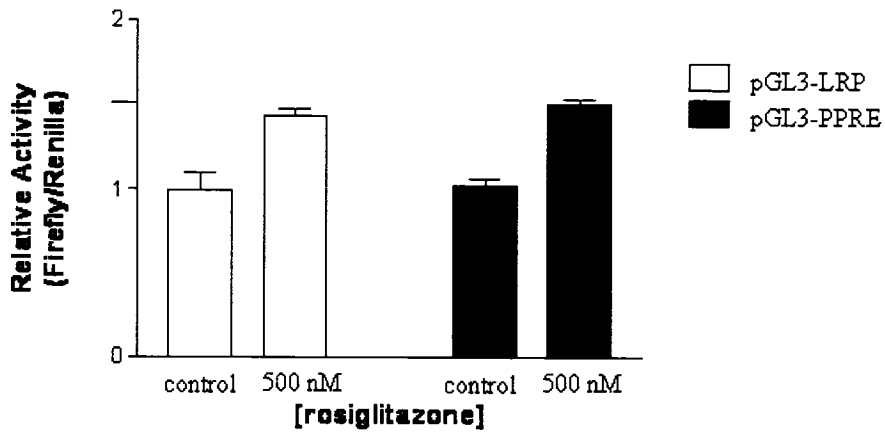
Figure 2.5. LRP promoter confers regulation of Luciferase activity by rosiglitazone.

A. The promoter region of human LRP (0 to –1200) was cloned into pGL3-Basic. This construct was then transiently co-transfected into SW872 cells along with the Renilla luciferase reporter vector pRL-CMV using the calcium-phosphate precipitation method. Luciferase activities for each of Firefly and Renilla luciferase were determined using the Dual Luciferase assay system. The intensity of Firefly luciferase is shown as a function of Renilla luciferase (relative activity). The relative intensity is normalized to the control (vehicle treatment) values. The means of triplicate experiments are graphed and the error bars represent the standard error of the mean. The one-tailed *p* values from paired *t*-tests are shown for significant differences. **B.** The sequence corresponding to the LRP PPRE and its immediate flanking sequences were cloned into the pGL3-promoter vector yielding pGL3-PPRE. This vector contains the SV40 promoter but no other enhancer elements such that we can test the LRP PPRE experimentally on its own. Transfections and assays were carried out as described above. Increases in luciferase activity were significant (2-tailed p-value <0.05). **C.** Three copies of the LRP PPRE and flanking sequences were cloned into the pGL3-promoter vector yielding pGL3-PPRE3. Transfections and assays were carried out as described above. Increases in luciferase activity were significant (2-tailed p-value <0.05).

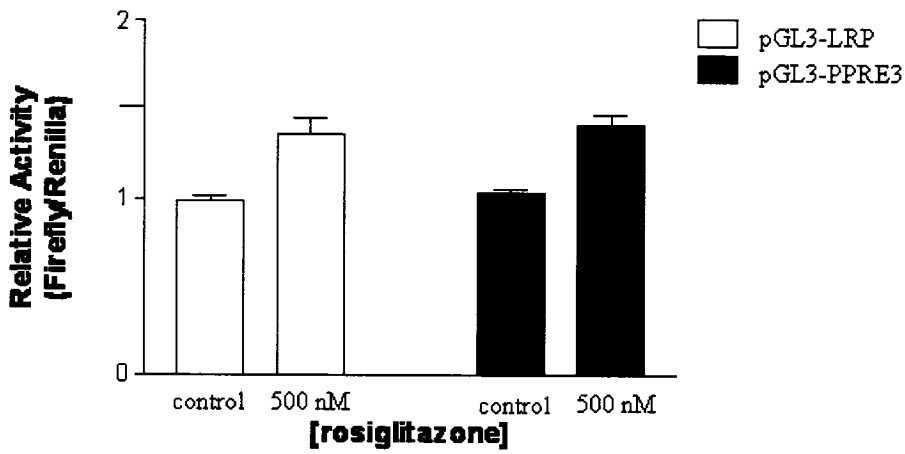
A


Relative Activity
(Firefly/Renilla)
2
1
0
p=0.02
p=0.03
Control
50 nM
250 nM
500 nM
750 nM
1 μM
[rosiglitazone]

B


Relative Activity
(Firefly/Renilla)
2
1
0
control
500 nM
control
500 nM
[rosiglitazone]
pGL3-LRP
pGL3-PPRE

C


Relative Activity
(Firefly/Renilla)
2
1
0
control
500 nM
control
500 nM
[rosiglitazone]
pGL3-LRP
pGL3-PPRE3

2.4.5 – Mutagenesis of the PPRE Results in the Loss of Enhancer Activity

A single half-site of the PPRE, to which PPARγ binds, was mutated using the Quikchange mutagenesis protocol (**Figure 2.6A**) and designated pGL3-LRP$_{\text{mutant PPRE}}$. This construct was sequenced and the point mutations within the PPRE were verified. The basal activity of this reporter construct (vehicle control) was similar to that of the pGL3-LRP construct (**Figure 2.6B**). In the presence of 500 nM rosiglitazone there was approximately a 1.5-fold increase in promoter activity of the pGL3-LRP construct. This increase, however, was lost in the pGL3-LRP$_{\text{mutant PPRE}}$ construct, indicating a role for this PPRE as a transcriptional enhancer.

2.4.6 – PPARγ:RXRα Heterodimers Selectively Bind the PPRE Identified in the LRP Promoter Region

The novel PPRE identified within the human LRP promoter is shown along with the previously characterized PPRE from the hACOX promoter which was used as a control for the assays below (**Figure 2.7A**). The TnT *in-vitro* transcription/translation of pSPORT1-PPARγ and pSG5-RXRα was first shown to express PPARγ and RXRα: when ^{35}S-methionine was added to the reaction mix, proteins of the correct molecular mass for RXRα, PPARγ and the luciferase control (48 kDa, 57 kDa and 62 kDa, respectively) were synthesized as verified by SDS-PAGE and autoradiography (**Figure 2.7B**).

For the EMSA, these constructs were subjected to the TnT reaction (without ^{35}S-methionine) and incubated with ^{32}P-end labeled oligomers corresponding to the PPRE of LRP. A clear shift was evident in the presence of these transcription factors (**Figure 2.7C,** lane 2) compared to when the TnT reaction was carried out with the empty

Figure 2.6. Site-directed mutagenesis of the PPRE abolishes the response to rosiglitazone.

A. pGL3-LRP was subjected to site-directed mutagenesis, in which the half-site of the DR-1 to which PPAR binds, was mutated so that 5 of 6 nucleotides no longer matched the consensus. PPREs of pGL3-LRP and pGL3-LRP$_{\text{mutant PPRE}}$ are shown with the consensus sequence for comparison. Point-mutations are underlined and mismatches present in endogenous PPRE are in italics. **B.** pGL3-LRP$_{\text{mutant PPRE}}$, was transiently co-transfected with pRL-CMV into SW872 cells and their respective luciferase activities were determined using the Dual Luciferase assay system. The relative intensity was calculated and the means of triplicate experiments are shown. The values of relative intensity are normalized to the control (vehicle treated) cells. The error bars represent the standard error of the mean. Rosiglitazone has a significant effect on pGL3-LRP, however, it is not significant for pGL3-LRP$_{\text{mutant PPRE}}$. The one-tailed p values from t-tests were 0.02 and 0.2 respectively.

A

TGAACTCAACGAT.....Mutant PPRE
TGAACTCTGACAT.....LRP PPRE
TGACCTnTGACCT.....consensus PPRE

B

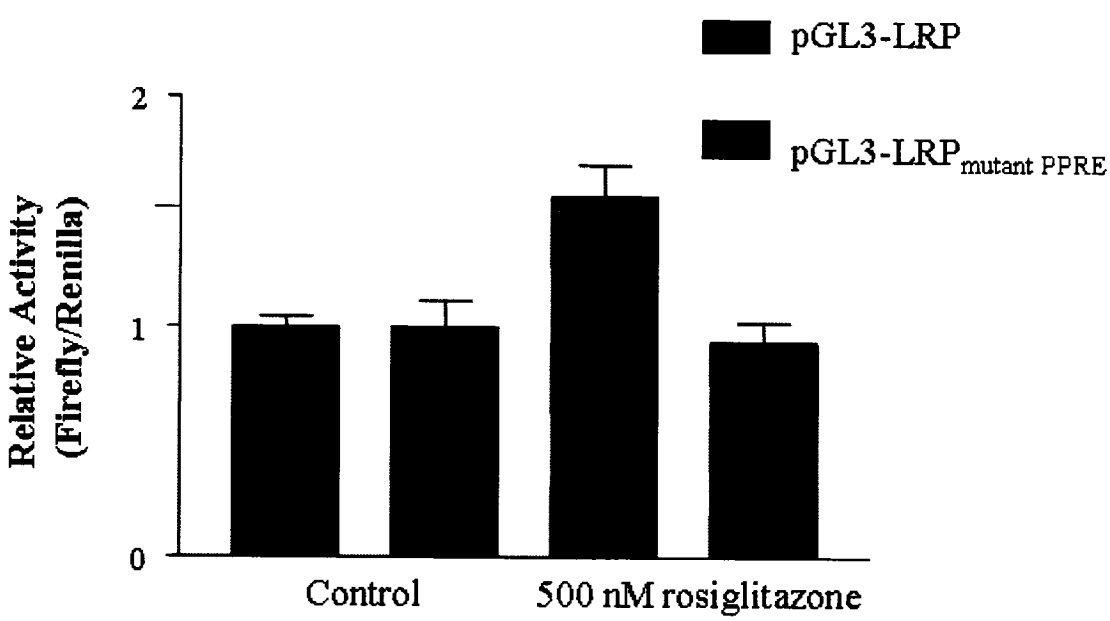
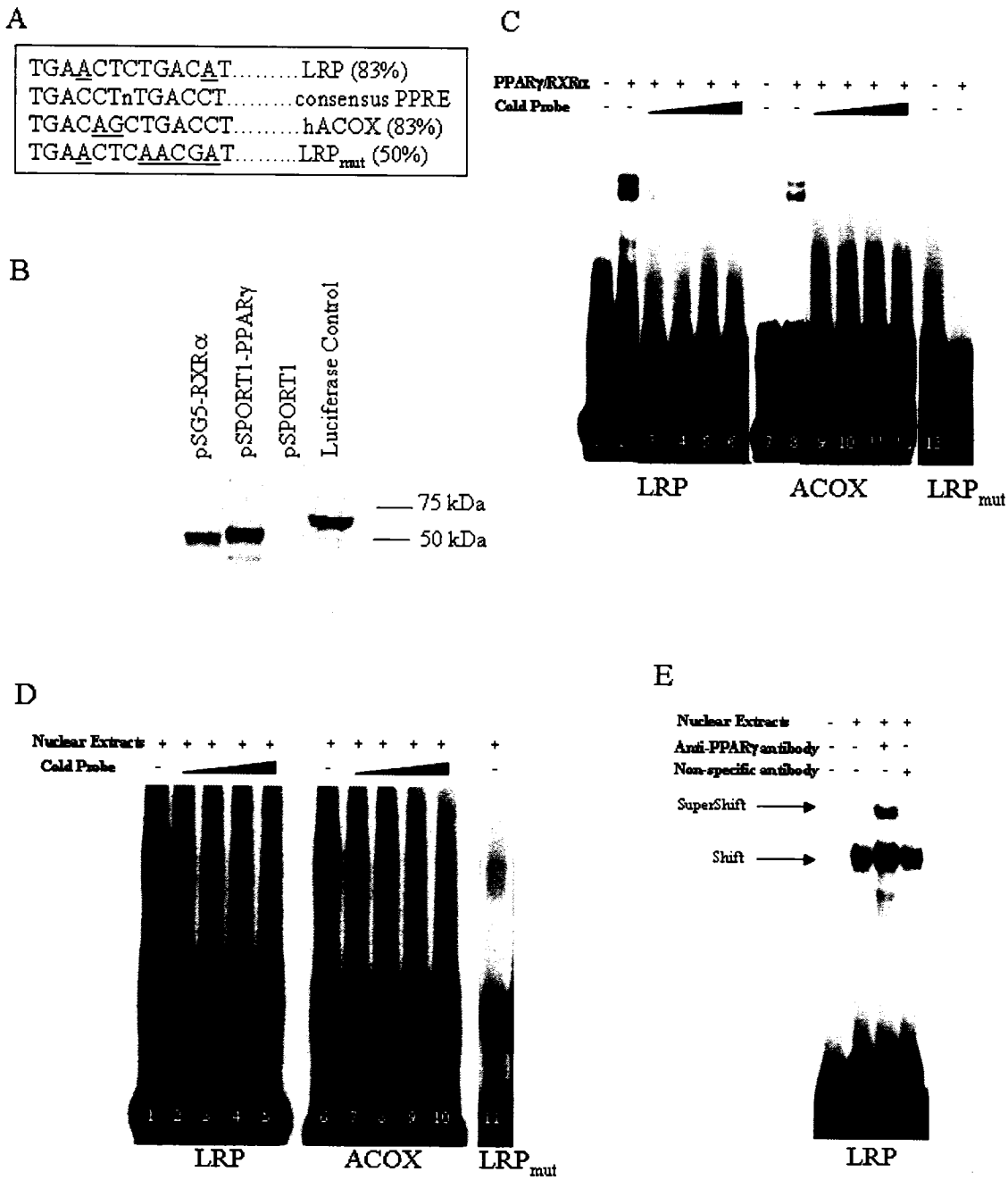


Figure 2.7. PPARγ:RXRα heterodimers selectively bind PPRE in the LRP promoter.

Electrophoretic mobility shift assays (EMSA) were performed on oligomers corresponding to the PPREs of LRP, hACOX and LRP_{mut} and a sequence comparison in shown in **A.** The mismatches for each PPRE are underlined. The mutant LRP oligomer is also shown with the mutated half-site underlined. **B.** Autoradiograph of TnT in-vitro transcribed translated PPARγ and RXRα. **C.** Oligomers were incubated on ice for 20 minutes with various components. Reticulocyte lysate generated with empty pSPORT1 vector was used as a negative control (lanes 1, 7 and 13). TnT *in-vitro* transcribed/translated PPARγ and RXRα were incubated with radiolabeled PPRE oligomers (lanes 2, 8 and 14). To control for specificity of binding, competition experiments were performed by adding increasing amounts of unlabeled oligomer to the binding reactions. LRP binding reactions were competed using cold hACOX oligomers at 5, 10, 25 and 50X excess (lanes 3-6) and hACOX binding reactions were competed using cold LRP oligomers at 5, 10, 25 and 50X excess (lanes 9-12). **D.** Nuclear extracts from primary human adipocytes were incubated with radiolabeled oligomer (lanes 1, 6 and 11) on ice for 20 minutes. Competition experiments were done as described in B (lanes 2-5 and 7-10). **E.** Gel super-shift analysis was used to determine if PPARγ was present in shift seen with nuclear extracts. Free probe (lane 1) was used as a control reaction. Nuclear extracts (18 μg) were incubated with labelled oligomer and run in lane 2. NuShift anti-PPARγ antibody (4 μl) was incubated with 18 μg of nuclear extracts from SW872 cells prior to being added to the EMSA reaction mix containing labelled oligomers (lane 3). A non-specific antibody (mouse monoclonal anti-β-actin) was used as a negative control for the supershift (lane 4). Arrows indicate the shift and the super-shift.


A
TGAACTCTGACAT.....LRP (83%)
TGACCTnTGACCT.....consensus PPRE
TGACAGCTGACCT.....hACOX (83%)
TGAACTCAACGAT.....LRPmut (50%)
B
pSG5-RXRα
pSPORT1-PPARγ
pSPORT1
Luciferase Control
75 kDa
50 kDa
C
PPARγ/RXRα
Cold Probe
LRP
ACOX
LRPmut
D
Nuclear Extracts
Cold Probe
LRP
ACOX
LRPmut
E
Nuclear Extracts
Anti-PPARγ antibody
Non-specific antibody
SuperShift
Shift
LRP

pSPORT1 vector (negative control) (**Figure 2.7C**, lane 1). The interaction between the protein complex and the DNA could be competed by the addition of increasing amounts of excess of unlabeled hACOX oligomer (**Figure 2.7C**, lanes 3-6). Identical experiments using the hACOX probe yielded very similar results (**Figure 2.7C**, lanes 7-12).

Incubation of the probes with nuclear protein extracts from primary adipocytes gave a shift of the same size as the TnT proteins (**Figure 2.7D**, lane 2). This shift could also be inhibited by increasing amounts of excess unlabeled hACOX oligomer (**Figure 2.7D**, lane 2-5). Nuclear extracts from the SW872 cell line yielded similar results. These results are comparable to those obtained using oligomers corresponding to the hACOX PPRE (**Figure 2.7D**, lanes 6-10). A probe containing a mutated PPRE half-site, LRP_{mut}, could no longer interact and bind TnT proteins (**Figure 7C**, lane 14) or nuclear extracts (**Figure 2.7D**, lane 11).

The shift caused by the nuclear extracts was further analyzed by gel super-shift analysis to confirm that PPARγ was a component of this complex (**Figure 2.7E**). Free probe was run in lane 1 as a control reaction, while a reaction containing nuclear protein extracts from primary human adipocytes is shown in lane 2. The nuclear protein extracts were pre-incubated with anti-PPARγ antibody prior to being added to the reaction containing labelled oligomers and this was run in lane 3. A band of the same size as that seen in lane 2 was present as well as a larger band that was absent in all other lanes (super-shift). The same results were obtained for the oligomer corresponding to the hACOX PPRE (data not shown). A supershift was not observed when a non-specific antibody was used in place of the anti-PPARγ antibody. Similar results were also

obtained using nuclear extracts from SW872 cells supporting the involvement of PPARγ in the protein/DNA complex (data not shown).

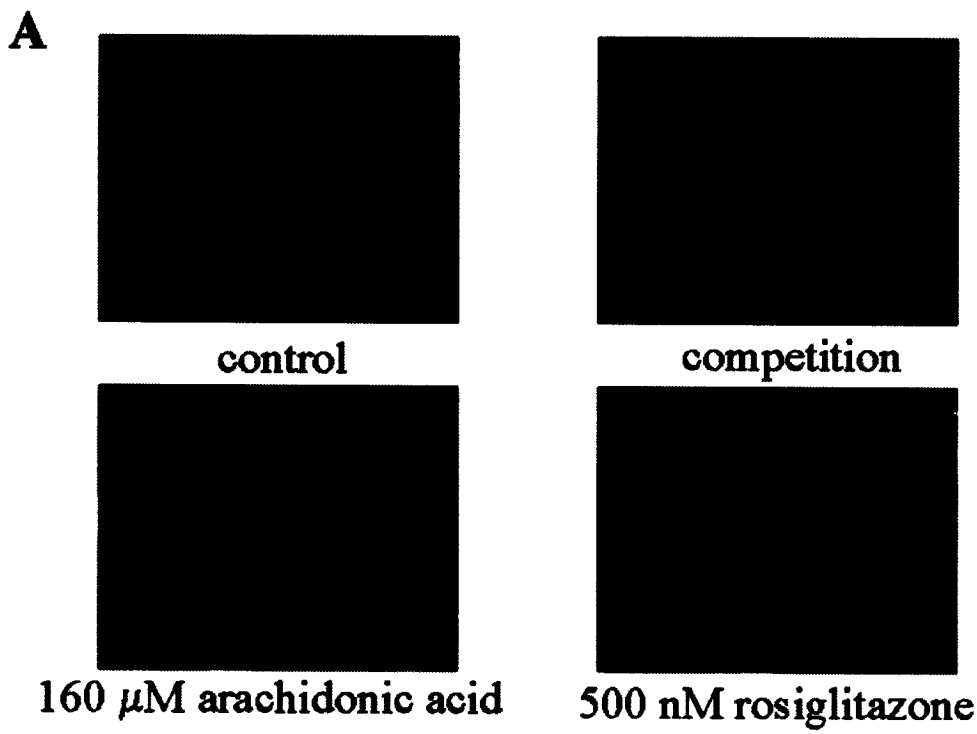
2.4.7 – Functional Cell Surface LRP is Increased Upon Exposure to PPARγ Ligands

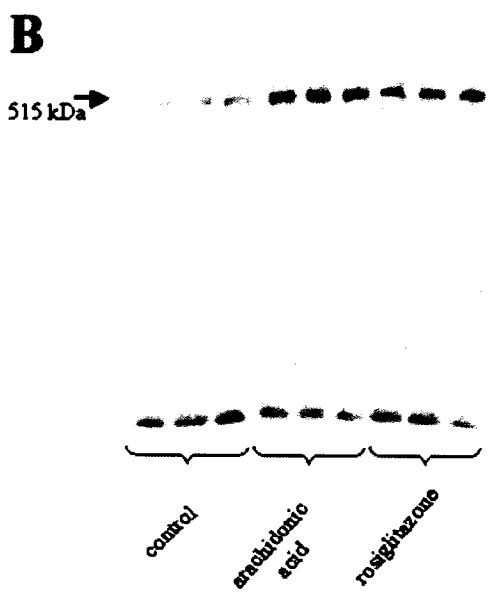
Cell surface LRP was increased in SW872 cells treated with 160 μM arachidonic acid or 500 nM rosiglitazone as determined by cell surface labelling experiments using fluorescently labelled α2M* (**Figure 2.8A**). In addition, total cellular LRP, which is represented by the 515 kDa band corresponding to the α-subunit, was increased in those cells as determined by Western blot analysis (**Figure 2.8B**). The increases seen in LRP using these methods were approximately 1.5 to 2 fold, supporting the increases seen in binding and degradation data below.

^{125}I-α2M* degradation assays were performed in the presence or absence of PPARγ ligands for the differentiated primary human adipocytes and SW872 cells to test LRP function. In primary adipocytes, there was a direct relationship between the amount of rosiglitazone added and the amount of α2M* degradation over 8 hours (**Figure 2.9A**) with very significant increases ranging from 1.2 to 1.7 fold over control ($P<0.009$). The receptor associated protein (RAP) is an antagonist of all identified LRP ligands including α2M* therefore purified RAP was used to block LRP function in these assays. When cells were treated with RAP, the amount of ^{125}I-α2M* degraded was diminished to background levels demonstrating that this process is LRP specific. Degradation assays were also performed in the SW872 cells treated with rosiglitazone (**Figure 2.9B**) or arachidonic acid (**Figure 2.9C**). There was a 1.5 fold increase in the amount of

Figure 2.8. LRP protein levels are increased in SW872 cells upon exposure to PPARγ ligands.

A. Cells were incubated with 1 μg/ml fluorescently labeled activated α2M (α2M*) (Cy3 Monofunctional reactive dye) on ice for 45 min in 3:1 DMEM/F12 supplemented with 2 mg/ml BSA buffered with 10 mM HEPES after an equilibration period of 1 h. The cells were washed with ice cold PBS 3 times prior to being fixed with 4% paraformaldehyde for 10 min at 0 °C. Cells were rinsed with PBS and kept in 2 ml of PBS at room temperature for fluorescence microscopy. Photographs are representative of cells treated with the ligands indicated. A 30 fold excess of unlabeled activated α2M (α2M*) was used to compete for binding with the fluorescently labelled α2M*. Photographs are normalized so that an increase in intensity on the cell surface and the cell circumference between photographs represents an increase in the fluorescence (i.e. total binding). **B.** Western blot of cells treated with DMSO (vehicle control), 160 μM arachidonic acid, or 500 nM rosiglitazone for 24 h. 5 μg of total protein was loaded in each lane. LRP was detected with a polyclonal anti-LRP antisera (Dr. G. Bu). The blot was developed using chemiluminescent techniques and bands were visualized using the ChemiDoc apparatus. The band at 515 kDa corresponds to the α-subunit of LRP. **C.** Quantification of Western blot. The 515 kDa band corresponding to LRP was quantified using Quantity One software. All samples are normalized to the vehicle treated cells (control). Results are shown as the mean of triplicate experiments and the error bars represent the standard error of the mean. (*) The two-tailed p value is 0.002; (**) The two-tailed p value is 0.003.


A
control
competition
160 μM arachidonic acid
500 nM rosiglitazone


B
515 kDa
control
arachidonic acid
rosiglitazone

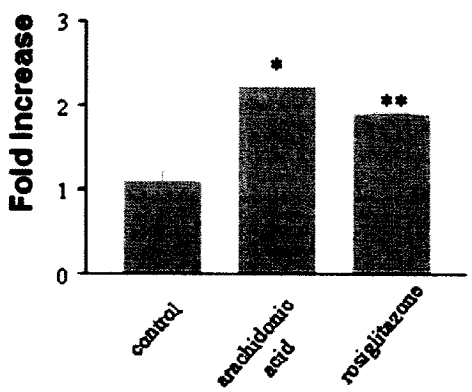
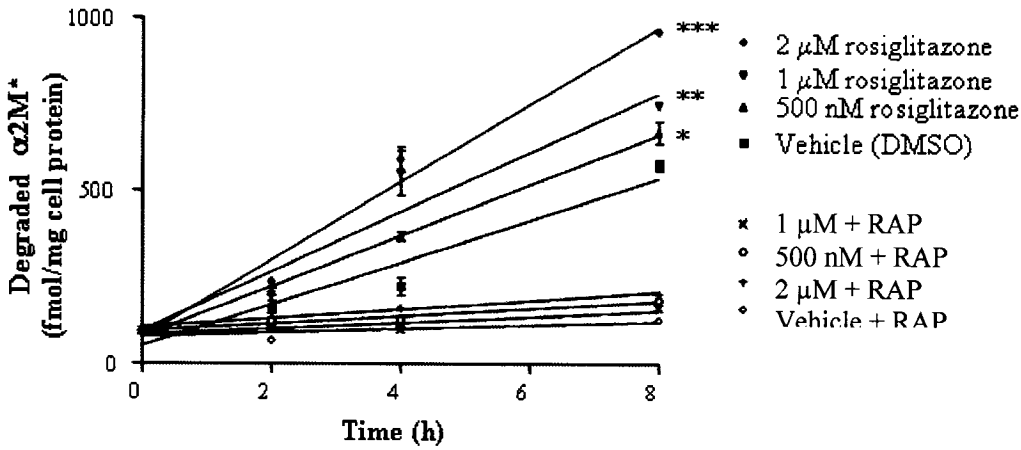

C
Fold Increase
3
2
1
0
*
**
control
arachidonic acid
rosiglitazone

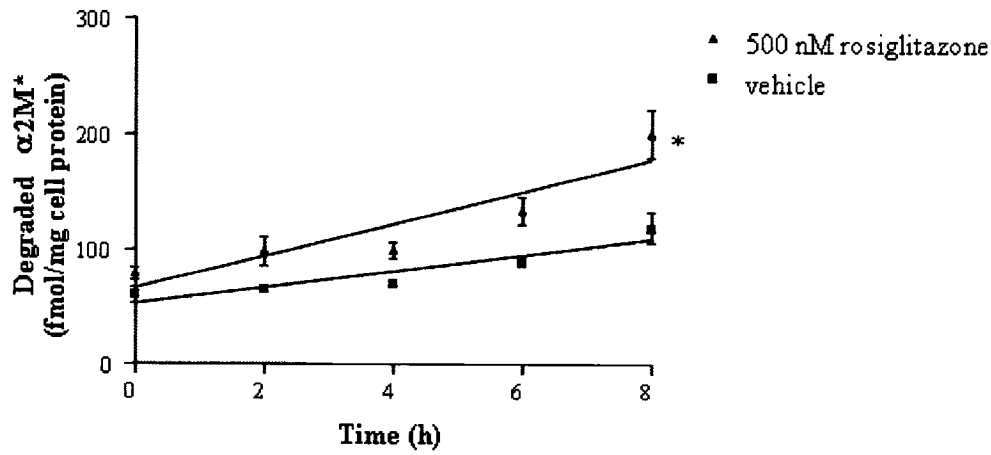
Figure 2.9. Increased cellular degradation of ^{125}I-α2M* by cells treated with PPARγ ligands.

A. Differentiated primary human adipocytes were treated with various concentrations of rosiglitazone in the absence of insulin for 24 h prior to the experiment. The cells were then incubated at 37°C with HBSS, 25 mM Hepes pH 7.45, 10 mg/ml BSA containing 1 μg/ml ^{125}I-α2M* with or without RAP (30 μg/ml). At the beginning of the incubation period and at each time point, the medium was precipitated with ice-cold tricarboxylic acid, and the tricarboxylic acid-soluble material was used as a measure of the degraded protein. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. (*) p=0.009; (**) p=0.007; (***) p=0.002 **B,C.** SW872 cells (vehicle, 500 nM rosiglitazone and 160 μM arachidonic acid treated) were washed at 37 °C to remove any residual fetal calf serum. The cells were incubated at 37 °C for the indicated times in HBSS, 25 mM Hepes pH 7.45, 10 mg/ml BSA containing 1 μg/ml ^{125}I-α2M*. At the beginning of the incubation period and at each time point, the medium was precipitated with ice-cold tricarboxylic acid, and the tricarboxylic acid-soluble material was used as a measure of the degraded protein. TCA soluble material from untreated cells (vehicle) was compared to the TCA soluble material from cells treated with (B) 500 nM rosiglitazone or (C) 160 μM arachidonic acid. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. (*) p=0.006; (**) p=0.03

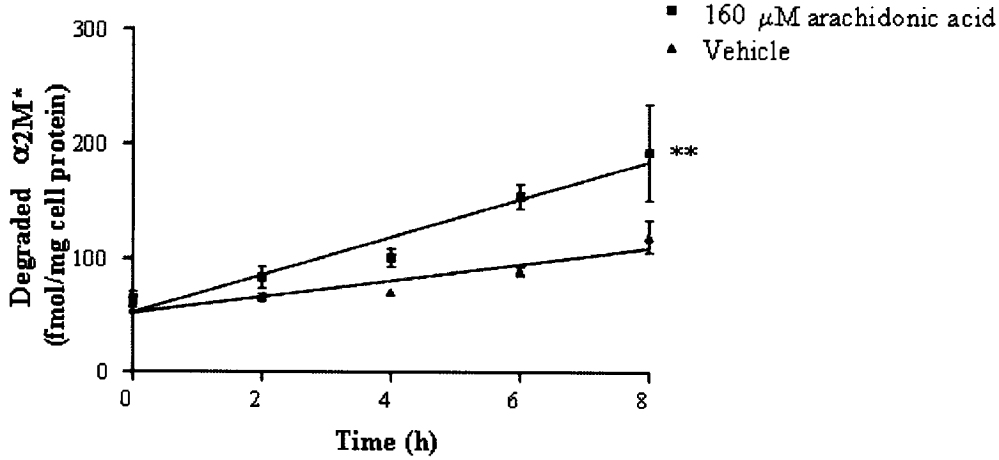
A


1000
500
0
0
2
4
6
8
Degraded α2M* (fmol/mg cell protein)
Time (h)
***
**
*
2 μM rosiglitazone
1 μM rosiglitazone
500 nM rosiglitazone
Vehicle (DMSO)
1 μM + RAP
500 nM + RAP
2 μM + RAP
Vehicle + RAP

B


300
200
100
0
0
2
4
6
8
Degraded α2M* (fmol/mg cell protein)
Time (h)
*
500 nM rosiglitazone
vehicle

C


300
200
100
0
0
2
4
6
8
Degraded α2M* (fmol/mg cell protein)
Time (h)
**
160 μM arachidonic acid
Vehicle

^{125}I-α2M* degraded over 8 hours in the treated cells versus the control cells for both of the PPARγ ligands.

The effects of PPARγ ligands on differentiated primary human adipocytes were further examined by ^{125}I-α2M* binding assays (**Figure 2.10A**). The B_{max} for cells incubated with 1 μM rosiglitazone (27.0 fmol/mg cell protein) is approximately 1.5 times greater than the vehicle treated cells (17.3 fmol/mg cell protein), indicating that there is an increase in the levels of functional cell surface LRP. The difference in binding was found to be highly significant with a two-tailed P value of less than 0.0001. When the cells were treated with RAP (30 μg/ml), the amount of binding was reduced to background levels demonstrating that this process is LRP specific. There was no statistically significant difference (2-tailed p value = 0.1292) in the K_d between the treated and control cells (0.1464 ± 0.03781 (SE) and 0.3243 ± 0.08513 (SE), respectively) as illustrated in the Scatchard plot (**Figure 2.10B**), indicating that the binding affinities have not changed.

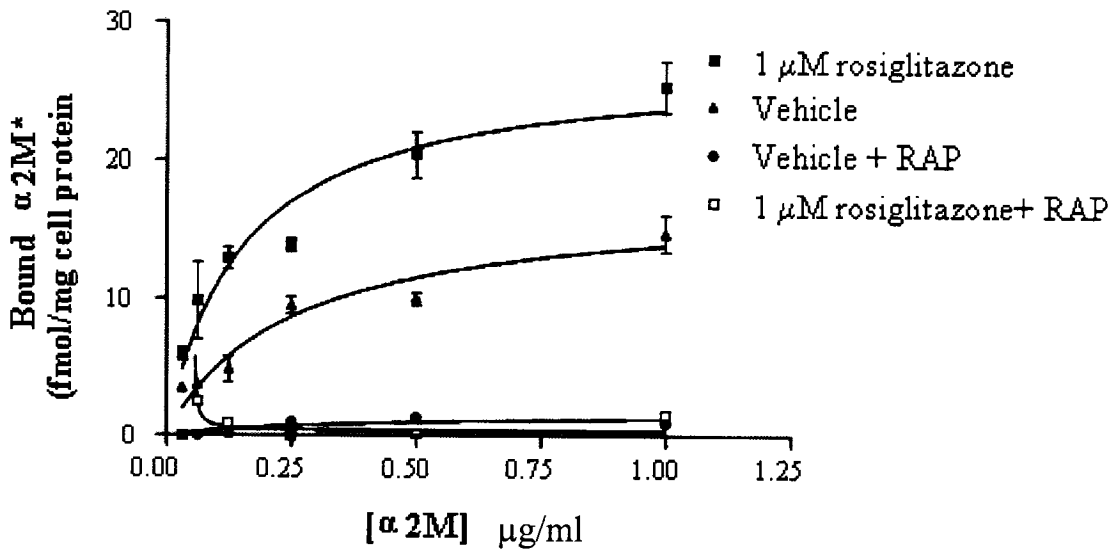
2.4.8 – ^{125}I-α2M Binding at 0°C is Increased in Primary Human Hepatocytes Treated with the PPARα Agonist Fenofibric Acid*

Primary human hepatocytes isolated from the victim of a motor vehicle accident, who was otherwise healthy, were purchased commercially. ^{125}I-α2M* binding at 0°C was measured following treatment with 250 μM fenofibric acid and is shown in **Figure 11A**. There was approximately a 50% increase in binding compared to the vehicle control and the Scatchard plot (**Figure 2.11B**) confirms that this effect is in the number of receptors on the cell surface and not an increase in binding affinity, similar to the observations made previously in the adipocyte experiments.

Figure 2.10. Increased cell surface binding of ^{125}I-α2M* by cells treated with a PPARγ ligand.

A. ^{125}I-α2M* binding curve for differentiated primary human adipocytes. Differentiated primary human adipocytes were pre-incubated for 24 h in differentiating media without insulin in either 1 μM rosiglitazone or vehicle alone (DMSO). Cells were then washed twice with HBSS, 25 mM Hepes pH 7.45, at 37 °C for 20 minutes each, placed on ice for 20 minutes to allow the cells to equilibrate and then ice cold HBSS, 25 mM Hepes pH 7.45, 10 mg/ml BSA containing various concentrations of ^{125}I-α2M (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 μg/ml) was added to the cells. Cells were also incubated in the presence or absence of RAP (30 μg/ml), a LRP ligand that acts as an antagonist to the binding of all other LRP ligands. Cells were further incubated on ice for 2 h and then washed six times with ice cold HBSS, 25 mM Hepes pH 7.45. Cells were then solubilized in 0.2 M NaOH and then cell associated radioactivity counted. **B.** Scatchard analysis of ^{125}I-α2M* binding curves. Scatchard analysis was performed on the data plotted in A to obtain information regarding the binding kinetics of ^{125}I-α2M on differentiated primary human adipocytes in the presence of vehicle alone or 1 μM rosiglitazone. This analysis is presented as a Scatchard plot with bound/free plotted against the bound ^{125}I-α2M*. The K_d is a measure of the binding affinity between the ligand and the receptor and is determined from the slope. The B_{max} is a measure of the number of receptors on the cell surface and is represented by the X-intercept. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. (*) The two-tailed p value is < 0.0001.

A


30
20
10
0
0.00
0.25
0.50
0.75
1.00
1.25
Bound α2M*
(fmol/mg cell protein
[α2M] μg/ml
1 μM rosiglitazone
Vehicle
Vehicle + RAP
1 μM rosiglitazone+ RAP

B

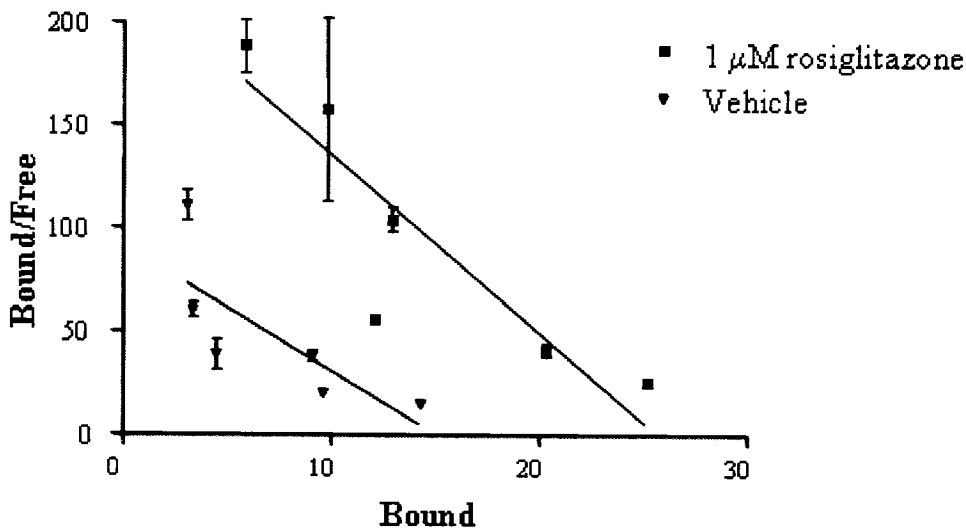
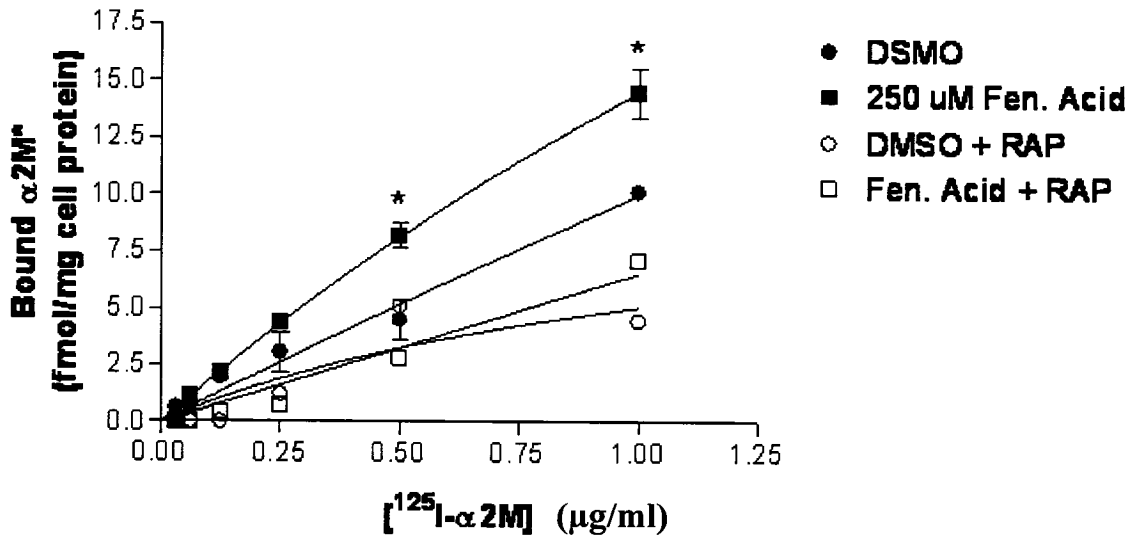

200
150
100
50
0
0
10
20
30
Bound/Free
Bound
1 μM rosiglitazone
Vehicle

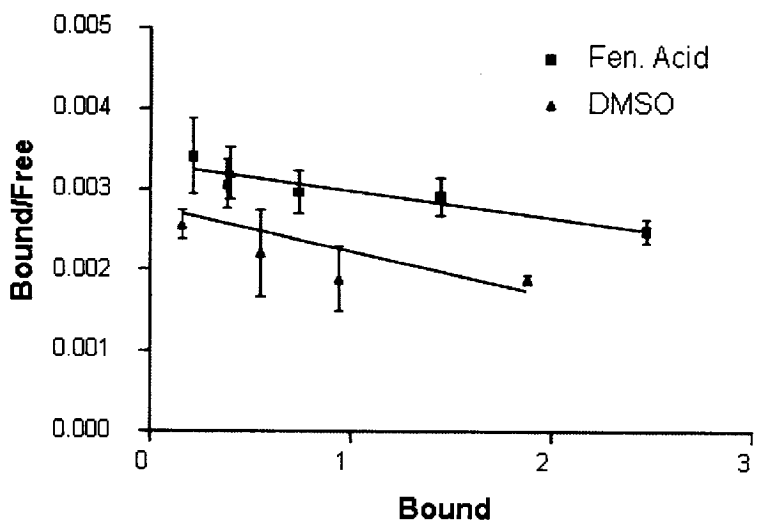
Figure 2.11. ^{125}I-α2M binding is increased by PPARα ligand fenofibric acid in cultured primary human hepatocytes.

A. ^{125}I-α2M* binding curve for cultured primary human hepatocytes. Cultured primary human hepatocytes were purchased commercially and pre-incubated for 24 h in media containing either 250 μM fenofibric acid or vehicle alone (DMSO). Cells were then washed twice with HBSS, 25 mM Hepes pH 7.45, at 37 °C for 20 minutes each, placed on ice for 20 minutes to allow the cells to equilibrate and then ice cold HBSS, 25 mM Hepes pH 7.45, 10 mg/ml BSA containing various concentrations of ^{125}I-α2M (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 μg/ml) was added to the cells. Cells were also incubated in the presence or absence of RAP (30 μg/ml), a LRP ligand that acts as an antagonist to the binding of all other LRP ligands. Cells were further incubated on ice for 2 h and then washed six times with ice cold HBSS, 25 mM Hepes pH 7.45. Cells were then solubilized in 0.2 M NaOH and then cell associated radioactivity counted. Data points represent means from triplicate samples and the error bars represent the standard error of the mean. * 2 tailed p-value<0.02 for unpaired t-test between fenofibric acid and DMSO. **B.** Scatchard analysis of ^{125}I-α2M* binding curves. Scatchard analysis was performed on the data plotted in A to obtain information regarding the binding kinetics of ^{125}I-α2M on cultured primary human hepatocytes in the presence of vehicle alone or 250 μM fenofibric acid. This analysis is presented as a Scatchard plot with bound/free plotted against the bound ^{125}I-α2M*. The K_d is a measure of the binding affinity between the ligand and the receptor and is determined from the slope. The B_{max} is a measure of the number of receptors on the cell surface and is represented by the X-intercept. Data points represent means from triplicate samples and the error bars represent the standard error of the mean. (*) The two-tailed p value is < 0.05.

A


Bound α2M*
(fmol/mg cell protein)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.00
0.25
0.50
0.75
1.00
1.25
[125I-α2M] (μg/ml)
DSMO
250 uM Fen. Acid
DMSO + RAP
Fen. Acid + RAP

B


Bound/Free
0.005
0.004
0.003
0.002
0.001
0.000
0
1
2
3
Bound
Fen. Acid
DMSO

2.4.9 – Mouse LRP mRNA Levels are not Increased Upon Stimulation by PPAR Agonists

In preliminary studies designed to test the efficacy of LRP upregulation in mouse, differentiated 3T3-L1 cells, a murine-derived adipocyte cell line, were treated with rosiglitazone for 24 hours and the levels of LRP mRNA were measured using real-time RT-PCR. Surprisingly, there was no change in LRP mRNA levels compared to the vehicle control cells however, the mRNA levels of the fatty acid transport protein (FATP), a known target of PPARγ and PPARα[220], was increased nearly 2 fold (**Figure 2.12A**). LRP mRNA was also measured in isolated primary mouse hepatocytes that were treated with the PPARα agonist fenofibric acid (**Figure 2.12B**). At concentrations of fenofibric acid known to induce the expression of PPARα target genes [221], there was no increase in LRP mRNA levels but there was again a 2 fold increase in the FATP mRNA levels. These observations do not indicate a problem with the chosen ligands since the control gene (FATP1), known to contain a functional PPRE specifically in mouse, responded appropriately.

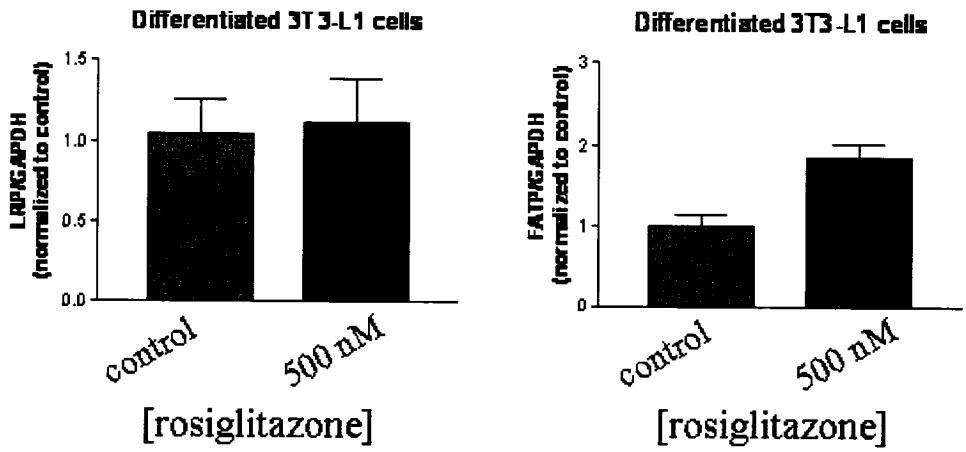
2.4.10 – LRP Promoter PPRE not Conserved Between Human and Mouse

Human LRP promoter sequence, accession Y18524, was aligned against the mouse chromosome 10 sequence, accession NT 039502.1, using Vector NTI software. The human LRP mRNA sequence, accession NM 002332, was also aligned against the mouse LRP mRNA sequence, accession NM 008512. The human LRP protein sequence, accession NP 002323, was aligned against the mouse LRP protein sequence, accession NP 035538 using blastp. Although the protein sequence, nucleotide coding sequence and

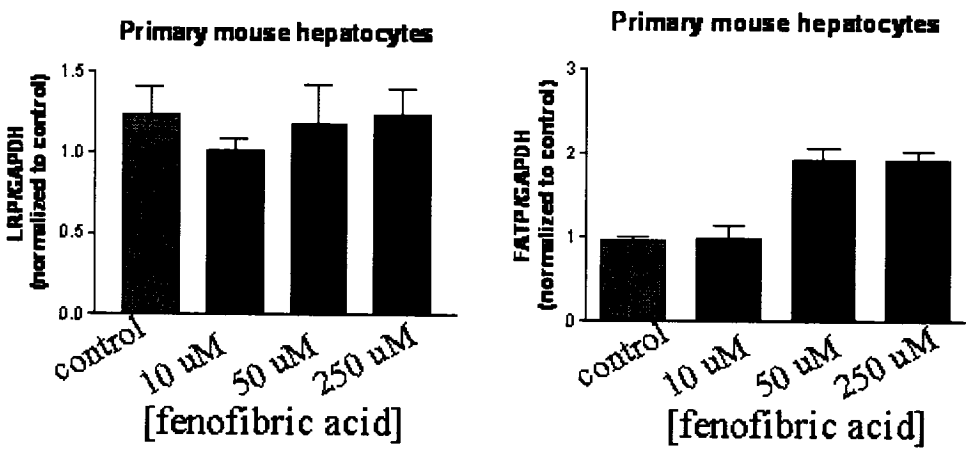
Figure 2.12. Endogenous LRP mRNA levels in murine derived cell lines are not affected by PPAR agonists.

A. Differentiated 3T3-L1 adipocytes, a mouse derived cell line, were treated with vehicle control (DMSO) or the PPARγ agonist rosiglitazone for 24 hours prior to RNA isolation and 2-step RT-PCR for LRP and FATP mRNA using the LightCycler. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. Increase in FATP mRNA was significant (2-tailed p-value=0.0138 in unpaired t-test). **B.** Primary mouse hepatocytes were isolated from C57Bl6J retired breeders and treated with various concentrations of the PPARα agonist fenofibric acid for 24 hours prior to RNA isolation and 2-step RT-PCR using the LightCycler. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. Increases in FATP mRNA for fenofibrate concentrations of 50 μM and 250 μM were significant (2-tailed p-values=0.0041 and 0.0015 for unpaired t-test, respectively).

A


Differentiated 3T3-L1 cells
LRPAGAPDH (normalized to control)
1.5
1.0
0.5
0.0
control
500 nM
[rosiglitazone]
Differentiated 3T3-L1 cells
FATPAGAPDH (normalized to control)
3
2
1
0
control
500 nM
[rosiglitazone]

B


Primary mouse hepatocytes
LRPAGAPDH (normalized to control)
1.5
1.0
0.5
0.0
control
10 uM
50 uM
250 uM
[fenofibric acid]
Primary mouse hepatocytes
FATPAGAPDH (normalized to control)
3
2
1
0
control
10 uM
50 uM
250 uM
[fenofibric acid]

5'-untranslated regions of the mRNA are highly conserved at 93%, 87% and 85% respectively, the promoters share much less homology at 75%. Additionally, the mouse contains 2 mismatches per half-site of the DR-1 consensus sequence compared to the single mismatch per half-site in the human (66% versus 83% homology to consensus DR-1 PPRE) (**Figure 2.13A**). Furthermore, when the PPRE and flanking sequences are examined together, there is only 51.5% homology between the human and mouse sequences suggesting that the two promoters might differ in their response to PPAR agonists.

2.4.11 – Nuclear Extracts Containing PPARγ do not Bind to Mouse PPRE Sequence in vitro

Electrophoretic mobility shift assays (EMSA) were performed using ^{32}P-labelled double-stranded oligomers corresponding to the human and mouse LRP PPRE and immediate flanking sequences. **Figure 2.13B** shows that the human oligomer binds nuclear extracts from THP-1 cells that are known to contain PPARγ and the complex is confirmed to contain PPARγ by super-shift. The mouse oligomer does not bind any proteins found within the nuclear extracts. Additionally, the cold mouse oligomer cannot compete for binding against the human sequence.

2.4.12 – LRP Function is not Stimulated by PPAR Agonists in Murine Derived Cells

To examine LRP function, activated α2M binding at 0°C was measured using differentiated 3T3-L1 mouse adipocytes (**Figure 2.14A**) and isolated primary mouse hepatocytes (**Figure 2.14B**). ^{125}I-α2M* degradation at 37°C was also measured in primary mouse hepatocytes (**Figure 2.14C**). Stimulation of mouse adipocytes with

Figure 2.13. Comparison of mouse and human LRP promoter sequences and evaluation of PPARγ binding by EMSA.

A. Human LRP promoter sequence (Y18524) was aligned against mouse chromosome 10 sequence (NT_039502.1). The PPRE sequences, including flanking regions, are shown and homologous sequences are indicated in yellow. The region of the PPRE is in bold and consensus matching nucleotides are underlined. The consensus PPRE sequence is given below. **B.** Double stranded oligomers corresponding to the sequences shown in A were radioactively end-labeled and subjected to EMSA using nuclear extracts from THP-1 cells provided in the Nushift PPARγ supershift kit. Conditions are as indicated below the figure.

A


PPRE
Homology to consensus
Human GTCGC-TGCCCCGCTC-CTTGAACTCTGACATGCAGACACCTAGAAAGTCA 83%
Mouse GTCGCCTGCTGAGCTTTCCAGAACTTTCACTTCAGGTCACAGAGAAAGTCA 66%
consensus TGACCTnTGACCT

B

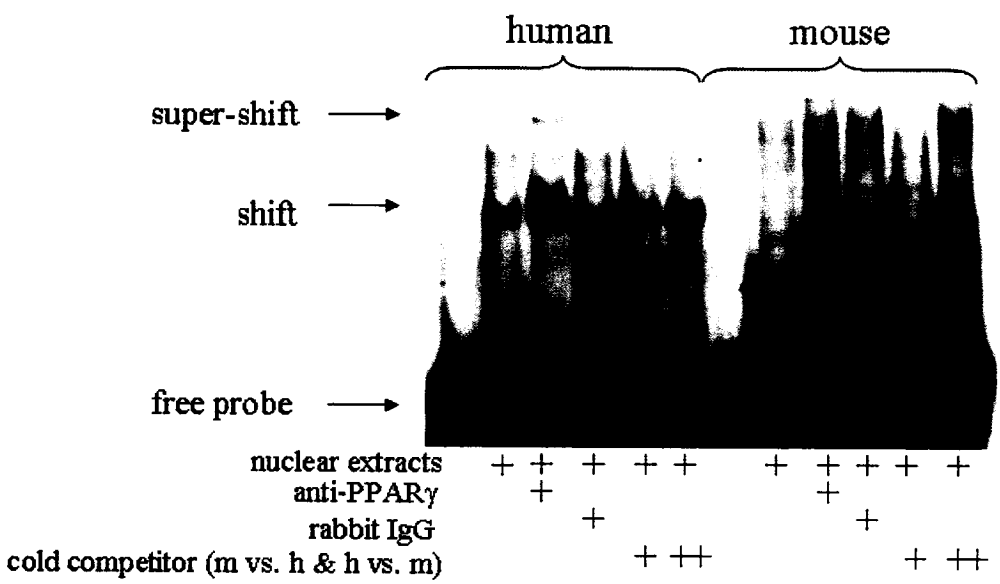
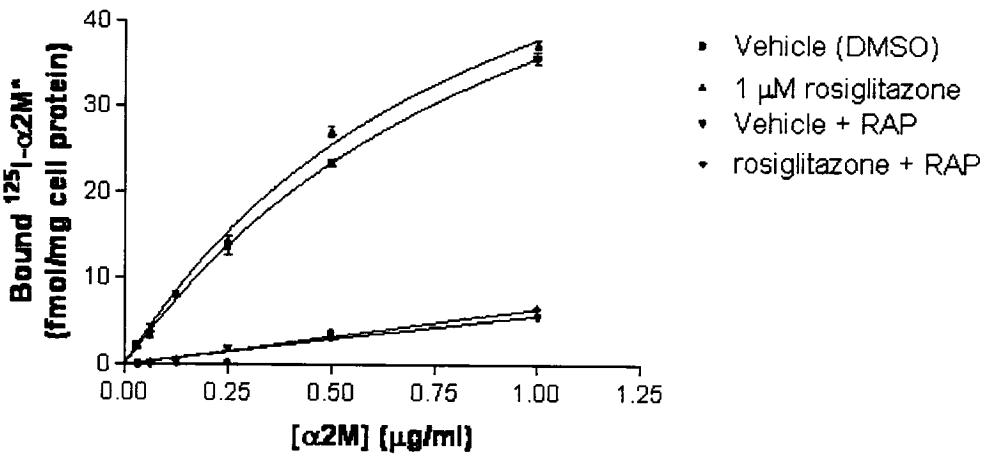

human
mouse
super-shift
shift
free probe
nuclear extracts + + + + + + + + + +
anti-PPARγ + +
rabbit IgG + +
cold competitor (m vs. h & h vs. m) + ++ + ++

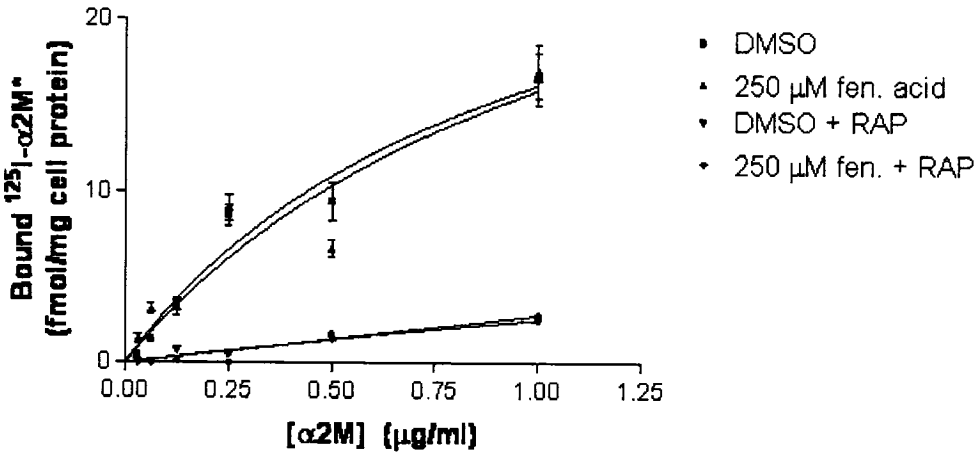
Figure 2.14. ^{125}I-α2M* binding and degradation are not affected by PPAR agonists in murine derived cells.

A. ^{125}I-α2M* binding curve at 0°C for differentiated 3T3-L1 mouse adipocytes. Differentiated 3T3-L1 mouse adipocytes were pre-incubated for 24 h in DMEM/10% fetal bovine serum without insulin in either 1 μM rosiglitazone or vehicle alone (DMSO). Cells were then washed twice with HBSS, 25 mM Hepes pH 7.45, at 37 °C for 20 minutes each, placed on ice for 20 minutes to allow the cells to equilibrate and then ice cold HBSS, 25 mM Hepes pH 7.45, 10 mg/ml BSA containing various concentrations of ^{125}I-α2M (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 μg/ml) was added to the cells. Cells were also incubated in the presence or absence of RAP (30 μg/ml), a LRP ligand that acts as an antagonist to the binding of all other LRP ligands. Cells were further incubated on ice for 2 h and then washed six times with ice cold HBSS, 25 mM Hepes pH 7.45. Cells were then solubilized in 0.2 M NaOH and then cell associated radioactivity counted. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. **B.** ^{125}I-α2M* binding curve at 0°C for isolated primary mouse hepatocytes. Primary mouse hepatocytes were pre-incubated for 24 h in DMEM containing various concentrations of fenofibric acid or vehicle alone (DMSO). RAP was used to block all LRP binding as a control. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean. **C.** ^{125}I-α2M* degradation at 37°C for isolated primary mouse hepatocytes. Primary mouse hepatocytes were pre-incubated for 24 h in DMEM containing various concentrations of fenofibric acid or vehicle alone (DMSO). RAP was used to block all LRP binding as a control. Data points represent means from triplicate experiments and the error bars represent the standard error of the mean.

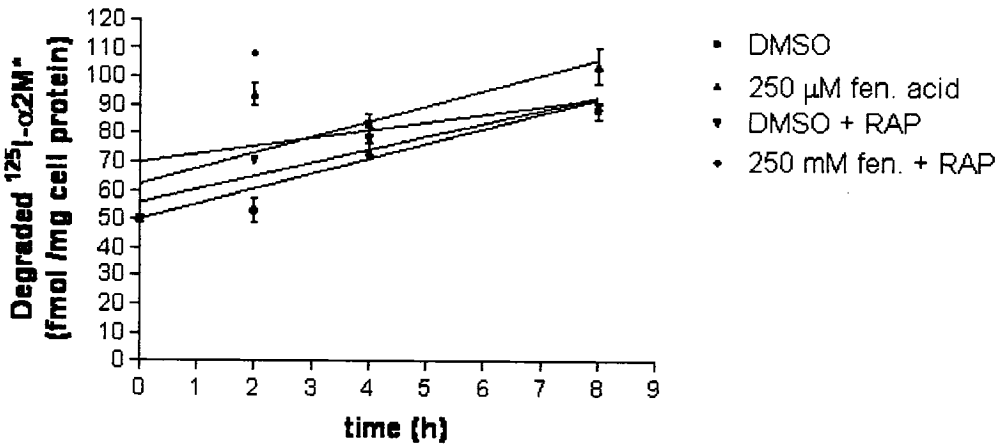
A


Bound ^{125}I-α2M* (fmol/mg cell protein)
40
30
20
10
0
0.00
0.25
0.50
0.75
1.00
1.25
[α2M] (μg/ml)
Vehicle (DMSO)
1 μM rosiglitazone
Vehicle + RAP
rosiglitazone + RAP

B


Bound ^{125}I-α2M* (fmol/mg cell protein)
20
10
0
0.00
0.25
0.50
0.75
1.00
1.25
[α2M] (μg/ml)
DMSO
250 μM fen. acid
DMSO + RAP
250 μM fen. + RAP

C


Degraded ^{125}I-α2M* (fmol /mg cell protein)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
time (h)
DMSO
250 μM fen. acid
DMSO + RAP
250 mM fen. + RAP

rosiglitazone (Bmax: 76.01 ± 4.409 versus 70.25 ± 3.538, 2-tailed p value = 0.1524; Kd: 1.133 ± 0.1054 versus 0.9514 ± 0.1004, 2-tailed p value = 0.1004) or hepatocytes with fenofibric acid (Bmax: 31.30 ± 6.922 versus 34.16 ± 13.44, 2-tailed p value = 0.7596; Kd: 0.9425 ± 0.3529 versus 1.159 ± 0.7255, 2-tailed p value = 0.4969) did not cause increased binding of 125I-labelled activated α2M at 0°C. Stimulation of mouse adipocytes with rosiglitazone did not increase 125I-α2M* degradation at 37°C either. These observations support the data presented above demonstrating that mouse LRP is not regulated by PPAR agonists due to the lack of a conserved PPRE in its promoter.

2.5 – Discussion

Despite the role of LRP in such important processes as lipoprotein metabolism and atherosclerosis, these studies are the first to demonstrate that human LRP gene expression is regulated at both the mRNA and protein levels via a discrete promoter element. The mechanism by which LRP gene expression is increased involves the ligand-induced upregulation of transcription via the activation of PPARγ:RXRα heterodimers which bind a newly identified PPRE in the promoter of the human LRP gene.

In human-derived SW872 cells or primary human adipocytes, LRP mRNA levels were modulated at the transcriptional level by ligands that activate PPARγ and this response was dose dependent. As anticipated, there was an inverse correlation between the amount of ligand required and the affinity of the ligand for PPARγ [195,222]. Rosiglitazone, the most potent ligand used, was found to have a maximal effect at approximately 500 nM in SW872 cells although there was significant up-regulation of LRP at 50 nM, a concentration much closer to rosiglitazone's reported K_d of 40 nM [195]. Rosiglitazone concentrations of 750 nM or higher did not alter LRP mRNA abundance or promoter activity of pGL3-LRP in SW872 cells. In differentiated primary human adipocytes, LRP mRNA levels were upregulated by rosiglitazone in a dose dependent manner at concentrations up to 1 μM, and only a slightly decreased efficacy was observed at a concentration of 2 μM. It has been suggested that rosiglitazone might act as a partial antagonist of PPARγ activation at these high concentrations [223]. In addition,

activation of PPARγ at the activation function 2 (AF2) domain enhances its degradation [224], which would explain the reduced efficacy of rosiglitazone at higher concentrations.

The intact full length LRP promoter conferred a measurable basal transcriptional rate of the luciferase reporter gene in dual luciferase activity assays in the absence of any other transcriptional signals. In the presence of rosiglitazone, the promoter yielded a 50% increase in luciferase activity, correlating with the increases in mRNA observed for SW872 cells. The isolated PPRE, alone or in tandem, acted as an enhancer for expression of luciferase driven by the SV40 ideal promoter, increasing luciferase activity by 50%, demonstrating that the LRP PPRE is a functional enhancer element. With a mutated LRP PPRE, luciferase activities were no longer increased in response to rosiglitazone, confirming that the LRP PPRE is the sole element responsible for this effect.

Rosiglitazone had a slightly larger effect on LRP mRNA levels in primary human adipocytes compared to SW872 cells (2 fold versus 1.5 fold) and primary human adipocytes responded to slightly higher concentrations of this ligand. This most likely reflects some inherent difference between the primary cell cultures and immortalized cell lines but is not due to decreased levels of PPARγ in SW872 cells since when PPARγ was co-transfected into SW872 cells with the luciferase reporter vectors, results were exactly the same as shown in Figure 2.5.

Direct binding of PPARγ:RXRα heterodimers to the PPRE identified in the human LRP promoter was demonstrated *in vitro* using electrophoretic mobility shift assays. The doublet bands that appeared in experiments using *in vitro* transcribed/translated PPARγ and RXRα have been observed in other studies where

rabbit reticulocyte lysate was used to produce these same proteins [225-227]. These doublets cannot be homodimers since PPARγ and RXRα TnT reactions, added individually to the EMSA reaction did not give shifts (data not shown). Proteins contained within the nuclear extracts of SW872 cells, primary human adipocytes or THP-1 cells could interact with the PPRE probe and PPARγ was shown as being one of the proteins bound. A probe corresponding to a mutated form of the LRP PPRE sequence (the same sequence found in pGL3-$LRP_{mutant\ PPRE}$ used in Figure 2.6B) could not interact with nuclear extracts containing PPARγ and RXRα confirming the importance of this sequence.

As the result of the increased LRP gene expression, levels of cell surface LRP were also increased in SW872 cells upon exposure to PPARγ ligands. This increased cell surface LRP resulted in increased LRP function in SW872 cells and primary human adipocytes upon exposure to rosiglitazone. Adipocyte LRP functions in chylomicron remnant cholesterol clearance both *in vitro* and *in vivo* [111] and also plays a role in the selective uptake of HDL-CE by human adipocytes [49,181] but the exact function of LRP in adipocytes is still unclear. The fact that it is co-ordinately regulated during pre-adipocyte differentiation by PPARγ along with many other adipocyte specific genes related to lipid metabolism such as LPL, FATP-1, ACS, CD36, and aP2 [175-178] (all of which contain at least one PPRE in their 5'-flanking sequences), suggests that LRP might serve in part to regulate cholesterol uptake to match TG synthesis during differentiation and maturation of the pre-adipocytes into fat cells.

Primary human hepatocytes treated with the PPARα ligand fenofibric acid also demonstrated increased LRP function. Although the increase in binding was slightly smaller compared to primary human adipocytes (1.4 fold and 1.5 fold, respectively), it

was significant. It is possible that this response was relatively low due to the lack of optimization of conditions for these cells. Adipocytes and hepatocytes are two very distinct cell types with varied physiological functions. The PPAR subtype expressed in each of these tissues is different (PPARγ and PPARα, respectively), yet stimulation with selective agonists resulted in similar increases in LRP function. This would suggest that the use of selective PPAR agonists in other tissues and cell types may yield similar results.

In all experiments, LRP function was determined by measuring binding and degradation of a well-characterized LRP ligand, α2M* [82,228]. Although this represents only one function of LRP, namely endocytosis of bound ligand, it is logical to conclude that all other potential functions that do not necessarily rely on endocytosis, such as intracellular signalling, would also be increased because they depend on extracellular ligand binding.

The increases in LRP expression and function observed in these studies were in the range of 1.5 to 2 fold and although not large in magnitude, were significant and similar to changes reported for other genes containing a PPRE in the promoter region [221,227,229]. Such small changes should not, and do not, reflect on their physiological importance. For example, the PPARα agonist fenofibrate increases apoA-I expression by only 1.5 to 2.0 fold, yet this translates into a clinically important HDL raising effect [230].

There are no studies in the literature examining the mouse or human LRP promoters and certainly no studies directly comparing the mouse and human LRP promoters. It was surprising to see that a functional enhancer element in the human promoter was not present in the mouse when the rest of the promoter sequences are so

similar. These differences could arise due to novel mutations in the human promoter rendering it a consensus PPRE or a loss of the enhancer sequence in mouse due to lack of selection against such mutations. These data emphasize that care should be taken when examining data from mouse studies and extrapolating it to humans since discrete promoter elements might not necessarily be conserved.

The studies presented here have important clinical significance since several PPAR ligands are already in widespread use. For example, type 2 diabetic patients administered rosiglitazone were found to have lower postprandial free fatty acid concentrations by increased metabolism of large triglyceride-rich lipoproteins [231]. Given the data presented above and our knowledge of LRP function in the adipocyte, this effect could be associated with increased LRP gene expression and function in adipocytes. In another study, administration of drugs of the thiazolidinedione class, including rosiglitazone and troglitazone, resulted in decreased progression of atherosclerosis in humans as demonstrated by decreased carotid intima-media thickness[232,233]. Rosiglitazone treatment in mice could also attenuate atherosclerosis and this was independent of its metabolic effects [234]. Fenofibrate, a widely used PPARα agonist of the fibric acid derivative class of drugs, is very effective at reducing plasma triglycerides in hypertriglyceridemic patients although the direct mechanism is not known. These studies suggest that a PPARα-mediated increase in hepatic LRP expression may explain in part the triglyceride-lowering effects of fibric acid derivatives. Finally, LRP has been shown to directly suppress atherosclerosis through inhibition of PDGF-dependent vascular smooth muscle cell migration and proliferation in mice [147]. This process required a ligand interaction between LRP apoE and interestingly, the apoE gene is regulated by

PPARγ through a PPRE contained within its promoter [235]. It is easy to speculate that upregulation of LRP and apoE by TZD administration could reduce atherosclerosis however, the direct effect of PPAR stimulation on LRP and apoE upregulation and ultimately on atherosclerosis will be difficult to address since current mouse models do not contain the human LRP promoter.

In conclusion, human LRP gene expression is clearly upregulated by PPARγ and PPARα ligands in two distinct cell types by a transcription dependent mechanism. The upregulation in gene expression results in increased protein function. Considering LRP is implicated in so many fundamentally important processes, these studies warrant further investigation into potential clinical effects of LRP upregulation, especially atherosclerosis.

CHAPTER 3 – CETP-MEDIATED HDL SELECTIVE UPTAKE IN THE LIVER

3.1 – Background

3.1.1 – Cholesteryl Ester Transfer Protein

Cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein of 476 amino acids with a molecular weight of 66 to 74 kDa [236]. It is a member of the lipid transfer protein/lipopolysaccharide binding protein (LTP/LBP) gene family which also includes phospholipid transfer protein (PLTP), lipopolysaccharide binding protein (LBP) and bactericidal/permeability increasing protein (BPI). Although these proteins share little polynucleotide or peptide sequence similarity [237,238], they share functional and structural similarities. Very little is known about the molecular structure of CETP since a crystal structure has not yet been resolved. The crystal structure for BPI however, has been solved [239] and immunochemical studies of CETP structure using the atomic coordinates of BPI support a common surface topology between BPI and CETP [240]. The putative structure function relationships of CETP and the other members of the lipid transfer/lipopolysaccharide binding protein family have been described exhaustively in the literature [241-244].

Several key regions and residues within CETP have critical importance in CETP function. Using a site-directed point mutagenesis approach, lysine 233 and arginine 259, two conserved residues found in all members of the LTP/LBP family, have been shown to be critical in the lipid transfer function of CETP and for HDL binding [245]. A mutant CETP protein carrying two point mutations at lysine residues 233 and 239 was reported

to have decreased CE transfer and decreased specific activity [245]. Compared to BPI, CETP has an additional 12 C-terminal amino acids which are predicted to form an amphiphilic helix critical for CE transfer [246] and possibly comprise the neutral lipid binding site directly involved in the lipid transfer mechanism [247]. This site is essential for transfer activity since neutral lipid transfer can be blocked by TP2, a monoclonal antibody against this epitope [248].

3.1.2 – CETP Gene Structure and Regulation

The human CETP gene contains 16 exons spanning approximately 25 kb [249] of chromosome 16 at locus 16q12-21 near the LCAT locus [250]. In humans, CETP is highly expressed in the liver, spleen and adipose tissue with lower levels of expression found in the small intestine, adrenal, kidney and heart [251,252]. In other species the levels of expression can vary between tissues but the site with the most conserved expression of CETP is adipose tissue [252]. cDNAs encoding CETP have been cloned from several species including monkey, rabbit, hamster, chicken and tree shrew with variations in homology of between 80-95% compared to the human cDNA [251-255]. Interestingly, mice and rats do not have the gene encoding CETP in their genomes [256].

A number of studies have demonstrated that CETP mRNA and protein expression are increased by cholesterol. Plasma CETP activity increases in rabbits in response to a high fat, high cholesterol diet [257], an effect attributed mainly to increased production of CETP in the liver [258]. Similarly, a high fat and high cholesterol diet induces increased plasma CETP transfer activity, plasma cholesterol and plasma triglycerides in hamsters [259]. In monocyte-derived macrophages, the secretion of CETP into the media was facilitated by cholesterol loading with acetylated LDL or free cholesterol and the

accumulation of CE is positively regulated with the secretion of CETP [260]. The human liposarcoma cell line, SW872, expresses 50-fold greater CETP than HepG2 cells and CETP mRNA and protein levels increased significantly in response to cholesterol loading [205]. In human adipose tissue, CETP expression and secretion were also increased by incubation with cholesterol-rich chylomicron remnants [203]. Furthermore, CETP expression is a function of membrane cholesterol content rather than lipid droplet cholesterol, that is, smaller less lipid loaded adipocytes secrete more CETP [203]. These data clearly demonstrate that cholesterol increases the expression of CETP.

In humans, increased plasma CETP levels are observed in the obese state resulting from increased CETP mRNA in the liver and adipose tissue [261]. In obese type 2 diabetic individuals however, plasma CETP levels were reportedly decreased, apparently the result of decreased CETP mRNA expression specifically in the liver, while adipose tissue expression remained unchanged. Sterol response element binding proteins (SREBP) -1 and -2 were lower in liver homogenates, suggesting that these transcription factors may mediate the effects of type 2 diabetes on hepatic CETP expression [262]. Although the liver is a major contributor of CETP, there is a strong correlation between adipose tissue CETP mRNA abundance and plasma CETP concentrations suggesting adipocytes contribute significantly to the plasma pool of CETP [201,263].

Much of our understanding surrounding the regulation of human CETP gene expression and activity comes from mouse models expressing the human CETP transgene. When human CETP with minimal 5' and 3' flanking sequences was placed under the control of the mouse metallothionein-1 promoter [264], moderate amounts of the CETP mRNA were detected in adipose tissue, heart and brain with smaller amounts

found in the liver, small intestine and muscle, similar to the expression pattern found in humans except for the liver expression [252]. No changes in CETP expression or activity were noted when mice were fed a high cholesterol diet. In contrast, when a large portion of the CETP 5' and 3' flanking sequences (3.2 kb upstream and 2 kb downstream) were used along with the CETP transgene to generate transgenic mice [265], the expression pattern of CETP again mimicked that of humans (with somewhat higher hepatic expression), and with its natural flanking sequences intact, CETP expression and activity were significantly increased by a high cholesterol diet. Increases in the mRNA levels upon exposure to the high cholesterol diet were greatest in the liver with only small increases observed in the spleen, small intestine and adipose tissue. Later studies demonstrated that the tissue specific regulation of CETP is mediated mainly by sequences found in the immediate 5' and 3' flanking sequences and that upregulation by cholesterol is mediated at several tissue specific sites in the upstream 5' flanking sequences up to – 570 bp [266]. Further examination of the 5' flanking sequences in the transgenic mice determined the regions containing specific elements yielding the tissue specific expression of CETP [266]. A sterol regulatory element (SRE)-like element in the region from -138 to -370 of the CETP 5' flanking sequence was identified to have homology to those found in other cholesterol responsive genes such as HMG CoA reductase. This element was shown to bind SREBP-1 and YY-1 by gel shift assays *in vitro*[267]. However, *in vivo* mutation of this element in the transgenic CETP mice demonstrated that the element is not involved in the upregulation by cholesterol, suggesting that there is an alternative distinct site containing a positive sterol response factor [267]. More recently, the transgenic mice described above [265] containing the natural human flanking sequences

demonstrated regulation of CETP gene expression in adipocytes and hepatocytes via a discrete DR-4 element within the promoter for LXR [268].

Several specific DNA elements have been identified within the 5' flanking sequence of the human CETP gene using cell based studies. A site at -93 to -118 contains a discrete element to which the nuclear hormone receptor apolipoprotein A1 regulatory protein-1 (ARP-1) can bind and transactivate CETP expression in HepG2 liver cells [269]. A functional cholesterol response element has been identified at position -186 to -213 of the human CETP promoter and this element conferred cholesterol regulation via transactivation from SREBP-1a and YY1 in the SW872 liposarcoma cell line [204]. Although the 5' flanking sequence up to 3.4 kb of the human CETP gene is sufficient for transcriptional activity and tissue-specific gene expression, distal promoter regions were also found to be important. An Alu repeat at -2153 to -2414 acts as a repressive element, whereas a binding site for the orphan nuclear receptor CYP7A promoter binding factor (CPF) at position -1042 facilitates activation of human CETP promoter activity suggesting a mechanism by which liver specific expression is achieved [270]. Finally, Sp1 and Sp3 sites have been identified at positions -37, -629 and -690 of the human CETP promoter. The Sp1/Sp3 site at -690 is a repressive element in reducing CETP promoter activity in reporter assays carried out in HepG2 cells working additively with the site at -629 when Sp3 levels are high. When Sp1 is present with lower levels of Sp3, there is activation at the -690 and -37 sites [271].

Interestingly, functional CETP expression appears to be regulated by the dominant negative suppression of an alternatively spliced transcript termed exon 9-deleted [272,273]. Additional studies in Caco-2 cells which normally have a low expression

of CETP activity found that these cells had an extremely high level of the exon 9-deleted mRNA suggesting tissue specific control over functional CETP expression through the exon 9-deleted transcript [274]. Furthermore, the expression of the functional full length transcript was increased with the addition of oleate but this did not affect the levels of the exon 9-deleted transcript suggesting that other positive elements exist within the promoter of CETP that can overcome the suppression [274].

3.1.3 – CETP Function

Early studies found that an unknown protein within the high density fraction of lipoproteins (>1.25 g/ml) from rabbit plasma could stimulate the exchange of cholesteryl esters between VLDL and LDL from hypercholesterolemic rabbits [275]. The same observation was later made for human plasma and the glycoprotein isolated from human plasma that was unlike any known apolipoprotein, and unable to esterify cholesterol or hydrolyze cholesteryl esters in lipoproteins, was termed cholesteryl ester transfer protein [276]. CETP is secreted from various tissues and the large majority of plasma CETP is associated with HDL, mostly within the pre-beta migrating fraction [277-279] although CETP can bind to all lipoprotein classes *in vitro* [280]. In plasma, CETP mediates the hetero- and homo-exchange of CE and TG between plasma lipoproteins resulting in the net transfer of CE from HDL to the triglyceride rich lipoproteins (TRLs), VLDL and chylomicron remnants, and to LDL. The net reciprocal transfer of TG to HDL results in a TG-enriched particle which is a better substrate for hepatic lipase [281,282]. CETP can also stimulate the exchange of LDL cholesteryl esters with VLDL triglycerides [281]. All neutral lipid exchange in human plasma is mediated by CETP [281,283].

The majority of studies measuring the kinetics of CETP lipid exchange between lipoprotein substrates support a mechanism whereby CETP acts as a shuttle [284-286]. Recently, the CETP inhibitor torcetrapib was reported to inhibit CETP transfer activity by creating a non-functional complex between HDL and CETP [287,288] supporting the shuttling model for neutral lipid transfer.

3.1.4 – CETP and Atherosclerosis

The controversy regarding the atherogenic potential of CETP relates to its multiple potential functions in lipoprotein metabolism and reverse cholesterol transport, the complex effects of CETP in genetically modified mouse models and the variable effects of polymorphisms in the CETP gene on coronary heart disease (CHD) risk in humans. On one hand, the transfer of CE from HDL to TRLs by CETP constitutes one of the pathways of the reverse cholesterol transport by which HDL cholesterol is returned to the liver (as discussed in section 1.2.2B). Additionally, CETP-mediated remodelling of HDL and subsequent action of hepatic lipase results in the production of smaller HDL particles, which can be a vehicle for both ABCG1-mediated and diffusional cholesterol efflux. For these reasons, CETP function has been proposed to be antiatherogenic. However, by increasing the cholesterol content of the TRLs, it has been proposed that CETP may promote atherogenesis [289]. Finally, much of the argument regarding a proatherogenic effect of CETP relates to the fact that high CETP activity is generally associated with low plasma concentrations of HDL, which is a risk factor for atherosclerosis.

Several mutations in the CETP gene in humans have been identified as a cause of CETP deficiency in individuals of Japanese descent [290-293]. In the homozygous state,

these mutations caused either a partial or a complete absence of CETP mass and activity in plasma resulting in an elevated level of HDL (lipid and apolipoprotein). In addition, LDL cholesterol and apoB are substantially reduced in individuals homozygous for these mutations. Although CETP deficiency results in an apparently antiatherogenic lipid profile, complete CETP deficiency does not clearly decrease susceptibility to atherosclerosis. A study of CHD susceptibility in men who were heterozygous carriers of functional mutations in CETP (Honolulu Heart Study) found a significantly increased risk of CHD in subjects with haploinsufficiency of CETP and average HDL cholesterol levels but not in men with very high HDL concentrations [294]. Overall, men with elevated HDL cholesterol levels due to partial CETP deficiency had the same rate of CHD as the control subjects with similar levels of HDL cholesterol not associated with a deficiency of CETP. However, more recently, 7-year prospective data from the participants of the Honolulu Heart Study reported no statistically significant relationship between heterozygosity for mutations in CETP and CHD incidence [295]. In a separate study of Japanese subjects, CETP deficiency was atheroprotective if the HDL concentrations were high [296]. Similar confusion has resulted from the studies of common single nucleotide polymorphisms (SNPs) in CETP and susceptibility to atherosclerosis (reviewed in [297,298]).

CETP expression in a variety of animal models of atherosclerosis has been extensively studied. These studies have also not provided a clear answer regarding CETP and atherosclerosis risk since the outcomes have varied, apparently contingent on the level of CETP expression and the genetic background of the animals studied. The introduction of the simian CETP transgene into cholesterol and bile salt-fed wild-type mice resulted in plasma CETP concentrations 8 to 10 fold greater than normal CETP

levels in humans, decreased HDL cholesterol and increased LDL and VLDL cholesterol and apoB and enhanced formation of fatty streak lesions compared with non-expressing controls [299]. When the human CETP gene was introduced into apoE-knockout mice or LDL receptor-knockout mice fed a Western diet, cholesterol esters were redistributed from HDL to VLDL/LDL and atherosclerosis was increased [300]. However, in mice overexpressing human apoA-1, the effect of CETP expression on lesion formation was not significant despite reduced HDL cholesterol levels [300]. Surprisingly, CETP transgene expression in mice made hypertriglyceridemic by overexpression of human apoC-III, actually reduced the extent of atherosclerotic lesions [301]. Finally, recent experiments on the effects of overexpression of reverse cholesterol transport genes (apoA-1, LCAT and CETP), in aged mice, individually or in combination, suggested that CETP is protective. While transgenic mice expressing CETP alone did not demonstrate a decrease in atherosclerotic lesion size, CETP expression in combination with LCAT overexpression only, or LCAT and apoA-1 overexpression, significantly decreased lesion size (back to non-transgenic control levels) compared to the deleterious effect LCAT and LCAT/ apoA-1 overexpression alone [302].

Rabbits express very high levels of CETP and are highly susceptible to diet-induced atherosclerosis. Inhibition of CETP expression in cholesterol fed rabbits resulted in decreased plasma CETP activity, increased HDL cholesterol levels and decreased LDL and VLDL cholesterol levels as well as increased hepatic LDL receptor expression [303,304]. These changes ultimately led to a decrease in atherosclerosis. In further attempts to inhibit CETP, cholesterol fed rabbits were vaccinated to produce neutralizing anti-CETP antibodies and were shown to have a marked reduction in atherosclerosis [305,306]. This

approach has been tested in humans and shown to be effective at generating anti-CETP antibodies but the effect on CETP activity or HDL cholesterol levels remains to be determined [307].

3.1.5 – Pharmacological Inhibition of CETP

Low HDL-cholesterol levels are highly predictive of CAD risk and high CETP levels are generally associated with reduced HDL-cholesterol concentrations. The marked increase in HDL cholesterol and apoA-1 associated with human deficiency of cholesteryl ester transfer protein (CETP) [292,308,309] has led to the suggestion that CETP inhibition might be a useful strategy aimed at raising HDL-C levels [310]. Several approaches have been used to inhibit CETP and include monoclonal antibodies [311,312], antisense oligonucleotides [304,313], and vaccines [305-307,314]. Another approach involves small molecule CETP inhibitors and several have been reported in the literature.

Dibenzodioxocinones are derivatives of the natural compound 11-hydroxy-3-[(S)-1-hydroxy-3-methylbutyl]-4-methoxy-9-methyl-5H,7H-dibenzo[b,g][1,5]dioxocin-5-one and several have been shown to act as CETP inhibitors *in vitro* with good stability in rat plasma [315].

A second small molecule CETP inhibitor, JTT-705, increased HDL cholesterol, decreased non-HDL cholesterol and inhibited the progression of atherosclerosis in cholesterol fed rabbits [316]. In a phase II safety and dose-response study in healthy individuals, the highest dose of JTT-705 (900 mg/d) was shown to be successful at decreasing plasma CETP activity by 37%, raising HDL-C by 34% all the while decreasing LDL-C levels by 7% [317]. The doses required to obtain reasonable effects were, however, quite high and the apparent occurrence of serious adverse effects raise

concerns regarding the safety of the drug. In a more recent study, 300 or 600 mg/d of JTT-705 was administered to patients with type II dyslipidemia in combination with 40 mg/d of pravastatin [318]. Four weeks of treatment with JTT-705 600 mg led to a 30% decrease in CETP activity, a similar increase in HDL-cholesterol and a small decrease in LDL-cholesterol concentrations.

Another compound under development by Pfizer, torcetrapib (CP-529,414), has undergone several clinical studies, the results of which were recently published. Reports from an initial phase 1 multidose study in healthy individuals [287], indicated a dose-dependent inhibition of CETP activity up to nearly 100% along with increases in HDL-C of 16% to 91% and decreases in LDL-C of 21% to 42%. There was also a significant increase in apoA1 and apoE concentrations (27% and 66%, respectively). In a single-blind study involving patients with low HDL-C levels [319], 120 mg/d of torcetrapib increased HDL-C levels by 41% while decreasing the levels of LDL-C by 8%. The effects of this lower dose of torcetrapib were greater in combination with atorvastatin (HDL increased by 61% and LDL decreased by 17%). Additional data regarding HDL composition, apoA-1 kinetics, bile acid synthesis and fecal sterol excretion (markers of reverse cholesterol transport) were published separately [320]. This report indicated that torcetrapib did not affect HDL apoA-1 production but reduced the fractional catabolic rate of apoA-1 in a dose-dependent fashion. No change was found in fecal sterol excretion or bile acid synthesis. In both trials, the therapy was very effective at raising HDL-C and lowering LDL-C, well tolerated and side effects were mild to moderate, giving promise for future clinical use of this drug.

3.1.6 – Selective Uptake

In the 1980s, a mechanism for the delivery of HDL cholesterol to cells was described that was very different from the classical mechanism of receptor-mediated endocytosis, the prototype being the LDL receptor [321,322]. Pittman and colleagues coined the term "selective lipid uptake" because following interaction of HDL with hepatocytes, cholesterol (primarily cholesteryl ester) was transferred into the hepatocyte, following which the lipid-depleted HDL particle dissociated from the cell to re-enter the circulation. Selective uptake has since been documented in many cell types including steroidogenic tissues (adrenal glands, testes and ovaries) [323], adipose tissue [53,324] and the liver. The major HDL selective uptake receptor in the liver is believed to be SR-B1 and was first identified in mouse-derived cell lines [325,326]. SR-B1 is clearly responsible for HDL-cholesterol clearance in the mouse [327], a species that lacks CETP [256]. The human homolog, CLA-1, has been identified [328] but its overall importance in HDL metabolism in humans remains unclear. Selective uptake represents the final step in HDL-mediated movement of cholesterol from the periphery to the liver and the net hepatic accumulation of this cholesterol is a final step in reverse cholesterol transport.

3.1.7 – CETP and Selective Uptake

Early studies investigating cholesterol and CE metabolism in adipocytes reported transfer of FC and CE from LDL and HDL to adipocytes[329,330], which was later attributed to CE selective uptake by an unknown component of the plasma membrane [331]. This laboratory later reported that human adipose tissue in organ culture or isolated primary cell culture accumulated significant amounts of HDL-derived CE without appreciable accumulation of the main protein component of HDL, apoA-1, and that the accumulation

of HDL-CE was enhanced by the addition of recombinant human CETP and partially inhibited by incubation of adipose tissue with TP2, an anti-CETP monoclonal antibody which inhibits neutral lipid transfer[53]. Other studies from this laboratory examined the role of CETP in selective uptake using CHO cells stably-expressing CETP (Zha and McPherson, unpublished data). These studies demonstrated that endogenously expressed CETP from these stably transfected cells associated with the plasma membrane by cell surface labeling with fluorescent TP2 anti-CETP antibody. Furthermore, CETP mediated the selective uptake of HDL-derived CE determined by the cellular uptake of fluorescent bodipy-CE independently of the fluorescently labeled apoA-1. More recent studies demonstrated that monensin treatment or energy depletion of the SW872 liposarcoma cells with 2-deoxyglucose and NaN3 had no effect on CETP-mediated selective uptake, indicating that endocytosis is not required [54].

3.2 – Rationale

The concept that CETP has a cellular function in mediating selective uptake of HDL-CE, analogous to that of SR-B1, is still not widely accepted. It has been proposed that CETP-mediated selective uptake observed in many cells is attributable to the CETP-mediated transfer of CE from HDL to newly secreted apolipoprotein B-containing lipoproteins, which are then internalized by the LDL receptor (LDL-R) as was first reported using HepG2 cells [332], however, this paradigm cannot explain our observations demonstrating CETP-mediated selective uptake in adipocytes since these cells do not synthesize or secrete apoB lipoproteins.

At the time these studies were initiated, there was no clear evidence that CETP mediated direct cellular uptake of cholesteryl esters but it was accepted that CETP played

an important role in indirect and quantitatively important pathways for the clearance of HDL-CE [333], both through its transfer of cholesterol from HDL to other lipoproteins for subsequent receptor-mediated endocytosis and in the remodelling of HDL rendering it a better substrate for SR-B1-mediated uptake [334]. The studies described below are an extension of the adipocyte work previously published from this laboratory. The primary objective was to determine the role of CETP in HDL-CE selective uptake in primary mouse hepatocytes and to establish a role for CETP in HDL-CE selective uptake *in vivo*. An important additional objective was to determine the effect of the CETP inhibitor torcetrapib on CETP-mediated selective uptake *in vitro* and *in vivo*. Based on this laboratory's previous observations that the anti-CETP monoclonal antibody, TP2, significantly reduced the ability of CETP to mediate selective uptake, we hypothesized that inhibition of CETP would lead to decreased CETP-mediated HDL-CE selective uptake and potentially impede a final step in reverse cholesterol transport.

3.3 – Materials & Methods

Reagents

Cell culture medium and reagents were purchased from Life Technologies and cell culture plasticware was purchased from Falcon. All common reagents were analytical grade and purchased from Fisher (Fair Lawn, NJ) and Sigma (St Louis, MO). Restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs. Taq DNA polymerase was purchased from Invitrogen. Maxiprep, miniprep and gel purification kits were purchased from Qiagen. Iodobeads and Super Signal chemiluminescence reagents were purchased from Pierce. Horseradish peroxidase-conjugated secondary antibodies and radioisotopes were from purchased from

Amersham. Partially purified recombinant CETP (rCETP) was purchased from Cardiovascular Targets, Inc (New York, NY).

Torcetrapib (CP-529,414) was kindly provided by Pfizer Inc. (Groton CT).

Generation of CETP adenoviruses

Wild type human CETP, in the pCMV5 expression mammalian expression vector (kindly provided by Dr Alan Tall), was subcloned into the pShuttle-CMV vector using the following PCR-based strategy. The following PCR primers (CETP SS/Kpn1-f; 5'-GCGGTACCATGCTGGCTGCCAC-3' and Not1/ECFP-r; 5'-TTCCCTTGCGGCCGCCCTTGTACAGCTC-3') were used to amplify the CETP sequence with the Kpn1 and HindIII restriction sites designed into the primers. This PCR fragment, along with pShuttle-CMV, were subsequently digested with the above restriction enzymes and both were gel purified prior to ligation and screening for the pShuttle-$CETP_{w/t}$ recombinant vectors.

A variant of the CETP, designated $CETP_{KD}$, which was previously shown to have very little transfer activity [245], was subcloned into pShuttle-CMV using restriction digests. The CETP cDNA was excised from pCMV4 (kindly provided by Dr Alan Tall) with Sma1 and cloned into the EcoRV site within the pShuttle vector. Screening of recombinants was performed to ensure that the CETP cDNA was inserted in the proper orientation.

An additional construct was generated in which an enhanced cyan fluorescent protein (eCFP) was added to the N-terminus of the mature CETP peptide. This was accomplished using a multi-step cloning approach described below. The CETP signal sequence (Met1 to Gly10 of mature CETP peptide) was generated by dimerizing

complementary oligos (CETPss 1; 5'-GATCTATGCTGGCTGCCACAGTCCTGACCCTGGCCCTGCTGGGCAATGCCCATGCCTGCTCCAAAGGCACCTCGCACGAGGCAGGG-3' & CETPss 2; 5'-GATCCCCTGCCTCGTGCGAGGTGCCTTTGGAGCAGGCATGGGCATTGCCCAGCAGGGCCAGGGTCAGGACTGTGGCAGCCAGCATA-3') corresponding to the CETP signal sequence with BglII and BamHI restriction sites designed into the 5' and 3' ends, respectively. The first 10 amino acids of the mature peptide were included in the signal sequence peptide so that the cell can recognize the cleavage site and properly process the mature eCFP-CETP. These first 10 amino acids will be repeated again after the eCFP as part of the mature CETP. This double stranded oligo was then subcloned into the BglII and BamHI sites in eCFP-N1 (kindly provided by Dr H McBride) to generate the plasmid CETPss/eCFP-N1. Meanwhile, pCMV5-CETP was used as a template to PCR generate the mature CETP sequence using the following primers: Not1/CETP-f; 5'-AATTGGCGGCCGCTGCTCCAAAGGC-3' & CETP/HindIII-r; 5'-CCGCCAAGCTTCTAGCTCAAGCTCTG-3'. These primers contained NotI and HindIII restriction sites so that once the PCR product was generated, it could be cut with these restriction enzymes and subcloned into the multiple cloning site of the pShuttle vector (Q-biogene) generating the plasmid pShuttle-$CETP_{mature}$. The following primers (CETP SS/Kpn1-f; 5'-GCGGTACCATGCTGGCTGCCAC-3' & Not1/ECFP-r; 5'-TTCCCTTGCGGCCGCCCTTGTACAGCTC-3') were then used to PCR generate the CETPss/eCFP with Kpn1 and NotI restriction sites from the CETPss/eCFP-N1 plasmid generated above without the stop codon of the eCFP. This PCR product was then digested and ligated into the corresponding sites in the pShuttle-

$CETP_{mature}$. The new vector generated above, designated pShuttle-CFP/CETP, now contains the CETP signal sequence upstream the eCFP which has now been attached to the sequence coding for the mature CETP peptide.

For the luciferase control adenovirus, the luciferase cDNA was excised by restriction digest from the pCA13-luciferase construct kindly provided by Dr Marcel and subcloned into the HindIII and XbaI sites of the pShuttle-CMV vector.

The pShuttle vectors generated above were transfected into Cos7 cells and Western blot analysis was performed using TP2 monoclonal anti-CETP antibodies to ensure that the CETP proteins were expressed correctly. Sequencing was also performed to ensure that there were no point mutations introduced during the cloning of the various constructs. The pShuttle vectors were used for subsequent generation of the adenovirus DNA construct via homologous recombination with the pAd-Easy1 adenovirus DNA vector in BJ5183 *E. coli* following the manufacturer's instructions (Qbiogene). The adenovirus was then generated and scaled-up in 293 cells, purified by cesium-chloride density ultracentrifugation and measured by OD following the manufacturer's instructions (Qbiogene). Virus particles per ml are converted to plaque forming units per ml (PFU/ml) by dividing by 18.

Immunoprecipitation and Western blot analysis

Cos7 cells were plated in 6-well culture dishes in DMEM containing 10% fetal bovine serum. Each well was infected with 1×10^8 PFU of each of the adenovirus constructs. Media was collected 2 days later and spun at 1000 rpm for 5 minutes to pellet cellular debris prior to the addition of 40 μg of purified TP2 IgG. The cells were scraped down in PBS and spun at 3000 rpm for 5 minutes to form a pellet which was

subsequently washed and spun with PBS twice more. The media samples were placed in a rotator overnight at 4°C. A 1:5 mix of protein G-sepharose was prepared with PBS and 150 µl of this slurry was added to the media samples the following morning and incubated at 4°C in the rotator. 24 hours later, the samples were spun at 300 rpm for 5 minutes and the pellets washed with 11 ml of cold PBS by rotating 5 minutes each. The final pellet was resuspended in 40 µl of denaturing/reducing loading buffer (10% SDS and 0.1% β-mercaptoethanol) and heated for 5 minutes at 95°C before being subjected to SDS-polacrylamide gel electrophoresis (8% gel with 4% stacking) at 100 V for 2 hours and transferred to nitrocellulose for 1 hour at 4°C [217]. Blocking of the blot was done with a 5% skim milk/1X Blotto solution for 1 hour followed by a 1:1000 dilution of the monoclonal anti-CETP antibody TP2 overnight. The blot was washed 3 times 5 minutes each in PBS and incubated with 1:5000 dilution secondary antibody conjugated to horseradish peroxidase. CETP bands were detected by chemiluminescent detection using the Super Signal solutions (Pierce Biochemicals).

Molecular biology techniques were essentially as described by Sambrook *et al.* [218].

Animals and primary hepatocyte isolation and culture

Retired breeders of the C57Bl6J (wild type) mouse strain were purchased from Charles River (Wilmington, MA). Mice do not express CETP [256], making them an ideal model for these studies. As indicated below for certain experiments, primary hepatocytes were isolated from SR-B1 and LDLR deficient mice. SR-B1 null mice (strain B6.129S2-Scarb1^{tm1Kri}) and LDLR null mice (strain B6.129S7-ldlr^{tm1Her}) were purchased from Jackson Laboratory (Bar Harbour, ME). The mice were maintained on a 12 h light/12 h

dark schedule on a normal chow diet. Primary hepatocytes were prepared from mice according to established protocols [208,209]. Briefly, mice were sedated and livers perfused with collagenase solution. The cells were seeded on fibronectin-coated (4 μg/well) 24-well plates at an initial density of 2.9 X 10^5 cells per well in William's medium containing penicillin (100 units/ml), streptomycin sulfate (100 units/ml), Fungizone (250 ng/ml; Invitrogen) and 10% fetal bovine serum (Sigma). 6 h following the initial plating, the cells were washed in William's medium without fetal bovine serum and fresh media (as above).

For experiments using adenovirus-mediated expression of CETP, CETP adenovirus (ad-CETP) or control luciferase adenovirus (ad-Luc) was added to the wells at a concentration of 2.175 X 10^7 PFU/well (75 MOI). The infected cells were left to express the transgene for 36 to 48 hours prior to the assay. Otherwise, cells were used 12-36 hours after initial wash.

Lipoprotein purification and labelling

Total HDL was purified from normolipemic plasma from a healthy male donor by density gradient ultracentrifugation [335] followed by dialysis X 3 against 4 L of nitrogen-sparged PBS, pH 7.4, with 2 g of Chelex (BioRad). The lipoproteins were labeled with ^{3}H-cholesteryl oleate using a modified protocol as described by Reaven [336]. Briefly, 100 μCi of ^{3}H-cholesteryl oleate ([^{3}H]-CE) (Amersham Biosciences), 0.92 μmol phosphatidylcholine, 0.24 μmol sphingomyelin, 0.24 μmol unesterified cholesterol, and 0.14 μmol triolein were dissolved in chloroform and dried under N_2 as a thin film. The mixture was resuspended in 2 ml of Tris-NaCl-EDTA buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.25 mM EDTA, pH 8.0) and sonicated on ice 3 X 10 minutes. 5 mg of total

HDL and 2 ml of lipoprotein-deficient serum were added to the lipid mixture and incubated overnight at 37°C. ^{3}H-CE HDL was separated by sequential gradient density ultracentrifugation and dialyzed against PBS as above.

Human apolipoprotein A-1 was kindly provided by Dr Daniel Sparks and labeled with ^{125}I as described [49]. ^{3}H-CE HDL was then incubated with ^{125}I-labeled apoA-1 or cold apoA-1 for 24 h at 37°C prior to being re-isolated by gradient density ultracentrifugation as described above. This results in an HDL particle that is labeled in the core with ^{3}H-CE and on the protein moiety with ^{125}I-apoA-1. The charge and size of the labeled HDL particles were verified to be normal by Lipogel analysis.

Selective uptake assay

Experiments were carried out essentially as outlined in detail previously [49]. Briefly, hepatocytes were seeded as described above, washed twice at 37°C in 1 ml of ligand buffer (HBSS, 25 mM Hepes, 5 mg/ml BSA, pH 7.45) and then incubated at 37°C for 30 min in this buffer. This buffer was replaced with 300 µl of ligand buffer containing ^{3}H-CE HDL or ^{125}I-apoA-1 HDL, with the addition of CETP and other compounds as indicated (concentrations as stated in figure and figures legends), and the cells were incubated at 37°C for 8 hours, unless otherwise indicated. At the end of the incubation, the extracellular buffer was removed, and the cells washed 5 times in HBSS, 25 mM Hepes, pH 7.45 at room temperature. If ^{125}I-apoA-1 HDL was used in the experiment, 250 µl of the extracellular buffer was subjected to TCA precipitation and TCA-soluble counts were determined by gamma counting to determine the amount of degraded ^{125}I-apoA-1 HDL. After the final wash, the remaining buffer was removed, and the cells were solubilized with 500 µl of 0.2 M NaOH overnight at room temperature

with gentle shaking. The protein content of 40 µl from each well was measured using a BSA standard and the BCA protein assay reagent according to the manufacturer's instructions (Pierce). The cell associated ^{3}H or ^{125}I radioactivity in 400 µl of each cell lysate was measured by liquid scintillation counting using Ecolite (ICN, Costa Mesa, CA) or by gamma counting, respectively. The cell-association of ^{3}H-CE or ^{125}I-apoA-1 or degradation of ^{125}I-apoA-1 are measured in units of the amount of these labels contained in 1 ng (protein content) of HDL and corrected for mg of cell protein in each well. Measured this way, an equivalent amount of ^{3}H-CE and ^{125}I-apoA-1 labels represents holoparticle uptake and any additional ^{3}H-CE cell association is attributed to selective uptake.

For experiments examining the reversible and irreversible components of selective uptake, hepatocytes were preincubated with 50 µg/ml ^{3}H-CE-HDL for 2 or 4 hours and then incubated for 4 h with 400 µg/ml cold HDL. The reversible phase was measured by counting the radioactivity found in the media that was removed from the cells. The irreversible phase was determined by measuring the cell-associated counts following 4 washes with HBSS.

CETP Transfer Activity Assays

CETP transfer activity assay was performed as follows. For each sample, done in duplicate, 2 µl of mouse plasma was diluted in 18 µl of PBS. 80 µl of TSE (50 mM tris, 150 mM NaCl, with azide and EDTA, pH 7.4) containing 50 µg LDL and 1 µg of ^{3}H-CE labeled HDL (labeling described above), based on total cholesterol content and not protein content, was added to each sample and incubated at 37°C for 3 hours with shaking. There are also 3 control samples in duplicate; 2 µl of human plasma to which

all the other samples will be compared, 2 µl of plasma that will not be precipitated (measures total counts) and the final control measures total precipitable counts in the absence of plasma (20 µl of PBS). 175 µl of a precipitate solution (4:1:1 mix of 20% BSA:$MnCl_2$:heparin) is added to each of the samples followed by 400 µl cold TSE. The samples are left on ice for 10 minutes followed by centrifugation at 4°C on maximum setting. The amount of radioactivity in 100 µl of the supernatant is determined and this represents the amount of ^{3}H-CE remaining in the HDL (^{3}H-CE transferred to LDL by CETP was precipitated and therefore removed from the supernatant).

In vivo Experiments

Preliminary experiments were carried out in 6-8 week-old C57Bl6J (wild-type) mice (Charles River). Wild-type mice (C57Bl6J) were injected with increasing amounts of $CETP_{w/t}$ and $CETP_{KD}$ adenoviruses (1.25×10^8, 2.5×10^8, 5×10^8 and 1×10^9 PFU). Blood was collected by saphenous vein puncture 6 days later and plasma obtained following centrifugation. CETP was detected by Western blot, as described above except for the following changes: electrophoresis conditions were denaturing only (10% SDS/non-reducing) and the anti-CETP monoclonal antibody was TP24 rather than TP2.

In some experiments, mice were subjected to tail vein injection of either ad-Luc or ad-$CETP_{KD}$ (1.25×10^8 PFU in 100 µl total volume). At baseline and 3, 6, and 10 days post injection, fasting blood samples were obtained for cholesterol measurements. Animals were sacrificed at 10 days and primary hepatocytes isolated for determination of CETP protein expression and ^{3}H-CE-HDL selective uptake assays.

In the final experiments, mice were injected via the tail vein with 2.5×10^8 PFU of ad-Luc or ad-$CETP_{w/t}$ (day 0) in a total volume of 100 µl. Mice were injected daily

via the tail vein with 100 μl of intralipid containing either vehicle (DMSO) or torcetrapib (0.125 mM; such that the final concentration in the mouse, assuming a 3 ml total blood volume, would be approximately 4 μM). Mice were fasted 8 hours and 100 μl of blood was collected in an EDTA collection vial via saphenous vein puncture on day 2, 4, 5, and 7 post-injection. The blood samples were centrifuged and plasma was aliquoted and frozen for assay at experiment completion. CETP expression in plasma samples was confirmed by Western blot. Samples were also assayed for total cholesterol using a colorimetric assay (Roche Diagnostics, Montreal, PQ). On day 7, the mice were bled using mild sedation and cardiac puncture to obtain approximately 1 ml whole blood. The blood samples were spun and sera were stored at 4°C prior to discontinuous density gradient ultracentrifugation in order to separate LDL and HDL subfractions. 500 μl aliquots were removed from the tubes after 18 hours at 45000 rpm and stored at 4°C prior to density measurement and total cholesterol determination using a colorimetric assay (Roche Diagnostics, Montreal, PQ).

Statistical Analysis

Results are expressed as the mean ± SEM. Where indicated, the statistical significance of the differences between groups was determined using two-tailed p values of the unpaired *t* test using GraphPad InStat v.3.06 statistical analysis software (GraphPad Software Inc.).

3.4 – Results

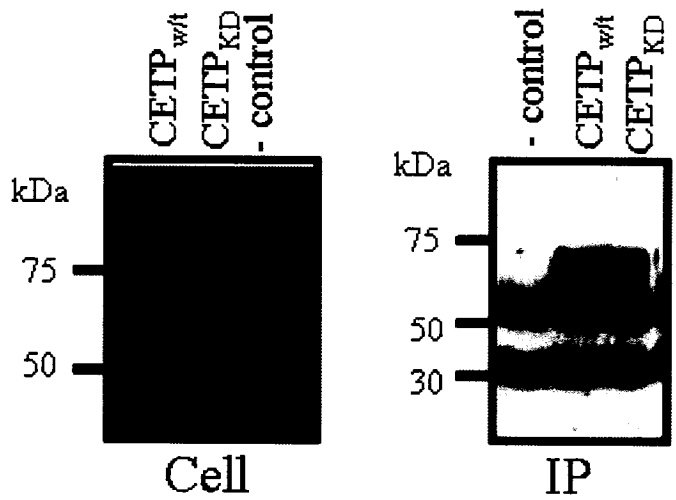
3.4.1 – Expression of CETP Using Adenovirus-mediated Gene Transfer

Cos7 cells, which are a monkey-derived cell line, do not express endogenous CETP making them a suitable cell line for preliminary experiments. Expression of CETP using adenovirus-mediated gene transfer resulted in equal expression levels of all three constructs (ad-$CETP_{w/t}$, ad-$CETP_{KD}$ and ad-CFP-CETP) as determined by Western blot analysis of cell lysates, using an anti-CETP monoclonal antibody TP2 (**Figure 3.1**). CETP and CFP-CETP were found at the expected molecular weights of approximately 74 kDa and 101 kDa (74 kDa + 27kDa (eCFP)), respectively. Ad-$CETP_{w/t}$ and ad-CFP-CETP were secreted into the media; in contrast $CETP_{KD}$ was poorly secreted despite having good expression in the cell. This was surprising since $CETP_{KD}$ was previously reported by Alan Tall's laboratory to exhibit normal secretion but impaired CETP transfer activity [245] and this was the main reason this particular combination of point mutations was chosen for the adenovirus construct. Furthermore, $CETP_{KD}$ demonstrated normal CETP activity in mouse plasma when expressed in the liver via tail vein injection of the adenovirus (shown below) contrary to the previous report using cell culture expression. Nonetheless, this construct was suitable for further experimentation since $CETP_{KD}$ was shown previously to associate with the cell in a similar fashion to wild-type CETP (Zha & McPherson, unpublished data) and was therefore used in experiments to examine the effects of cell-associated CETP on selective uptake.

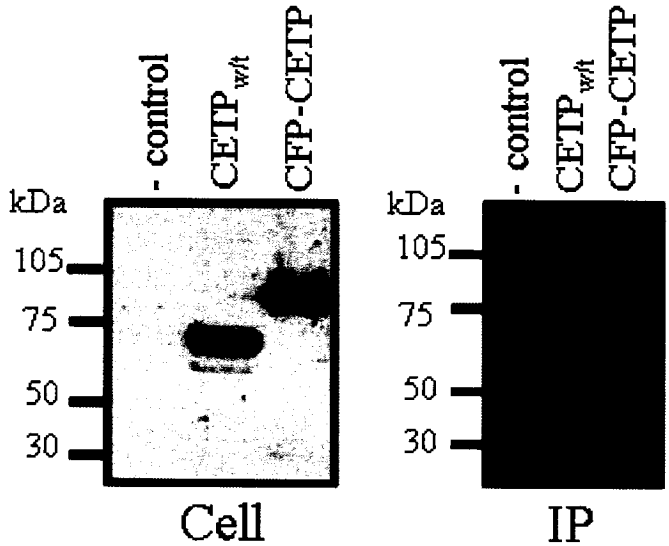
Figure 3.1. Expression of CETP transgenes in Cos7 cells following adenovirus-mediated gene transfer.

Cos7 cells, cultured to a confluent monolayer in a 6-well dish, were infected with 1 x 10^8 PFU of adenovirus. Cells were left to express the transgenes for 2 days prior to Western blot analysis of the cell lysate and immunoprecipitation of CETP from the media. **A.** Western blot of cell lysate and immunoprecipitable CETP from the media of Cos7 cells infected with ad-$CETP_{w/t}$ or ad-$CETP_{KD}$. **B.** Western blot of cell lysate and immunoprecipitable CETP from the media of Cos7 cells infected with ad-$CETP_{w/t}$ or ad-CFP-CETP.

A


CETP w/t
CETP KD
- control
kDa
75
50
Cell
- control
CETP w/t
CETP KD
kDa
75
50
30
IP

B


- control
CETP w/t
CFP-CETP
kDa
105
75
50
30
Cell
- control
CETP w/t
CFP-CETP
kDa
105
75
50
30
IP

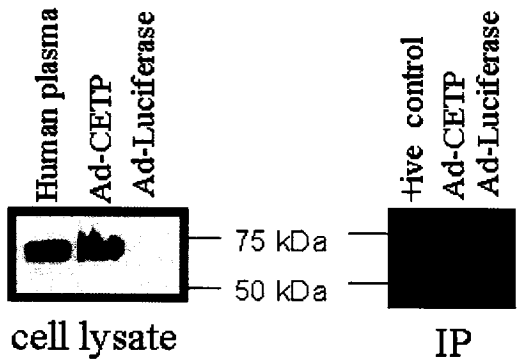
3.4.2 – CETP Mediates Selective Uptake of HDL-derived CE in Primary Mouse Hepatocytes

Western blot analysis of wild-type mouse hepatocytes infected in culture with CETP adenovirus (ad-$CETP_{w/t}$) or the control luciferase adenovirus (ad-Luc) demonstrates that CETP is expressed at the expected molecular weight in cell lysates from ad-$CETP_{w/t}$-infected hepatocytes in contrast to control (**Figure 3.2A**). For these experiments, in order to specifically address the effects of cell associated CETP as opposed to CETP secreted into the media, a moderate viral titer of ad-$CETP_{w/t}$ (2.175 x 10^7 PFU/well) was used to test expression and secretion into the media over an 8 hour time period, the incubation time used for the selective uptake experiments. Under these conditions, hepatocyte secretion of CETP was negligible as demonstrated by the absence of CETP in the media by Western blot following immunoprecipitation (IP) with TP2 anti-CETP monoclonal antibody (mAb) at the end of the 8h time course (**Figure 3.2A**) and no detectable transfer of ^{3}H-CE from HDL to LDL in an *in vitro* transfer assay (data not shown). Cells were subsequently isolated from additional wild-type mice and infected with a low viral titer of ad-$CETP_{w/t}$, 7.25 x 10^6 PFU/well (**Figure 3.2B**), which corresponds to a multiplicity of infection of approximately 25 PFU per cell (MOI 25). Cells were also infected with a moderate dose of ad-$CETP_{w/t}$ (2.175 x 10^7 PFU/well), which corresponds to an MOI of 75 (**Figure 3.2C**). As expected in luciferase-transfected hepatocytes, ^{3}H-CE cellular incorporation was greater than that of ^{125}I-apoA-I, consistent with SR-BI mediated selective uptake of HDL-CE by primary hepatocytes (**Figure 3.2A&B**). However, even in the absence of detectable CE transfer activity in the media, adenovirus-mediated expression of CETP in mouse primary hepatocytes resulted in a

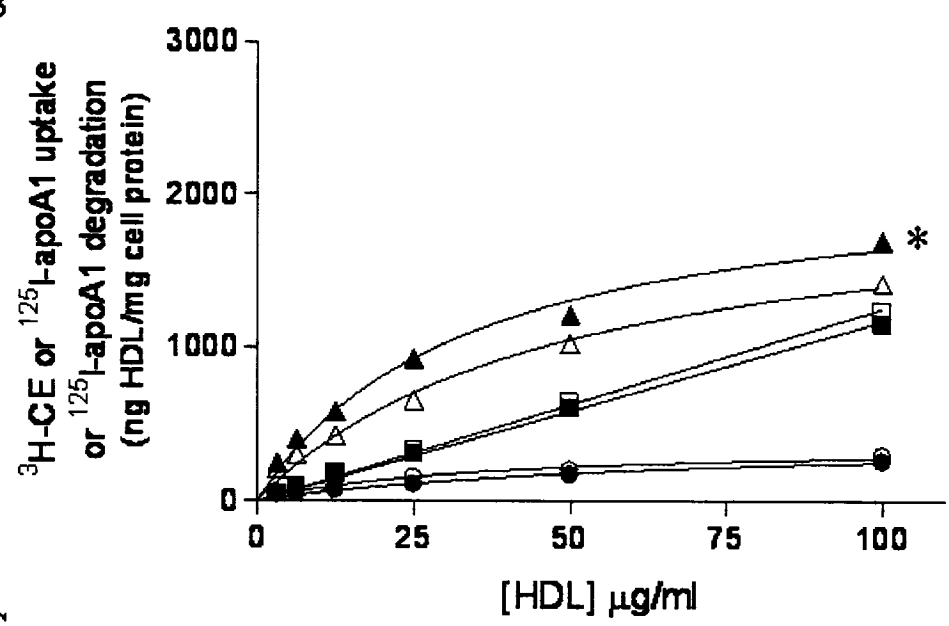
Figure 3.2. $CETP_{w/t}$ transgene expression mediates selective uptake of HDL-derived ^{3}H-CE in primary mouse hepatocytes.

A. Western blot (post selective uptake assay) for CETP in cell lysate from isolated primary hepatocytes (C57Bl6J) infected with ad-$CETP_{w/t}$ (2.175×10^{7} PFU/well) for 2 days and IP from the media collected following the 8 hour incubation time. **B.** Selective uptake assay on primary mouse hepatocytes (C57Bl6J) infected with 7.25×10^{6} PFU/well (multiplicity of infection of 25 (MOI)) of ad-$CETP_{w/t}$ or ad-Luc 2 days prior. ^{3}H-CE uptake in cells infected with ad-$CETP_{w/t}$ (▲) or ad-Luc (Δ); ^{125}I-apoA-I cell uptake in cells infected with ad-$CETP_{w/t}$ (●) or ad-Luc (○); ^{125}I-apoA-I degradation in cells infected with ad-$CETP_{w/t}$ (■) or ad-Luc (□). *2-tailed p values < 0.05 for all values compared to luciferase for all concentrations of HDL. **C.** Selective uptake assay on primary mouse hepatocytes (C57Bl6J) infected with 2.175×10^{7} PFU/well (75 MOI) of ad-$CETP_{w/t}$ or ad-Luc 2 days prior. ^{3}H-CE uptake in cells infected with ad-$CETP_{w/t}$ (▲) or ad-Luc (Δ); ^{125}I-apoA-I cell uptake in cells infected with ad-$CETP_{w/t}$ (●) or ad-Luc (○); ^{125}I-apoA-I degradation in cells infected with ad-$CETP_{w/t}$ (■) or ad-Luc (□). * 2-tailed p values $<$ 0.0007 compared to luciferase for all concentrations of HDL.

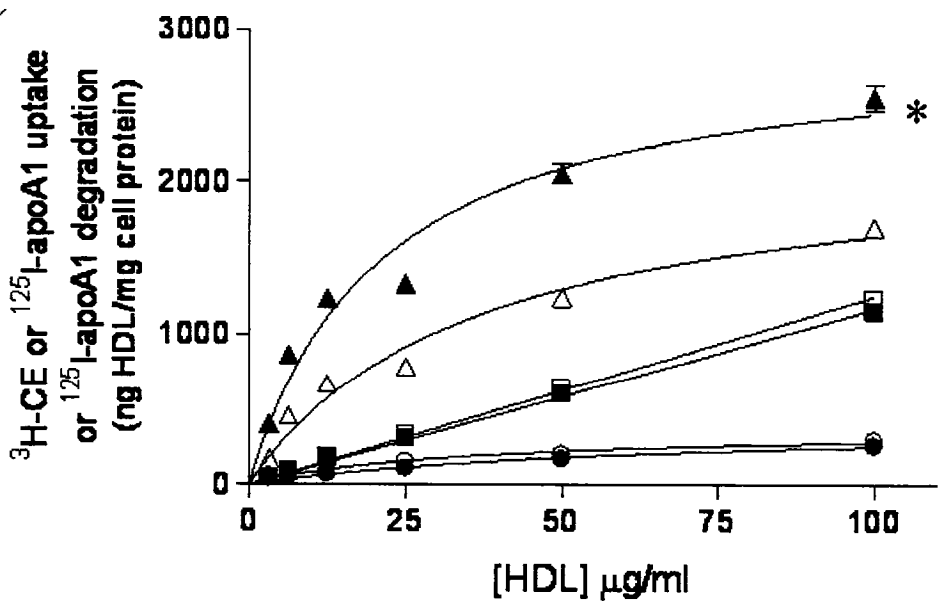
A


Human plasma
Ad-CETP
Ad-Luciferase
+ive control
Ad-CETP
Ad-Luciferase
75 kDa
50 kDa
cell lysate
IP

B


^{3}H-CE or ^{125}I-apoA1 uptake
or ^{125}I-apoA1 degradation
(ng HDL/mg cell protein)
3000
2000
1000
0
0
25
50
75
100
[HDL] µg/ml
*

C


^{3}H-CE or ^{125}I-apoA1 uptake
or ^{125}I-apoA1 degradation
(ng HDL/mg cell protein)
3000
2000
1000
0
0
25
50
75
100
[HDL] µg/ml
*

viral dose-dependent increase in ^{3}H-CE cell-association as compared to ad-Luc control (20% and 65% increase, respectively). This effect began to saturate at 50 μg/ml of HDL and therefore further experiments used this concentration of HDL. For future experiments, the MOI of 75 was chosen because of the more robust effect on ^{3}H-CE uptake. The cell-association of ^{125}I-apoA-1 or the cell-mediated degradation of ^{125}I-apoA-1 was unchanged with ad-CETP expression compared to ad-Luc control cells, indicating that although CETP increases CE uptake, it does not alter HDL particle uptake demonstrating true selective uptake.

3.4.3 – CETP-mediated Selective Uptake Does Not Depend on CETP Secretion into the Media

This laboratory has shown previously that $CETP_{KD}$, when expressed endogenously by stable transfection of Cos7 cells, associates with the cell plasma membrane where it is thought to participate in the selective uptake of HDL-derived CE (Zha & McPherson, unpublished data). Although ad-$CETP_{KD}$ is poorly secreted (Figure 3.1A), it had equivalent cell uptake of HDL-derived ^{3}H-CE to the other wild-type constructs tested (ad-$CETP_{w/t}$ and ad-CFP-CETP) (**Figure 3.3A**).

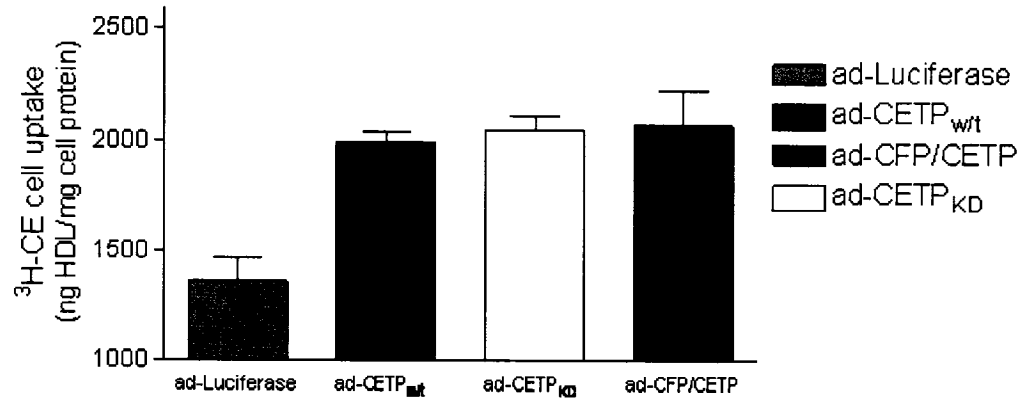
3.4.4 – CETP-mediated Selective Uptake is Independent of Other Lipoprotein Receptors

Ad-CETP expression in isolated primary mouse hepatocytes isolated from SR-B1 -/- animals resulted in increased selective uptake of HDL-derived ^{3}H-CE compared to ad-Luc-infected cells (**Figure 3.3B**). Although the absolute levels of uptake were lower in

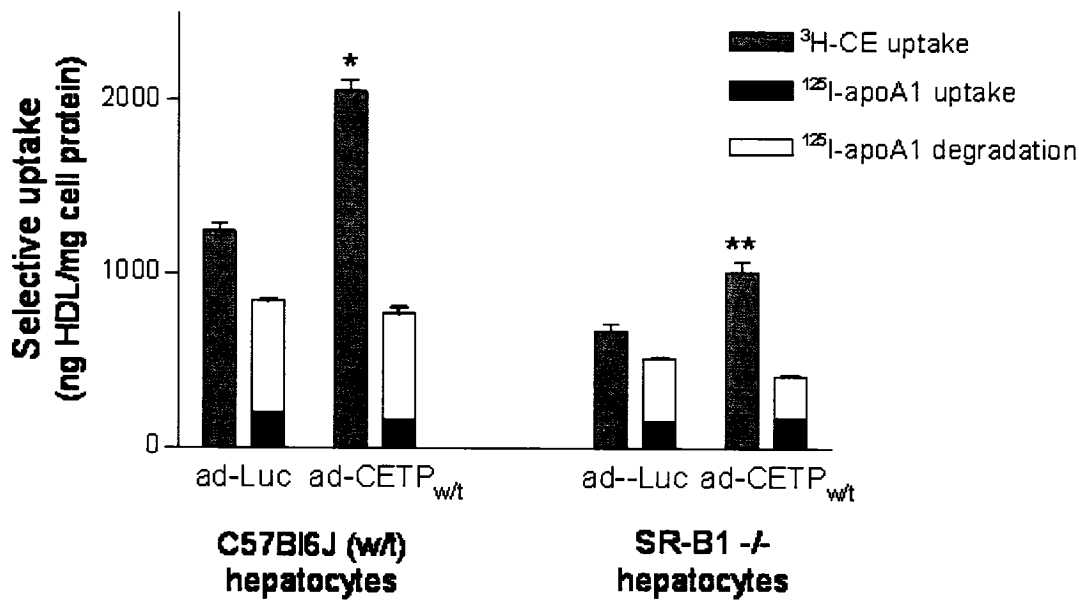
Figure 3.3. CETP-mediated selective uptake is independent of secretion, SR-B1 or LDLR family members.

A. Selective uptake assay (50 μg/ml ^{3}H-CE-HDL) on isolated primary mouse hepatocytes (C57Bl6J) infected with 2.175 x 10^{7} PFU/ml of each of the adenovirus constructs (ad-$CETP_{w/t}$, ad-$CETP_{KD}$ and ad-CFP-CETP) 2 days prior. * 2-tailed p values < 0.002 for all compared to luciferase. **B.** Selective uptake assay (50 μg/ml ^{3}H-CE-HDL or ^{125}I-apoA1-HDL) on isolated primary hepatocytes from a wild type (C57Bl6J) mouse and an SR-B1 -/- mouse infected with 2.175 x 10^{7} PFU/ml of each ad-$CETP_{w/t}$ 2 days prior. * 2 tailed p value < 0.0001; ** 2 tailed p value = 0.004. **C.** Selective uptake assay (50μg/ml ^{3}H-CE-HDL) on isolated primary mouse hepatocytes (C57Bl6J) infected with 2.175 x 10^{7} PFU/ml of each of ad-$CETP_{w/t}$ 2 days prior. Some of the cells were infected with a RAP-expressing adenovirus 24 hours prior to the selective uptake assay (2.175 x 10^{7} PFU/ml). * 2 tailed p value = 0.03; ** 2 tailed p value = 0.003; n.s. = not significant.

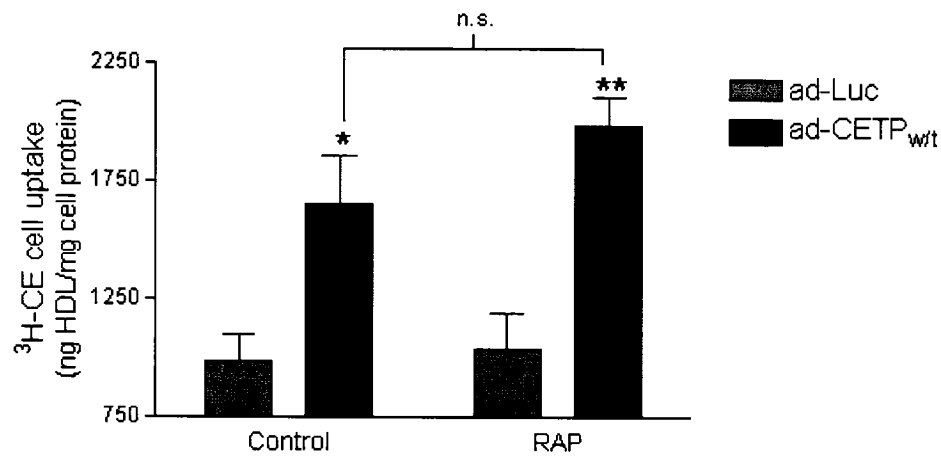
A


2500
2000
1500
1000
^{3}H-CE cell uptake
(ng HDL/mg cell protein)
ad-Luciferase
ad-CETP$_{w/t}$
ad-CFP/CETP
ad-CETP$_{KD}$
ad-Luciferase
ad-CETP$_{w/t}$
ad-CETP$_{KD}$
ad-CFP/CETP

B


Selective uptake
(ng HDL/mg cell protein)
2000
1000
0
*
**
^{3}H-CE uptake
^{125}I-apoA1 uptake
^{125}I-apoA1 degradation
ad-Luc
ad-CETP$_{w/t}$
ad--Luc
ad-CETP$_{w/t}$
C57Bl6J (w/t)
hepatocytes
SR-B1 -/-
hepatocytes

C


n.s.
2250
1750
1250
750
^{3}H-CE cell uptake
(ng HDL/mg cell protein)
*
**
ad-Luc
ad-CETP$_{w/t}$
Control
RAP

the ad-$CETP_{w/t}$-infected SR-B1-/- hepatocytes compared to wild-type hepatocytes, the increase in selective uptake was the same (approximately 65%). These results were similar with ad-$CETP_{KD}$ infection (data not shown).

Adenovirus-mediated expression of RAP, which has been shown to impede the expression, presentation and function of LDLR family members [118,128], did not affect CETP-mediated HDL-derived ^{3}H-CE uptake (**Figure 3.3C**). The results were similar when cells were infected with ad-$CETP_{KD}$ rather than ad-$CETP_{w/t}$ (data not shown).

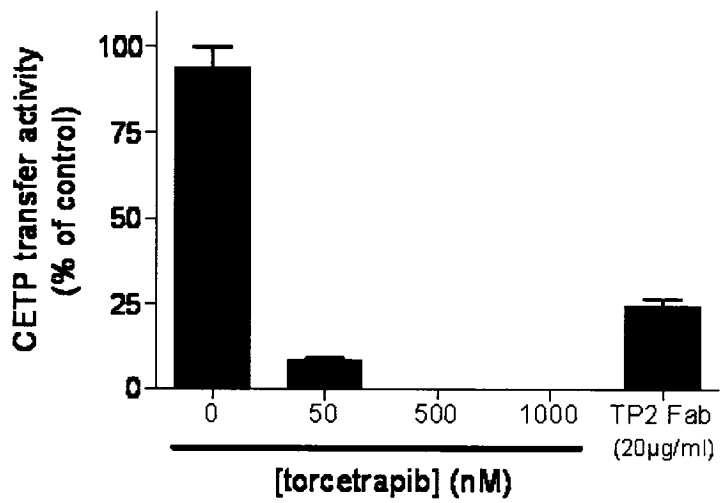
3.4.5 – CETP-mediated Selective Uptake is Independent of CETP Transfer Activity

To verify that torcetrapib could inhibit CETP transfer activity, a transfer activity assay was performed and results are shown in **Figure 3.4A**. Torcetrapib was very effective at inhibiting CETP transfer activity of 2 µl of fresh human plasma at extremely low concentrations. TP2 Fab, on the other hand, was only 75% effective at inhibiting CETP transfer activity. This laboratory has shown previously that TP2 Fab can inhibit CETP-mediated selective uptake by nearly 80% in adipocytes[53], but we show here complete inhibition of CETP-mediated ^{3}H-CE uptake (**Figure 3.4B**). This difference is likely due to different concentrations of CETP present on the adipocyte versus the mouse hepatocyte infected with ad-$CETP_{w/t}$ (2.175×10^7 PFU/well). Surprisingly, torcetrapib was not able to inhibit ^{3}H-CE uptake mediated by cell-associated CETP (**Figure 3.4B**), even at concentrations much higher than those shown to be effective for inhibiting transfer activity. Identical data was obtained using primary mouse hepatocytes infected with ad-$CETP_{KD}$ rather than ad-$CETP_{w/t}$ (data not shown).

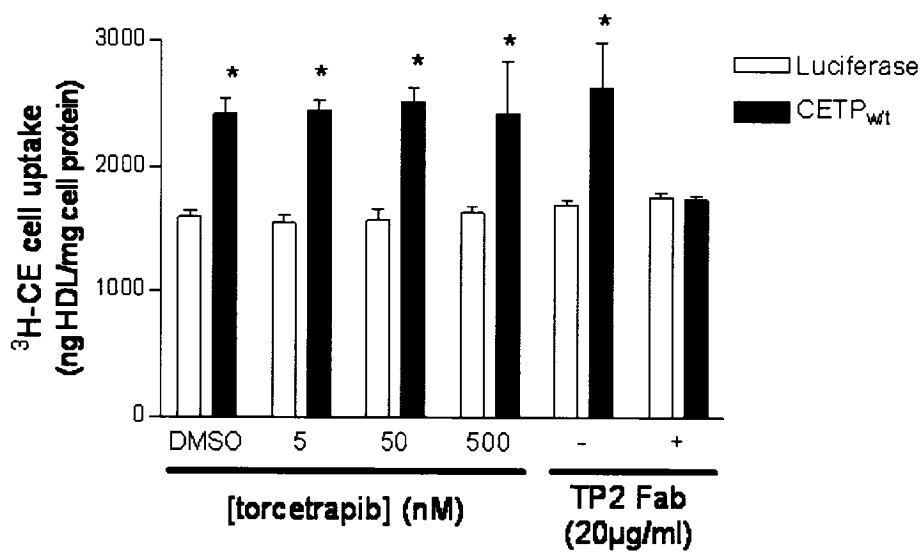
Figure 3.4. Selective uptake mediated by cell-associated CETP does not require transfer activity.

A. CETP transfer activity assay measuring transfer of HDL-derived ^{3}H-CE to LDL in the presence of increasing concentrations of the CETP inhibitor torcetrapib or TP2 Fab. Values are expressed as a percentage of the assay control sample (2 µl human plasma without torcetrapib). **B.** Selective uptake assay (50 µg/ml ^{3}H-CE-HDL) on isolated primary mouse hepatocytes (C57Bl6J) infected with 2.175 x 10^{7} PFU/ml of ad-CETP$_{w/t}$ 2 days prior. Assay was performed in the absence or presence of increasing amounts of torcetrapib (vehicle was DMSO) or TP2 Fab. * 2 tailed p values < 0.05 for all CETP values compared to luciferase.

A


CETP transfer activity
(% of control)
100
75
50
25
0
0
50
500
1000
TP2 Fab
(20µg/ml)
[torcetrapib] (nM)

B


^{3}H-CE cell uptake
(ng HDL/mg cell protein)
3000
2000
1000
0
*
Luciferase
CETP$_{wt}$
DMSO
5
50
500
-
+
[torcetrapib] (nM)
TP2 Fab
(20µg/ml)

3.4.6 – Exogenously Added Recombinant CETP Mediates HDL-derived ^{3}H-CE Selective Uptake, an Effect Which is Partially Inhibitable by Torcetrapib

Exogenously added recombinant CETP (rCETP) can mediate the selective uptake of HDL-derived CE in wild type (C57Bl6J) primary mouse hepatocytes. As shown in **Figure 3.5A**, rCETP, which was added to the media at a concentration of 0.48 μg/ml, increased the selective uptake of HDL-derived CE by 400%. Surprisingly, torcetrapib only partially inhibited rCETP-mediated selective uptake of ^{3}H-CE (70% increase). There were no significant differences observed in the cellular uptake or degradation of ^{125}I-apoA-1 in the rCETP treated cells in the presence or absence of torcetrapib. To exclude the possibility that there was insufficient inhibition of CETP transfer activity, rCETP(0.48 μg/ml) was added to the media with or without increasing concentrations of torcetrapib. As shown in **Figure 3.5B**, ^{3}H-CE uptake increased by 500-600% in mouse hepatocytes treated with rCETP, and incubation of cells with increasing concentrations of torcetrapib reduced the ability of exogenously added CETP to enhance ^{3}H-CE uptake by up to 80%. Identical results were obtained when rCETP, ^{3}H-CE HDL and torcetrapib were pre-incubated for 2 hours prior to being added to the primary hepatocytes (data not shown).

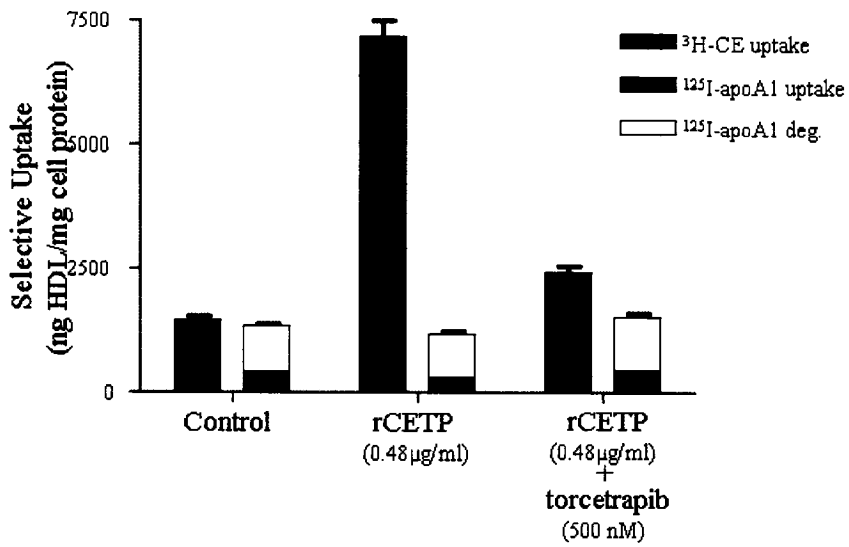
3.4.7 – Exogenously added rCETP can Associate with Primary Hepatocytes in Culture and Mediate Uptake of HDL-derived ^{3}H-CE

To further investigate the mechanism by which exogenously added rCETP mediates selective uptake, cells were incubated with 0.48 μg/ml of rCETP for 2 h at 4°C and 37°C and then probed for CETP by Western blot to determine whether CETP added

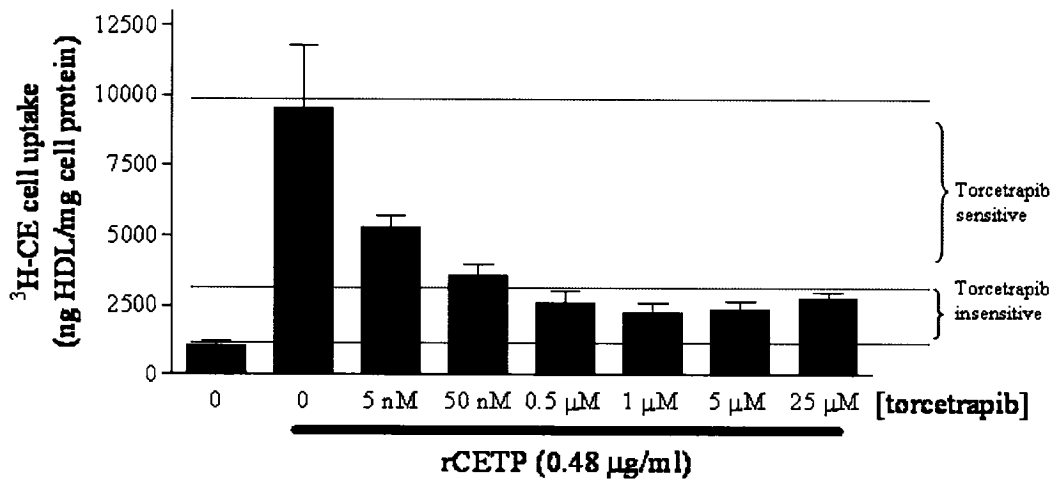
Figure 3.5. Exogenously added CETP mediates selective uptake in primary mouse hepatocytes by 2 distinct mechanisms.

A. Selective uptake assay on primary mouse hepatocytes (C57Bl6J) incubated with ^{3}H-CE HDL or ^{125}I-apoA-1 HDL (50 μg/ml), with or without the addition of recombinant CETP (rCETP) at a concentration of 0.48 μg/ml. * 2 tailed p value < 0.0001; ** 2 tailed p value = 0.0007. **B.** Selective uptake assay on primary mouse hepatocytes (C57Bl6J) incubated with ^{3}H-CE HDL (50 μg/ml), with or without the addition of recombinant CETP (rCETP) at a concentration of 0.48 μg/ml and 0 to 25 μM torcetrapib. 2-tailed p-values < 0.04 for all rCETP values compared to control.

A


7500
5000
2500
0
Selective Uptake
(ng HDL/mg cell protein)
3H-CE uptake
125I-apoA1 uptake
125I-apoA1 deg.
Control
rCETP
(0.48µg/ml)
rCETP
(0.48µg/ml)
+
torcetrapib
(500 nM)

B


12500
10000
7500
5000
2500
0
3H-CE cell uptake
(ng HDL/mg cell protein)
Torcetrapib
sensitive
Torcetrapib
insensitive
0
0
5 nM
50 nM
0.5 µM
1 µM
5 µM
25 µM
[torcetrapib]
rCETP (0.48 µg/ml)

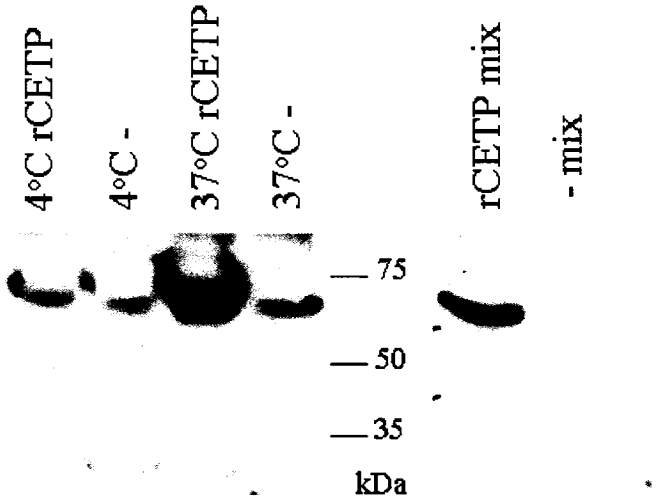
to the media could associate with the cells (**Figure 3.6A**). Western blot of cell lysates shows the presence of immunoreactive CETP in the cell lysate of the cells pre-incubated with CETP at 37°C. The light bands in the other lanes are likely cross-reactivity or overflow during filling. The media sample (control) represents approximately 12 ng of rCETP loaded on the gel and this band is approximately 50-75% the intensity of the 37°C cell lysate sample. Half the cell lysate was loaded onto the gel indicating that approximately 36-48 ng, or one-third of rCETP added to the media, became cell-associated at 37°C.

Cells preincubated with rCETP at 37°C for 2 hours prior to the selective uptake, in which no additional rCETP was added, had an increase in ^{3}H-CE uptake of 122% and this was insensitive to torcetrapib (**Figure 3.6B**). This is similar to the increase observed when cells were incubated with rCETP during the assay in the presence of torcetrapib (155% increase). Interestingly, the increase in ^{3}H-CE uptake of 122% is approximately 30% of that of cells incubated with rCETP during the selective uptake assay (406% increase) and this correlates to the 33% of the rCETP that associated with the cells during a 2-hour pre-incubation in another experiment; the small difference could be due to cell to cell differences from different hepatocyte preparations. The difference in ^{3}H-CE uptake observed between rCETP + torcetrapib incubation during the assay versus rCETP pre-incubation was significant (2-tailed p value = 0.001) and likely reflects the longer incubation time for CETP cell association (8 hour assay versus 2 hour pre-incubation).

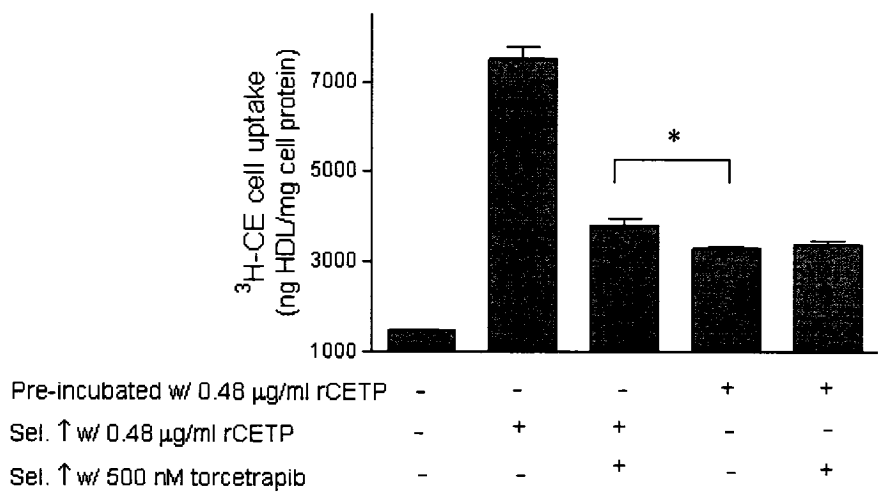
Figure 3.6. CETP added to media can associate with primary mouse hepatocytes and mediate selective uptake.

A. Western blot analysis of isolated primary mouse hepatocytes (C57Bl6J) incubated with rCETP (0.48 μg/ml) for 2 hours at 4°C or 37°C. Cells were washed twice with either cold or room temperature HBSS, respectively, following the 2 hour incubation and scraped down in SDS-loading buffer (denaturing/reducing) and half of the sample was used to detect CETP by Western analysis. A portion of the ligand mix was run on the same Western analysis as a positive control. **B.** Selective uptake assay (50 μg/ml ^{3}H-CE HDL) on primary mouse hepatocytes (C57Bl6J) that were pre-incubated or not with 0.48 μg/ml rCETP for 2 hours at 37°C. Cells were washed twice with room temperature HBSS prior to being subjected to a selective uptake assay, during which some wells received an additional 0.48 μg/ml of rCETP. Some cells also received 500 nM of torcetrapib. All results are significant compared to control (2-tailed p value < 0.0001). * 2-tailed p-value = 0.001.

A


4°C rCETP
4°C -
37°C rCETP
37°C -
rCETP mix
- mix
75
50
35
kDa

B


^{3}H-CE cell uptake
(ng HDL/mg cell protein)
7000
5000
3000
1000
*
Pre-incubated w/ 0.48 µg/ml rCETP - - - + +
Sel. ↑ w/ 0.48 µg/ml rCETP - + + - -
Sel. ↑ w/ 500 nM torcetrapib - - + - +

3.4.8 – ^{3}H-CE Uptake, Mediated by Exogenous rCETP, is Independent of Other Classical Selective Uptake Pathways

As shown in Figure 3.3 for ad-CETP, neither SR-B1 nor LDL receptor family members were required for exogenously added rCETP (0.48 μg/ml) to mediate uptake of HDL-derived ^{3}H-CE (**Figure 3.7A&B**). In both cases, torcetrapib only partially inhibited ^{3}H-CE uptake to similar magnitudes observed in Figure 3.5.

Additional studies were carried out in hepatocytes from SR-B1 deficient mice. As expected, the overall levels of HDL-CE uptake were lower in hepatocytes from mice lacking SR-B1. However, exogenous rCETP (0.48μg/ml) enhanced hepatocyte incorporation of ^{3}H-CE to a similar extent irrespective of SR-BI expression (**Figure 3.7B**).

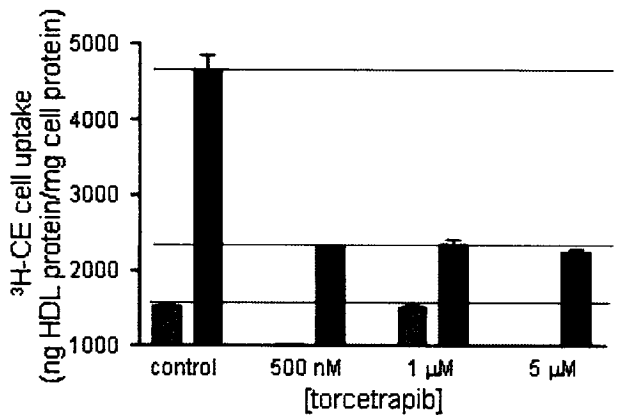
Hepatocytes were incubated with or without 30 μg/ml of receptor associated protein (RAP) to block the members of the LDLR family through competitive binding. Incubation with RAP did not affect rCETP-mediated selective uptake and both torcetrapib-sensitive and insensitive pathways were again observed (**Figure 3.7C**).

Finally, studies were carried out in the presence of heparin (100 U/ml), which disrupts binding of hepatic lipase and lipoprotein lipase to the HSPGs. Heparin treatment did not reduce the ability of exogenous rCETP to enhance hepatocyte accumulation of ^{3}H-CE nor alter the torcetrapib-sensitive or insensitive components of rCETP-mediated selective uptake (**Figure 3.7D**).

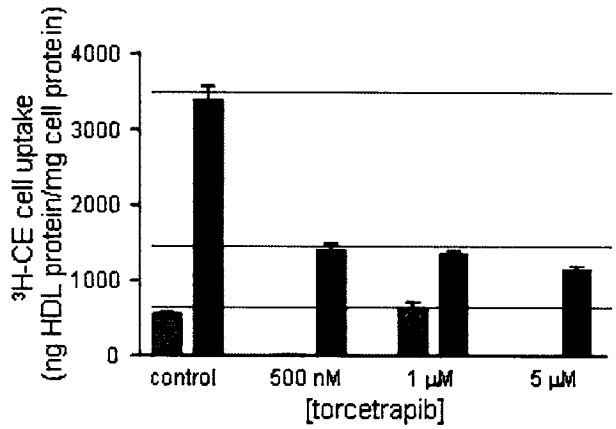
Fig 3.7. Selective uptake mediated by exogenously added CETP is independent of classical lipoprotein receptors.

Uptake of ^{3}H-CE HDL (50μg/ml) with or without the addition of CETP (0.48 μg/ml) and in the presence of 0 to 5 μM torcetrapib by primary mouse hepatocytes prepared from LDLR null mice (**A**), SR-BI-/- mice (**B**) or from wild type mice and incubated with RAP (30mg/ml) (**C**) or from wildtype mice and incubated with heparin (100 U/ml) (**D**). The 2-tailed p values < 0.05 for all CETP samples compared to control.

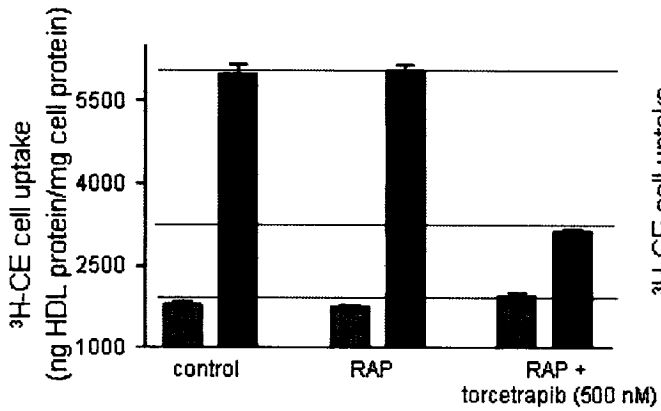
A. LDL-receptor deficient



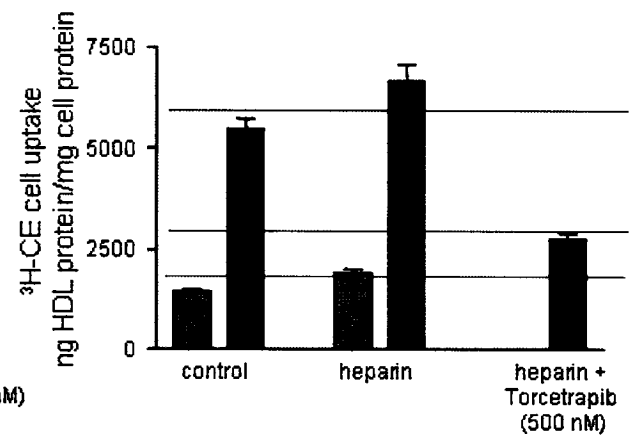
B. SR-B1 deficient



C. RAP (30 μg/ml)



C. Heparin (100 U/ml)



Legend

control

rCETP (0.48 μg/ml)

3.4.9 – Effects of Torcetrapib on Reversible and Irreversible Components of CETP Selective Uptake

The reversible and irreversible phases of CE-uptake at 2 and 4 hours in the presence or absence of torcetrapib (500 nM) were determined to further investigate the mechanism by which exogenous rCETP and cell-associated ad-CETP mediate selective uptake. As shown in **Figure 3.8A**, when primary hepatocytes were incubated with 0.48 μg/ml of rCETP, entry of ^{3}H-CE into a reversible compartment was completely inhibited by torcetrapib whereas accumulation of ^{3}H-CE in the irreversible compartment was partially but not completely inhibited by torcetrapib (**Figure 3.8B**). In contrast, when this experiment was carried out using hepatocytes infected with a moderate level of ad-$CETP_{w/t}$ (2.175×10^{7} PFU/ml) there was no effect of torcetrapib on entry of ^{3}H-CE into either the reversible or irreversible compartments (**Figure 3.8C**), further demonstrating that selective uptake mediated by cell-associated CETP (ad-CETP) is not susceptible to inhibition by torcetrapib.

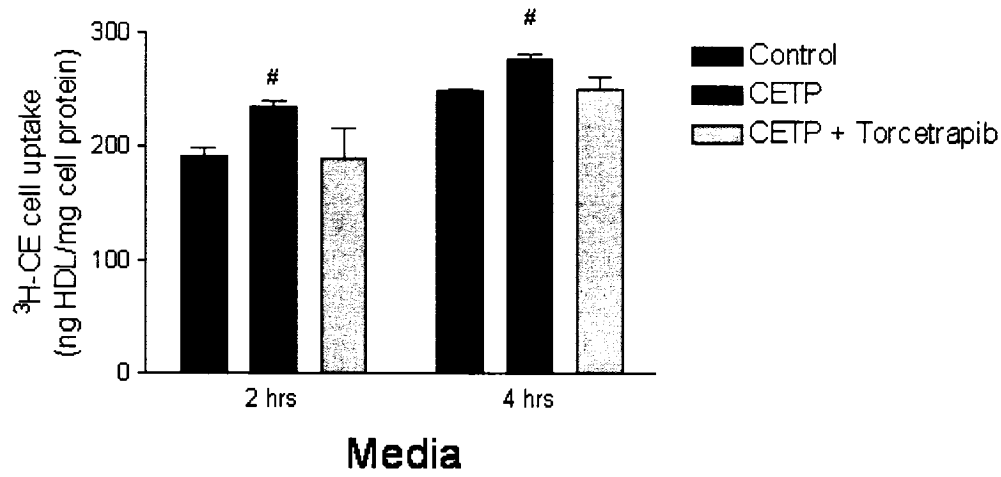
3.4.10 – Ad-$CETP_{w/t}$ and ad-$CETP_{KD}$ Expression in vivo

Wild type mice (C57Bl6J) at 6-8 weeks of age were injected with increasing titres of either ad-$CETP_{w/t}$ or ad-$CETP_{KD}$ via tail vein injection. Plasma samples from each mouse were obtained 6 days after the injections and subjected to Western blot analysis for CETP. As shown in **Figure 3.9A**, $CETP_{w/t}$ expression *in vivo* was dependent on the dose of adenovirus administered. Almost no CETP was detectable in the plasma of the 1.25×10^{8} PFU dose whereas the plasma sample from the 2.5×10^{8} PFU dose appeared to contain the same amount of immunoreactive CETP as human plasma. $CETP_{KD}$

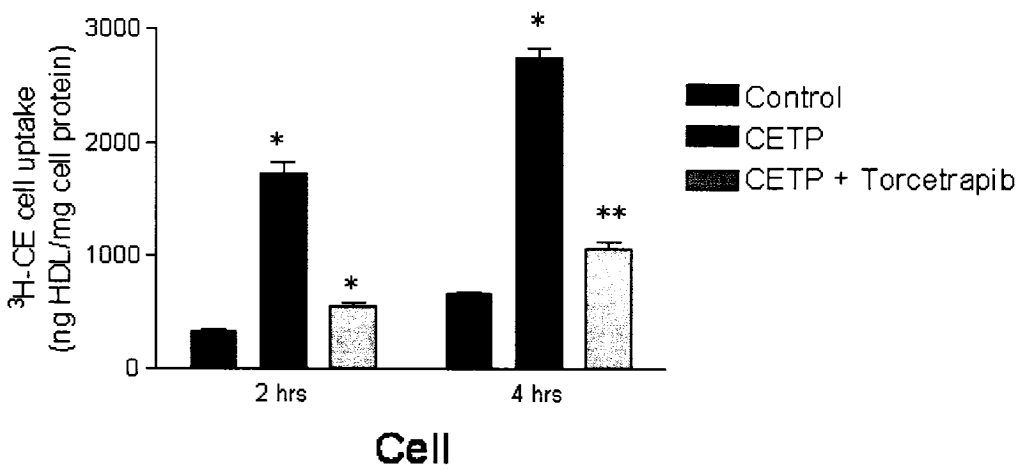
Figure 3.8. Effects of torcetrapib on reversible and irreversible components of selective uptake.

A and B. The reversible and irreversible components of selective uptake mediated by exogenously added rCETP (0.48 μg/ml). Hepatocytes were preincubated with 50μg/ml ^{3}H-CE-HDL for the indicated times and then incubated for 4 h with 400 μg/ml cold HDL. Cells were treated with either vehicle (DMSO) or torcetrapib (1 μM). Removal of ^{3}H-CE label from the reversible phase is represented by the media counts in A. The irreversible phase was determined by measuring the cell-associated counts following 4 washes with HBSS and is represented by the cell counts in B. **C.** The reversible and irreversible components of CETP selective uptake in mouse hepatocytes infected with ad-$CETP_{w/t}$ (2.175×10^{7} PFU/well). Cells were treated as above. # 2-tailed p value = 0.007; * 2-tailed p value < 0.0001; ** 2-tailed p value = 0.005.

A


300
200
100
0
^{3}H-CE cell uptake
(ng HDL/mg cell protein)
#
#
2 hrs
4 hrs
Media
Control
CETP
CETP + Torcetrapib

B


3000
2000
1000
0
^{3}H-CE cell uptake
(ng HDL/mg cell protein)
*
*
*
**
2 hrs
4 hrs
Cell
Control
CETP
CETP + Torcetrapib

C

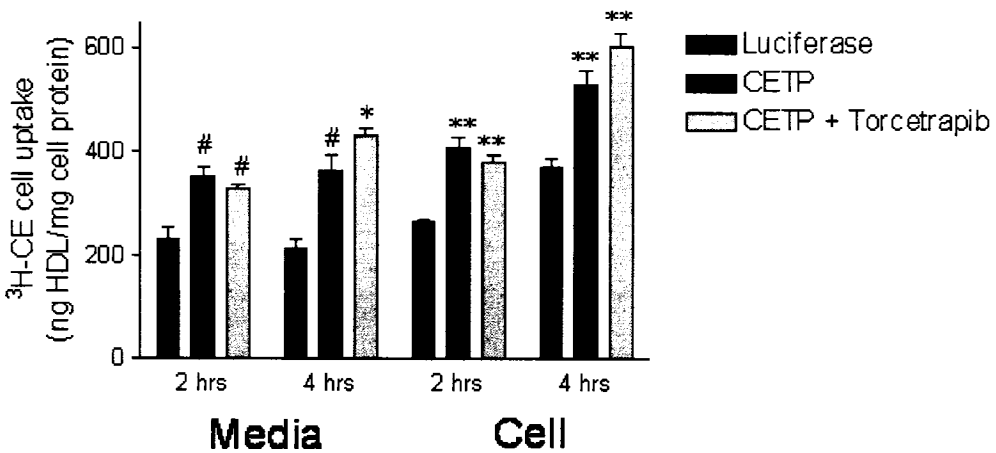
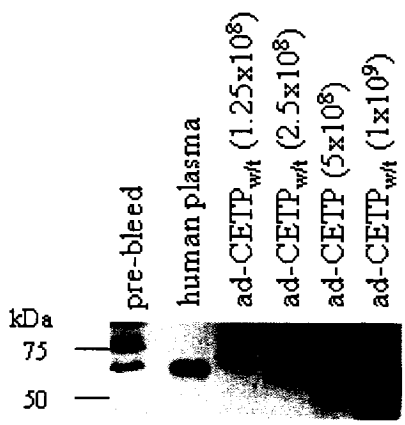

600
400
200
0
^{3}H-CE cell uptake
(ng HDL/mg cell protein)
#
#
#
*
**
**
**
**
2 hrs
4 hrs
2 hrs
4 hrs
Media
Cell
Luciferase
CETP
CETP + Torcetrapib

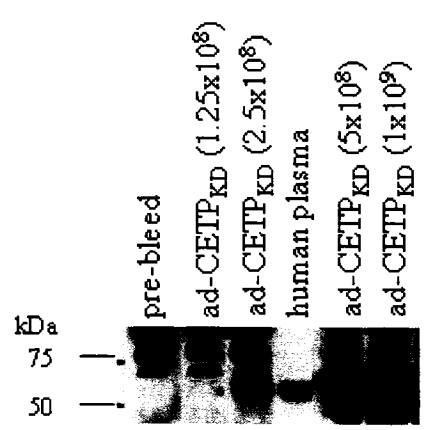
Figure 3.9. CETPw/t and $CETP_{KD}$ are secreted into plasma *in vivo*.

A&B. Western blot analysis of plasma from C57Bl6J mice 6 days after they were injected with various concentrations of either ad-$CETP_{w/t}$ (A) or ad-$CETP_{KD}$ (B). 2μl of human plasma was loaded as a control. **C&D.** CETP transfer activity assays of plasma samples from each of the ad-$CETP_{w/t}$ or ad-$CETP_{KD}$ mice 6 days after the injections. 2 μl of plasma was used from each mouse and compared to the transfer activity of 2μl of human plasma. Plasma samples were tested in duplicates and the mean +/- SEM is shown.

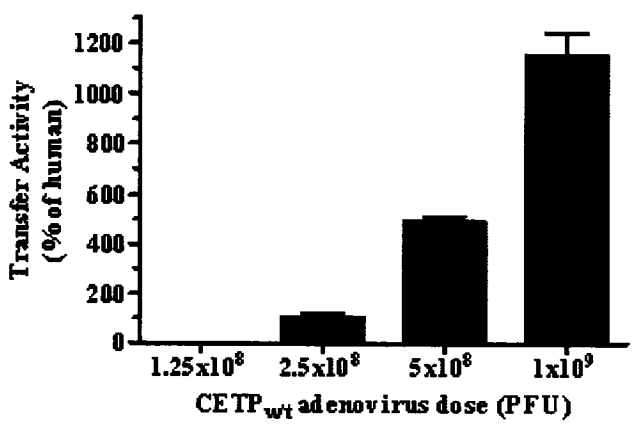
A


kDa
75
50
pre-bleed
human plasma
ad-CETP$_{wt}$ (1.25x10^8)
ad-CETP$_{wt}$ (2.5x10^8)
ad-CETP (5x10^8)
ad-CETP$_{wt}$ (1x10^9)

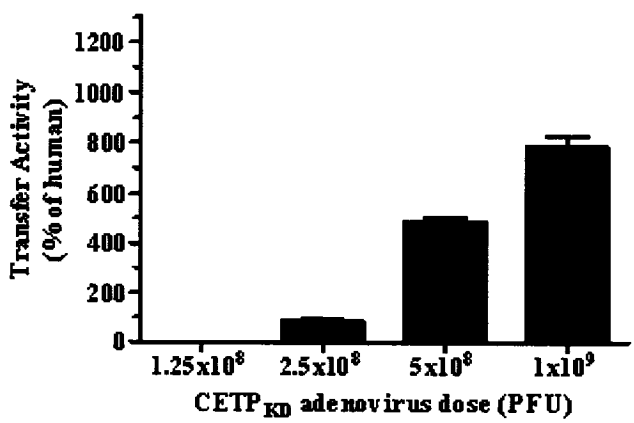
B


kDa
75
50
pre-bleed
ad-CETP$_{KD}$ (1.25x10^8)
ad-CETP$_{KD}$ (2.5x10^8)
human plasma
ad-CETP$_{KD}$ (5x10^8)
ad-CETP$_{KD}$ (1x10^9)

C


Transfer Activity
(% of human)
1200
1000
800
600
400
200
0
1.25x10^8
2.5x10^8
5x10^8
1x10^9
CETP$_{wt}$ adenovirus dose (PFU)

D


Transfer Activity
(% of human)
1200
1000
800
600
400
200
0
1.25x10^8
2.5x10^8
5x10^8
1x10^9
CETP$_{KD}$ adenovirus dose (PFU)

expression also showed dose dependent expression (**Figure 3.9B**), except that no CETP was detected in the plasma from the 1.25 x 10^8 dose. The plasma samples from day 6 of each of the mice were also subjected to a CETP transfer activity assay that measures the transfer of HDL-derived ^{3}H-CE to LDL (**Figure 3.9C & D**). The transfer activities measured correlated well with the amount of immunoreactive CETP detected by Western blot analysis. Surprisingly, $CETP_{KD}$ transfer activities correlated well with the mass detected by Western blot and it had comparable activity levels to $CETP_{w/t}$. This is contradictory to the previous reports stating limited transfer activity of $CETP_{KD}$ secreted into the media by transfected Cos7 cells [245].

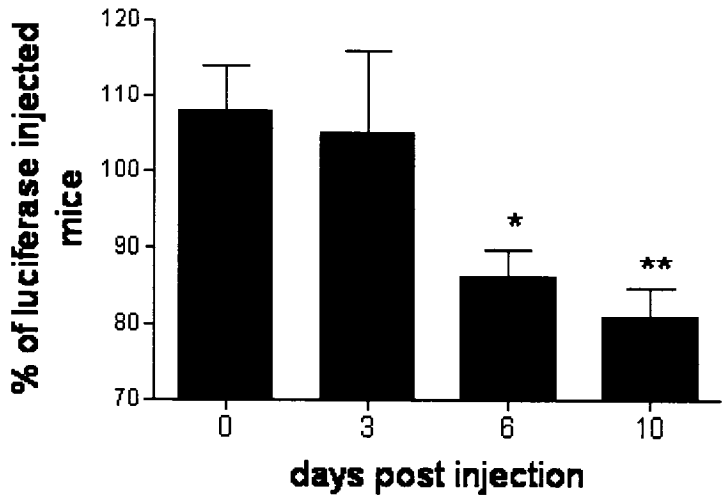
3.4.11 – $CETP_{KD}$ Expression in vivo Decreases Total Plasma Cholesterol and Total Cholesterol of Lipoprotein Fractions Corresponding to HDL

Based on the data in Figure 3.9, ad-$CETP_{KD}$ was injected at a dose of 1.25 x 10^8 PFU/mouse. At this dose, no CETP was detected in plasma by either Western blot or transfer activity assay thus reducing the potentially confounding effects of CETP secretion into plasma. In parallel, mice were also injected with 1.25 X 10^8 PFU of ad-Luc (n=4 for each) and all mice were sacrificed on day 10. Plasma total cholesterol was significantly lower in mice infected with ad-CETP on day 6 and 10 (20% and 35%, respectively) (**Figure 3.10A**). This was due to a selective decrease in cholesterol in particles within the HDL density range (**Figure 3.10B**).

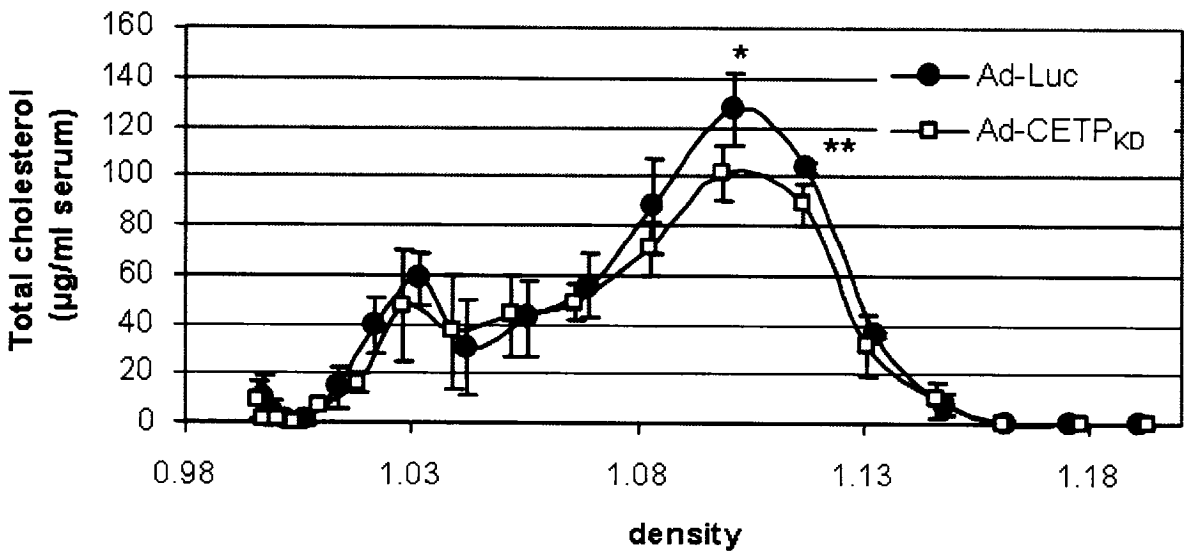
Figure 3.10. Effects of hepatic ad-CETP$_{KD}$ expression on selective uptake in vivo.

A. Plasma total cholesterol from mice injected with 1.25 X 10^8 PFU of ad- CETP$_{KD}$ compared to ad-Luc injected mice (n=4 for each). Mice were bled via saphenous vein puncture on days 3, 6 and 10 and plasma obtained following centrifugation. * 2-tailed p value = 0.013; ** 2-tailed p value = 0.006. **B.** Lipid profiles of ad-CETP$_{KD}$ and ad-Luc injected mice (1.25 x 10^7 PFU). The mice above were exsanguinated on day 10 and serum obtained following centrifugation. Total cholesterol was measured in each of the fractions separated by discontinuous density gradient ultracentrifugation. The 2 tailed p values for ad-CETP mice compared to ad-Luc mice at the HDL peak are * 0.047 and **0.033.

A


% of luciferase injected mice
120
110
100
90
80
70
*
**
0
3
6
10
days post injection

B


Total cholesterol (µg/ml serum)
160
140
120
100
80
60
40
20
0
*
**
Ad-Luc
Ad-CETP$_{KD}$
0.98
1.03
1.08
1.13
1.18
density

3.4.12 – In vivo $CETP_{KD}$ Expression Mediates Selective Uptake of HDL-derived ^{3}H-CE in vitro

Mice were injected with 1.25 X 10^8 PFU of either ad-Luc or ad-$CETP_{KD}$. Plasma total cholesterol was measured and results were similar to those presented in Figure 3.10A. The mice were sacrificed on day10 and primary hepatocytes were isolated from each. CETP was detectable by Western blot in the cell lysate of the hepatocytes from ad-$CETP_{KD}$ infected mice (**Figure 3.11A**). ^{3}H-CE cell uptake was increased by 25% as compared to hepatocytes from ad-Luc infected mice (**Figure 3.11B**), which correlates closely with the decrease in total plasma cholesterol and HDL-cholesterol seen in Figure 3.10.

3.4.13 – $CETP_{w/t}$ Mediates Hepatic Selective Uptake of HDL-derived CE in vivo

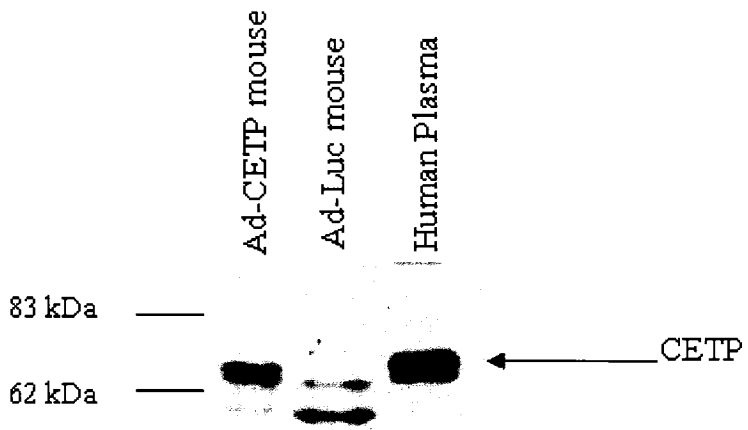
To confirm the functional importance of CETP in hepatocyte selective uptake of HDL-CE *in vivo*, we determined changes in plasma total cholesterol and lipoprotein sub-fractions 7 days post tail vein injection of ad-$CETP_{w/t}$ or ad-Luc. Ad-$CETP_{w/t}$ and ad-Luc were used at a dose of 2.5 x 10^8 PFU/mouse such that the expression level and activity in plasma is roughly that of humans as demonstrated in Figure 3.9. As shown in **Figure 3.12A**, hepatic expression of CETP at levels sufficient to elicit $CETP_{w/t}$ secretion into plasma, in a species lacking endogenous CETP, reduced plasma total cholesterol by 50% relative to luciferase control. Importantly, this decrease was entirely due to a decrease in cholesterol in particles in the HDL density range (**Figure 3.12B**).

Since torcetrapib was found to reduce selective uptake mediated by exogenously added rCETP *in vitro* (Figure 3.5A), studies were also carried out to determine the effects

Figure 3.11. Effects of *in vivo* hepatic expression of ad-$CETP_{KD}$ on selective uptake *in vitro*.

A. Western blot of the cell lysate of hepatocytes isolated from a mouse (C57Bl6J) infected with ad-$CETP_{KD}$ or ad-Luc (1.25×10^8 PFU) 10 days prior. **B.** Effects of *in vivo* expressed $CETP_{KD}$ on selective uptake *in vitro*. Primary hepatocytes isolated from mice 10 days post ad-Luc or ad-$CETP_{KD}$ infection (1.25×10^8 PFU) were incubated at 37°C for 8 h with ^{3}H-CE HDL or ^{125}I-apoA-1 HDL (50 µg/ml). ^{3}H-CE uptake in cells infected with ad-$CETP_{w/t}$ (▲) or ad-Luc (Δ); ^{125}I-apoA-I cell uptake in cells infected with ad-$CETP_{w/t}$ (●) or ad-Luc (○); ^{125}I-apoA-I degradation in cells infected with ad-$CETP_{w/t}$ (■) or ad-Luc (□). ** 2-tailed p value = 0.01.

A


Ad-CETP mouse
Ad-Luc mouse
Human Plasma
83 kDa
62 kDa
CETP

B

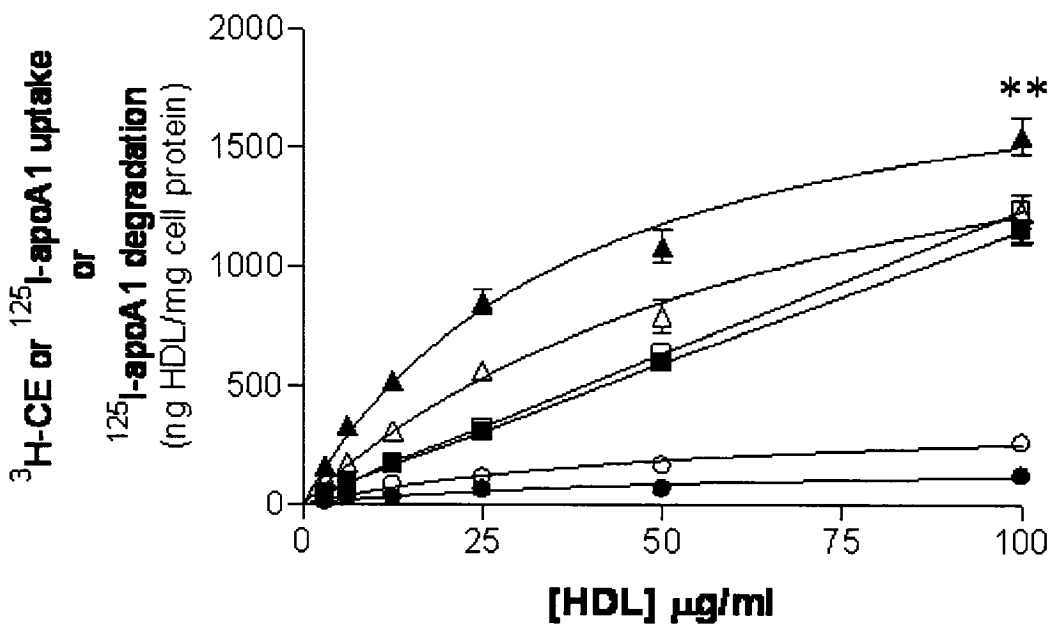
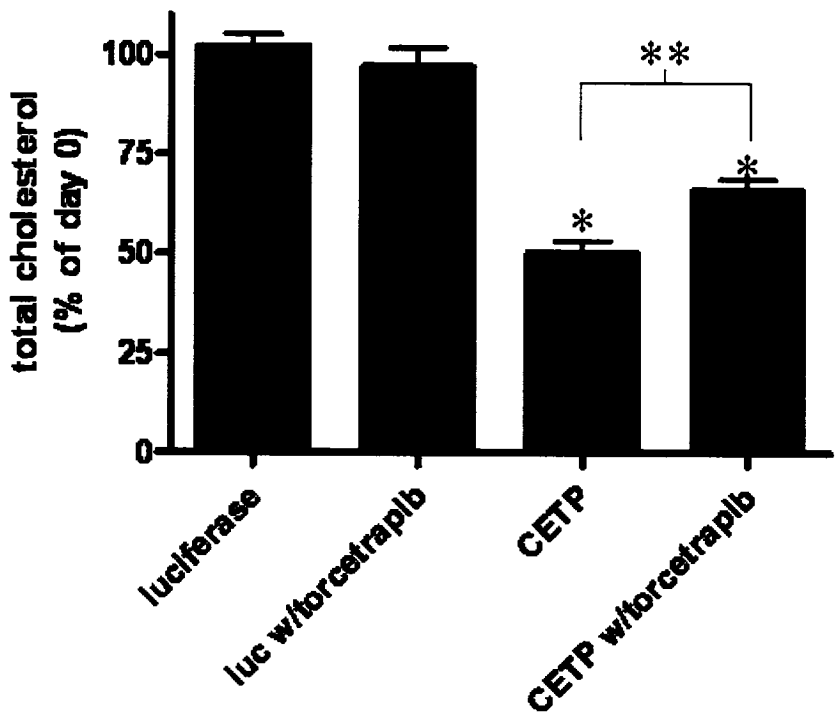

^{3}H-CE or ^{125}I-apoA1 uptake
or
^{125}I-apoA1 degradation
(ng HDL/mg cell protein)
2000
1500
1000
500
0
0
25
50
75
100
**
[HDL] µg/ml

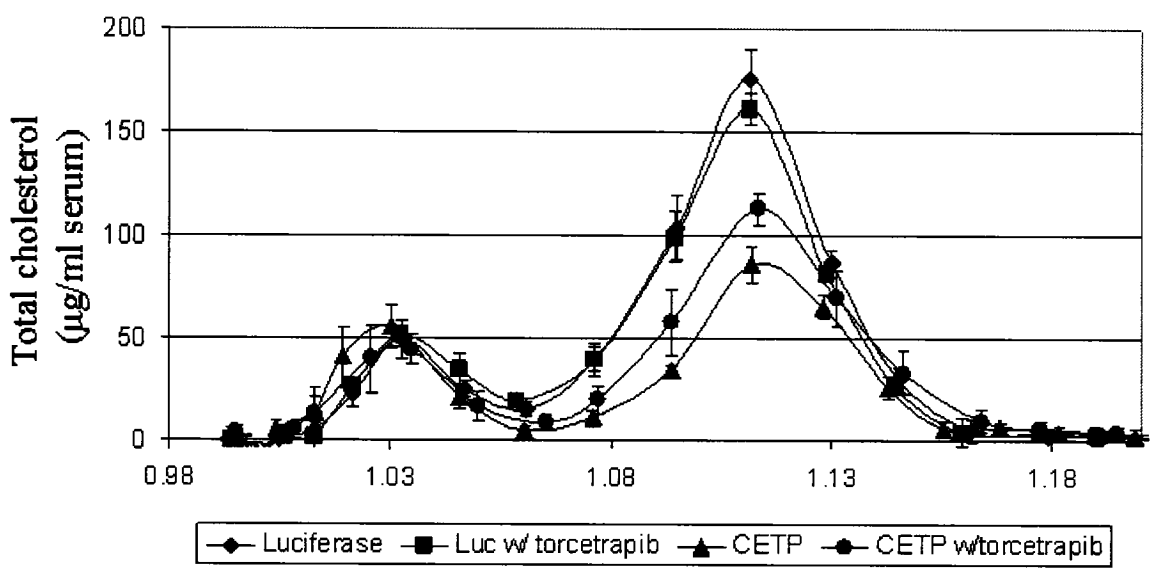
Figure 3.12. Effects of hepatic expression of ad-CETPw/t on selective uptake *in vivo*.

Mice were injected via tail vein with 2.5 x 10^8 PFU of ad-$CETP_{w/t}$ or ad-Luc and treated with daily IV injections of Intralipid® containing vehicle (DMSO) or torcetrapib X 7d. **A.** Fasting plasma total cholesterol from day 7 post injection. Data is presented as a % of the day 0 total plasma cholesterol measurements. * 2-tailed p value < 0.001 compared to luciferase control; ** 2-tailed p value = 0.02 comparing vehicle and torcetrapib treated ad-$CETP_{w/t}$ mice. **B.** Total cholesterol in lipoprotein fractions separated by discontinuous density gradient ultracentrifugation. The 2 tailed p values for ad-CETP mice compared to ad-Luc mice at the main peak (corresponding to HDL) are < 0.0001. The 2-tailed p value comparing vehicle and torcetrapib ad-CETP mice = 0.002.

A


total cholesterol (% of day 0)
100
75
50
25
0
**
*
*
luciferase
luc w/torcetrapib
CETP
CETP w/torcetrapib

B


Total cholesterol (µg/ml serum)
200
150
100
50
0
0.98
1.03
1.08
1.13
1.18
Luciferase
Luc w/ torcetrapib
CETP
CETP w/torcetrapib

of concomitant administration of the CETP inhibitor, torcetrapib, on CETP-mediated selective uptake *in vivo*. Torcetrapib is poorly absorbed by the oral route in rodents and thus mice received daily tail vein injections of vehicle (DMSO) or torcetrapib (0.125 mM) in 100 µl of Intralipid®. Torcetrapib treated mice had slightly smaller decreases in total plasma cholesterol and in total serum cholesterol in lipoprotein particles of the HDL density range (**Figure 3.12A and B**). Overall, ad-$CETP_{w/t}$ expression acutely reduced total cholesterol (mainly in particles in the HDL density range) by 50% in mice not treated with torcetrapib and by 33% in animals subjected to concomitant torcetrapib treatment.

3.5 – Discussion

The studies presented here are the first to demonstrate that hepatocyte CETP directly mediates the selective uptake of HDL-derived cholesteryl esters. An increase in cellular accumulation of ^{3}H-CE was observed in an ad-CETP viral dose-dependent manner (up to 100%) while cellular or degraded ^{125}I-apoA-1 did not change with ad-CETP expression. In contrast to the above experiments using adenovirus-driven expression of CETP, recombinant CETP added exogenously to cells at a concentration much lower than that normally found in human plasma (0.48 μg/ml versus ~1.8 μg/ml), caused a 500 to 700% increase in ^{3}H-CE selective uptake.

CETP's established role in the plasma is to mediate the transfer of HDL-CE to apoB containing lipoproteins. It was believed that the major role of CETP in the return of HDL-CE to the liver consisted of the selective transfer of CE to apoB lipoproteins for subsequent uptake into the cell by receptor-mediated endocytosis involving the LDLR or other members of the LDLR family. However, the present studies demonstrate that members of the LDLR family of receptors are not required for CETP-mediated selective uptake since this process occurs in LDLR-deficient hepatocytes and blocking of LRP and VLDLR by expression of RAP following adenovirus infection did not alter CETP-mediated uptake of HDL-derived ^{3}H-CE. The only well established receptor for selective uptake of HDL-CE and free cholesterol is SR-B1. It was proposed that CETP remodels the HDL particle such that it becomes a better substrate for SR-B1-mediated selective uptake [334]. To exclude this possibility, experiments were performed in primary mouse hepatocytes isolated from SR-B1 -/- animals. As expected, selective uptake of HDL-

derived ^{3}H-CE in luciferase infected cells was decreased in SR-B1 -/- hepatocytes compared to wild type (C57Bl6J) supporting a role for SR-B1 in this process. However, when CETP was expressed using adenovirus or added exogenously to cells, there was a similar increase in HDL-derived ^{3}H-CE selective uptake irrespective of SR-B1 expression.

To exclude a role for other members of the LDL receptor gene family, we incubated cells with 30 μg/ml of RAP to competitively block those receptors. Uptake of ^{3}H-CE was unaffected by RAP and both the torcetrapib-sensitive and insensitive components were observed. To exclude a role of other molecules identified to mediate selective uptake, cells were incubated with heparin thus preventing HL- and LPL-mediated selective uptake [51,52]. Heparin treatment did not reduce the ability of exogenous rCETP to enhance hepatocyte accumulation of ^{3}H-CE nor alter the torcetrapib-sensitive or insensitive components of rCETP-mediated selective uptake, ruling out a requirement for hepatic lipase or lipoprotein lipase in this process (Figure 3.7D). In fact, heparin increased the CE uptake in both control and rCETP treated cells. One explanation is the ability of heparin to release bound HL from the surface thus allowing more of the HDL to interact with other cell surface proteins not displaced by heparin, such as the rCETP. Thus, the torcetrapib-sensitive and insensitive components of exogenously added rCETP selective uptake do not require the LDLR or any other LDLR family member, SR-B1, HL or LPL. These studies demonstrate that in hepatocytes, as in adipocytes, CETP directly mediates selective uptake independently of known lipoprotein receptors.

To gain further insight into the mechanism of CETP-mediated selective uptake, ad-CETP$_{w/t}$ and ad-CETP$_{KD}$ selective uptake were compared. Secretion of CETP into the

media, which is severely impaired in the case of ad-$CETP_{KD}$, was not required for CETP to mediate the uptake of HDL-derived ^{3}H-CE.

Using the CETP inhibitor torcetrapib as a tool, selective uptake mediated by CETP expressed at a low dose such that CETP was not detectable in the media over the time frame of the assay, was examined. Under these conditions, cell-associated CETP increased selective uptake irrespective of torcetrapib treatment, even though the torcetrapib dose was sufficiently high to completely prevent CE transfer [287]. In contrast, the Fab fragment of the anti-CETP monoclonal antibody, TP2, completely blocked selective uptake in hepatocytes. Although TP2 Fab completely blocked selective uptake, it was only partially capable of inhibiting transfer activity, whereas torcetrapib completely inhibited transfer activity but has no effect on selective uptake mediated by cell-associated CETP. TP2 inhibition of selective uptake demonstrates that the C terminus of CETP is required for selective uptake but the lack of effect of torcetrapib confirms that this effect is not contingent on inhibition of transfer activity.

In other studies, torcetrapib was used to examine exogenous rCETP-mediated selective uptake, and up to 80% of rCETP-mediated selective uptake was inhibited by torcetrapib. The rCETP-mediated ^{3}H-CE uptake that remained despite torcetrapib treatment, corresponded to an increase in ^{3}H-CE uptake of approximately 70-155% versus control. Notably, this torcetrapib-independent component of selective uptake in the presence of exogenous CETP was observable even at concentrations far exceeding the clinically effective dose [287,319]. The torcetrapib-insensitive fraction of selective uptake does not appear to be due to inaccessibility of CETP to torcetrapib since preincubation of HDL and CETP with torcetrapib for 2 hours prior to the assay failed to reduce the

fraction of selective uptake not inhibitable by torcetrapib. These data suggest that two distinct mechanisms are responsible for rCETP-mediated selective uptake: one that is torcetrapib-sensitive and the other torcetrapib-insensitive. Furthermore, rCETP added to the media of primary hepatocytes associated with the cells and mediated the uptake of HDL-derived ^{3}H-CE irrespective of torcetrapib suggesting that the torcetrapib-insensitive component of rCETP-mediated selective uptake represents CETP which has become cell associated and functions similarly to CETP endogenously expressed by low dose ad-CETP.

To further investigate the mechanism by which exogenous rCETP and cell-associated ad-CETP mediate selective uptake, the reversible and irreversible phases of CE-uptake at 2 and 4 hrs were measured in the presence or absence of torcetrapib. Selective uptake of HDL-derived CE is characterized by its entry into reversible and irreversible compartments[49,337]. The reversible compartment is proposed to include CE that has entered the plasma membrane and remains accessible to extraction by extracellular, unlabeled HDL [49]. This reversible pool attains a plateau value after 2 h, possibly because the plasma membrane has a limited capacity to store CE or because the incorporation of CE into the plasma membrane is in equilibrium with the transfer out of this compartment. The plasma membrane CE is subsequently transferred to an irreversible compartment as it is internalized and becomes inaccessible to extraction by extracellular, unlabeled HDL [338]. Torcetrapib has been shown to increase the association of CETP with its lipoprotein substrates creating a non-functional complex [287], which would be expected to impair the ability of CETP to shuttle CE directly to the plasma membrane and this could account for the torcetrapib-sensitive component of CETP-

mediated selective uptake. In contrast, the torcetrapib-insensitive pathway may represent CETP which has become cell associated. Neither the reversible nor irreversible phases of selective uptake mediated by cell-associated CETP were affected by torcetrapib.

Based on the data presented above and previous published studies in adipocytes [54], the following model of CETP-mediated selective uptake in the hepatocyte is proposed, in which 2 mechanisms are functioning simultaneously. First, circulating CETP mediates selective uptake by shuttling CE directly from HDL to the plasma membrane. It has been previously proposed that CETP may transfer lipids to particular membrane structures such as microvilli or protrusions that have a high curvature, comparable to a lipoprotein at the molecular level [54]. This portion of selective uptake depends on CETP transfer activity and thus is inhibited by torcetrapib. Second, cell-associated CETP, either endogenously expressed CETP that is not released by the cell during secretion or circulating CETP re-associating with the cell, mediates the transient fusion of HDL with the cell membrane allowing transfer of CE into the membrane by a concentration gradient. CETP-mediated HDL particle fusion has been demonstrated previously [339]. It is possible that a similar fusion process is occurring between the phospholipid bilayer of the plasma membrane and HDL phospholipids. This fusion process however must be transient since no binding of particles (^{125}I-apoA-1-HDL) was observed at 0°C in primary mouse hepatocytes when tested. Furthermore, this process requires the C-terminal amino acids of CETP since it can be blocked by TP2 Fab but does not require transfer activity since it is unaffected by torcetrapib.

To establish the physiological significance of CETP-mediated selective uptake, the effect of hepatic CETP expression *in vivo* was examined in mice. Recombinant

adenoviruses infect mouse hepatocytes very well *in vitro* and *in vivo*. It has been demonstrated that greater than 99% of recombinant adenoviruses injected into mice via tail vein injection are targeted to the liver [340]. The cellular uptake of the adenovirus is mediated by coxsackievirus receptor binding [341], which is highly expressed in murine hepatocytes [342]. The principal drawback of first generation recombinant adenoviruses is that they may elicit non-specific effects on HDL metabolism due to the immune response that results from the viral protein expression. To control for these potentially confounding effects, a firefly luciferase reporter adenovirus (ad-Luc) in the same Ad5 gene as that in the CETP transgenes, was used as an injection control. Additionally, experiments were carried out at early time points after the adenovirus injections (0-10 days) when the immune responses would be less significant.

In preliminary experiments, serial doses of ad-$CETP_{w/t}$ and ad-$CETP_{KD}$ were injected into mice via the tail vein. The result was a dose-dependent expression of CETP as demonstrated by CETP detected in the plasma. The CETP transfer activity correlated well with the amount of immunoreactive CETP detected in the plasma.

The effect of a low dose injection of ad-$CETP_{KD}$, in which no CETP was detected in the plasma by Western blot or activity assay, was decreased total plasma cholesterol over the 10 days, with a drop specifically in the density range of HDL.

When hepatocytes were isolated from a different mouse treated in exactly the same manner, CETP expression was detected in the cell lysate. CETP expression, resulting from *in vivo* injection of adenovirus 10 days previous, was sufficient to mediate the selective uptake of HDL-derived ^{3}H-CE. The 25% increase in selective uptake

correlated well with the 35% drop in plasma total cholesterol and HDL total cholesterol observed *in vivo*.

Finally, the effects of torcetrapib on CETP-mediated selective uptake of HDL-CE were determined under conditions designed to achieve plasma levels of CETP expression similar to those in healthy human subjects. Using a dose of ad-$CETP_{w/t}$ or ad-Luc (2.5 x 10^8 PFU) that yielded approximately 100% of human plasma CETP mass (detected by Western blot) and activity, changes in plasma total cholesterol and lipoprotein sub-fractions 7 days post tail vein injection were determined. This model allowed the measurement of both pools of CETP; (i) a cell associated pool which corresponds to the cell-associated CETP in the *in vitro* experiments, and (ii) a plasma pool of secreted CETP which corresponds to exogenously added rCETP in the *in vitro* experiments. CETP expression resulted in a 50% reduction in total plasma cholesterol compared to ad-Luc injected controls and this decrease was entirely due a decrease in cholesterol in particles in the HDL density range (Figure 3.12), consistent with an acute effect of hepatic CETP expression on selective uptake *in vivo*. CETP expressing animals were treated with 0.125 mM torcetrapib. Assuming a 3 ml total blood volume, the final plasma concentration in the mouse would be approximately 4 μM which is several fold higher than those achieved with clinically effective doses of this agent (0.1-1μM) [287,319,343]. Consistent with the results of the *in vitro* experiments, torcetrapib was found to partially attenuate the ability of adenovirus-driven hepatocyte expressed CETP to mediate selective uptake as indicated by smaller decreases in total cholesterol in lipoprotein particles of the HDL density range compared to non-torcetrapib treated animals (Figure 3.11A and B).

Overall, these data suggest that CETP can mediate the selective uptake of HDL-derived CE *in vivo* and that this process is independent of transfer activity.

The evidence provided above using CETP expression in primary mouse hepatocytes supports the role of CETP as a direct mediator of selective uptake. Furthermore, the data demonstrating increased selective uptake *ex vivo* and decreased HDL total cholesterol *in vivo* following liver-specific CETP expression in mice supports a role for CETP in the selective uptake of HDL-CE *in vivo*. Unlike humans and several other species including the rabbit, mice do not express CETP and exhibit low plasma concentrations of apoB-containing lipoproteins. Although endogenous CETP has been shown to mediate selective uptake in adipose tissue *in vitro* [53], it is not known whether endogenous hepatic CETP could mediate selective uptake *in vivo* in humans. Interestingly, the composition of HDL in humans treated with torcetrapib differs from that associated with homozygous CETP deficiency. In torcetrapib-treated subjects, there was not the significant increase in the CE content of HDL noted in patients that are CETP-deficient [287], suggesting that CETP-mediated selective uptake of HDL-CE may in fact occur *in vivo* in humans although its overall importance remains to be determined. Clearly, in humans, the established role for CETP is the transfer of neutral lipids between HDL and apoB-containing lipoproteins in the plasma compartment. The potential atherogenicity of this process has resulted in the development of CETP inhibitors. The implication of the data presented here is that although the potentially atherogenic function of CETP is inhibited by torcetrapib, this agent has much smaller effects on the ability of CETP to mediate selective uptake.

CHAPTER 4 – CONCLUSIONS & FUTURE DIRECTIONS

Atherosclerosis is a complex disease with many contributing factors. This work has attempted to address potential mechanisms related to atherosclerosis through the study of two distinct biological pathways.

The first pathway investigated was the regulation of LRP gene expression. LRP is a molecule with wide tissue distribution and biological function and understanding its regulation at the molecular level is important for a number of disease processes. The studies presented here have demonstrated that LRP gene expression and function are upregulated by ligands for PPAR, specifically the PPARγ agonist rosiglitazone and the PPARα agonist fenofibric acid, in a cell type specific manner.

An important clinical observation made in patients on PPARγ agonist therapy is increased central adiposity due to the differentiation of preadipocytes into small mature adipocytes. This effect is beneficial for glycemic control since small adipocytes are more insulin sensitive as compared to large adipocytes and small fat cells can sequester and store large amounts of triglycerides and cholesterol. LRP may have several potential functions in adipocyte physiology. First, upregulation of LRP by PPARγ agonists could have a functional role in adipocyte differentiation. Second, LRP is an important receptor for TG rich remnant lipoproteins thereby assisting in the clearance of TG from the circulation and improving the insulin sensitivity of skeletal muscle and liver. An additional mechanism whereby LRP upregulation may alter the risk of atherosclerosis is via the hepatic clearance of triglyceride-rich lipoproteins. Furthermore, HDL levels are inversely related to TG-rich lipoproteins therefore strategies aimed to increase the clearance of TG-rich lipoproteins, such as upregulation of LRP gene expression in

adipocytes and hepatocytes, may be of benefit. More recently, LRP has been implicated directly in preventing the formation of atherosclerotic lesions through inhibition of PDGF-induced vascular smooth muscle cell proliferation and migration. LRP upregulation in vascular smooth muscle cells could be beneficial in attenuating atherosclerosis by further inhibiting PDGF signaling. Interestingly, the co-activator required for this process is apoE, which is itself up-regulated by PPAR agonists, thus supporting an overall beneficial role for PPAR agonists.

Future experiments in the laboratory are focused on determining factors responsible for the temporal regulation of LRP gene expression during differentiation of primary human adipocytes. By better understanding the differentiation-dependent expression of LRP, insights should be gained into its potential functions, possibly leading to the development of more specific pharmacological targets for upregulating LRP. In ongoing studies, the role of LRP in adipocyte differentiation is being elucidated using primary mouse adipocytes from LRP^{flox} mice. Using this model, the LRP gene is deactivated in isolated preadipocytes *in vitro* with cre-expressing adenovirus prior to induction of differentiation. If a difference in the ability to differentiate is established, further experiments will focus on the role of LRP as an endocytic receptor or a signaling receptor.

The second pathway studied was that of CETP-mediated selective uptake. These studies demonstrated for the first time that CETP could mediate the selective uptake of HDL-CE in the liver. This finding is of major physiological importance since selective uptake of HDL-CE is the final step in the reverse cholesterol transport pathway. Studies presented in this thesis demonstrate that CETP-mediated selective uptake is only partially

inhibited by the CETP inhibitor torcetrapib. Thus, the potentially atherogenic transfer activity of CE and TG between HDL and apoB-containing lipoproteins can be inhibited by torcetrapib without significantly impairing the potentially antiatherogenic process of HDL-CE selective uptake.

Ongoing studies in the laboratory are determining the effect of CETP transgene expression in the SR-B1-knockout background. Relevant to the above findings, that CETP can function in the liver in an analogous fashion to SR-B1, it will be of interest to determine whether CETP over-expression can attenuate the moderate atherosclerosis that develops in SR-B1-deficient mice fed a high fat diet.

Future experiments in the laboratory are focused on further defining the mechanism of CETP-mediated selective uptake of HDL-CE. As described above (3.3 Materials and Methods), we have generated a fluorescent CETP fusion protein by adding a eCFP tag (engineered to contain the proper CETP signal sequence) to the N-terminus of the mature CETP peptide. This protein was expressed at the correct molecular weight and secreted into the media (Figure 3.1). In addition, CFP-CETP was shown to mediate the uptake of ^{3}H-CE from HDL (Figure 3.3). Future studies will use live cell microscopy to explore intracellular trafficking of CETP and HDL-cholesterol.

REFERENCES

1. World Health Organization. World Health Report. 2003.

2. Heart and Stroke Foundation of Canada. The Growing Burden of Heart Disease and Stroke in Canada 2003. 2003. Ottawa, Canada.

3. Health Canada. Economic Burden of Illness in Canada, 1998. 2002. Ottawa, Canada.

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

5. Scott,J. 2004. Pathophysiology and biochemistry of cardiovascular disease. *Curr.Opin.Genet.Dev.* 14:271-279.

6. Rasouli,M.L., K.Nasir, R.S.Blumenthal, R.Park, D.C.Aziz, and M.J.Budoff. 2005. Plasma homocysteine predicts progression of atherosclerosis. *Atherosclerosis* 181:159-165.

7. Willerson,J.T. and P.M.Ridker. 2004. Inflammation as a cardiovascular risk factor. *Circulation* 109:II2-10.

8. Vaughan,D.E. 2005. PAI-1 and atherothrombosis. *J.Thromb.Haemost.* 3:1879-1883.

9. Schoen,F.J. 2005. Blood Vessels. *In* Robbins and Cotran Pathologic Basis of Disease. V.Kuman, A.K.Abas, and N.Fausto, editors. Elsevier Saunders, Philadelphia, Pennsylvania. 511-554.

10. Stary,H.C., A.B.Chandler, R.E.Dinsmore, V.Fuster, S.Glagov, W.Insull, Jr., M.E.Rosenfeld, C.J.Schwartz, W.D.Wagner, and R.W.Wissler. 1995. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 92:1355-1374.

11. Ross,R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801-809.

12. Stary,H.C. 1989. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 9:I19-I32.

13. Gimbrone,M.A., Jr. 1999. Endothelial dysfunction, hemodynamic forces, and atherosclerosis. *Thromb.Haemost.* 82:722-726.

14. Gustafsson,M. and J.Boren. 2004. Mechanism of lipoprotein retention by the extracellular matrix. *Curr.Opin.Lipidol.* 15:505-514.

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

16. Havel,R.J. and J.P.Kane. 1995. Introduction: Structure and Metabolism of Plasma Lipoproteins. *In* The metabolic and molecular bases of inherited disease. C.R.Scriver, A.L.Beaudet, W.S.Sly, and D.Valle, editors. McGraw-Hill, Inc., New York. 1841-1851.

17. Mosca,L. 2004. Therapy of Hyperlipidemia. *In* Cardiology. M.H.Crawford, J.P.DiMarco, and W.J.Paulus, editors. Mosby, Toronto, ON. 73-90.

18. Lange,Y., M.H.Swaisgood, B.V.Ramos, and T.L.Steck. 1989. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J.Biol.Chem.* 264:3786-3793.

19. Yeagle,P.L. 1989. Lipid regulation of cell membrane structure and function. *FASEB J.* 3:1833-1842.

20. Yeagle,P.L. 1991. Modulation of membrane function by cholesterol. *Biochimie* 73:1303-1310.

21. Repa,J.J. and D.J.Mangelsdorf. 2000. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu.Rev.Cell Dev.Biol.* 16:459-81.:459-481.

22. Cullis,P., D.B.Fenske, and M.J.Hope. 1996. Physical properties and functional roles of lipids in membranes. *In* Biochemistry of Lipids, Lipoproteins and Membranes. D.E.Vance and J.E.Vance, editors. Elsevier, New York. 1-11.

23. Bastiaanse,E.M., K.M.Hold, and L.A.Van der. 1997. The effect of membrane cholesterol content on ion transport processes in plasma membranes. *Cardiovasc.Res.* 33:272-283.

24. Grundy,S.M. 1983. Mechanism of cholesterol gallstones formation. *Semin.Liver Dis.* 3:97-111.

25. Rader,D.J. and H.H.Hobbs. 2005. Disorders of Lipoprotein Metabolism. *In* Harrison's Principles of Internal Medicine. D.L.Kasper, E.Braunwald, A.S.Fauci, S.L.Hauser, D.L.Longo, and J.L.Jameson, editors. McGraw-Hill, Toronto. 2286-2298.

26. Turley,S.D. and J.M.Dietschy. 2003. Sterol absorption by the small intestine. *Curr.Opin.Lipidol.* 14:233-240.

27. Grundy,S.M. 1983. Absorption and metabolism of dietary cholesterol. *Annu.Rev.Nutr.* 3:71-96.:71-96.

28. Altmann,S.W., H.R.Davis, Jr., L.J.Zhu, X.Yao, L.M.Hoos, G.Tetzloff, S.P.Iyer, M.Maguire, A.Golovko, M.Zeng, L.Wang, N.Murgolo, and M.P.Graziano. 2004.

Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* %20;303:1201-1204.

29. Garcia-Calvo,M., J.Lisnock, H.G.Bull, B.E.Hawes, D.A.Burnett, M.P.Braun, J.H.Crona, H.R.Davis, Jr., D.C.Dean, P.A.Detmers, M.P.Graziano, M.Hughes, D.E.Macintyre, A.Ogawa, K.A.O'neill, S.P.Iyer, D.E.Shevell, M.M.Smith, Y.S.Tang, A.M.Makarewicz, F.Ujjainwalla, S.W.Altmann, K.T.Chapman, and N.A.Thornberry. 2005. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc.Natl.Acad.Sci.U.S.A* 102:8132-8137.

30. Duan,L.P., H.H.Wang, and D.Q.Wang. 2004. Cholesterol absorption is mainly regulated by the jejunal and ileal ATP-binding cassette sterol efflux transporters Abcg5 and Abcg8 in mice. *J.Lipid Res.* 45:1312-1323.

31. Altmann,S.W., H.R.Davis, Jr., X.Yao, M.Laverty, D.S.Compton, L.J.Zhu, J.H.Crona, M.A.Caplen, L.M.Hoos, G.Tetzloff, T.Priestley, D.A.Burnett, C.D.Strader, and M.P.Graziano. 2002. The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption. *Biochim.Biophys.Acta* 1580:77-93.

32. Cai,L., E.R.Eckhardt, W.Shi, Z.Zhao, M.Nasser, W.J.de Villiers, and D.R.van der Westhuyzen. 2004. Scavenger receptor class B type I reduces cholesterol absorption in cultured enterocyte CaCo-2 cells. *J.Lipid Res.* 45:253-262.

33. Chang,C.C., N.Sakashita, K.Ornvold, O.Lee, E.T.Chang, R.Dong, S.Lin, C.Y.Lee, S.C.Strom, R.Kashyap, J.J.Fung, R.V.Farese, Jr., J.F.Patoiseau, A.Delhon, and T.Y.Chang. 2000. Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. *J.Biol.Chem.* 275:28083-28092.

34. Zannis,V.I., A.Chroni, K.E.Kypreos, H.Y.Kan, T.B.Cesar, E.E.Zanni, and D.Kardassis. 2004. Probing the pathways of chylomicron and HDL metabolism using adenovirus-mediated gene transfer. *Curr.Opin.Lipidol.* 15:151-166.

35. Verine,A., P.Salers, and J.Boyer. 1982. Effects of apoproteins C on lipoprotein lipase activity bound to rat fat cells. *Am.J.Physiol* 243:E175-E181.

36. Mahley,R.W. and Z.S.Ji. 1999. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J.Lipid Res.* 40:1-16.

37. Yang,L.Y., A.Kuksis, J.J.Myher, and G.Steiner. 1995. Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies. *J.Lipid Res.* 36:125-136.

38. Gibbons,G.F., D.Wiggins, A.M.Brown, and A.M.Hebbachi. 2004. Synthesis and function of hepatic very-low-density lipoprotein. *Biochem.Soc.Trans.* 32:59-64.

39. Murayama,T., M.Yokode, H.Horiuchi, H.Yoshida, H.Sano, and T.Kita. 2000. Overexpression of low density lipoprotein receptor eliminates apolipoprotein B100-containing lipoproteins from circulation and markedly prevents early atherogenesis in apolipoprotein E-deficient mice. *Atherosclerosis* 153:295-302.

40. Groen,A.K., R.P.Oude Elferink, H.J.Verkade, and F.Kuipers. 2004. The ins and outs of reverse cholesterol transport. *Ann.Med.* 36:135-145.

41. Gotto,A.M., Jr. and E.A.Brinton. 2004. Assessing low levels of high-density lipoprotein cholesterol as a risk factor in coronary heart disease: a working group report and update. *J.Am.Coll.Cardiol.* 43:717-724.

42. Asztalos,B.F. 2004. High-density lipoprotein metabolism and progression of atherosclerosis: new insights from the HDL Atherosclerosis Treatment Study. *Curr.Opin.Cardiol.* 19:385-391.

43. Brewer,H.B., Jr., A.T.Remaley, E.B.Neufeld, F.Basso, and C.Joyce. 2004. Regulation of plasma high-density lipoprotein levels by the ABCA1 transporter and the emerging role of high-density lipoprotein in the treatment of cardiovascular disease. *Arterioscler.Thromb.Vasc.Biol.* 24:1755-1760.

44. Lee,J.Y. and J.S.Parks. 2005. ATP-binding cassette transporter AI and its role in HDL formation. *Curr.Opin.Lipidol.* 16:19-25.

45. von Eckardstein,A., M.Hersberger, and L.Rohrer. 2005. Current understanding of the metabolism and biological actions of HDL. *Curr.Opin.Clin.Nutr.Metab Care* 8:147-152.

46. Lewis,G.F. and D.J.Rader. 2005. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ.Res.* 96:1221-1232.

47. Wang,N., D.Lan, W.Chen, F.Matsuura, and A.R.Tall. 2004. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc.Natl.Acad.Sci.U.S.A* 101:9774-9779.

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

49. Vassiliou,G., F.Benoist, P.Lau, G.N.Kavaslar, and R.McPherson. 2001. The Low Density Lipoprotein Receptor-related Protein Contributes to Selective Uptake of High Density Lipoprotein Cholesteryl Esters by SW872 Liposarcoma Cells and Primary Human Adipocytes. *J.Biol.Chem.* 276:48823-48830.

50. Vassiliou,G. and R.McPherson. 2004. A novel efflux-recapture process underlies the mechanism of high-density lipoprotein cholesteryl ester-selective uptake

mediated by the low-density lipoprotein receptor-related protein. *Arterioscler.Thromb.Vasc.Biol.* 24:1669-1675.

51. Brundert,M., J.Heeren, H.Greten, and F.Rinninger. 2003. Hepatic lipase mediates an increase in selective uptake of HDL-associated cholesteryl esters by cells in culture independent from SR-BI. *J.Lipid Res.* 44:1020-1032.

52. Seo,T., M.Al Haideri, E.Treskova, T.S.Worgall, Y.Kako, I.J.Goldberg, and R.J.Deckelbaum. 2000. Lipoprotein lipase-mediated selective uptake from low density lipoprotein requires cell surface proteoglycans and is independent of scavenger receptor class B type 1. *J.Biol.Chem.* 275:30355-30362.

53. Benoist,F., P.Lau, M.McDonnell, H.Doelle, R.Milne, and R.McPherson. 1997. Cholesteryl ester transfer protein mediates selective uptake of high density lipoprotein cholesteryl esters by human adipose tissue. *J.Biol.Chem.* 272:23572-23577.

54. Vassiliou,G. and R.McPherson. 2004. Role of cholesteryl ester transfer protein in selective uptake of high density lipoprotein cholesteryl esters by adipocytes. *J.Lipid Res.* 45:1683-1693.

55. Breslow,J.L. 2000. Genetics of lipoprotein abnormalities associated with coronary artery disease susceptibility. *Annu.Rev.Genet.* 34:233-254.:233-254.

56. Wilson,P.W., R.B.D'Agostino, D.Levy, A.M.Belanger, H.Silbershatz, and W.B.Kannel. 1998. Prediction of coronary heart disease using risk factor categories. *Circulation* 97:1837-1847.

57. Gordon,D.J., J.L.Probstfield, R.J.Garrison, J.D.Neaton, W.P.Castelli, J.D.Knoke, D.R.Jacobs, Jr., S.Bangdiwala, and H.A.Tyroler. 1989. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* 79:8-15.

58. Libby,P. 2005. Prevention and Treatment of Atherosclerosis. *In* Harrison's Principles of Internal Medicine. D.L.Kasper, E.Braunwald, A.S.Fauci, S.L.Hauser, D.L.Longo, and J.L.Jameson, editors. McGraw-Hill, Toronto. 1430-1433.

59. Navab,M., G.M.Anantharamaiah, S.Hama, D.W.Garber, M.Chaddha, G.Hough, R.Lallone, and A.M.Fogelman. 2002. Oral administration of an Apo A-I mimetic Peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. *Circulation* 105:290-292.

60. Navab,M., G.M.Anantharamaiah, S.T.Reddy, S.Hama, G.Hough, J.S.Frank, V.R.Grijalva, V.K.Ganesh, V.K.Mishra, M.N.Palgunachari, and A.M.Fogelman. 2005. Oral Small Peptides Render HDL Antiinflammatory in Mice and Monkeys and Reduce Atherosclerosis in ApoE Null Mice. *Circ.Res.* ..

61. Barter,P.J., P.W.Baker, and K.A.Rye. 2002. Effect of high-density lipoproteins on the expression of adhesion molecules in endothelial cells. *Curr.Opin.Lipidol.* 13:285-288.

62. Calabresi,L., M.Gomaraschi, and G.Franceschini. 2003. Endothelial protection by high-density lipoproteins: from bench to bedside. *Arterioscler.Thromb.Vasc.Biol.* 23:1724-1731.

63. Wadham,C., N.Albanese, J.Roberts, L.Wang, C.J.Bagley, J.R.Gamble, K.A.Rye, P.J.Barter, M.A.Vadas, and P.Xia. 2004. High-density lipoproteins neutralize C-reactive protein proinflammatory activity. *Circulation* 109:2116-2122.

64. Mineo,C. and P.W.Shaul. 2003. HDL stimulation of endothelial nitric oxide synthase: a novel mechanism of HDL action. *Trends Cardiovasc.Med.* 13:226-231.

65. Norata,G.D., E.Callegari, H.Inoue, and A.L.Catapano. 2004. HDL3 induces cyclooxygenase-2 expression and prostacyclin release in human endothelial cells via a p38 MAPK/CRE-dependent pathway: effects on COX-2/PGI-synthase coupling. *Arterioscler.Thromb.Vasc.Biol.* 24:871-877.

66. Turkoski,B.B. 2004. An ounce of prevention. Drugs used to treat hyperlipidemia (Part 1). *Orthop.Nurs.* 23:58-61.

67. Turkoski,B. 2004. An ounce of prevention. Drugs used to treat hyperlipidemia (Part 2). *Orthop.Nurs.* 23:184-189.

68. Carlson,L.A. 2005. Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *J.Intern.Med.* 258:94-114.

69. Nissen,S.E., T.Tsunoda, E.M.Tuzcu, P.Schoenhagen, C.J.Cooper, M.Yasin, G.M.Eaton, M.A.Lauer, W.S.Sheldon, C.L.Grines, S.Halpern, T.Crowe, J.C.Blankenship, and R.Kerensky. 2003. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA* 290:2292-2300.

70. Brewer,H.B., Jr. 2004. Increasing HDL Cholesterol Levels. *N.Engl.J.Med.* 350:1491-1494.

71. Goldstein,J.L., H.H.Hobbs, and M.S.Brown. 2001. Familial hypercholesteremia. *In* Metabolic and molecular bases of inherited disease. C.R.e.al.Scriver, editor. McGraw-Hill Publishing Co., New York, New York, USA. 2863-2913.

72. Herz,J. and D.K.Strickland. 2001. LRP: a multifunctional scavenger and signaling receptor. *J.Clin.Invest* 108:779-784.

73. Willnow,T.E., J.M.Moehring, N.M.Inocencio, T.J.Moehring, and J.Herz. 1996. The low-density-lipoprotein receptor-related protein (LRP) is processed by furin in vivo and in vitro. *Biochem.J.* 313:71-76.

74. Herz,J., R.C.Kowal, J.L.Goldstein, and M.S.Brown. 1990. Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *EMBO J.* 9:1769-1776.

75. Moestrup,S.K. and J.Gliemann. 1991. Analysis of ligand recognition by the purified alpha 2-macroglobulin receptor (low density lipoprotein receptor-related protein). Evidence that high affinity of alpha 2-macroglobulin-proteinase complex is achieved by binding to adjacent receptors. *J.Biol.Chem.* 266:14011-14017.

76. Huang,W., K.Dolmer, and P.G.Gettins. 1999. NMR solution structure of complement-like repeat CR8 from the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 274:14130-14136.

77. Strickland,D.K., M.Z.Kounnas, and W.S.Argraves. 1995. LDL receptor-related protein: a multiligand receptor for lipoprotein and proteinase catabolism. *FASEB J.* 9:890-898.

78. Strickland,D.K. and S.Ranganathan. 2003. Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. *J.Thromb.Haemost.* 1:1663-1670.

79. Herz,J. 2003. LRP: a bright beacon at the blood-brain barrier. *J.Clin.Invest* 112:1483-1485.

80. Mikhailenko,I., M.Z.Kounnas, and D.K.Strickland. 1995. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor mediates the cellular internalization and degradation of thrombospondin. A process facilitated by cell-surface proteoglycans. *J.Biol.Chem.* 270:9543-9549.

81. Kounnas,M.Z., F.C.Church, W.S.Argraves, and D.K.Strickland. 1996. Cellular internalization and degradation of antithrombin III-thrombin, heparin cofactor II-thrombin, and alpha 1-antitrypsin-trypsin complexes is mediated by the low density lipoprotein receptor-related protein. *J.Biol.Chem.* 271:6523-6529.

82. Kristensen,T., S.K.Moestrup, J.Gliemann, L.Bendtsen, O.Sand, and L.Sottrup-Jensen. 1990. Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the alpha 2-macroglobulin receptor. *FEBS Lett.* 276:151-155.

83. Meilinger,M., C.Gschwentner, I.Burger, M.Haumer, M.Wahrmann, L.Szollar, J.Nimpf, and M.Huettinger. 1999. Metabolism of activated complement component C3 is mediated by the low density lipoprotein receptor-related protein/alpha(2)-macroglobulin receptor. *J.Biol.Chem.* 274:38091-38096.

84. Bovenschen,N., R.C.Boertjes, G.van Stempvoort, J.Voorberg, P.J.Lenting, A.B.Meijer, and K.Mertens. 2003. Low density lipoprotein receptor-related protein and factor IXa share structural requirements for binding to the A3 domain of coagulation factor VIII. *J.Biol.Chem.* 278:9370-9377.

85. Neels,J.G., B.M.van den Berg, K.Mertens, H.ter Maat, H.Pannekoek, A.J.van Zonneveld, and P.J.Lenting. 2000. Activation of factor IX zymogen results in exposure of a binding site for low-density lipoprotein receptor-related protein. *Blood* 96:3459-3465.

86. Warshawsky,I., G.J.Broze, Jr., and A.L.Schwartz. 1994. The low density lipoprotein receptor-related protein mediates the cellular degradation of tissue factor pathway inhibitor. *Proc.Natl.Acad.Sci.U.S.A* 91:6664-6668.

87. Nykjaer,A., G.Bengtsson-Olivecrona, A.Lookene, S.K.Moestrup, C.M.Petersen, W.Weber, U.Beisiegel, and J.Gliemann. 1993. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds lipoprotein lipase and beta-migrating very low density lipoprotein associated with the lipase. *J.Biol.Chem.* 268:15048-15055.

88. Kounnas,M.Z., D.A.Chappell, H.Wong, W.S.Argraves, and D.K.Strickland. 1995. The cellular internalization and degradation of hepatic lipase is mediated by low density lipoprotein receptor-related protein and requires cell surface proteoglycans. *J.Biol.Chem.* 270:9307-9312.

89. Beisiegel,U., W.Weber, G.Ihrke, J.Herz, and K.K.Stanley. 1989. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* 341:162-164.

90. Kowal,R.C., J.Herz, K.H.Weisgraber, R.W.Mahley, M.S.Brown, and J.L.Goldstein. 1990. Opposing effects of apolipoproteins E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J.Biol.Chem.* 265:10771-10779.

91. Gotthardt,M., M.Trommsdorff, M.F.Nevitt, J.Shelton, J.A.Richardson, W.Stockinger, J.Nimpf, and J.Herz. 2000. Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. *J.Biol.Chem.* 275:25616-25624.

92. Trommsdorff,M., J.P.Borg, B.Margolis, and J.Herz. 1998. Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J.Biol.Chem.* 273:33556-33560.

93. Barnes,H., B.Larsen, M.Tyers, and G.P.van der. 2001. Tyrosine-phosphorylated low density lipoprotein receptor-related protein 1 (Lrp1) associates with the adaptor protein SHC in SRC-transformed cells. *J.Biol.Chem.* 276:19119-19125.

94. Barnes,H., E.J.Ackermann, and G.P.van der. 2003. v-Src induces Shc binding to tyrosine 63 in the cytoplasmic domain of the LDL receptor-related protein 1. *Oncogene* 22:3589-3597.

95. Yasuda,J., A.J.Whitmarsh, J.Cavanagh, M.Sharma, and R.J.Davis. 1999. The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol.Cell Biol.* 19:7245-7254.

96. Hofer,F., M.Gruenberger, H.Kowalski, H.Machat, M.Huettinger, E.Kuechler, and D.Blass. 1994. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proc.Natl.Acad.Sci.U.S.A* 91:1839-1842.

97. Kounnas,M.Z., R.E.Morris, M.R.Thompson, D.J.FitzGerald, D.K.Strickland, and C.B.Saelinger. 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J.Biol.Chem.* 267:12420-12423.

98. Kounnas,M.Z., R.D.Moir, G.W.Rebeck, A.I.Bush, W.S.Argraves, R.E.Tanzi, B.T.Hyman, and D.K.Strickland. 1995. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* 82:331-340.

99. Kounnas,M.Z., W.S.Argraves, and D.K.Strickland. 1992. The 39-kDa receptor-associated protein interacts with two members of the low density lipoprotein receptor family, alpha 2-macroglobulin receptor and glycoprotein 330. *J.Biol.Chem.* 267:21162-21166.

100. Willnow,T.E., A.Rohlmann, J.Horton, H.Otani, J.R.Braun, R.E.Hammer, and J.Herz. 1996. RAP, a specialized chaperone, prevents ligand-induced ER retention and degradation of LDL receptor-related endocytic receptors. *EMBO J.* 15:2632-2639.

101. Willnow,T.E., S.A.Armstrong, R.E.Hammer, and J.Herz. 1995. Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo. *Proc.Natl.Acad.Sci.U.S.A.* 92:4537-4541.

102. Andersen,O.M., L.L.Christensen, P.A.Christensen, E.S.Sorensen, C.Jacobsen, S.K.Moestrup, M.Etzerodt, and H.C.Thogersen. 2000. Identification of the minimal functional unit in the low density lipoprotein receptor-related protein for binding the receptor-associated protein (RAP). A conserved acidic residue in the complement-type repeats is important for recognition of RAP. *J.Biol.Chem.* 275:21017-21024.

103. Herz,J., U.Hamann, S.Rogne, O.Myklebost, H.Gausepohl, and K.K.Stanley. 1988. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J.* 7:4119-4127.

104. van der,Z.A., L.Stas, C.Hilleker, F.Van Leuven, K.W.van Dijk, L.Havekes, R.Frants, and M.Hofker. 1994. Genomic cloning of the mouse LDL receptor related protein/alpha 2-macroglobulin receptor gene. *Genomics* 23:256-259.

105. Nimpf,J., S.Stifani, P.T.Bilous, and W.J.Schneider. 1994. The somatic cell-specific low density lipoprotein receptor-related protein of the chicken. Close kinship to mammalian low density lipoprotein receptor gene family members. *J.Biol.Chem.* 269:212-219.

106. Yochem,J. and I.Greenwald. 1993. A gene for a low density lipoprotein receptor-related protein in the nematode Caenorhabditis elegans. *Proc.Natl.Acad.Sci.U.S.A* 90:4572-4576.

107. Herz,J., D.E.Clouthier, and R.E.Hammer. 1992. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation [published erratum appears in Cell 1993 May 7;73(3):428]. *Cell* 71:411-421.

108. Herz,J., D.E.Couthier, and R.E.Hammer. 1993. Correction: LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell.* 73:428.

109. Moestrup,S.K., J.Gliemann, and G.Pallesen. 1992. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* 269:375-382.

110. Tooyama,I., T.Kawamata, H.Akiyama, H.Kimura, S.K.Moestrup, J.Gliemann, A.Matsuo, and P.L.McGeer. 1995. Subcellular localization of the low density lipoprotein receptor-related protein (alpha 2-macroglobulin receptor) in human brain. *Brain Res.* 691:235-238.

111. Descamps,O., D.Bilheimer, and J.Herz. 1993. Insulin stimulates receptor-mediated uptake of apoE-enriched lipoproteins and activated alpha 2-macroglobulin in adipocytes. *J.Biol.Chem.* 268:974-981.

112. Schreibman,P.H. and R.B.Dell. 1975. Human adipocyte cholesterol. Concentration, localization, synthesis, and turnover. *J.Clin.Invest* 55:986-993.

113. Angel,A. and J.Farkas. 1974. Regulation of cholesterol storage in adipose tissue. *J.Lipid Res.* 15:491-499.

114. Zhang,H., P.H.Links, J.K.Ngsee, K.Tran, Z.Cui, K.W.Ko, and Z.Yao. 2004. Localization of low density lipoprotein receptor-related protein 1 to caveolae in 3T3-L1 adipocytes in response to insulin treatment. *J.Biol.Chem.* 279:2221-2230.

115. Herz,J. 1999. Low-density lipoprotein receptor-related protein. *In* Lipoproteins in Health and Disease. Betterridge DJ, Illingworth DR, and Shepherd J, editors. Arnold, London. 333-359.

116. Hussain,M.M., F.R.Maxfield, J.Mas-Oliva, I.Tabas, Z.S.Ji, T.L.Innerarity, and R.W.Mahley. 1991. Clearance of chylomicron remnants by the low density

lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J.Biol.Chem.* 266:13936-13940.

117. Ji,Z.S., H.L.Dichek, R.D.Miranda, and R.W.Mahley. 1997. Heparan sulfate proteoglycans participate in hepatic lipaseand apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. *J.Biol.Chem.* 272:31285-31292.

118. Willnow,T.E., Z.Sheng, S.Ishibashi, and J.Herz. 1994. Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist. *Science* 264:1471-1474.

119. Ishibashi,S., J.Herz, N.Maeda, J.L.Goldstein, and M.S.Brown. 1994. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc.Natl.Acad.Sci.U.S.A* 91:4431-4435.

120. Herz,J., S.Q.Qiu, A.Oesterle, H.V.DeSilva, S.Shafi, and R.J.Havel. 1995. Initial hepatic removal of chylomicron remnants is unaffected but endocytosis is delayed in mice lacking the low density lipoprotein receptor. *Proc.Natl.Acad.Sci.U.S.A* 92:4611-4615.

121. Veniant,M.M., C.H.Zlot, R.L.Walzem, V.Pierotti, R.Driscoll, D.Dichek, J.Herz, and S.G.Young. 1998. Lipoprotein clearance mechanisms in LDL receptor-deficient "Apo-B48- only" and "Apo-B100-only" mice. *J.Clin.Invest* 102:1559-1568.

122. Rohlmann,A., M.Gotthardt, R.E.Hammer, and J.Herz. 1998. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J.Clin.Invest* 101:689-695.

123. Martins,I.J., E.Hone, C.Chi, U.Seydel, R.N.Martins, and T.G.Redgrave. 2000. Relative roles of LDLr and LRP in the metabolism of chylomicron remnants in genetically manipulated mice. *J.Lipid Res.* 41:205-213.

124. Mortimer,B.C., D.J.Beveridge, I.J.Martins, and T.G.Redgrave. 1995. Intracellular localization and metabolism of chylomicron remnants in the livers of low density lipoprotein receptor-deficient mice and apoE- deficient mice. Evidence for slow metabolism via an alternative apoE- dependent pathway. *J.Biol.Chem.* 270:28767-28776.

125. Zeng,B.J., B.C.Mortimer, I.J.Martins, U.Seydel, and T.G.Redgrave. 1998. Chylomicron remnant uptake is regulated by the expression and function of heparan sulfate proteoglycan in hepatocytes. *J.Lipid Res.* 39:845-860.

126. Choi,S.Y. and A.D.Cooper. 1993. A comparison of the roles of the low density lipoprotein (LDL) receptor and the LDL receptor-related protein/alpha 2-macroglobulin receptor in chylomicron remnant removal in the mouse in vivo. *J.Biol.Chem.* 268:15804-15811.

127. Jackle,S., C.Huber, S.Moestrup, J.Gliemann, and U.Beisiegel. 1993. In vivo removal of beta-VLDL, chylomicron remnants, and alpha 2- macroglobulin in the rat. *J.Lipid Res.* 34:309-315.

128. Jong,M.C., V.E.Dahlmans, P.J.van Gorp, K.W.van Dijk, M.L.Breuer, M.H.Hofker, and L.M.Havekes. 1996. In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor- associated protein-sensitive pathway. *J.Clin.Invest* 98:2259-2267.

129. Linton,M.F., A.H.Hasty, V.R.Babaev, and S.Fazio. 1998. Hepatic apo E expression is required for remnant lipoprotein clearance in the absence of the low density lipoprotein receptor. *J.Clin.Invest* 101:1726-1736.

130. Beisiegel,U., W.Weber, and G.Bengtsson-Olivecrona. 1991. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proc.Natl.Acad.Sci.U.S.A* 88:8342-8346.

131. Wilsie,L.C. and R.A.Orlando. 2003. The low density lipoprotein receptor-related protein complexes with cell surface heparan sulfate proteoglycans to regulate proteoglycan-mediated lipoprotein catabolism. *J.Biol.Chem.* 278:15758-15764.

132. Rubinsztein,D.C., J.C.Cohen, G.M.Berger, D.R.van der Westhuyzen, G.A.Coetzee, and W.Gevers. 1990. Chylomicron remnant clearance from the plasma is normal in familial hypercholesterolemic homozygotes with defined receptor defects. *J.Clin.Invest* 86:1306-1312.

133. Schneider,W.J. and J.Nimpf. 2003. LDL receptor relatives at the crossroad of endocytosis and signaling. *Cell Mol.Life Sci.* 60:892-903.

134. Duilio,A., R.Faraonio, G.Minopoli, N.Zambrano, and T.Russo. 1998. Fe65L2: a new member of the Fe65 protein family interacting with the intracellular domain of the Alzheimer's beta-amyloid precursor protein. *Biochem.J.* 330:513-519.

135. Duilio,A., N.Zambrano, A.R.Mogavero, R.Ammendola, F.Cimino, and T.Russo. 1991. A rat brain mRNA encoding a transcriptional activator homologous to the DNA binding domain of retroviral integrases. *Nucleic Acids Res.* 19:5269-5274.

136. May,P., Y.K.Reddy, and J.Herz. 2002. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. *J.Biol.Chem.* 277:18736-18743.

137. Kinoshita,A., T.Shah, M.T.Tangredi, D.K.Strickland, and B.T.Hyman. 2003. The intracellular domain of the low density lipoprotein receptor-related protein (LRP) modulates transactivation mediated by APP and Fe65. *J.Biol.Chem.* ..

138. Orr,A.W., C.E.Pedraza, M.A.Pallero, C.A.Elzie, S.Goicoechea, D.K.Strickland, and J.E.Murphy-Ullrich. 2003. Low density lipoprotein receptor-related protein is a

calreticulin coreceptor that signals focal adhesion disassembly. *J.Cell Biol.* 161:1179-1189.

139. Yepes,M., M.Sandkvist, E.G.Moore, T.H.Bugge, D.K.Strickland, and D.A.Lawrence. 2003. Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. *J.Clin.Invest* 112:1533-1540.

140. Nassar,T., S.Akkawi, A.Shina, A.Haj-Yehia, K.Bdeir, M.Tarshis, S.N.Heyman, and A.A.Higazi. 2004. In vitro and in vivo effects of tPA and PAI-1 on blood vessel tone. *Blood* 103:897-902.

141. Wang,X., S.R.Lee, K.Arai, S.R.Lee, K.Tsuji, G.W.Rebeck, and E.H.Lo. 2003. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. *Nat.Med.* 9:1313-1317.

142. Lutz,C., J.Nimpf, M.Jenny, K.Boecklinger, C.Enzinger, G.Utermann, G.Baier-Bitterlich, and G.Baier. 2002. Evidence of functional modulation of the MEKK/JNK/cJun signaling cascade by the low density lipoprotein receptor-related protein (LRP). *J.Biol.Chem.* 277:43143-43151.

143. Loukinova,E., S.Ranganathan, S.Kuznetsov, N.Gorlatova, M.M.Migliorini, D.Loukinov, P.G.Ulery, I.Mikhailenko, D.A.Lawrence, and D.K.Strickland. 2002. Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function betwenn LRP and the PDGF. *J.Biol.Chem.* 277:15499-15506.

144. Boucher,P., P.Liu, M.Gotthardt, T.Hiesberger, R.G.Anderson, and J.Herz. 2002. Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low Density lipoprotein receptor-related protein in caveolae. *J.Biol.Chem.* 277:15507-15513.

145. Ishigami,M., D.K.Swertfeger, N.A.Granholm, and D.Y.Hui. 1998. Apolipoprotein E inhibits platelet-derived growth factor-induced vascular smooth muscle cell migration and proliferation by suppressing signal transduction and preventing cell entry to G1 phase. *J.Biol.Chem.* 273:20156-20161.

146. Swertfeger,D.K., G.Bu, and D.Y.Hui. 2002. Low density lipoprotein receptor-related protein mediates apolipoprotein E inhibition of smooth muscle cell migration. *J.Biol.Chem.* 277:4141-4146.

147. Boucher,P., M.Gotthardt, W.P.Li, R.G.Anderson, and J.Herz. 2003. LRP: role in vascular wall integrity and protection from atherosclerosis. *Science* 300:329-332.

148. Van Leuven,F., L.Stas, C.Hilliker, K.Lorent, L.Umans, L.Serneels, L.Overbergh, S.Torrekens, D.Moechars, and B.De Strooper. 1994. Structure of the gene (LRP1) coding for the human alpha 2-macroglobulin receptor lipoprotein receptor-related protein. *Genomics* 24:78-89.

149. Kutt,H., J.Herz, and K.K.Stanley. 1989. Structure of the low-density lipoprotein receptor-related protein (LRP) promoter. *Biochim.Biophys.Acta* 1009:229-236.

150. Gaeta,B.A., I.Borthwick, and K.K.Stanley. 1994. The 5'-flanking region of the alpha 2MR/LRP gene contains an enhancer-like cluster of Sp1 binding sites. *Biochim.Biophys.Acta* 1219:307-313.

151. Smith,J.L., S.R.Lear, and S.K.Erickson. 1995. Developmental expression of elements of hepatic cholesterol metabolism in the rat. *J.Lipid Res.* 36:641-652.

152. Ishiguro,M., Y.Imai, and S.Kohsaka. 1995. Expression and distribution of low density lipoprotein receptor-related protein mRNA in the rat central nervous system. *Brain Res.Mol.Brain Res.* 33:37-46.

153. Kim,S., C.H.Kim, and N.D.Vaziri. 2005. Upregulation of hepatic LDL receptor-related protein in nephrotic syndrome: response to statin therapy. *Am.J.Physiol Endocrinol.Metab* 288:E813-E817.

154. Kim,C. and N.D.Vaziri. 2005. Down-regulation of hepatic LDL receptor-related protein (LRP) in chronic renal failure. *Kidney Int.* 67:1028-1032.

155. Gafvels,M.E., G.Coukos, R.Sayegh, C.Coutifaris, D.K.Strickland, and J.F.Strauss. 1992. Regulated expression of the trophoblast alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Differentiation and cAMP modulate protein and mRNA levels. *J.Biol.Chem.* 267:21230-21234.

156. Kancha,R.K., M.E.Stearns, and M.M.Hussain. 1994. Decreased expression of the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor in invasive cell clones derived from human prostate and breast tumor cells. *Oncol.Res.* 6:365-372.

157. Yamamoto,M., K.Ikeda, K.Ohshima, H.Tsugu, H.Kimura, and M.Tomonaga. 1997. Increased expression of low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor in human malignant astrocytomas. *Cancer Res.* 57:2799-2805.

158. Hussaini,I.M., M.D.Brown, L.R.Karns, J.Carpenter, G.T.Redpath, S.L.Gonias, and S.R.VandenBerg. 1999. Epidermal growth factor differentially regulates low density lipoprotein receptor-related protein gene expression in neoplastic and fetal human astrocytes. *Glia* 25:71-84.

159. Kancha,R.K. and M.M.Hussain. 1996. Up-regulation of the low density lipoprotein receptor-related protein by dexamethasone in HepG2 cells. *Biochim.Biophys.Acta* 1301:213-220.

160. Tran,H., S.Robinson, I.Mikhailenko, and D.K.Strickland. 2003. Modulation of the LDL receptor and LRP levels by HIV protease inhibitors. *J.Lipid Res.* 44:1859-1869.

161. Hussaini,I.M., J.LaMarre, J.J.Lysiak, L.R.Karns, S.R.VandenBerg, and S.L.Gonias. 1996. Transcriptional regulation of LDL receptor-related protein by IFN-gamma and the antagonistic activity of TGF-beta(1) in the RAW 264.7 macrophage-like cell line. *J.Leukoc.Biol.* 59:733-739.

162. Businaro,R., C.Fabrizi, T.Persichini, G.Starace, M.G.Ennas, L.Fumagalli, and G.M.Lauro. 1997. Modulation of the alpha 2 macroglobulin receptor/low density lipoprotein receptor related protein by interferon-gamma in human astroglial cells. *J.Neuroimmunol.* 72:75-81.

163. Misra,U.K., G.Gawdi, M.Gonzalez-Gronow, and S.V.Pizzo. 1999. Coordinate regulation of the alpha(2)-macroglobulin signaling receptor and the low density lipoprotein receptor-related protein/alpha(2)-macroglobulin receptor by insulin. *J.Biol.Chem.* 274:25785-25791.

164. Boucher,P., P.H.Ducluzeau, P.Davelu, F.Andreelli, P.Vallier, J.P.Riou, M.Laville, and H.Vidal. 2002. Expression and regulation by insulin of low-density lipoprotein receptor-related protein mRNA in human skeletal muscle. *Biochim.Biophys.Acta* 1588:226-231.

165. Watanabe,Y., T.Inaba, H.Shimano, T.Gotoda, K.Yamamoto, H.Mokuno, H.Sato, Y.Yazaki, and N.Yamada. 1994. Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages. Possible involvement in the atherosclerotic process. *Arterioscler.Thromb.* 14:1000-1006.

166. Palmer,A.M., E.Nova, E.Anil, K.Jackson, P.Bateman, E.Wolstencroft, C.M.Williams, and P.Yaqoob. 2005. Differential uptake of subfractions of triglyceride-rich lipoproteins by THP-1 macrophages. *Atherosclerosis* 180:233-244.

167. Qu,S., F.Wu, J.Tian, Y.Li, Y.Wang, Y.Wang, and Y.Zong. 2004. Role of VLDL receptor in the process of foam cell formation. *J.Huazhong.Univ Sci.Technolog.Med.Sci.* 24:1-4, 8.

168. Lupu,F., D.Heim, F.Bachmann, and E.K.Kruithof. 1994. Expression of LDL receptor-related protein/alpha 2-macroglobulin receptor in human normal and atherosclerotic arteries. *Arterioscler.Thromb.* 14:1438-1444.

169. Luoma,J., T.Hiltunen, T.Sarkioja, S.K.Moestrup, J.Gliemann, T.Kodama, T.Nikkari, and S.Yla-Herttuala. 1994. Expression of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein and scavenger receptor in human atherosclerotic lesions. *J.Clin.Invest* 93:2014-2021.

170. Llorente-Cortes,V., M.Otero-Vinas, S.Sanchez, C.Rodriguez, and L.Badimon. 2002. Low-density lipoprotein upregulates low-density lipoprotein receptor-related protein expression in vascular smooth muscle cells: possible involvement of sterol regulatory element binding protein-2-dependent mechanism. *Circulation* 106:3104-3110.

171. Llorente-Cortes,V., M.Otero-Vinas, M.Berrozpe, and L.Badimon. 2004. Intracellular lipid accumulation, low-density lipoprotein receptor-related protein expression, and cell survival in vascular smooth muscle cells derived from normal and atherosclerotic human coronaries. *Eur.J.Clin.Invest* 34:182-190.

172. Boucher,P., M.de Lorgeril, P.Salen, P.Crozier, J.Delaye, J.J.Vallon, A.Geyssant, and R.Dante. 1998. Effect of dietary cholesterol on low density lipoprotein-receptor, 3-hydroxy-3-methylglutaryl-CoA reductase, and low density lipoprotein receptor-related protein mRNA expression in healthy humans. *Lipids* 33:1177-1186.

173. Schulz,S., G.Birkenmeier, U.Schagdarsurengin, K.Wenzel, U.Muller-Werdan, D.Rehfeld, T.Suss, A.Kabisch, K.Werdan, and C.Glaser. 2003. Role of LDL receptor-related protein (LRP) in coronary atherosclerosis. *Int.J.Cardiol.* 92:137-144.

174. Wu,Z., Y.Xie, N.L.Bucher, and S.R.Farmer. 1995. Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. *Genes Dev.* 9:2350-2363.

175. Cornelius,P., O.A.MacDougald, and M.D.Lane. 1994. Regulation of adipocyte development. *Annu.Rev.Nutr.* 14:99-129.

176. Mandrup,S. and M.D.Lane. 1997. Regulating adipogenesis. *J.Biol.Chem.* 272:5367-5370.

177. Spiegelman,B.M. and J.S.Flier. 1996. Adipogenesis and obesity: rounding out the big picture. *Cell* 87:377-389.

178. Tontonoz,P., E.Hu, and B.M.Spiegelman. 1995. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor gamma. *Curr.Opin.Genet.Dev.* 5:571-576.

179. Farkas,J., A.Angel, and M.I.Avigan. 1973. Studies on the compartmentation of lipid in adipose cells. II. Cholesterol accumulation and distribution in adipose tissue components. *J.Lipid Res.* 14:344-356.

180. Krause,B.R. and A.D.Hartman. 1976. Relationship between cell size, plasma cholesterol and rat adipocyte cholesterol storage. *Biochim.Biophys.Acta* 450:197-205.

181. Benoist,F., P.Lau, and R.McPherson. 1997. A new role for adipocyte LRP in selective uptake of HDL-CE. *Circulation* 96:I-485. (Abstr.)

182. Schoonjans,K., B.Staels, and J.Auwerx. 1996. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J.Lipid Res.* 37:907-925.

183. Kersten,S., B.Desvergne, and W.Wahli. 2000. Roles of PPARs in health and disease. *Nature* 405:421-424.

184. DiRenzo,J., M.Soderstrom, R.Kurokawa, M.H.Ogliastro, M.Ricote, S.Ingrey, A.Horlein, M.G.Rosenfeld, and C.K.Glass. 1997. Peroxisome proliferator-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptor heterodimers with ligands, coactivators, and corepressors. *Mol.Cell Biol.* 17:2166-2176.

185. Mukherjee,R., L.Jow, G.E.Croston, and J.R.J.Paterniti. 1997. Identification, characterization, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARgamma2 versus PPARgamma1 and activation with retinoid X receptor agonists and antagonists. *J.Biol.Chem.* 272:8071-8076.

186. Juge-Aubry,C., A.Pernin, T.Favez, A.G.Burger, W.Wahli, C.A.Meier, and B.Desvergne. 1997. DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5'-flanking region. *J.Biol.Chem.* 272:25252-25259.

187. Desvergne,B. and W.Wahli. 1995. Inducible Gene Expression. Birkhauser, Boston. 142-176 pp.

188. Luquet,S., C.Gaudel, D.Holst, J.Lopez-Soriano, C.Jehl-Pietri, A.Fredenrich, and P.A.Grimaldi. 2005. Roles of PPAR delta in lipid absorption and metabolism: a new target for the treatment of type 2 diabetes. *Biochim.Biophys.Acta* 1740:313-317.

189. Lefebvre,A.M., J.Peinado-Onsurbe, I.Leitersdorf, M.R.Briggs, J.R.Paterniti, J.C.Fruchart, C.Fievet, J.Auwerx, and B.Staels. 1997. Regulation of lipoprotein metabolism by thiazolidinediones occurs through a distinct but complementary mechanism relative to fibrates. *Arterioscler.Thromb.Vasc.Biol.* 17:1756-1764.

190. Edvardsson,U., M.Bergstrom, M.Alexandersson, K.Bamberg, B.Ljung, and B.Dahllof. 1999. Rosiglitazone (BRL49653), a PPARgamma-selective agonist, causes peroxisome proliferator-like liver effects in obese mice. *J.Lipid Res.* 40:1177-1184.

191. Kliewer,S.A., S.S.Sundseth, S.A.Jones, P.J.Brown, G.B.Wisely, C.S.Koble, P.Devchand, W.Wahli, T.M.Willson, J.M.Lenhard, and J.M.Lehmann. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc.Natl.Acad.Sci.U.S.A.* 94:4318-4323.

192. Keller,H., C.Dreyer, J.Medin, A.Mahfoudi, K.Ozato, and W.Wahli. 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc.Natl.Acad.Sci.U.S.A.* 90:2160-2164.

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

194. Devchand,P.R., H.Keller, J.M.Peters, M.Vazquez, F.J.Gonzalez, and W.Wahli. 1996. The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature* 384:39-43.

195. Lehmann,J.M., L.B.Moore, T.A.Smith-Oliver, W.O.Wilkison, T.M.Willson, and S.A.Kliewer. 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J.Biol.Chem.* 270:12953-12956.

196. De Vos,P., A.M.Lefebvre, S.G.Miller, M.Guerre-Millo, K.Wong, R.Saladin, L.G.Hamann, B.Staels, M.R.Briggs, and J.Auwerx. 1996. Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. *J.Clin.Invest.* 98:1004-1009.

197. Nolan,J.J., B.Ludvik, P.Beerdsen, M.Joyce, and J.Olefsky. 1994. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N.Engl.J.Med.* 331:1188-1193.

198. Okuno,A., H.Tamemoto, K.Tobe, K.Ueki, Y.Mori, K.Iwamoto, K.Umesono, Y.Akanuma, T.Fujiwara, H.Horikoshi, Y.Yazaki, and T.Kadowaki. 1998. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J.Clin.Invest* 101:1354-1361.

199. de Souza,C.J., M.Eckhardt, K.Gagen, M.Dong, W.Chen, D.Laurent, and B.F.Burkey. 2001. Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. *Diabetes* 50:1863-1871.

200. Mahley,R.W., Y.Huang, and S.C.Rall, Jr. 1999. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. *J.Lipid Res.* 40:1933-1949.

201. Radeau,T., M.Robb, P.Lau, J.Borthwick, and R.McPherson. 1998. Relationship of adipose tissue cholesteryl ester transfer protein (CETP) mRNA to plasma concentrations of CETP in man. *Atherosclerosis* 139:369-376.

202. Radeau,T., M.Robb, M.McDonnell, and R.McPherson. 1998. Preferential expression of cholesteryl ester transfer protein mRNA by stromal-vascular cells of human adipose tissue. *Biochim.Biophys.Acta* 1392:245-253.

203. Radeau,T., P.Lau, M.Robb, M.McDonnell, G.Ailhaud, and R.McPherson. 1995. Cholesteryl ester transfer protein (CETP) mRNA abundance in human adipose tissue: relationship to cell size and membrane cholesterol content. *J.Lipid Res.* 36:2552-2561.

204. Gauthier,B., M.Robb, F.Gaudet, G.S.Ginsburg, and R.McPherson. 1999. Characterization of a cholesterol response element (CRE) in the promoter of the cholesteryl ester transfer protein gene: functional role of the transcription factors SREBP-1a, -2, and YY1. *J.Lipid Res.* 40:1284-1293.

205. Richardson,M.A., D.T.Berg, P.A.Johnston, D.McClure, and B.W.Grinnell. 1996. Human liposarcoma cell line, SW872, secretes cholesteryl ester transfer protein in response to cholesterol. *J.Lipid Res.* 37:1162-1166.

206. Izem,L. and R.E.Morton. 2001. Cholesteryl ester transfer protein biosynthesis and cellular cholesterol homeostasis are tightly interconnected. *J.Biol.Chem.* 276:26534-26541.

207. Schumaker,V.N. and D.L.Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* 128:155-170.

208. Subrahmanyan,L. and R.Kisilevsky. 1988. Effects of culture substrates and normal hepatic sinusoidal cells on in vitro hepatocyte synthesis of Apo-SAA. *Scand.J.Immunol.* 27:251-260.

209. Thomas,S.S., J.Plenkiewicz, E.R.Ison, M.Bols, W.Zou, W.A.Szarek, and R.Kisilevsky. 1995. Influence of monosaccharide derivatives on liver cell glycosaminoglycan synthesis: 3-deoxy-D-xylo-hexose (3-deoxy-D-galactose) and methyl (methyl 4-chloro-4-deoxy-beta-D-galactopyranosid) uronate. *Biochim.Biophys.Acta* 1272:37-48.

210. Dodd,F., M.Limoges, R.T.Boudreau, G.Rowden, P.R.Murphy, and C.K.Too. 2000. L-arginine inhibits apoptosis via a NO-dependent mechanism in Nb2 lymphoma cells. *J.Cell Biochem.* 77:624-634.

211. Adolph,S., S.Brusselbach, and R.Muller. 1993. Inhibition of transcription blocks cell cycle progression of NIH3T3 fibroblasts specifically in G1. *J.Cell Sci.* 105:113-122.

212. Boren,J., I.Lee, M.J.Callow, E.M.Rubin, and T.L.Innerarity. 1996. A simple and efficient method for making site-directed mutants, deletions, and fusions of large DNA such as P1 and BAC clones. *Genome Res.* 6:1123-1130.

213. Blackhart,B.D., Z.M.Yao, and B.J.McCarthy. 1990. An expression system for human apolipoprotein B100 in a rat hepatoma cell line. *J.Biol.Chem.* 265:8358-8360.

214. Slomiany,B.A., M.M.Kelly, and D.T.Kurtz. 2000. Extraction of nuclear proteins with increased DNA binding activity. *Biotechniques* 28:938-942.

215. Dugail,I. 2001. Transfection of Adipocytes and Preparation of Nuclear Extracts. *In* Adipose Tissue Protocols. G.Ailhaud, editor. Humana Press, Totawa, NJ. 141-147.

216. Varanasi,U., R.Chu, Q.Huang, R.Castellon, A.V.Yeldandi, and J.K.Reddy. 1996. Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl coenzyme A oxidase gene. *J.Biol.Chem.* 271:2147-2155.

217. Hames B.D. 1981. *In* Gel Electrophoresis of Proteins: A Practical Approach. B.D.Hames and D.Rickwood, editors. IRL Press at Oxford University Press, Oxford. 1-86.

218. Sambrook,J., E.F.Fritsch, and T.Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

219. Vassiliou,G. and K.K.Stanley. 1994. Exogenous receptor-associated protein binds to two distinct sites on human fibroblasts but does not bind to the glycosaminoglycan residues of heparan sulfate proteoglycans. *J.Biol.Chem.* 269:15172-15178.

220. Frohnert,B.I., T.Y.Hui, and D.A.Bernlohr. 1999. Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *J.Biol.Chem.* 274:3970-3977.

221. Martin,G., H.Poirier, N.Hennuyer, D.Crombie, J.C.Fruchart, R.A.Heyman, P.Besnard, and J.Auwerx. 2000. Induction of the fatty acid transport protein 1 and acyl-CoA synthase genes by dimer-selective rexinoids suggests that the peroxisome proliferator-activated receptor-retinoid X receptor heterodimer is their molecular target. *J.Biol.Chem.* 275:12612-12618.

222. Hill,M.R., M.D.Young, C.M.McCurdy, and J.M.Gimble. 1997. Decreased expression of murine PPARgamma in adipose tissue during endotoxemia. *Endocrinology* 138:3073-3076.

223. Miles,P.D., Y.Barak, W.He, R.M.Evans, and J.M.Olefsky. 2000. Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency. *J.Clin.Invest.* 105:287-292.

224. Hauser,S., G.Adelmant, P.Sarraf, H.M.Wright, E.Mueller, and B.M.Spiegelman. 2000. Degradation of the peroxisome proliferator-activated receptor gamma is linked to ligand-dependent activation [In Process Citation]. *J.Biol.Chem.* 275:18527-18533.

225. Schoonjans,K., J.Peinado-Onsurbe, A.M.Lefebvre, R.A.Heyman, M.Briggs, S.Deeb, B.Staels, and J.Auwerx. 1996. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* 15:5336-5348.

226. Devine,J.H., D.W.Eubank, D.E.Clouthier, P.Tontonoz, B.M.Spiegelman, R.E.Hammer, and E.G.Beale. 1999. Adipose expression of the phosphoenolpyruvate carboxykinase promoter requires peroxisome proliferator-

activated receptor gamma and 9-cis-retinoic acid receptor binding to an adipocyte-specific enhancer in vivo. *J.Biol.Chem.* 274:13604-13612.

227. Baumann,C.A., N.Chokshi, A.R.Saltiel, and V.Ribon. 2000. Cloning and characterization of a functional peroxisome proliferator activator receptor-gamma-responsive element in the promoter of the CAP gene. *J.Biol.Chem.* 275:9131-9135.

228. Strickland,D.K., J.D.Ashcom, S.Williams, W.H.Burgess, M.Migliorini, and W.S.Argraves. 1990. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J.Biol.Chem.* 265:17401-17404.

229. Medvedev,A.V., S.K.Snedden, S.Raimbault, D.Ricquier, and S.Collins. 2001. Transcriptional regulation of the mouse uncoupling protein-2 gene. Double E-box motif is required for peroxisome proliferator-activated receptor-gamma-dependent activation. *J.Biol.Chem.* 276:10817-10823.

230. Berthou,L., N.Duverger, F.Emmanuel, S.Langouet, J.Auwerx, A.Guillouzo, J.C.Fruchart, E.Rubin, P.Denefle, B.Staels, and D.Branellec. 1996. Opposite regulation of human versus mouse apolipoprotein A-I by fibrates in human apolipoprotein A-I transgenic mice. *J.Clin.Invest* 97:2408-2416.

231. van Wijk,J.P., E.J.de Koning, C.M.Castro, and T.J.Rabelink. 2005. Rosiglitazone improves postprandial triglyceride and free fatty acid metabolism in type 2 diabetes. *Diabetes Care* 28:844-849.

232. Sidhu,J.S., Z.Kaposzta, H.S.Markus, and J.C.Kaski. 2004. Effect of rosiglitazone on common carotid intima-media thickness progression in coronary artery disease patients without diabetes mellitus. *Arterioscler.Thromb.Vasc.Biol.* 24:930-934.

233. Xiang,A.H., R.K.Peters, S.L.Kjos, C.Ochoa, A.Marroquin, J.Goico, S.Tan, C.Wang, S.P.Azen, C.R.Liu, C.H.Liu, H.N.Hodis, and T.A.Buchanan. 2005. Effect of thiazolidinedione treatment on progression of subclinical atherosclerosis in premenopausal women at high risk for type 2 diabetes. *J.Clin.Endocrinol.Metab* 90:1986-1991.

234. Calkin,A.C., J.M.Forbes, C.M.Smith, M.Lassila, M.E.Cooper, K.A.Jandeleit-Dahm, and T.J.Allen. 2005. Rosiglitazone Attenuates Atherosclerosis in a Model of Insulin Insufficiency Independent of Its Metabolic Effects. *Arterioscler.Thromb.Vasc.Biol.* ..

235. Galetto,R., M.Albajar, J.I.Polanco, M.M.Zakin, and J.C.Rodriguez-Rey. 2001. Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region. *Biochem.J.* 357:521-527.

236. Hesler,C.B., T.L.Swenson, and A.R.Tall. 1987. Purification and characterization of a human plasma cholesteryl ester transfer protein. *J.Biol.Chem.* 262:2275-2282.

237. Schumann,R.R., S.R.Leong, G.W.Flaggs, P.W.Gray, S.D.Wright, J.C.Mathison, P.S.Tobias, and R.J.Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science* 249:1429-1431.

238. Day,J.R., J.J.Albers, C.E.Lofton-Day, T.L.Gilbert, A.F.Ching, F.J.Grant, P.J.O'Hara, S.M.Marcovina, and J.L.Adolphson. 1994. Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. *J.Biol.Chem.* 269:9388-9391.

239. Beamer,L.J., S.F.Carroll, and D.Eisenberg. 1997. Crystal structure of human BPI and two bound phospholipids at 2.4 angstrom resolution. *Science* %20;276:1861-1864.

240. Guyard-Dangremont,V., V.Tenekjian, V.Chauhan, S.Walter, P.Roy, E.Rassart, and A.R.Milne. 1999. Immunochemical evidence that cholesteryl ester transfer protein and bactericidal/permeability-increasing protein share a similar tertiary structure. *Protein Sci.* 8:2392-2398.

241. Barter,P. and K.A.Rye. 1994. Cholesteryl ester transfer protein: its role in plasma lipid transport. *Clin.Exp.Pharmacol.Physiol* 21:663-672.

242. Tall,A. 1995. Plasma lipid transfer proteins. *Annu.Rev.Biochem.* 64:235-57.:235-257.

243. Bruce,C., R.A.Chouinard, Jr., and A.R.Tall. 1998. Plasma lipid transfer proteins, high-density lipoproteins, and reverse cholesterol transport. *Annu.Rev.Nutr.* 18:297-330.:297-330.

244. Bruce,C., L.J.Beamer, and A.R.Tall. 1998. The implications of the structure of the bactericidal/permeability-increasing protein on the lipid-transfer function of the cholesteryl ester transfer protein. *Curr.Opin.Struct.Biol.* 8:426-434.

245. Jiang,X.C., C.Bruce, T.Cocke, S.Wang, M.Boguski, and A.R.Tall. 1995. Point mutagenesis of positively charged amino acids of cholesteryl ester transfer protein: conserved residues within the lipid transfer/lipopolysaccharide binding protein gene family essential for function. *Biochemistry* 34:7258-7263.

246. Wang,S., X.Wang, L.Deng, E.Rassart, R.W.Milne, and A.R.Tall. 1993. Point mutagenesis of carboxyl-terminal amino acids of cholesteryl ester transfer protein. Opposite faces of an amphipathic helix important for cholesteryl ester transfer or for binding neutralizing antibody. *J.Biol.Chem.* 268:1955-1959.

247. Wang,S., P.Kussie, L.Deng, and A.Tall. 1995. Defective binding of neutral lipids by a carboxyl-terminal deletion mutant of cholesteryl ester transfer protein. Evidence for a carboxyl-terminal cholesteryl ester binding site essential for neutral lipid transfer activity. *J.Biol.Chem.* 270:612-618.

248. Wang,S., L.Deng, R.W.Milne, and A.R.Tall. 1992. Identification of a sequence within the C-terminal 26 amino acids of cholesteryl ester transfer protein responsible for binding a neutralizing monoclonal antibody and necessary for neutral lipid transfer activity. *J.Biol.Chem.* 267:17487-17490.

249. Agellon,L.B., E.M.Quinet, T.G.Gillette, D.T.Drayna, M.L.Brown, and A.R.Tall. 1990. Organization of the human cholesteryl ester transfer protein gene. *Biochemistry* 29:1372-1376.

250. Lusis,A.J., S.Zollman, R.S.Sparkes, I.Klisak, T.Mohandas, D.Drayna, and R.M.Lawn. 1987. Assignment of the human gene for cholesteryl ester transfer protein to chromosome 16q12-16q21. *Genomics* 1:232-235.

251. Drayna,D., A.S.Jarnagin, J.McLean, W.Henzel, W.Kohr, C.Fielding, and R.Lawn. 1987. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature* 327:632-634.

252. Jiang,X.C., P.Moulin, E.Quinet, I.J.Goldberg, L.K.Yacoub, L.B.Agellon, D.Compton, R.Schnitzer-Polokoff, and A.R.Tall. 1991. Mammalian adipose tissue and muscle are major sources of lipid transfer protein mRNA. *J.Biol.Chem.* 266:4631-4639.

253. Nagashima,M., J.W.McLean, and R.M.Lawn. 1988. Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J.Lipid Res.* 29:1643-1649.

254. Pape,M.E., E.F.Rehberg, K.R.Marotti, and G.W.Melchior. 1991. Molecular cloning, sequence, and expression of cynomolgus monkey cholesteryl ester transfer protein. Inverse correlation between hepatic cholesteryl ester transfer protein mRNA levels and plasma high density lipoprotein levels. *Arterioscler.Thromb.* 11:1759-1771.

255. Zeng,W., J.Zhang, B.Chen, G.Wu, and H.Xue. 2003. Cloning and characterization of cholesteryl ester transfer transfer protein isolated from the tree shrew. *Chin Med.J.(Engl.)* 116:928-931.

256. Hogarth,C.A., A.Roy, and D.L.Ebert. 2003. Genomic evidence for the absence of a functional cholesteryl ester transfer protein gene in mice and rats. *Comp Biochem.Physiol B Biochem.Mol.Biol.* 135:219-229.

257. Son,Y.S. and D.B.Zilversmit. 1986. Increased lipid transfer activities in hyperlipidemic rabbit plasma. *Arteriosclerosis* 6:345-351.

258. Quinet,E.M., L.B.Agellon, P.A.Kroon, Y.L.Marcel, Y.C.Lee, M.E.Whitlock, and A.R.Tall. 1990. Atherogenic diet increases cholesteryl ester transfer protein messenger RNA levels in rabbit liver. *J.Clin.Invest* 85:357-363.

259. Stein,Y., Y.Dabach, G.Hollander, and O.Stein. 1990. Cholesteryl ester transfer activity in hamster plasma: increase by fat and cholesterol rich diets. *Biochim.Biophys.Acta* 1042:138-141.

260. Faust,R.A., J.H.Tollefson, A.Chait, and J.J.Albers. 1990. Regulation of LTP-I secretion from human monocyte-derived macrophages by differentiation and cholesterol accumulation in vitro. *Biochim.Biophys.Acta* 1042:404-409.

261. Arai,T., S.Yamashita, K.Hirano, N.Sakai, K.Kotani, S.Fujioka, S.Nozaki, Y.Keno, M.Yamane, E.Shinohara, and . 1994. Increased plasma cholesteryl ester transfer protein in obese subjects. A possible mechanism for the reduction of serum HDL cholesterol levels in obesity. *Arterioscler.Thromb.* 14:1129-1136.

262. MacLean,P.S., S.Vadlamudi, K.G.MacDonald, W.J.Pories, and H.A.Barakat. 2005. Suppression of hepatic cholesteryl ester transfer protein expression in obese humans with the development of type 2 diabetes mellitus. *J.Clin.Endocrinol.Metab* 90:2250-2258.

263. Quinet,E.M., P.Huerta, D.Nancoo, A.R.Tall, Y.L.Marcel, and R.McPherson. 1993. Adipose tissue cholesteryl ester transfer protein mRNA in response to probucol treatment: cholesterol and species dependence. *J.Lipid Res.* 34:845-852.

264. Agellon,L.B., A.Walsh, T.Hayek, P.Moulin, X.C.Jiang, S.A.Shelanski, J.L.Breslow, and A.R.Tall. 1991. Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice. *J.Biol.Chem.* 266:10796-10801.

265. Jiang,X.C., L.B.Agellon, A.Walsh, J.L.Breslow, and A.Tall. 1992. Dietary cholesterol increases transcription of the human cholesteryl ester transfer protein gene in transgenic mice. Dependence on natural flanking sequences. *J.Clin.Invest* 90:1290-1295.

266. Oliveira,H.C., R.A.Chouinard, L.B.Agellon, C.Bruce, L.Ma, A.Walsh, J.L.Breslow, and A.R.Tall. 1996. Human cholesteryl ester transfer protein gene proximal promoter contains dietary cholesterol positive responsive elements and mediates expression in small intestine and periphery while predominant liver and spleen expression is controlled by 5'-distal sequences. Cis-acting sequences mapped in transgenic mice. *J.Biol.Chem.* 271:31831-31838.

267. Chouinard,R.A., Jr., Y.Luo, T.F.Osborne, A.Walsh, and A.R.Tall. 1998. Sterol regulatory element binding protein-1 activates the cholesteryl ester transfer protein gene in vivo but is not required for sterol up-regulation of gene expression. *J.Biol.Chem.* 273:22409-22414.

268. Luo,Y. and A.R.Tall. 2000. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *J.Clin.Invest* 105:513-520.

269. Gaudet,F. and G.S.Ginsburg. 1995. Transcriptional regulation of the cholesteryl ester transfer protein gene by the orphan nuclear hormone receptor apolipoprotein AI regulatory protein-1. *J.Biol.Chem.* 270:29916-29922.

270. Le Goff,W., M.Guerin, M.J.Chapman, and J.Thillet. 2003. A CYP7A promoter binding factor site and Alu repeat in the distal promoter region are implicated in regulation of human CETP gene expression. *J.Lipid Res.* 44:902-910.

271. Le Goff,W., M.Guerin, L.Petit, M.J.Chapman, and J.Thillet. 2003. Regulation of human CETP gene expression: role of SP1 and SP3 transcription factors at promoter sites -690, -629, and -37. *J.Lipid Res.* 44:1322-1331.

272. Inazu,A., E.M.Quinet, S.Wang, M.L.Brown, S.Stevenson, M.L.Barr, P.Moulin, and A.R.Tall. 1992. Alternative splicing of the mRNA encoding the human cholesteryl ester transfer protein. *Biochemistry* 31:2352-2358.

273. Quinet,E., T.P.Yang, C.Marinos, and A.Tall. 1993. Inhibition of the cellular secretion of cholesteryl ester transfer protein by a variant protein formed by alternative splicing of mRNA. *J.Biol.Chem.* 268:16891-16894.

274. Dessi,M., C.Motti, C.Cortese, E.Leonardis, C.Giovannini, G.Federici, and F.Piemonte. 1997. Alternative splicing of human plasma cholesteryl ester transfer protein mRNA in Caco-2 cells and its modulation by oleic acid. *Mol.Cell Biochem.* 177:107-112.

275. Zilversmit,D.B., L.B.Hughes, and J.Balmer. 1975. Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. *Biochim.Biophys.Acta* 409:393-398.

276. Pattnaik,N.M., A.Montes, L.B.Hughes, and D.B.Zilversmit. 1978. Cholesteryl ester exchange protein in human plasma isolation and characterization. *Biochim.Biophys.Acta* 530:428-438.

277. Moulin,P., M.C.Cheung, C.Bruce, S.Zhong, T.Cocke, H.Richardson, and A.R.Tall. 1994. Gender effects on the distribution of the cholesteryl ester transfer protein in apolipoprotein A-I-defined lipoprotein subpopulations. *J.Lipid Res.* 35:793-802.

278. Marcel,Y.L., R.McPherson, M.Hogue, H.Czarnecka, Z.Zawadzki, P.K.Weech, M.E.Whitlock, A.R.Tall, and R.W.Milne. 1990. Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipemic subjects. *J.Clin.Invest* 85:10-17.

279. Francone,O.L., A.Gurakar, and C.Fielding. 1989. Distribution and functions of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in plasma lipoproteins. Evidence for a functional unit containing these activities together with apolipoproteins A-I and D that catalyzes the esterification and transfer of cell-derived cholesterol. *J.Biol.Chem.* 264:7066-7072.

280. Nishida,H.I., H.Arai, and T.Nishida. 1993. Cholesterol ester transfer mediated by lipid transfer protein as influenced by changes in the charge characteristics of plasma lipoproteins. *J.Biol.Chem.* 268:16352-16360.

281. Yen,F.T., R.J.Deckelbaum, C.J.Mann, Y.L.Marcel, R.W.Milne, and A.R.Tall. 1989. Inhibition of cholesteryl ester transfer protein activity by monoclonal antibody. Effects on cholesteryl ester formation and neutral lipid mass transfer in human plasma. *J.Clin.Invest* 83:2018-2024.

282. Morton,R.E. and D.B.Zilversmit. 1983. Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. *J.Biol.Chem.* 258:11751-11757.

283. Hesler,C.B., A.R.Tall, T.L.Swenson, P.K.Weech, Y.L.Marcel, and R.W.Milne. 1988. Monoclonal antibodies to the Mr 74,000 cholesteryl ester transfer protein neutralize all of the cholesteryl ester and triglyceride transfer activities in human plasma. *J.Biol.Chem.* 263:5020-5023.

284. Barter,P.J. and M.E.Jones. 1980. Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. *J.Lipid Res.* 21:238-249.

285. Connolly,D.T., J.McIntyre, D.Heuvelman, E.E.Remsen, R.E.McKinnie, L.Vu, M.Melton, R.Monsell, E.S.Krul, and K.Glenn. 1996. Physical and kinetic characterization of recombinant human cholesteryl ester transfer protein. *Biochem.J.* 320:39-47.

286. Epps,D.E., K.A.Greenlee, J.S.Harris, E.W.Thomas, C.K.Castle, J.F.Fisher, R.R.Hozak, C.K.Marschke, G.W.Melchior, and F.J.Kezdy. 1995. Kinetics and inhibition of lipid exchange catalyzed by plasma cholesteryl ester transfer protein (lipid transfer protein). *Biochemistry* 34:12560-12569.

287. Clark,R.W., T.A.Sutfin, R.B.Ruggeri, A.T.Willauer, E.D.Sugarman, G.Magnus-Aryitey, P.G.Cosgrove, T.M.Sand, R.T.Wester, J.A.Williams, M.E.Perlman, and M.J.Bamberger. 2004. Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. *Arterioscler.Thromb.Vasc.Biol.* 24:490-497.

288. Clark,R.W., R.B.Ruggeri, D.Cunningham, and M.J.Bamberger. 2005. Description of the torcetrapib series of cholesteryl ester transfer protein inhibitors, including mechanism of action. *J.Lipid Res.* ..

289. Mann,C.J., F.T.Yen, A.M.Grant, and B.E.Bihain. 1991. Mechanism of plasma cholesteryl ester transfer in hypertriglyceridemia. *J.Clin.Invest* 88:2059-2066.

290. Koizumi,J., H.Mabuchi, A.Yoshimura, I.Michishita, M.Takeda, H.Itoh, Y.Sakai, T.Sakai, K.Ueda, and R.Takeda. 1985. Deficiency of serum cholesteryl-ester

transfer activity in patients with familial hyperalphalipoproteinaemia. *Atherosclerosis* 58:175-186.

291. Brown,M.L., A.Inazu, C.B.Hesler, L.B.Agellon, C.Mann, M.E.Whitlock, Y.L.Marcel, R.W.Milne, J.Koizumi, H.Mabuchi, and . 1989. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature* 342:448-451.

292. Inazu,A., M.L.Brown, C.B.Hesler, L.B.Agellon, J.Koizumi, K.Takata, Y.Maruhama, H.Mabuchi, and A.R.Tall. 1990. Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *N.Engl.J.Med.* 323:1234-1238.

293. Inazu,A., X.C.Jiang, T.Haraki, K.Yagi, N.Kamon, J.Koizumi, H.Mabuchi, R.Takeda, K.Takata, Y.Moriyama, and . 1994. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J.Clin.Invest* 94:1872-1882.

294. Zhong,S., D.S.Sharp, J.S.Grove, C.Bruce, K.Yano, J.D.Curb, and A.R.Tall. 1996. Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. *J.Clin.Invest* 97:2917-2923.

295. Curb,J.D., R.D.Abbott, B.L.Rodriguez, K.Masaki, R.Chen, D.S.Sharp, and A.R.Tall. 2004. A prospective study of HDL-C and cholesteryl ester transfer protein gene mutations and the risk of coronary heart disease in the elderly. *J.Lipid Res.* 45:948-953.

296. Moriyama,Y., T.Okamura, A.Inazu, M.Doi, H.Iso, Y.Mouri, Y.Ishikawa, H.Suzuki, M.Iida, J.Koizumi, H.Mabuchi, and Y.Komachi. 1998. A low prevalence of coronary heart disease among subjects with increased high-density lipoprotein cholesterol levels, including those with plasma cholesteryl ester transfer protein deficiency. *Prev.Med.* 27:659-667.

297. Barter,P.J., H.B.Brewer, Jr., M.J.Chapman, C.H.Hennekens, D.J.Rader, and A.R.Tall. 2003. Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.* 23:160-167.

298. Stein,O. and Y.Stein. 2005. Lipid transfer proteins (LTP) and atherosclerosis. *Atherosclerosis* 178:217-230.

299. Marotti,K.R., C.K.Castle, T.P.Boyle, A.H.Lin, R.W.Murray, and G.W.Melchior. 1993. Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. *Nature* 364:73-75.

300. Plump,A.S., L.Masucci-Magoulas, C.Bruce, C.L.Bisgaier, J.L.Breslow, and A.R.Tall. 1999. Increased atherosclerosis in ApoE and LDL receptor gene knock-

out mice as a result of human cholesteryl ester transfer protein transgene expression. *Arterioscler.Thromb.Vasc.Biol.* 19:1105-1110.

301. Hayek,T., L.Masucci-Magoulas, X.Jiang, A.Walsh, E.Rubin, J.L.Breslow, and A.R.Tall. 1995. Decreased early atherosclerotic lesions in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *J.Clin.Invest* 96:2071-2074.

302. Berti,J.A., E.C.de Faria, and H.C.Oliveira. 2005. Atherosclerosis in aged mice over-expressing the reverse cholesterol transport genes. *Braz.J.Med.Biol.Res.* 38:391-398.

303. Sugano,M. and N.Makino. 1996. Changes in plasma lipoprotein cholesterol levels by antisense oligodeoxynucleotides against cholesteryl ester transfer protein in cholesterol-fed rabbits. *J.Biol.Chem.* 271:19080-19083.

304. Sugano,M., N.Makino, S.Sawada, S.Otsuka, M.Watanabe, H.Okamoto, M.Kamada, and A.Mizushima. 1998. Effect of antisense oligonucleotides against cholesteryl ester transfer protein on the development of atherosclerosis in cholesterol-fed rabbits. *J.Biol.Chem.* 273:5033-5036.

305. Rittershaus,C.W., D.P.Miller, L.J.Thomas, M.D.Picard, C.M.Honan, C.D.Emmett, C.L.Pettey, H.Adari, R.A.Hammond, D.T.Beattie, A.D.Callow, H.C.Marsh, and U.S.Ryan. 2000. Vaccine-induced antibodies inhibit CETP activity in vivo and reduce aortic lesions in a rabbit model of atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.* 20:2106-2112.

306. Gaofu,Q., L.Jun, Z.Xiuyun, L.Wentao, W.Jie, and L.Jingjing. 2005. Antibody against cholesteryl ester transfer protein (CETP) elicited by a recombinant chimeric enzyme vaccine attenuated atherosclerosis in a rabbit model. *Life Sci.* ..

307. Davidson,M.H., K.Maki, D.Umporowicz, A.Wheeler, C.Rittershaus, and U.Ryan. 2003. The safety and immunogenicity of a CETP vaccine in healthy adults. *Atherosclerosis* 169:113-120.

308. Koizumi,J., H.Mabuchi, A.Yoshimura, I.Michishita, M.Takeda, H.Itoh, Y.Sakai, T.Sakai, K.Ueda, and R.Takeda. 1985. Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyperalphalipoproteinaemia. *Atherosclerosis* 58:175-186.

309. Brown,M.L., A.Inazu, C.B.Hesler, L.B.Agellon, C.Mann, M.E.Whitlock, Y.L.Marcel, R.W.Milne, J.Koizumi, H.Mabuchi, and . 1989. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature* 342:448-451.

310. Forrester,J.S., R.Makkar, and P.K.Shah. 2005. Increasing high-density lipoprotein cholesterol in dyslipidemia by cholesteryl ester transfer protein inhibition: an update for clinicians. *Circulation* 111:1847-1854.

311. Whitlock,M.E., T.L.Swenson, R.Ramakrishnan, M.T.Leonard, Y.L.Marcel, R.W.Milne, and A.R.Tall. 1989. Monoclonal antibody inhibition of cholesteryl ester transfer protein activity in the rabbit. Effects on lipoprotein composition and high density lipoprotein cholesteryl ester metabolism. *J.Clin.Invest* 84:129-137.

312. Swenson,T.L., C.B.Hesler, M.L.Brown, E.Quinet, P.P.Trotta, M.F.Haslanger, F.C.Gaeta, Y.L.Marcel, R.W.Milne, and A.R.Tall. 1989. Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J.Biol.Chem.* 264:14318-14326.

313. Sugano,M. and N.Makino. 1996. Changes in plasma lipoprotein cholesterol levels by antisense oligodeoxynucleotides against cholesteryl ester transfer protein in cholesterol-fed rabbits. *J.Biol.Chem.* 271:19080-19083.

314. Gaofu,Q., L.Jun, Y.Xin, L.Wentao, W.Jie, Z.Xiuyun, and L.Jingjing. 2005. Vaccinating rabbits with a cholesteryl ester transfer protein (CETP) B-Cell epitope carried by heat shock protein-65 (HSP65) for inducing anti-CETP antibodies and reducing aortic lesions in vivo. *J.Cardiovasc.Pharmacol.* 45:591-598.

315. Bruckner,D., F.T.Hafner, V.Li, C.Schmeck, J.Telser, A.Vakalopoulos, and G.Wirtz. 2005. Dibenzodioxocinones--a new class of CETP inhibitors. *Bioorg.Med.Chem.Lett.* 15:3611-3614.

316. Okamoto,H., F.Yonemori, K.Wakitani, T.Minowa, K.Maeda, and H.Shinkai. 2000. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature* 406:203-207.

317. de Grooth,G.J., J.A.Kuivenhoven, A.F.Stalenhoef, J.de Graaf, A.H.Zwinderman, J.L.Posma, A.van Tol, and J.J.Kastelein. 2002. Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study. *Circulation* 105:2159-2165.

318. Kuivenhoven,J.A., G.J.de Grooth, H.Kawamura, A.H.Klerkx, F.Wilhelm, M.D.Trip, and J.J.Kastelein. 2005. Effectiveness of inhibition of cholesteryl ester transfer protein by JTT-705 in combination with pravastatin in type II dyslipidemia. *Am.J.Cardiol.* 95:1085-1088.

319. Brousseau,M.E., E.J.Schaefer, M.L.Wolfe, L.T.Bloedon, A.G.Digenio, R.W.Clark, J.P.Mancuso, and D.J.Rader. 2004. Effects of an inhibitor of cholesteryl ester transfer protein on HDL cholesterol. *N.Engl.J.Med.* 350:1505-1515.

320. Brousseau,M.E., M.R.Diffenderfer, J.S.Millar, C.Nartsupha, B.F.Asztalos, F.K.Welty, M.L.Wolfe, M.Rudling, I.Bjorkhem, B.Angelin, J.P.Mancuso, A.G.Digenio, D.J.Rader, and E.J.Schaefer. 2005. Effects of cholesteryl ester transfer protein inhibition on high-density lipoprotein subspecies, apolipoprotein A-I metabolism, and fecal sterol excretion. *Arterioscler.Thromb.Vasc.Biol.* 25:1057-1064.

321. Brown,M.S. and J.L.Goldstein. 1979. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc.Natl.Acad.Sci.U.S.A* 76:3330-3337.

322. Goldstein,J.L., R.G.Anderson, and M.S.Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* 279:679-685.

323. Glass,C., R.C.Pittman, M.Civen, and D.Steinberg. 1985. Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro. *J.Biol.Chem.* 260:744-750.

324. Despres,J.P., B.S.Fong, J.Jimenez, P.Julien, and A.Angel. 1988. Selective uptake of HDL cholesterol ester by human fat cells. *Am.J.Physiol* 254:E667-E675.

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

326. Xu,S., M.Laccotripe, X.Huang, A.Rigotti, V.I.Zannis, and M.Krieger. 1997. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *J.Lipid Res.* 38:1289-1298.

327. Brundert,M., A.Ewert, J.Heeren, B.Behrendt, R.Ramakrishnan, H.Greten, M.Merkel, and F.Rinninger. 2005. Scavenger receptor class B type I mediates the selective uptake of high-density lipoprotein-associated cholesteryl ester by the liver in mice. *Arterioscler.Thromb.Vasc.Biol.* 25:143-148.

328. Calvo,D. and M.A.Vega. 1993. Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. *J.Biol.Chem.* 268:18929-18935.

329. Despres,J.P., B.S.Fong, J.Jimenez, P.Julien, and A.Angel. 1988. Selective uptake of HDL cholesterol ester by human fat cells. *Am.J.Physiol.* 254:E667-E675.

330. Fong,B.S. and A.Angel. 1989. Transfer of free and esterified cholesterol from low-density lipoproteins and high-density lipoproteins to human adipocytes. *Biochim.Biophys.Acta.* 1004:53-60.

331. Parkes,J.G. and A.Angel. 1990. Selective uptake of cholesteryl ester from high density lipoproteins by plasma membranes of adipose tissue. *Biochem.Cell Biol.* 68:870-879.

332. Rinninger,F. and R.C.Pittman. 1989. Mechanism of the cholesteryl ester transfer protein-mediated uptake of high density lipoprotein cholesteryl esters by Hep G2 cells. *J.Biol.Chem.* 264:6111-6118.

333. Goldberg,D.I., W.F.Beltz, and R.C.Pittman. 1991. Evaluation of pathways for the cellular uptake of high density lipoprotein cholesterol esters in rabbits. *J.Clin.Invest* 87:331-346.

334. Collet,X., A.R.Tall, H.Serajuddin, K.Guendouzi, L.Royer, H.Oliveira, R.Barbaras, X.C.Jiang, and O.L.Francone. 1999. Remodeling of HDL by CETP in vivo and by CETP and hepatic lipase in vitro results in enhanced uptake of HDL CE by cells expressing scavenger receptor B-I. *J.Lipid Res.* 40:1185-1193.

335. Sattler,W., D.Mohr, and R.Stocker. 1994. Rapid isolation of lipoproteins and assessment of their peroxidation by high-performance liquid chromatography postcolumn chemiluminescence. *Methods Enzymol.* 233:469-89.:469-489.

336. Reaven,E., L.Tsai, and S.Azhar. 1996. Intracellular events in the "selective" transport of lipoprotein-derived cholesteryl esters. *J.Biol.Chem.* 271:16208-16217.

337. Rinninger,F. and R.C.Pittman. 1987. Regulation of the selective uptake of high density lipoprotein-associated cholesteryl esters. *J.Lipid Res.* 28:1313-1325.

338. Knecht,T.P. and R.C.Pittman. 1989. A plasma membrane pool of cholesteryl esters that may mediate the selective uptake of cholesteryl esters from high-density lipoproteins. *Biochim.Biophys.Acta* 1002:365-375.

339. Rye,K.A., N.J.Hime, and P.J.Barter. 1997. Evidence that cholesteryl ester transfer protein-mediated reductions in reconstituted high density lipoprotein size involve particle fusion. *J.Biol.Chem.* 272:3953-3960.

340. Herz,J. and R.D.Gerard. 1993. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc.Natl.Acad.Sci.U.S.A* 90:2812-2816.

341. Bergelson,J.M., J.A.Cunningham, G.Droguett, E.A.Kurt-Jones, A.Krithivas, J.S.Hong, M.S.Horwitz, R.L.Crowell, and R.W.Finberg. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275:1320-1323.

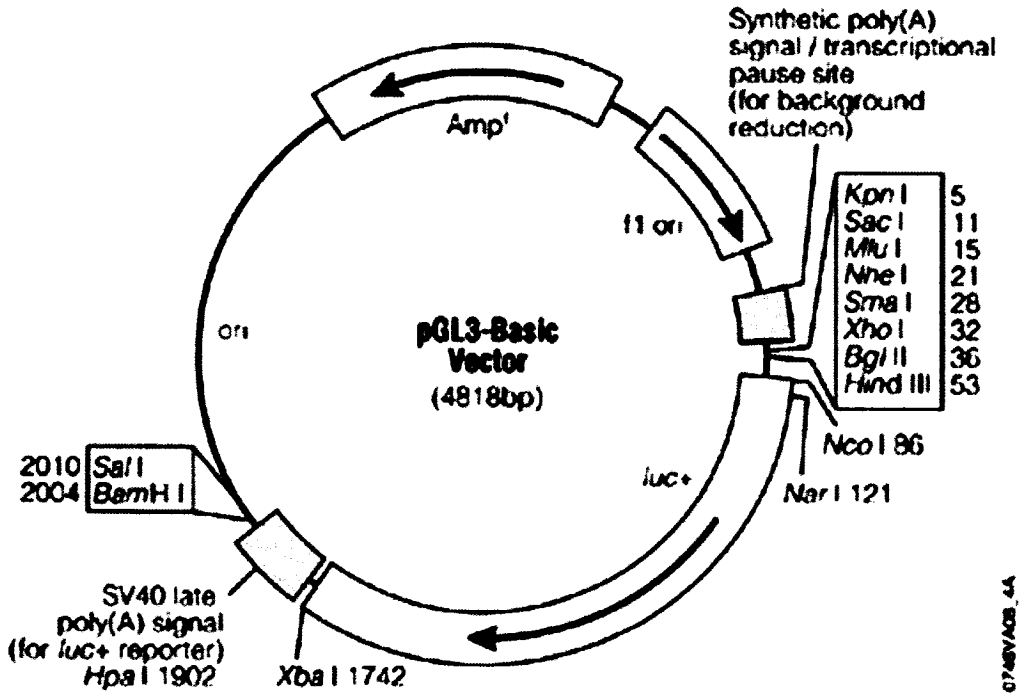
342. Bergelson,J.M., A.Krithivas, L.Celi, G.Droguett, M.S.Horwitz, T.Wickham, R.L.Crowell, and R.W.Finberg. 1998. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J.Virol.* 72:415-419.

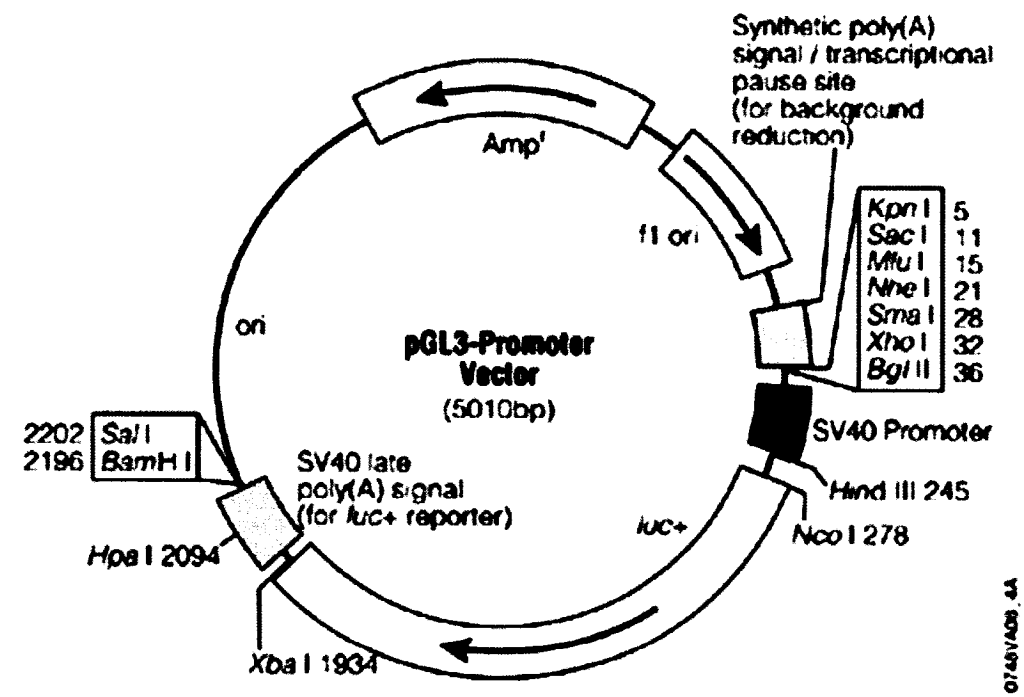
343. Lloyd,D.B., M.E.Lira, L.S.Wood, L.K.Durham, T.B.Freeman, G.M.Preston, X.Qiu, E.Sugarman, P.Bonnette, A.Lanzetti, P.M.Milos, and J.F.Thompson. 2005. Cholesteryl ester transfer protein variants have differential stability but uniform inhibition by torcetrapib. *J.Biol.Chem.* 280:14918-14922.

CONTRIBUTIONS OF COLLABORATORS

All experiments and results presented in this thesis were the work of the thesis author, André Gauthier except for the following: (1) figure 1 was the work of Dr Benoit Gauthier; (2) experiments depicted in figure 2.9B and 2.9C were performed by Dr Gerard Vassiliou; (3) the collection of raw data from the *in vivo* mouse experiments shown in figures 3.9, 3.10, 3.11, and 3.12 was completed with the help of Paulina Lau as well as optimization of the CETP Western blot protocol.

APPENDIX I


Synthetic poly(A)
signal / transcriptional
pause site
(for background
reduction)
Amp'
f1 ori
ori
pGL3-Basic
Vector
(4818bp)
Kpn I 5
Sac I 11
Mlu I 15
Nhe I 21
Sma I 28
Xho I 32
Bgl II 36
Hind III 53
Nco I 86
Nar I 121
luc+
2010 Sal I
2004 BamH I
SV40 late
poly(A) signal
(for luc+ reporter)
Hpa I 1902
Xba I 1742


Synthetic poly(A)
signal / transcriptional
pause site
(for background
reduction)
Amp'
f1 ori
ori
pGL3-Promoter
Vector
(5010bp)
Kpn I 5
Sac I 11
Mlu I 15
Nhe I 21
Sma I 28
Xho I 32
Bgl II 36
SV40 Promoter
Hind III 245
Nco I 278
luc+
2202 Sal I
2196 BamH I
SV40 late
poly(A) signal
(for luc+ reporter)
Hpa I 2094
Xba I 1934

CURRICULUM VITAE

Name: André Gauthier

Date of Birth:

Address:

Education:

Doctorate in Medicine (2nd year)
University of Ottawa, 2008 (expected)

Ph.D. Biochemistry
University of Ottawa, 2006

B.Sc. Honours Biology / Biotechnology Option
University of Ottawa, 1999

Awards & Distinctions:

CIHR Canada Graduate Scholarship (Ph.D.)
May 2004-September 2004 and May 2005-September 2005

University of Ottawa Excellence Scholarship
September 2002-May 2004 and May 2005-September 2005

Silver Award, Canadian Student Health Research Forum (CSHRF) CIHR National Health Research Poster Competition
June 2004 Winnipeg, Manitoba

1st place (tie), Best Poster (Ph.D.), BMI department poster day
April 2004

Merck Frosst Schering Award for best basic science presentation
University of Ottawa Heart Institute Research Day, April 2004

Ontario Graduate Scholarship (Ph.D.)
September 2002-May 2004

Runner-up, Best Poster, BMI department poster day
April 2002

Ontario Graduate Scholarship in Science & Technology (Ph.D.)
September 2001-September 2002

Ontario Graduate Scholarship in Science & Technology (M.Sc.)
September 2000-September 2001)

Dean's Honour List, University of Ottawa
May 1999

Magna cum laude, University of Ottawa
May 1999

Peer-Reviewed Publications:

1. **Gauthier, A.**, Lau, P., Zha, X., Milne, R., and McPherson, R. (2005). Cholesteryl Ester Transfer Protein Directly Mediates Selective Uptake of High Density Lipoprotein Cholesteryl Esters by the Liver. Arterioscler.Thromb.Vasc.Biol. *25*, 2177-2184.

2. Brown,R.J., **Gauthier,A.**, Parks,R.J., McPherson,R., Sparks,D.L., Schultz,J.R., and Yao,Z. (2004). Severe hypoalphalipoproteinemia in mice expressing human hepatic lipase deficient in binding to heparan sulfate proteoglycan. J. Biol. Chem. *279*, 42403-42409.

3. McPherson,R. and **Gauthier,A.** (2004). Molecular regulation of SREBP function: the Insig-SCAP connection and isoform-specific modulation of lipid synthesis. Biochem. Cell Biol. *82*, 201-211.

4. **Gauthier,A.**, Vassiliou,G., Benoist,F., and McPherson,R. (2003). Adipocyte low density lipoprotein receptor-related protein gene expression and function is regulated by peroxisome proliferator-activated receptor gamma. J. Biol. Chem. *278*, 11945-11953.

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

6. Harper,M.E., Dent,R., Monemdjou,S., Bezaire,V., Van Wyck,L., Wells,G., Kavaslar,G.N., **Gauthier,A.**, Tesson,F., and McPherson,R. (2002). Decreased mitochondrial proton leak and reduced expression of uncoupling protein 3 in skeletal muscle of obese diet-resistant women. Diabetes *51*, 2459-2466.

7. Harper,M.E., Dent,R.M., Bezaire,V., Antoniou,A., **Gauthier,A.**, Monemdjou,S., and McPherson,R. (2001). UCP3 and its putative function: consistencies and controversies. Biochem. Soc. Trans. *29*, 768-773.

Peer-Reviewed Abstracts:

1. Zha X, **Gauthier A**, Milne R and McPherson R. Surface-Anchored Cholesteryl Ester Transfer Protein (CETP) Functions as an HDL Receptor and Mediates Selective Uptake of Cholesteryl Esters from HDL. (2002) Circulation; 106; II-76. Abstract

2. Zha X, **Gauthier A**, Genest J and McPherson R. Cholesterol Trafficking During Efflux is Mediated by Both Vesicular and Non-Vesicular Transport Processes: ABCA1 Influences Vesicular Transport from the Golgi to the Plasma Membrane. (2002) Circulation; 106; II-219. Abstract

Peer-Reviewed Conference Presentations:

1. **Gauthier A** & McPherson R. Hepatocyte CETP Mediates HDL-CE Selective Uptake by a Process Which Is Only Partially Susceptible to Inhibition by Torcetrapib. 6th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology, Grand Hyatt Washington, Washington, DC. April 28 - 30, 2005. Poster (Dr McPherson was the presenting author since I was not available)

2. Zha X, **Gauthier A***, Milne R and McPherson R. Surface-Anchored Cholesteryl Ester Transfer Protein (CETP) Functions as an HDL Receptor and Mediates Selective Uptake of Cholesteryl Esters from HDL. American Heart Association Scientific Sessions 2002, Chicago Illinois, November 17-20. Poster (*indicates presenting author)

2. **Gauthier A**, Benoist F, Vassiliou G and McPherson R. Adipocyte LRP gene expression is Regulated by PPAR gamma. XIV International Symposium on Drugs Affecting Lipid Metabolism 2001, New York, NY, September 9-12. Oral

Non Peer-Reviewed Conference Presentations:

1. **Gauthier A** & McPherson R. Inhibition of Cholesteryl Ester Transfer Protein (CETP) activity by Torcetrapib Does Not Affect CETP-mediated Selective Uptake of HDL-derived Cholesteryl Esters. Canadian Student Health Research Forum (CSHRF) CIHR National Health Research Poster Competition 2004, Winnipeg, Manitoba, June 8-9. Poster

2. **Gauthier A**, Vassiliou G and McPherson R. Regulation of LDL Receptor-related Protein (LRP) Gene Expression by Peroxisome Proliferator Activated Receptor (PPAR) Ligands. Canadian Lipoprotein Conference (CLC) 2000, White Rock, BC, October 26-29. Oral