UNIVERSITÀ DEGLI STUDI DI PALERMO

STUDIES ON FACTORS INFLUENCING HIGH-DENSITY LIPOPROTEIN FUNCTIONALITY AND REVERSE CHOLESTEROL TRANSPORT

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STUDIES ON FACTORS INFLUENCING HIGH-DENSITY LIPOPROTEIN FUNCTIONALITY AND REVERSE CHOLESTEROL TRANSPORT

by

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To

My Parents

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1. ACKNOWELEDGEMENTS

Working on my PhD thesis has been a journey filled with challenges, excitements, joy, hurdles, and at times a mixed feeling of how to accomplish this herculean task of not simply completing for the sake of the PhD degree, but also to learn along the way what life teaches in the midst of so many challenges that life presents, especially when a mother uses all her skills to balance family, children, job and studies.

To foster and flourish my passion of pursuing science as a career and then to proceed to understand complexity of biology has not come easy, and there has been several personalities along this path who have, at times, provided guidance and encouragement to accept the challenges and excel. I am thankful to my husband, Ajit, who has been a continuous source of encouragement since the start of my research career at the Washington University. He has been very patience in mentoring me throughout my research career. It is because of him that I am able to complete and submit this dissertation despite a number of hurdles and challenges.

It was at the Washington University School of Medicine where I had an opportunity to work with Prof Maurizio Averna, MD and Dr Angelo Baldassare Cefalù, M.D., PhD, and Late Prof Gustav Schonfeld, a renowned lipidoogist. I developed interest in lipids and cardiovascular research and pursued my passion in this field. Despite working in various industries, I continued my interest in high-density lipoproteins and players in the reverse cholesterol transport, mainly ATP binding cassette transporter A1. Because of my interest in this field, I authored review articles on HDL in 2000 and on ABCA1 in 2002. In collaboration with Prof Maurizio Averna and Dr Angelo Baldassare Cefalù I continued working in this area whenever I had an opportunity to do so.

This dissertation is a compilation of my work carried out over a span of several years with the help of a number of researchers and laboratories, including the laboratory of my supervisors, Prof Maurizio Averna and Dr Angelo Baldassare

Cefalù. Many of the studies included in this dissertation were designed with the help of my supervisors and carried out in various laboratories.

I sincerely thank my supervisors, Prof Maurizio Averna and Dr Angelo Baldassare Cefalù to mentor me throughout the course of this study and allowing me to submit this dissertation towards the fulfillment of the PhD degree.

Neelam Srivastava, MA, MS

2. SUMMARY

Coronary artery disease (CAD) in dyslipidemic and diabetic subjects remains the leading cause of death in the Western society. Current therapeutic strategies to prevent cardiovascular diseases are primarily based on the use of statins, which inhibit key enzyme in the cholesterol synthesis, HMG-CoA reductase. Another prominent risk factor for developing premature atherosclerosis is the low levels of high-density lipoprotein cholesterol (HDL-C). Despite documented benefits of statins a good proportion of individuals still remain at a higher risk of developing CAD. Therefore, focus has shifted on HDL-raising therapeutics to further improve the CV outcome. While Niacin and fenofibrate have not shown clinical benefits in two separate trials, results from CETP inhibitors, Torcetrapib and Dalcetrapib, in phase 3 clinical studies have been disappointing despite substantial increase in HDL-C. One of the challenges encountered with measuring plasma HDL as a biomarker for cardiovascular risk is the fact that the antiinflammatory HDL becomes pro-inflammatory after it undergoes oxidative modification in diabetics. Pro-inflammatory states have also been shown to reduce HDL functionality. Thus, the lack of translatability of animal results to humans has further added challenges in the HDL therapeutics area. These findings necessitated rethinking about HDL therapeutics leading to transition in HDL therapy concept that seems to focus more around HDL functionality rather than HDL level. This PhD dissertation focuses on factors that influence HDL functionality and analyzes a correlation between HDL functionality and aortic lipid deposition. Several agents that raise HDL levels have been utilized to dissect HDL functionality. In addition, animal model of diabetes and atherosclerosis was employed to establish a correlation between HDL functionality, hyperglycemia and inflammation. Through a series of studies, it is shown that HDL function correlates with beneficial effects, and diabetes and inflammation dampens HDL functionality leading to increased accumulation of aortic lesion formation. Agents that attenuate hyperlipidemia, glycemic index and proinflammatory state improved HDL functionality leading to reductions in atherosclerotic burden.

3. HYPOTHESES

Despite the well-documented benefits of statin treatment, cardiovascular disease remains a major risk factor for developing coronary artery disease. To further impact the CV outcome, efforts have been made towards HDL-raising therapeutics, including niacin and PPAR-α activators. However, clinical benefits of these therapies remain uncertain because of the failure of CETP inhibitors, Torcetrapib and Dalcetrapib, in phase 3 clinical trials. Low levels of HDL remain a prominent risk factor for developing premature atherosclerosis in diabetics. It is therefore hypothesized that:

a) HDL functionality, not the HDL level, is important in removing cholesterol from periphery by a process called reverse cholesterol transport
b) HDL functionality correlates with extent of aortic lipid deposition
c) HDL functionality is compromized in animal models of hyperlipidemia, oxidative stress and hyperglycemia
d) Proinflammatory state modifies HDL functionality
e) Attenuation of insulin resistance, hyperglycemia, and inflammation restores HDL functionality and promotes plaque regression.

4. APPROACH

The above hypotheses were tested in established and widely used animal models using available tool molecules shown to influence RCT in animal models. The hypotheses were tested in the following steps:

a) Since ABCA1 is the most important player in the maturation of HDL and promotion of cellular cholesterol efflux, a stable cell line was developed to evaluate direct correlation between ABCA1 expression and cholesterol efflux to show that ABCA1 deinduction abolishes cholesterol efflux

b) Optimization of in vivo cholesterol efflux in C57Bl mice using both radioisotopic and nonradioisotopic method

c) Evaluation of ABCA1 inducers on cholesterol efflux in Rader's assay

d) Demonstration of HDL functionality and RCT pathway gene induction in WT and genetically altered mouse models

e) Investigation of HDL functionality in animal models of diabetes, hyperlipidemia and atherosclerosis.

f) Demonstration of inverse correlation between HDL functionality and aortic lipid deposition.

5. ABBREVIATIONS

ABBREVIATION	DEFINITION
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ABCG5	ATP-binding cassette transporter G5
Apo	Apolipoprotein
ApoA-I	Apolipoprotein A-I
AMPK	Adenosine monophoasphate kinase
ApoB	Apolipoprotein B
AUC	Area under the curve
BHK	Baby hamster kidney
b.i.d.	Twice daily dose
8-Br-cAMP	8-bromo cyclic adenosine monophosphate
CAD	Coronary artery disease
cAMP	Cyclic AMP
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
ChreBP	Carbohydrate response element binding protein
CRE-BP	Cyclic AMP response element binding protein
COE	Cholestery oleyl ester
CRP	C-reactive protein
Cyp7A1	Cholesterol 7-alpha hydroxylase
CV	Cardiovascular
CVD	Cardiovascular disease
Chol	Cholesterol
CMC	Carboxymethylcellulose
DKO	Double knockout
DMSO	Dimethylsulfoxide
FAS	Fatty acid synthase
FC	Free cholesterol
FPLC	Fast protein liquid chromatography
FXR	Farnexoid X receptot
g, gm	Gram(s)
h, hr	Hour(s)
HAEC	Human aortic endothelial cell
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HPLC	High performance liquid chromatography
HMGCoA	Hydroxymethyl glutarate CoA
IACUC	Institutional animal care and use committee

IL-1β	Interleukin 1-β
IL-6	Interleukin-6
IFN-γ	Interferon-gamma
IMT	Intima media thickness
kg	Kilogram(s)
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LDLr	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
LUV	Large unilamellar vesicles
LXR	Liver X- receptor
MCH	Mean corpuscular hemoglobin
MCP-1	Macrophage chemo-attractant protein 1
MetS	Metabolic syndrome
MIP1b	Macrophage inflammatory protein 1b
mg	Milligram(s)
mRNA	Messenger RNA
mL	Milliliter(s)
MPO	Myloperoxidase
MS-RCT	Macrophage-specific reverse cholesterol transport
MS	Metabolic syndrome
MUV	Medium unilamellar vesicles
N	Number
ND	Not determined
PBS	Phosphate-buffered saline
PD	Pharmacodynamics
PK	Pharmacokinetics
PL	Phospholipids
PO	Oral dosing
PPAR	Peroxisome proliferator-activated receptor
rHDL	Reconstituted HDL
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase- polymerase chain reaction
RXR	Retinoid X-receptor
SAA	Serum Amyloid A
SD	Standard deviation
SEM	Standard error of the mean
SAA	Serum amyloid
SCD1	Steroyl CoA desaturase
SHP	Small heterodimer parner
SR-B1	Sacvenger receptor class B type I

SREBP1c	Sterol response element binding protein 1c
SREBP2	Sterol response element binding protein 2
SUV	Small unilamellar vesicles
TC	Total cholesterol
TD	Tangier's disease
Tg	Transgenic
TG	Triglycerides
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor- α
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar
UTR	Untranslated region
USF1	Upstream stimulatory factor1
VCAM-1	Vascular adhesion molecule-1
VLDL-C	Very low-density lipoprtoein
WK	Week
WHAM	Wisconsin Hypoalpha Mutant
XBP1	X-box binding protein 1
ZNF202	Zinc finger protein nuclear factor

6. INTRODUCTION

6.1 Cardiovascular Disease and HDL-C

Coronary artery disease (CAD) remains the leading cause of death in the United States and other developed countries (1). While elevated levels of low-density lipoprotein cholesterol (LDL-C) and triglycerides are risk factors for developing coronary artery disease (2), a pathological hallmark of atherosclerosis is the excessive accumulation of cholesterol by macrophages, leading to their conversion to foam cells (3,4). Since LDL-C has been linked to CAD, current therapeutic strategies to prevent atherosclerosis are primarily based on the use of statins, which inhibit HMG-CoA reductase, a key enzyme in the de-novo cholesterol synthesis, and thereby leading to decreased serum LDL-C (2,5,6). Another prominent risk factor for developing premature atherosclerosis is the low levels of high-density lipoprotein cholesterol (HDL-C) (7). Despite documented benefits of statins (2), a good proportion of individuals still remain at a higher risk of developing CAD (8). HDL-C levels are inversely correlated with the risk of coronary artery diseases (8-11) as evidenced by clinical trial results (12-14). There has been a marked increase in the disease risk factor referred to as Metabolic Syndrome (MS) and characterized by a clustering of risk factors leading to developing cardiovascular diseases (15-17). Low HDL-C is characterized as one of the features of MS. The other risk factors include dyslipidemia, hypertriglyceridemia, hypertension, and impaired glucose tolerance. A recent report suggests that MS is becoming pandemic and the number of individuals suffering from MS is likely to double by 2030 worldwide (18). Therefore, aiming to correct dyslipidemia and increase HDL-C may reduce the risks of developing atherosclerotic lesion formation. Low HDL-C levels are the most common lipid abnormalities observed in men with CAD (11). ApoA-I, the major protein component of HDL, determines the blood levels of HDL-C (19) and promotes cholesterol efflux, which in turn promotes reverse cholesterol transport. Therefore, elevating HDL-C may have protective effects on the development of CAD. The current hypothesis of HDL's protective role depicts that HDL inhibits atherogenesis by promoting cholesterol efflux from peripheral tissues and from lipid-laden macrophages and smooth muscle cells. HDL

is suggested to inhibit atherogenesis through other pathways where it exerts direct effect on the vessel wall, and inhibits lipoprotein oxidation. The most important atheroprotective function, however, is the HDL-mediated enhancement of reverse cholesterol transport, a process in which HDL receives excess cholesterol from the peripheral tissues, including macrophages in the arterial wall, which is subsequently delivered to the liver for biliary excretion. The understanding of reverse cholesterol transport and the molecular mechanisms that control serum HDL cholesterol levels have been dramatically increased by the discovery of scavenger receptor- BI (SR-BI) (20,21), ATP-binding cassette transporter A1 (ABCA1) (22,23), and ATP-binding cassette transporter G1 (ABCG1) (24).

6.2. HDL Pathway and Reverse Cholesterol Transport

Cholesterol is transported as two main lipoprotein particles in our body. One is the low density lipoproteins (LDL) or the 'bad cholesterol' synthesized and secreted by the liver, and subsequently cholesterol in LDL particles are taken to the peripheral tissues for cellular needs, and the other is HDL or 'good cholesterol' that brings back cholesterol from the peripheral tissues to the liver for excretion as bile. The main protein component of the LDL particle is the apolipoprotein B, a 550 kDa ampipathic protein, which serves as a ligand for the LDL receptor-mediated uptake by various tissues (25,26). The main protein component of HDL particles is the apolipoprotein A-I (27-29). Several metabolic pathways determine the net concentration of the HDL in the circulation. As depicted in Fig. 1, HDL particles are synthesized and secreted by the liver and intestine. The HDL precursors, also called as nascent HDL or HDL discs, are generated through the action of lipoprotein lipase on chylomicrons and very low-density lipoproteins as well as by the direct secretion by the liver. In the plasma, HDL undergoes structural and compositional changes by the action of lipid transfer proteins and lipases, and by the exchange of apolipoproteins and lipids between circulating lipoprotein particles. HDL particles bring back cholesterol from the peripheral tissues to the liver for excretion (Fig. 2). Scavenger receptor class B type I (SR-B1) (21) and cubulin and megalin (30) have been suggested to be involved in the uptake of HDL particles. Thus, plasma levels of HDL represent a

net result of the rates of production into and the rates of clearance from plasma. Reverse cholesterol transport refers to the process by which cholesterol is removed from the tissues and returned to the liver. HDL is the key lipoprotein involved in reverse cholesterol transport and the transfer of cholesteryl esters between lipoproteins. The smallest and most dense lipoprotein particle is HDL, which undergoes maturation when precursor particles (nascent HDL), secreted by the liver and intestine, proceed through a series of conversions (known as the "HDL cycle") to attract cholesterol from cell membranes and free cholesterol to the core of the HDL particle. The action of cholesteryl ester transfer protein (31), which transforms HDL into a TG-rich particle that interacts with hepatic-triglyceride lipase. Cholesterol ester-rich HDL may also be taken up directly by the receptors in the liver. Another mechanism may be that cholesterol esters are delivered directly to the liver for uptake without catabolism of the HDL cholesterol particle (21).

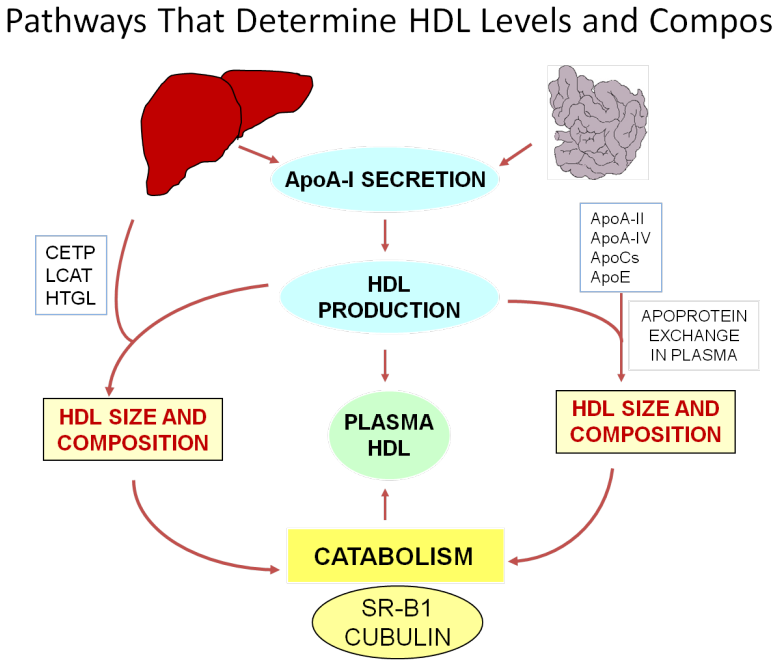


Fig. 1. A general scheme showing metabolic pathways regulating plasma levels of HDL. As shown, plasma levels of HDL are controlled by its rates of production into and the rates of removal from plasma. Production rates of HDL, as depicted, are determined mainly by the production rates of apoA-I, while the removal of HDL from plasma is regulated by structural and compositional changes of circulating HDL particles as well as receptor-mediated uptake by the liver and other peripheral tissues.

Among other players, SR-BI (21), ABCA1 (23), and hepatic lipase (32) appear to be important in the reverse cholesterol pathway. In ultracentrifugation, HDL floats at a density of 1.09–1.21, g/ml. HDL particles can also be separated by their size using gel permeation chromatography that separates particles based on their size (25,27). Since VLDL and LDL particles are larger than HDL, these particles elute from the FPLC column in the order of: VLDL, LDL, and HDL. HDL particle sizes range in diameter between 70 and 100 Å and their molecular mass ranges from 200–400 × 103 daltons (25). The concentrations of plasma HDL are expressed by their cholesterol content, although the cholesterol content of the HDL particles is only about 15%. HDL circulates in plasma as spherical particles. Cholesteryl esters form the 'core-lipid', and depending upon the action of lipid transfer proteins, variable amounts of triglycerides are present in the HDL particles. The outer surface of the HDL particle contains free cholesterol, phospholipids, and apolipoproteins. In human plasma, the presence of several discrete classes of HDL sub-populations has been identified. These HDL subclasses vary in their size, and composition. HDL_2 and HDL_3 are the major HDL subpopulations present in plasma of most animal species. HDL_2 is about 50% larger than HDL_3 with 3-4-fold more cholesteryl esters and 2-fold more phospholipids than HDL_3. Several other discrete HDL subclasses have been identified in human plasma including HDL_1, which can be separated from other HDL sub-fractions. Majority of the plasma apolipoprotein E in rats and mice are associated with HDL_1. A number of apolipoproteins are associated with HDL. They are apolipoprotein AI, constituting 70% of the total protein in HDL particles, apoA-II, apoA-IV, apoE, apoC-I, C-II, and C-III. HDL particles also carry other proteins like LCAT, lecithin cholesterol acyltransferase (33). Throughout their circulating lifetime, the apoA-I molecules appear to be released and transferred between HDL particles by replacement of apoA-I molecule. Both apoA-I and A-II have been shown to exchange between HDL_2 and HDL_3 (34). ApoA-I and apoA-II can also exchange from free solution by replacing apoA-I and A-II molecules on the HDL particles. However, it is not possible to exchange all the apoA-I molecules on the HDL particles. Other

apoproteins can replace apoA-I from the HDL particles, the most important among those being apoA-II and apoCs (35).

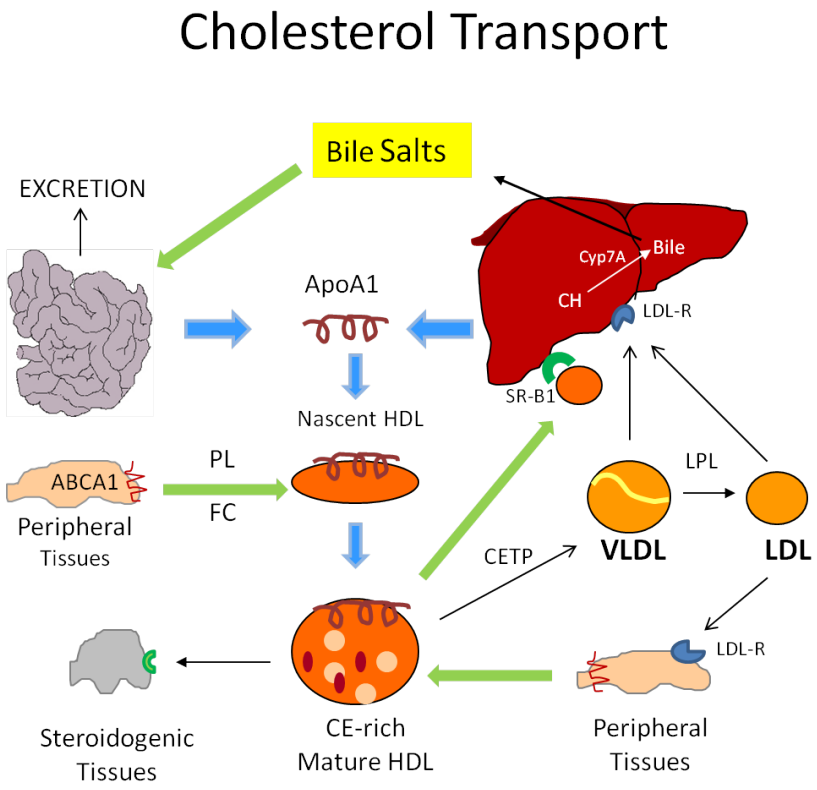


Fig. 2. Cholesterol transport. Apolipoprotein A-I, synthesized from the liver and the gut, forms nascent HDL particles. The nascent discoidal HDL particle accepts cholesterol and phospholipids from the peripheral tissues in ABCA1-dependent manner, and gets converted into cholesteryl ester-rich mature HDL particles. The mature HDL particles are then taken to the liver in a process called reverse cholesterol transport in which SR-B1 plays an important role in docking and accepting the cholesterol esters from HDL particles. The cholesterols delivered to the liver is converted into bile by cholesterol 7-α hydroxylase and excreted to the gut.

6.2.1. ApoA-I and Coronary Artery Disease

ApoA-I is the major protein component of HDL and shows a strong correlation with the plasma levels of HDL (36,37). Human mature apoA-I consists of 243 aa with a molecular size of 28-kDa. It is synthesized as a precursor protein, preproapoA-I, of 267 aa. The major sites of apoAI synthesis are the liver and the small intestine (19). Several studies suggest that apoA-I protect against developing premature atherosclerosis, and acts

as an antiatherogenic agent. Feeding high fat high cholesterol diet to African green monkeys resulted modest hypercholesterolemia and less atherosclerosis as comparedto cynomolgus monkeys, which was attributed to the higher basal levels of HDL and apoA-I in African green monkeys compared to cynomolgus monkeys (38). African green monkeys showed more than two fold circulating plasma HDL levels compared to cynomolgus monkeys. Measurements of apoA-I synthesis in the liver perfusates correlated with the plasma levels of HDL (Fig. 1). Subsequently, these authors found that the lower rates of apoA-I synthesis in cynomolgus monkeys were due to low levels of hepatic and intestinal apoA-I mRNA, suggesting that apoAI mRNA and apoA-I production rates determine the plasma levels of apoA-I, and hence plasma levels of HDL. This study suggested that higher plasma levels of HDL due to higher production rates of apoA-I protects against diet-induced atherosclerosis. Similar results with regard to the protective effects of HDL and apoA-I have been obtained in several mutant mouse models of atherosclerosis.

Transgenic mouse lines over expressing human apoA-I have been developed by several groups (39). These apoA-I transgenic mice have higher levels of human apoA-I, but showed reductions in plasma levels of mouse apoA-I, and appearance of discrete sub-populations of HDL particles (39,40). Since mouse apoA-I mRNA did not change in these transgenic mouse lines expressing human apoA-I, it was concluded that the over-expression of human apoA-I possibly displaced mouse apoAI from the HDL particles, resulting the appearance of two subclasses of HDL populations (39). Another group of researchers that developed apoA-I transgenic mouse line reported increased plasma levels of high-density lipoproteins as a result of over-expression of human apoA-I, but the HDL particle sizes were smaller as compared to human HDL_3 (40). They further reported that the alteration in the HDL particle size distribution affected selective uptake of HDL cholesteryl esters (40). Since higher levels of HDL and its major apoprotein component, apoA-I, have been implicated in lowering the risk of premature coronary artery disease, it is safe to hypothesize that mice over-expressing apoA-I, and secreting high levels of HDL should be protected from developing high fat diet-induced premature

atherosclerotic lesion formation. This hypothesis was tested in the apoA-I transgenic mice-fed high fat diet. As compared to their nontransgenic littermates, the transgenic mice inhibited high fat diet-induced early atherogenic fatty streak formation (41), confirming the antiatherogenic properties of apoA-I. The role of apoA-I and HDL in atherosclerosis risk was also studied in mice over-expressing human apoB and lacking apoA-I (42). Feeding these mice Western-type high fat diet developed lesion formation twice as much in mice over-expressing human apoB gene and lacking apoA-I than in mice over-expressing human apoB gene. These observations were further supported by studies carried out in the apoE-deficient mouse (43). Thus, these findings confirm the role of apoA-I in reducing the atheroslcerotic lesion formation, and hence the risk of developing premature atherosclerosis. The over-expression of apo[a] promotes lesion formation, while over-expression of human apoA-I in apo[a] transgenic mice lowered the risk of atherosclerosis by 20-fold (44). Thus, HDL is associated with the decreased atherosclerosis risk, and plasma apoA-I level is a major determinant of HDL concentrations. The role of apoA-I as an antiatherogenic agent has been established in animal models (43,45,46) as well as in humans (47).

6.2.2. Cholesterol Efflux, ABCA1 and Atherosclerosis

Bailey (48) first observed in 1965 in an *in vitro* model that cholesterol can be removed from the cells by exposing them to serum. Subsequently, Glomset (33)formulated the concept of 'reverse cholesterol transport', in which cholesterol from the peripheral tissues are brought to the liver by HDL particles for excretion as bile salts. Most of the earlier studies relating to cholesterol efflux were done on movement of cholesterol between serum lipoproteins and cells in a bidirectional manner. In addition to whole serum, several cholesterol acceptors like cyclodextrins (49), phospholipid vesicles such as SUV, LUV, and MUV (50), lipid-poor apolipoproteins (51,52), and reconstituted and native HDL (53), induced cholesterol efflux from cholesterol-loaded cells. Fournier *et al.* (54) first showed a correlation between cholesterol efflux from macrophages and serum apoA1 concentration, suggesting a role of apoA-I in cellular cholesterol efflux. Later studies in a variety of cell-types showed that other apoproteins like AII, E, and AIV

can also function as cholesterol acceptors (55). Smith *et al.* (56) as well as Sakr *et al.* (57) demonstrated that cAMP treatment of macrophages resulted in several-fold increase in cholesterol efflux to acceptor lipid-poor apolipoproteins. These observations together with other studies (51,58) suggested the existence of an interaction between the acceptor apolipoproteins and the cell membrane component(s).

The assembly of apoB-containing particles is quite different compared to the assembly of apoA-I-containing particles. The assembly of apoB particles takes place intracellularly in a highly regulated manner involving several intracellular components that determine how much apoB particles to be assembled and secreted (26,59,60). The assembly of apoA-I particles, on the other hand, takes place extracellularly, in which each component necessary for the assembly are secreted separately and assembled in the circulation. That other players, in addition to those secreted by liver and intestine, are also involved in the assembly of HDL particles became apparent from the studies with WHAM chickens (61). These mutant chickens have normal secretion rates of apoA-I yet have only 5% of the HDL compared to normal. Later kinetic studies with labeled apoA-I solved this puzzle, showing that the secreted apoA-I, if not assembled into HDL particles, are rapidly catabolized. These studies proved to be the hall-mark in the process of HDL assembly, suggesting that secretion rates of apoA-I alone is not sufficient to set the plasma levels of HDL, as has been proposed by many investigators in the past (reviewed in (19) . Although the process of cellular cholesterol efflux and the roles of apoproteins in this process have been known for decades, it was not until 1999 when the specific role of ABCA1 was identified in the cellular cholesterol trafficking. Three separate groups simultaneously identified mutations in the *Abca1* linked to TD (62-64) with incredibly low levels of circulating HDL, cholesterol ester storage in tissues, and susceptibility to premature atherosclerosis. As shown in Fig. 2, the lipid-poor apoA-I particle functions as a cholesterol acceptor from peripheral tissues, and gets converted into mature HDL particle, which then delivers this cholesterol to the liver and steroidogenic tissues via scavenger receptor class B type 1 (SR-B1)-mediated pathway. ABCA1 participates in the reverse cholesterol transport by facilitating the efflux of cholesterol from cells to the

acceptor lipid-poor apoA-I-particles (Fig. 2), which are then taken to the liver for excretion as bile salts (Fig. 1 & 2). In addition to apoA-I, other apoproteins also mediate cholesterol efflux by functioning as a cholesterol acceptor. The roles of various apolipoproteins in the ABCA1- mediated lipid efflux were recently evaluated in stably transfected Hela cells expressing ABCA1-GFP fusion protein (65). The apoproteins A-I, A-II, A-IV, C-I, C-II, C-III and E all showed more than 3-fold increase in cholesterol efflux compared to control cells, suggesting that other apoproteins are also capable of inducing cholesterol and phospholipid efflux. Thus, amphipathic helical domains appear to be an important feature of the acceptor molecule in the cholesterol efflux process. Indeed, amphipathic peptides do induce cholesterol efflux in ABCA1-dependent and ABCA1-independent manner. Despite other apoproteins being able to induce cholesterol efflux, lipid-poor apoA-I appears to be the preferred acceptor of ABCA1-mediated lipid efflux. Since apoA-I has been shown to specifically bind to ABCA1 (66), the lipid-poor apoA1 (preβ-HDL) functions as an acceptor of cholesterol and phospholipid in an ABCA1-dependent manner resulting the formation of mature cholesterol ester rich spherical α-HDL particles following the action of LCAT. SR-BI interacts with the mature α-HDL and facilitates the uptake of CE from the HDL particles (Fig. 2).

ABCA1 transporters constitute a large family of proteins, and participate in the translocation of many different substrates across the membrane (67). The majority of ABC transporters are membrane proteins with two membrane domains and two ATP subunits, either present in one molecule (fullsize transporters) or in two polypeptide molecules (half-size transporters). The ABC proteins bind ATP and use the energy to drive the transport of various molecules across the membranes. ABCA1 is a full-size transporter, and one of the members of seven subfamilies having a total of 48 known members. ABCA1 is ubiquitously present in variety of tissue (68), notably, liver, brain, and steroidogenic tissues.

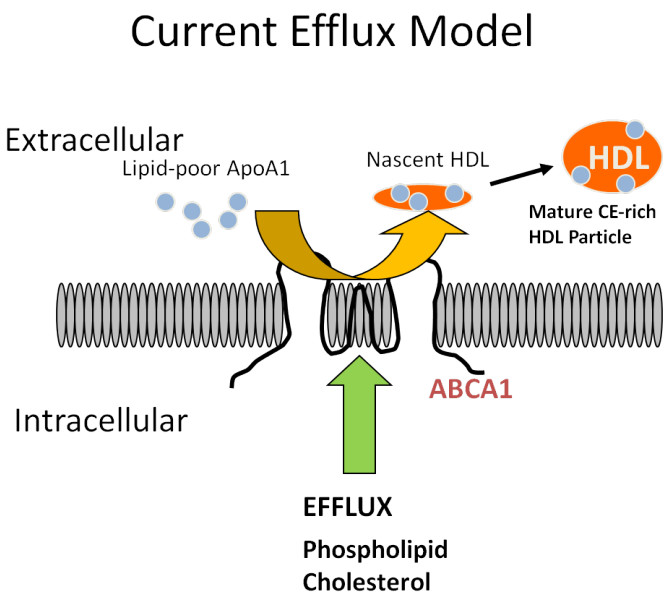


Fig. 3. ABCA1-mediated cholesterol efflux. Lipid-poor discoidal HDL particle in the circulation accepts cholesterol and phospholipids from the tissues via ABCA1-mediated pathway. ABCA1 is a membrane protein that facilitates the transport of cholesterol and phospholipids from the tissues to the lipid-poor HDL particles and, as a result, the nascent HDL particles get converted into mature cholesterol ester-rich HDL particles capable of transporting cholesterol to the liver for excretion. Any defect in the membrane-associated ABCA1 protein renders them unable to mediate the cellular cholesterol efflux resulting into the deposition of cholesterol within the tissues.

6.2.3. ABCA1 and HDL

ABCA1 is a 2261 amino acid integral membrane protein. Several mutations in ABCA1 have been identified that cause a severe HDL deficiency syndrome, known as Tangier's disease (TD) (62-64,69). The fibroblasts of these patients are unable to facilitate the efflux of cholesterol and phospholipids, consistent with a defective ABCA1 in facilitating lipid trafficking across the membrane. The TD patients, among other abnormalities, are characterized by the accumulation of cholesteryl esters in reticuloendothelial cells, lipid deposits in neuronal Schwann cells, smooth muscle cells, and fibroblasts together with hyper-cholesterolemia and cardiovascular disease. The only other animal species in which functional mutation in ABCA1 gene was identified is Wisconsin Hypoalpha Mutant (WHAM) chicken (61). These WHAM chickens have very low levels of HDL due to hypercatabolism of lipid-poor apoA-I resulting in the accumulation of cholesteryl esters in tissues. Further insights on the role of ABCA1 were

gained by the development of *Abca1* knockout mice by two separate groups (70,71). These mice lacking ABCA1 exhibited phenotypes similar to TD, and showed pronounced accumulation of lipids in the tissues, consistent with the reported function of ABCA1 in the cholesterol trafficking across the membrane. *Abca1*-deficient mice were severely hypolipidemic with almost complete absence of HDL, a condition observed in TD. Interestingly, the *Abca1*$^{-/-}$ mice also showed decreased absorption of cholesterol, suggesting a role of ABCA1 in cholesterol absorption. The loss of function of ABCA1 and the absence of HDL in *Abca1*$^{-/-}$ was also shown to significantly affect the steroidogenesis (72), corroborating earlier studies that the primary source of cholesterol to the steroidogenic tissues is HDL via SR-B1-mediated uptake. SR-B1 is highly expressed in steroidogenic tissues (20).

Recently, it was shown that ABCA1 is also involved in the trafficking of α-tocopherol (73). Since α-tocopherol is lipid-soluble, and hence associated with lipoprotein particles, and since ABCA1 plays an important role in cholesterol and phospholipid trafficking, these authors hypothesized that ABCA1may be involved in the trafficking of α-tocopherol between tissues. Both in the unstimulated and in cAMP-stimulated macrophages, α-tocopherol was found to be effluxed from the cells. Since α-tocopherol efflux from Tangier disease fibroblast was impaired, it was concluded that cellular cholesterol and α-tocopherol efflux occurred via ABCA1-mediated mechanism. Further evidence of ABCA1-mediated α-tocopherol trafficking between tissues came from BHK cells transfected with ABCA1. In the transfected cells, the α-tocopherol efflux occurred in the presence of apoA-I, HDL, or trypsinized HDL, suggesting that ABCA1 is indeed involved in the trafficking of α-tocopherol.

Since fibroblasts from TD patients and from *Abca1*$^{-/-}$ mice showed significantly reduced apoA-I-dependent efflux, it was anticipated that overexpression of ABCA1 in cells and in animal species should enhance the apoAI-dependent cholesterol and phospholipid efflux. Indeed, overexpression of ABCA1 in mouse macrophages enhanced cholesterol efflux (74) further supporting the key role of ABCA1 in apoA-I-dependent cholesterol efflux from cells. *Abca1* gene located on chromosome 9 has 50 exons and 49

introns (75). Two separate groups of researchers used Bac clones containing *Abca1* for the generation of transgenic mice (76,77), whereas one group used ABCA1 cDNA for making transgenic mouse line (78).

Two transgenic mouse lines were produced by Cavalier *et al.* (76), one containing 255 kb genomic fragment having 36 kb 5′ upstream region from the transcription start site and 15 kb 3′UTR region. The other mouse line was made from a 171 kb genomic fragment containing the same 15 kb 3′ UTR region, but had only exon 1 sequences in the 5′ region. They used FVB mouse for making transgenic line. The mouse expressing 255 kb transgene showed expression in many tissues with macrophage showing enhanced cholesterol efflux. The 171 kb transgene mouse line showed expression in liver, but not in macrophages, suggesting the presence of a regulatory element in the 5' upstream promoter of the *Abca1* responsible for macrophage-specific expression of ABCA1 and cholesterol efflux. Most notably, the total- and HDL cholesterol in both ABCA1 transgenic lines did not change. Singarajaja *et al.* (77) used genomic DNA with natural ABCA1 promoter (10 kb) in C57BL background, and showed that the overexpression of ABCA1 increased HDL and cholesterol efflux in macrophages. They also observed increased ABCA1 mRNA and HDL in non-transgenic and transgenic mice fed atherogenic diet. Vaisman *et al.* (78) cloned full-length ABCA1 cDNA in pLIV.11 expression vector with apoE enhancer. Using this transgenic line in C57BL/6 mouse was made. Overexpression of ABCA1 increased plasma levels of HDL and increased apoA-I-mediated cholesterol efflux from macrophages. In an elegant study, they further showed that increased plasma levels of HDL occurred by decreased catabolism of HDL. The apoA-I production rates did not contribute to increased levels of HDL.

It should be noted that chow diet used by Singaraja *et al.* (77) contained cholic acid, which was evident from low HDL levels on this diet (HDL 36 mg/dl; non-HDL-C 28 mg/dL). The other group (78) reported HDL level of 80 mg/dL and non-HDL-chol 7–10 mg/dl, which makes sense given the published data on HDL levels in several mouse strains fed either a chow (no cholic acid) or a high fat (no cholic acid) diet (25,27). Given that cholic acid containing diet decreases HDL particles by transcriptional mechanism

(79), and increases apoB-containing particles (80), it is not surprising that Singaraja *et al.* (77) noted lower than expected levels of HDL on cholic acid containing chow diet, and higher than expected levels of apoB particles. Because bile acids may independently influence cholesterol homeostasis via FXR (81) and also via LXR (82), it is possible that cholic acid may influence ABCA1 expression. Nevertheless, studies from Singaraja *et al.* (77) demonstrated that atherogenic diet increased ABCA1 mRNA and protein, which makes sense given the upregulation of ABCA1 in cholesterol-loaded cells, and increased lipid levels in the livers of mice-fed high fat diet (27). These observations were further corroborated by another study in wild-type and A-I-CIII-AIV transgenic mice (83). Feeding high fat diet for 20 weeks increased hepatic ABCA1 mRNA levels in A-I-CIII-AIV transgenic mice, which was associated with the atheroprotective effects. The reason(s) why two groups noticed HDL elevation in their transgenic mouse lines, while the other did not, despite increased cholesterol efflux by isolated macrophages, could be due to the differences in the genetic background of mice used for making transgenic line.

Inbred strains of mice differ in their responsiveness to dietary and hormonal interventions (84). Also, it is not necessary that increased ABCA1 expression in macrophages would proportionally increase circulating HDL particles (85). An elegant study by Haghpassand *et al.* (86) evaluated the role of macrophage ABCA1 in contributing to plasma levels of HDL. They used ABCA1$^{-/-}$ mice created in DBA1Lac/J background (70). Bone marrow transplantation was carried out from WT to WT and ABCA1$^{-/-}$ mice, and ABCA1$^{-/-}$ to WT and ABCA1$^{-/-}$ mice, resulting into four groups as follows: WT → WT, WT → ABCA1$^{-/-}$, ABCA1$^{-/-}$ → WT, ABCA1$^{-/-}$ → ABCA1$^{-/-}$. Bone marrow transplantation from WT mice resulted increased ABCA1 expression in WT and ABCA1$^{-/-}$ macrophages, and bone marrow transplantation from ABCA1$^{-/-}$ mice resulted lack of ABCA1 expression in WT and ABCA1$^{-/-}$ recipient macrophages. Despite increased levels of macrophage ABCA1 expression there was no change in the plasma levels of HDL, suggesting that macrophage ABCA1 contributes very little to maintaining plasma HDL levels. Therefore, hepatic ABCA1 expression gives rise to the bulk HDL particles. These results combined with other studies suggest that macrophage cholesterol

efflux can be a determinant in the prevention of atherosclerosis, but may not contribute to the bulk HDL particles.

6.2.4. ABCG Transporters and Reverse Cholesterol Transport

In addition to ABCA1, which effluxes cholesterol to the nascent, discoidal HDL particles, there is another player in the pathway of HDL maturation that effluxes cholesterol to the doiscoidal HDL particles generated by the action of ABCA1. This transporter is known as ABCG1 (24,87,88). ABCG1, one of the half-type ATP binding cassette proteins, mediates the efflux of cholesterol to HDL and functions in the reverse cholesterol transport from peripheral cells to the liver. ABCG1 also mediate phospholipid efflux to human serum and HDL. ABCG1-mediated free cholecterol efflux correlated significantly with a number of HDL subfractions and components in serum collected from normolipidemic individuals (89). It did not enhance influx of cholesterol or cholesteryl oleyl ether (COE) when cells were incubated with radiolabeled HDL and expression of ABCG1 did not increase the association of HDL with cells.

Wang et al (90,91) showed that ABCG1 promotes efflux of cholesterol to a variety of acceptors, including HDL and phospholipid vesicles without increasing the binding of lipoproteins to cells. ABCG1 also promoted efflux of a broader spectrum of sterols including oxysterols, whereas ABCG1 and ABCG4 together promote efflux of sterol intermediates from the cholesterol synthesis pathway such as desmosterol (92). A number of ABCs involved in lipid transport are regulated through activation by LXR, and these include ABCA1 (93), ABCG1 (90), and ABCG4 (90). However, studies with ABCG4 $^{-/-}$ mice suggested that ABCG4 is not expressed in macrophages and does not contribute to HDL-mediated cholesterol efflux (94).

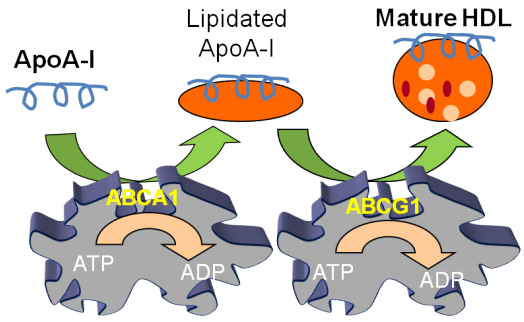


Fig. 4. ABCA1-and ABCG1-mediated cholesterol efflux requires energy in the two-step HDL maturation. Lipid-poor discoidal HDL particle in the circulation becomes lipidated by ABCA1using ATP followed by ATP-dependent maturation of HDL by ABCG1.

Interestingly, overexpression of ABCG1 increases efflux of not only cellular sterols but also, to some extent, cellular phospholipids. (90,95,96). Overexpression of ABCG1 results in increased cell membrane cholesterol pools available for efflux and increases the rate constant for efflux; efflux appears to be diffusional and unidirectional and is more efficient for smaller HDL particles (89). Different models have been proposed to explain how ABCG1 promotes cholesterol efflux to HDL. One suggests that ABCG1 helps sterol molecules to overcome the energy barrier for entry into the hydrophilic water layer, perhaps by using ATP to promote protrusion of the cholesterol molecule into water, followed by a transient collision with an acceptor (97). A second model points to a function of ABCG1 as a phospholipid floppase (95), promoting changes in the organization of plasma membrane phospholipids and subsequent attraction of sterols to the outer leaflet for diffusional efflux. Another possibility is that ABCG1 promotes flopping of cholesterol in liquid ordered regions of plasma membrane such that spontaneous flipping of sterol back to the inner membrane is not possible.

The incubation of apoA-I with J774 mouse macrophages expressing ABCA1 (66) leads to the efflux of cholesterol and phospholipid and the subsequent formation of nascent high density lipoprotein (HDL) particles (98). ABCG1 transcription is highly up-regulated in cholesterol loaded macrophages (99), and ABCG1 facilitates the efflux of cholesterol from cells to HDL, rather than to free apoA-I (90,100,101). The peroxisomal proliferator activated receptor-γ (PPAR-γ) activators also increase ABCG1 transcription

in macrophages and stimulate cholesterol efflux to HDL independent of their induction of LXR (102). Like ABCG1, ABCG4 also facilitates the efflux of cholesterol from cells to HDL (87,90), although there is evidence that ABCG1 and ABCG4 can form a heterodimer (103). ABCA1 and ABCG1 have been shown to synergize to mediate the efflux of cholesterol to apoA-I (88). These findings suggest a significant role for ABCG1 and possibly ABCG4 in the release of cholesterol from cells into the reverse cholesterol transport pathway. The importance of macrophage ABCA1 expression in atherosclerosis has been demonstrated in mice, where the bone marrow transplantation of ABCA1 null macrophages was shown to lead increased atherosclerosis in hyperlipidemic animals (104). Conversely, the over-expression of macrophage ABCA1 in mice reduces atherosclerotic lesion development in low-density lipoprotein receptor deficient mice (105). The targeted disruption of ABCG1 in mice on a high fat and high cholesterol diet causes the massive accumulation of both neutral lipids and phospholipids in hepatocytes and macrophages of multiple tissues (24). Conversely, tissues in ABCG1 transgenic mice are protected from dietary fat-induced lipid accumulation (24). The efflux of cholesterol to these particles is dependent both on apoA-I concentration used to efflux lipid from ABCA1 and on the time the particles are incubated with ABCG1 and ABCG4 expressing cells. The cholesterol released from the ABCG1 and ABCG4 expressing cells by HDL comes mainly from a cell-surface cholesterol domain that is formed upon expression of the transporters (87).

6.2.5. ABCA1 Regulation

Considerable work has been done on the regulation of ABCA1. Most of the studies focused on the transcriptional regulation, although some attempts have been made to investigate posttranscriptional regulation of the ABCA1. A number of researchers have shown that cholesterol efflux to apoA-I in macrophages is highly inducible by cyclic AMP analogs, which was attributed to the transcriptional upregulation of ABCA1 (106,107). Oram *et al.* (66) showed that incubation of macrophages with 8-Br-cAMP stimulated ABCA1 protein and mRNA, with highest level achieved after 24 h incubation. Withdrawal of 8-Br-cAMP resulted in bringing the ABCA1 protein and mRNA to the

basal level within 2–6 h, suggesting that ABCA1 protein is highly unstable and gets degraded in the absence of 8-Br-cAMP. First study showing cAMP-mediated induction of cholesterol efflux through ABCA1 phosphorylation was demonstrated in fibroblasts (108). These authors further showed that apoA-I activated cellular cAMP signaling through ABCA1(109). Further studies with ABCA1 promoter in the presence and absence of cAMP response elements confirmed (110) the results of Oram *et al.* (66) and Haider et al (108). These authors also demonstrated (110) the role of PKA in cAMP-mediated upregulation of ABCA1 gene expression, since protein kinase A has been implicated in the cAMP-mediated regulation of target genes via phosphorylating a transcription factor, CRE-BP1 (cAMP response element binding protein 1), which binds to the cAMP response element, and drives the transcription of target genes. Experiments with non-macrophage cells with cloned ABCA1 gene in front of heterologous promoter with CRE-BP1 element will be needed to prove the hypothesis that cAMP-mediated response occurs through PKA-mediated pathway primarily at the transcription level.

The cloning of ABCA1 gene and its promoter enabled the analysis of ABCA1 structure and function. In addition to functional regulatory domains in the exon 1, an LXR element was recently reported in the intron 1 sequences (77). The nalternative splicing of ABCA1 gene gives rise to two splice variants. The ABCA1 promoter, in addition to TATA box and CAAT box, also contains other potential regulatory sites. An imperfect direct repeat of the nuclear receptor half sitenTGACCT spaced by 4 nucleotides (DR4) bind to the LXR/ RXR heterodimers, and mutation in the DR4 abolished the oxysterol-responsive ABCA1 activation, suggesting that DR4 is important in LXR/RXR-mediated upregulation of ABCA1, as well as for oxysterol-induced activation of ABCA1 (85,111). Two separate regions of ABCA1 promoter appears to be important in the transcriptional activations of ABCA1, the E-Box motif located –147 bp upstream that binds to transcriptional factors USF1 and 2. It also binds to the transcriptional repressor Fra2. LXR-α is stimulated by its ligand, oxysterols, and binds as a heterodimer (LXR/RXR) to the DR4 and activates the transcription of ABCA1. *In vivo* studies in mice have confirmed that LXR is an important activator of ABCA1 (85).

cAMP has been shown by several researchers as an activator of ABCA1 by transcriptional mechanism, since increased ABCA1 mRNA by cAMP does not involve ABCA1 mRNA stability (66). This implies that a cAMP response element should be present in the ABCA1 promoter. Indeed, a cAMP response element in the ABCA1 promoter has been identified (110), which appears to drive effect of cAMP on the transcriptional activation of ABCA1, although detailed study showing involvement of CREBP on transcription of ABCA1 gene was not shown. In order for cAMP to regulate transcription of the target gene in a given cell line, the expression of CRE-BP (cAMP response element binding protein) in that cell line appears to be a prerequisite. Future researches will focus on how regulation of ABCA1 gene by cAMP is coordinated at the transcription, translation and post-translation level. The transcriptional repressor ZNF202 (112) was mapped to chromosome 11q23 and was found to be associated with hypoalphalipoproteinemia. Since mutations in ABCA1 were also shown to be associated with hypoalphaproteinemia, it was anticipated that a link between ABCA1 and ZNF202 exists. Using transfection and mutational studies, it was demonstrated that ZNF202 binds to the GnT motif of ABCA1 promoter and mediates the transcriptional repression (112). Therefore, ZNF202 is an interesting candidate to modulate cholesterol efflux via ABCA1-mediated pathway.

6.2.6. ABCA1 and Atherosclerosis

The removal of excess cholesterol from macrophage foam cells by HDL and its principal apolipoprotein, apoA-I, is thought to be one of the key mechanisms underlying the atheroprotective properties of HDL. Insights gained from ABCA1 heterozygotes are indicative of ABCA1's cardioprotective effects (113). An analysis of 77 individuals from 11 families suggested that ABCA1 heterozygotes had about 40–45% decrease in HDL levels and 40% increase in triglyceride level compared to unaffected controls. The decreased levels of HDL were also found to be associated with the decreased cholesterol efflux by fibroblasts, suggesting a direct association between circulating HDL levels and cholesterol efflux. These researchers went on further to explore a correlation between HDL cholesterol/cholesterol efflux and intima-media complex thickness (IMT) of

peripheral arteries in these heterozygotes. Interestingly, the increased IMT was correlated with decreased cholesterol efflux and about 25 years earlier onset of progression of atherosclerosis than the unaffected controls. These data clearly demonstrate that the removal of cholesterol from artery wall (both ABCA1- dependent and ABCA1-independent) is associated with atheroprotection, and defect in cholesterol efflux predisposes an individual to premature atherosclerosis. Thus, small increases in cholesterol efflux may have significant impact on the extent of atherosclerosis as measured by the intimal thickness (61,113). Therefore, given the data that more than 60% of patients with cardiovascular disease have normal LDL levels, the cholesterol efflux is a good target to reduce the risk of atherosclerosis, and given the important role of ABCA1 in apoprotein-mediated cholesterol efflux, it is possible to modulate cholesterol efflux by modulating ABCA1 expression.

The data from two different groups (77,78) are in agreement with regard to the elevated levels of HDL, cholesterol efflux and recently with regard to the inhibition of atherogenesis in ABCA1 transgenic mice (22), and in A1-CIII-AIV transgenic mice (83). Overexpression of ABCA1 in diet-induced atherosclerosis susceptible mice C57BL/6 resulted in decreased atherogenic particle by half and increased HDL levels 2.8-fold. ApoE and apoA1 levels also increased in these mice by more than 2-fold (22). These changes were associated with significantly lower (65%) aortic atherosclerosis, demonstrating that ABCA1 has antiatherogenic properties, possibly via removing cholesterol from the arterial wall, since HDL levels increased more than 2-fold and LDL levels decreased 2-fold. In addition, increased apoE levels in ABCA1- transgenic mice may have further contributed to the inhibition of progression of atherosclerosis via removal of the atherogenic particles from the circulation, and cholesterol from lipid-laden macrophages. However, when these mice were crossed with $apoE^{-/-}$ mice, the protective effect of ABCA1 was lost, and as a result, ABCA1 transgenic mice having $apoE^{-/-}$ background showed more atherosclerosis (22). Given the enhanced cholesterol efflux in ABCA1 over-expressing mice, one would expect the double mutant mice ABCA1-Tg x $apoE^{-/-}$ to show inhibition of atherosclerosis. To further have insights into the role of

ABCA1 in the inhibition of atherosclerotic lesion formation, bone marrow transplantation technique was used. They used either WT or ABCA1$^{-/-}$ mouse line for bone marrow transplantation into irradiated LDLr$^{-/-}$ mice similar to a recent study (86). As determined by PCR, they showed more than 90% of bone marrow cells were from donor origin. No changes in the lipoprotein profiles were noticed either on chow or on high fat diets. These results clearly established that ABCA1 is protective against development of atherosclerotic lesion formation, and the absence of ABCA1 promotes lesion formation in LDLr deficient mice fed high cholesterol high fat diet for 12 weeks. These results are consistent with another study showing no changes in the circulating levels of HDL following bone marrow transplantation from WT to ABCA1$^{-/-}$ mice (86). Clee *et al.* (113) showed that ABCA1 heterozygotes and homozygotes had more prevalence and severity of atherosclerosis than the unaffected controls. They also showed that heterozygotes had an earlier onset of coronary artery disease. These studies together with ABCA1 transgenic mice suggest that ABCA1 protects individuals from developing premature atherosclerosis. Based on these results the role of ABCA1 in atheroprotection is schematically depicted in Fig. 5.

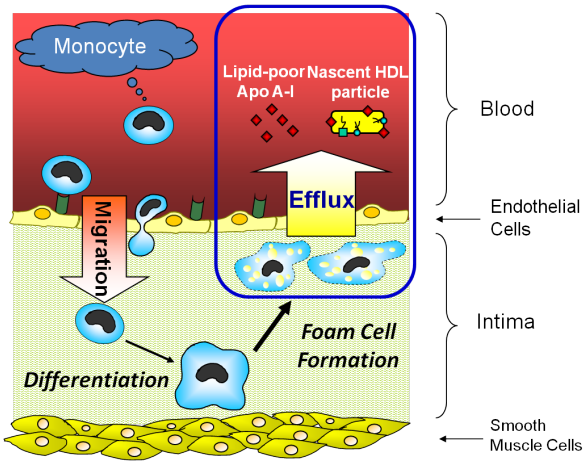


Fig. 5. Schematic representation of atheroprotective effects of ABCA1. Lipid-laden macrophages in the arterial walls set the stage for the initiation of atherosclerosis via a variety of mechanisms. The removal of lipids form the macrophages are therefore a prerequisite in the process of inhibition of atherosclerosis. ABCA1-elevating agents and stimuli accelerate the removal of cholesterol and phospholipids from the lipid-laden macrophages, and reduce the risk of predisposition to atherosclerosis.

6.3 Nuclear Hormone Receptors Mediated Cholesterol Efflux

Incubation of human and murine macrophages with lipoproteins, oxysterols, and oxidized LDL induced ABCA1 mRNA concomitant with increased levels of LXRα expression (93,111,114,115). Both RXR as well as LXR agonists induce ABCA1 expression as measured by ABCA1 mRNA levels. Repa *et al.* (85) identified the heterodimeric partner of LXRα responsible for the induction of ABCA1 mRNA by LXRα agonists, and showed that both RXR and LXR agonists induce ABCA1 mRNA in duodenum, jejunum and ileum, but not in the liver. Treatments of macrophages with the RXR and LXR-selective agonists induced ABCA1 expression, suggesting that ABCA1 is the direct target gene of RXR/LXR activation. LXR agonist, T0901317, did show induction of ABCA1 in cultured cells (116) and in animal models (117,118). This was also corroborated by studies in LXRα and LXRβ KO mice, which failed to show effects on ABCA1 following treatments with RXR and LXR agonists. Thus, RXR and LXR agonists, in addition to influencing cholesterol efflux, also inhibit cholesterol absorption by inducing ABCA1 in the gut. 9-cis retinoic acid alone or in combination with oxysterols induces ABCA1 mRNA (93). Retinoic acid receptor mediated induction of ABCA1 has been reported in macrophages (119). RXR agonists ((85,93), LXR-α agonists (85), and PPAR-α and PPAR-γ agonists (120,121) induce the transcription of ABCA1. Addition of PPAR and LXR-α agonists showed additive effects on ABCA1 upregulation, suggesting that these agonists influence ABCA1 transcription via independent mechanism. PPARα and PPARγ receptors are nuclear receptors that heterodimerize with RXR to modulate the expression of target genes involved in lipid and glucose metabolism. The ligands for PPARα and PPARγ induce ABCA1 mRNA in primary human macrophages (120). Since LXRα was also induced by PPARα and γ agonists, and since ABCA1 promoter harbors LXR element, it suggests that PPARα and γ agonists induce ABCA1 gene expression via LXR-mediated pathway (120). Although studies with macrophages isolated from LXR KO mice will be necessary to confirm that indeed PPARα and γ agonists induce ABCA1 gene expression via LXR-mediated pathway, the absence of PPAR element in ABCA1 promoter and the presence of PPAR

element in the LXR promoter (122) do suggest the likelihood of LXRmediated upregulation of ABCA1 by PPARα and γ agonists. Indeed, another PPAR receptor, PPARδ, expressed in many tissues, has been implicated in the regulation of ABCA1 gene expression (123). A recent study (124) with PPARδ-specific agonist, demonstrated upregulation of ABCA1 mRNA, which was associated with increased plasma levels of HDL.

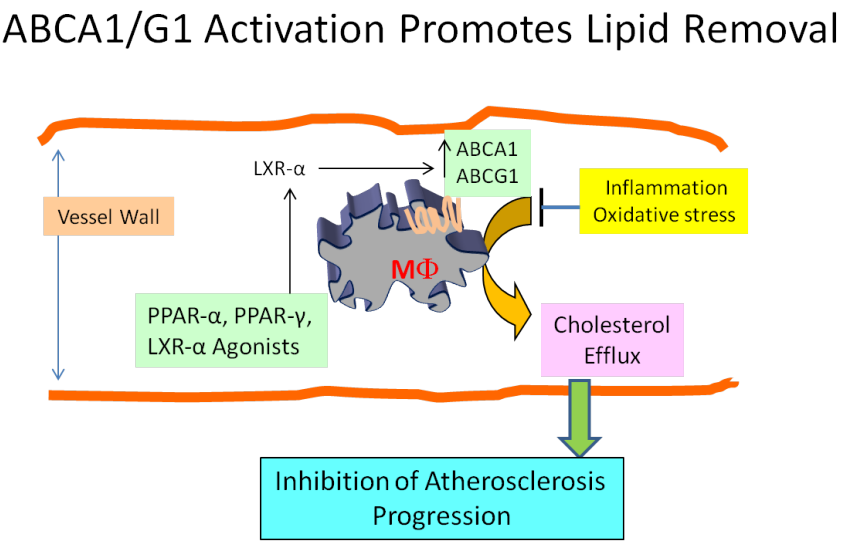


Fig. 6. Nuclear hormone receptor upregulates ABCA1 and G1, which effluxes cholesterol from lipid-laden macrophages in the arterial walls and inhibits atherosclerosis. Inflammation and oxidative stress impairs cholesterol efflux.

At least in two studies ABCA1 was found to be down-regulated. In one study, treatments of macrophages with IFN-γ reduced ABCA1 mRNA and cholesterol efflux to apoA-I acceptor (125), suggesting that IFN-γ may promote foam cell formation and accelerate the progression of atherosclerosis. A recent study demonstrated that geranylgeranyl pyrophosphate, a major metabolite in the mevalonate pathway, is a potent suppressor of ABCA1 by acting as an antagonist of LXR, and also by Rho GTP binding proteins (126). Several studies have indeed shown that LXR agonists inhibit progression of atherosclerosis in LDLr deficient (127-129) and apoE-deficinet (130) mice, and promote regression of preformed lesion in the aorta of apoE3*Leiden (128) and

hyperlipidemic hamsters (131). Similarly PPAR-α and PPAR- γ agonists also inhibit atherosclerosis (102,132,133) and regresses preformed lesion (131). The antiatherogenic effect of LXR agonists appears to occur via induction of the macrophage ABCA1 (116) (Figure 6).

6.4. Diabetes, HDL, Oxidative Stress, and ABCA1

Type 2 diabetes mellitus and the cluster of pathologies characteristics of metabolic syndrome including insulin resistance, obesity, and high plasma triglycerides are often associated with low HDL and shown to be dysfunctional as a result of the formation of advanced glycation endproducts (134,135). In contrast, individuals doing intensive exercise have high HDL and also show improved glucose tolerance (136), suggesting a link between low HDL and energy metabolism. Given the higher prevalence of cardiovascular morbidity and mortality in diabetics, this is an important area to pay attention to. Recent cell-based studies suggest that HDL may modulate plasma glucose through both insulin-dependent (137,138) and –independent and AMPK-mediated mechanisms (139). The ATP-binding cassette transporter A1 (ABCA1) has been shown to modulate insulin secretion (138), and HDL can reverse the deleterious effects of oxidized low-density lipoprotein (LDL) on insulin secretion by pancreatic beta cells (137). In addition, HDL may also increase glucose disposal through direct effects in skeletal muscle, the major site of glucose disposal in the body. It was reported that HDL and its major apolipoprotein, apolipoprotein A-I (apoA-I), activate the key metabolic regulatory enzyme AMP-activated protein kinase (AMPK) in endothelial cells and are critical for the nitric oxide–mediated vasodilatory effects of HDL (140). Infusion studies with recombinant and reconstituted HDL (rHDL) demonstrated modest effects on coronary plaque morphology and volume (47,141) and also showed improved endothelial function in type 2 diabetes mellitus (142).

The systemic metabolic disturbances of diabetes, including hyperglycemia and hyperlipidemia, likely play a central role in the pathogenesis of diabetes-associated atherosclerosis through the generation of oxidative stress and inflammation. Hyperglycemia causes increased flux through the polyol pathway, formation of advanced

glycation end products, activation of protein kinase C isoforms, and increased hexosamine pathway flux, all of which may contribute to increased oxidative stress (143-145). Excessive free fatty acids delivered to nonadipose tissues can lead to reactive oxygen species (ROS) formation through cycles of oxidative phosphorylation, activation of NADPH oxidase, and alterations in mitochondrial structure that precipitate ROS production (146-148). In addition to evidence for activation of these pathways in cultured endothelial cells, human studies support the notion of increased systemic oxidative stress in diabetic subjects in whom increased circulating levels of adhesion molecules and oxidized lipids correlate with increases in HbA1c and hypertriglyceridemia (149). The effects of oxidative stress in diabetes on both the vascular wall and lipoproteins in the circulation may promote atherogenesis. Jaleel et al. (150) provided intriguing evidence that poor glycemic control in type-1 diabetes is associated with accelerated oxidative damage to apoA-I, the main protein component of HDL. These investigators adapted a pulse-chase approach, classically used in cell culture experiments, to label newly synthesized proteins with ^{13}C-phenylalanine in human subjects. They then analyzed various plasma apoA-I isoforms by two-dimensional gel separation and mass spectrometry. This approach enabled quantification of isotopic enrichment in newly synthesized forms of the protein containing the propeptide and in more mature cleaved forms, which together form a charge train of five spots in two-dimensional gel analyses. The older forms of apoA-I accumulated significantly more, suggesting damage as a result of deamidation, oxidation, and carbonylation of amino acids that likely contributed to their altered migration in isoelectric focusing.

Given that apoA-I is a major component of HDLs, which protect against atherosclerosis by facilitating the removal of cholesterol from macrophages in the arterial wall and promoting reverse cholesterol transport, obvious extensions of this work will be to determine if the changes observed by Jaleel et al. (150) in apoA-I forms are due directly to oxidative stress. Indeed, recent studies demonstrated the presenc of significant amounts of oxidation products of apoA-I in human atherosclerotic plaques (151,152). Additionally, studies just published showed that myeloperoxidase enzyme that causes

oxidation of apoA-I, predict accelerated progression of atherosclerosis in diabetics (153). Thus, these studies provide mechanistic insight into the etiology of the oxidative modification of apoA-I and also how the functionally of HDL is linked to increased cardiovascular risk in diabetes.

Diabetic individuals often have higher non-esterified fatty acids that may impact ABCA1-mediated cholesterol efflux. Indeed, Wang and Oram (154) demonstrated that unsaturated fatty acids inhibit ABCA1-mediated cholesterol efflux. This finding is very important given the elevated levels of fatty acids in diabetics (155), and enhanced apoB secretion by fatty acids as a result of impaired presecretory degradation of apoB (26,60,156). It therefore appears that ABCA1 could be important not only in the inhibition of progression of atherosclerosis, but also in metabolic diseases. Wang and Oram (154) studied the effects of fatty acids, ranging in carbon chain length from 8 to 20, on cholesterol and phospholipid efflux in murine J774 and RAW 264.7 cells. The saturated fatty acids, palmitate and stearate, neither inhibited ABCA1- mediated cholesterol and phospholipid efflux nor they influenced ABCA1 protein. However, unsaturated fatty acids, oleate and linoleate, reduced cholesterol efflux as well as ABCA1 protein in a dose-dependent manner. Interestingly, oleate and stearate did not alter ABCA1 mRNA. As determined from ABCA1 turnover studies, it was concluded that unsaturated fatty acids enhanced the degradation of ABCA1 protein. These authors looked into the mechanism of fatty acid-mediated degradation of ABCA1 and carried elegant studies to demonstrate that unsaturated fatty acids phosphorylate and destabilize ABCA1 through a phospholipase D2 pathway (157). Further studies revealed that protein kinase C delta pathway is also involved in this process (158). Thus, it appears that the triggering of the the ABCA1 degradation by fatty acids possibly occurs via a mechanism distinct from the one observed with the cAMP withdrawal (66).

6.4.1. HDL, ApoA-I and AMPK

The role of apoA-I, the main protein component of HDL lipoproteins, on energy and glucose metabolism was first investigated by Han et al. (139) in C2C12 myocytes. These investigators reported AMPK phosphorylation at Thr-172 following treatments

with apoA-I, and this effect was found to be specific to apoA-I protein since treatment with apoB did not result into AMPK phosphprylation. ApoA-I also increased glucose uptake by C2C12 cells similar to AMPK activatos (159). These effects were similar to the activation of AMPK by adiponectin leading to increased glucose uptake (160). These authors extended their cell-based studies into apoA-I $^{-/-}$ mice to support their hypothesis that apoA-I is involved in glucose and energy metabolism. Indeed, apoA-I $^{-/-}$ mice had higher circulating glucose and impaired glucose tolerarce. Based on these findings Drew et al (161) extended these studies in human primary skeletal muscle cells isolated from type 2 diabetic patients infused with either a placebo or reconstituted HDL. They reported reductions in the fasting glucose in the rHDL treated group as compared to the placebo group. In cultured primary human skeletal muscle cells, apoA-I, the main protein component of HDL increased glucose uptake by 50%, which was associated with the activation os AMPK as measured by the AMPK phosphorylation at Thr-172. To further gain insights into the mechanism of apoA-I/HDL-mediated AMPK activation, these authors looked into two primary pathways of AMPK activation, i.e. LKB1 and CaMKK, the two upstream kinases known to phosphorylate AMPK (162,163). They found that HDL-mediated induction of AMPK phosphoryltion occurs via CaMKK-mediated pathway, since the CaMKK inhibitor STO609 abolished HDL-mediated phosphorylation of AMPK. Interestingly, the HDL-mediated induction of skeletal muscle glucose uptake occurred in ABCA1-dependent manner, since ABCA1 blocking antibody inhibited apoA-I and HDL-mediated uptake of glucose (161).

6.5 HDL, Inflammation, and Reverse Cholesterol Transport

Atherosclerosis has been characterized as a chronic inflammatory response to cholesterol deposition in arteries, but the mechanisms linking cholesterol accumulation in macrophage foam cells to inflammation are not completely understood. One of the mechanisms to protect cells from free cholesterol and oxysterol-induced toxicity during progression of atherosclerosis is the macrophage cholesterol efflux. During the cholesterol efflux process, the ATP-binding cassette transporters ABCA1 and ABCG1 are important players responsible for the major part of macrophage cholesterol efflux to

HDL in macrophage foam cells. Recent studies have shown that the sterol efflux activities of ABCA1 and ABCG1 modulate macrophage expression of inflammatory cytokines and chemokines as well as lymphocyte proliferative responses. Accumulating evidence suggests that by promoting cholesterol and oxysterol efflux, HDL regulates all these cellular responses in macrophage foam cells. Indeed, several studies demonstrated that native and reconstituted HDL, apoA-I and apoA-I mimetic peptides, all show anti-inflammatory activity (164-169). ABCA1 and ABCG1 deficiency in macrophages causes increased signaling via various Toll-like receptors including TLR4. These studies have also shown that the primary function of HDL and ABC transporters in cholesterol efflux and reverse cholesterol transport are also mechanistically linked to antiinflammatory and immunosuppressive functions of HDL. The potent antiinflammatory properties of HDL and apoA-I occurs throrugh a number of mechanisms, including the ability of HDL to bind and sequester lipopolysaccharide (170,171), resulting in the suppression of Toll-like receptor 4 (TLR4) signaling (172). HDL-mediated cholesterol efflux inhibits cellular inflammatory signaling leading to inhibition of MCP-1 and CD11b expression and monocyte transmigration.

Mice lacking ABCA1 and ABCG1 accumulate inflammatory macrophage foam cells not only in the myocardium but also in various tissues such as in the lung, liver, spleen, or thymus. Several studies have shown that the increased lipid raft formation in macrophages with genetic deficiencies of ABCA1 (173-175) or ABCG1 (176) could account for the enhanced inflammatory responses, especially after treatment with LPS or other TLR ligands leading to enhanced signaling via Myd88-NFkB. Replenishment or removal of cholesterol using cyclodextrin modulates the inflammatory response of macrophages deficient in ABCA1 or ABCG1, indicating that the increased inflammatory response is likely attributable to cholesterol accumulation in membranes (Figure). Yuvan-Charvet et al (175) showed that by modulating membrane cholesterol, deficiency of ABCA1 or ABCG1 increased TLR4 cell surface expression. Recently, Bensinger et al (177) reported that Liver X Receptor (LXR) signaling is involved in T-cell lymphocyte proliferation in an ABCG1-dependent fashion. ApoA-I KO mice also develop T-cell

proliferation and activation and features of autoimmunity when backcrossed into an LDL receptor– deficient background (178). These studies strongly suggest that HDL-mediated cholesterol efflux via LXR-regulated ABC transporters plays a key role in dampening lymphocyte proliferation and activation.

Poor glycemic control in type-1 diabetes is associated with accelerated oxidative damage to apolipoprotein (apo) A-I, the main protein component of HDL (150) and advanced glycated albumin diminishes anti-inflammatory properties of HDL (179,180). Reconstituted HDL (rHDL) shows anti-inflammatory activity in humans (164,168,181). ABCA1-mediated cholesterol efflux capability of HDL is compromised in type 2 diabetes patients (182), possibly as a result of oxidative damage of apoA-I and increased inflammation (183,184). Since antioxidative and antiinflammatory properties of HDL are impaired in diabetics (184), oxidative stress and inflammation may contribute to HDL dysfunction (185). HDL undergoes modification and multiple structural changes in an inflammatory condition and transforms normal functional HDL into “acute phase HDL”, enriched in free fatty acids, triglycerides, serum amyloid A (SAA), and decreased antiinflammatory enzymes, including paraoxanase 1 (186,187). In addition, inflammation induces secretion of myeloperoxidase (MPO), which has been shown to modify apolipoprotein A-I and impair its ability to accept cholesterol (151,188-191). MPO-mediated oxidation of apoA-I makes it proinflammatory (192). Tryptophan substitution in apoA-I renders it resistance to MPO oxidation (193). All these studies suggest that increase in inflammation leads to HDL dysfunction.

7. MATERIALS & METHODS

7.1 Materials

7.1.1 Clones, Vectors and Probes

ABCA1 expressing stable human embryonic kidney 293T cell line was generated by Flip-In system (Invitrogen). Full-length human ABCA1 cDNA was isolated from cultured human skin fibroblast cells and cloned into pcDNA5/FRT vector (Invitrogen).

Following probes were used for the quantitation of mRNA in mouse tissues.

Species	Gene	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence	Amplicon Length
MOUSE	Cyclophilin	CGATGACGAGCCCTTGG	TCTGCTGTCTTTGGAACTTTG	CGCGTCTCCTTTGAGCTGTTTGCA	64
MOUSE	ABCA1	GCTCTCAGGTGGGATGCA	GCTCGTCCAGAATGACAA	CTTGGCCTTCGTGGGTGGATCC	78
MOUSE	ABCG1	ATCTGAGGGATCTGGGTCT	CCTGATGCCACTTCCAT	CTGCCCTACCTACCACAACCCAGCA	67
MOUSE	ABCG5	TACACCGGCATGCTCAATG	GGCCATCCTGACTCTCCTG	TCTGTTTCCCATGCTGAGAGCCGT	76
MOUSE	ABCG8	GTCGTCAGATTTCCAATGAC	AGGGACATCAGGCAGGCTT	GGGACCTGCCCACGCTG	75
MOUSE	SREBP1c	CGTCTGCACGCCCTAGG	CTGGAGCATGTCTTCAAATGTG	AATCCATGGCTCCGTGGTCCG	70
MOUSE	FAS	CGGAAACTTCAGGAAATGTCC	TCAGAGACGTGTCACTCCTGG	CCAAGACTGACTCGGCTACTGACACGAC	82
MOUSE	SCD-1	TGTACGGGATCATACTGGTTCC	CCCGGCTGTGATGCC	TGCCTCTTCGGGATTTTCTACTACATGAC	95

To generate ABCA1 monoclonal stable cell line, 293 cells were co-transfected with pcDNA5/FRT-ABCA1 and pOG44 (Invitrogen) using Fugene-6 (Roche) in the growth medium without zeocin according to manufacturer's recommendations. After 24 h

following transfection, cells were maintained in DMEM with 10%FBS and 50 μg/ml hygromycin. Individual colonies were examined for the expression of ABCA1 by Western blotting.

7.1.2 Growth media

The complete D-MEM medium containing 10% FBS supplemented with 0.1 mM MEM non-Eessential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine was used to culture 293 cells.

7.1.3 Animal Models

All studies were carried out in C57Bl male mice until and unless indicated. Other animal models used in this study were $LDLr^{-/-}$, ob/ob, apoA-I transgenic, $LDLr^{-/-}$/ob/ob double knockout.

7.2 Methods

7.2.1 Cell culturing and Transient Transfection

WT human embryonic kidney 293 cells maintained in D-MEM with 10% FBS, 50U/ml penicillin streptomycin was used for transfection of ABCA1 construct. The details of the stable transfection have been described (194). HepG2 cells were transfected with 12 mg plasmid containing apoA-I promoter, and cotransfected with 5 mg of a-gal plasmid to account for the transfection efficiency. Transfection was performed using a commercial kit obtained from Promega, and by following the protocol provided by the manufacturer. Chloremphenicol acetyl transferase activity in the hepatocytes was normalized with Gal activity as described previously (194).

7.2.2 Cholesterol Efflux Assay

For cholesterol efflux assay, cells ($4x10^4$ /ml) were seeded in 24 well plates. Next day labeling media (culture medium plus 1% FBS with 2 mCi/ml [^{3}H] cholesterol) was added to the cells at 0.5 ml/well. After 24 h of incubation, the cells were switched to 0.2% BSA (defated, Sigma) in serum-free medium for 3 h to equilibrate radiolabeled-free cholesterol. Medium was removed and cells were incubated in serum-free culture medium with or without ApoAI (final concentration 10 mg/ml) and with or without 8-Br-cAMP (0.3 mM) for 24 hours. One hundred microliter of the medium was removed and

counted on a liquid scintillation counter. For cholesterol efflux quantitation, cells were first solubilized by adding 500 μl of 0.1N NaOH to each culture plate well, and then 100 μl of supernatant was removed and counted on a liquid scintillation counter. The amount of cholesterol efflux was calculated as follows: Cholesterol efflux = Total radioactivity in the medium/(Total radioactivity in the medium + Total radioactivity in the cells)

7.2.3 ABCA1 Protein Assay

After each experiment, the cells were collected by spinning, and lysed in lysis buffer (20 mM Hepes, 5 mM KCl, 5mM $MgCl_2$, 0.5% (v/v) Triton X-100 and complete protease inhibitor). Cell debris was removed by centrifugation at 8000 rpm for 10 min at 4°C. Protein concentration in the supernatant was determined by BioRad protein assay kit. 25 microgram of protein was separated on a 8-16% Tris- Glycine gel (Pre Cast Invitrogen gels) and electrophoretically transferred to nitrocellulose membrane (Invitrogen). Membranes were probed with either ABCA1 antibody or a polyclonal anti-beta actin antibody (Santa Cruz Biotechnology) as a control for equal loading. Immunoreactivity was detected by Super signal (Pierce).

7.2.4 ABCA1 mRNA Quantitation

Gene- specific primers and probe were designed using the Primer Express software (Perkin Elmer Life Sciences). The real time RT-PCR reaction was performed essentially following the manufacture's protocol with RT-PCR mix and 200 nM FAM-probe and 600 nM of both forward and reverse primers, and was analyzed in ABI prism 7900HT sequence detection system (Applied Biosystems). Relative amounts of ABCA1 mRNA levels were plotted as fold-change compared to no treatment. For normalization, 18S ribosomal RNA was used. Taqman RT-PCR was performed in triplicate and experiment was independently repeated three times

7.2.5. Experiments with 8-Br-cAMP and Protein Kinase Inhibitors

Effects of addition of 8-Br-cAMP and protein kinase inhibitors were studied in nontransfected as well as in the stably transfected 293 cells. Cells were treated with 8-Br-cAMP (Sigma) (0.3 mM final concentration) for 24 hr as described (22). To perform experiment with PK inhibitors, cells were first treated for 3 h with PK inhibitors, H7, H8

or H9 (Sigma) at the final concentration of 15 μM followed by the addition cAMP together with PK inhibitors for 24 hrs. Parallel experiments were performed without PK inhibitor and without 8-Br-cAMP. After each treatment, cholesterol efflux, ABCA1 protein, and ABCA1 mRNA were measured.

7.2.6. Fecal Cholesterol Measurements in Non-radioisotopic Studies

Fecal cholesterol were measured using a shortened method of Homan et al. (195). In brief, 100mg of each fecal sample was homogenized for 20 seconds in 0.50 mL (150 mM NaCl/5mM MOPS/1 mM EDTA/0.01% PMSF) followed by the addition of 0.5 mL of (150 mM NaCl 5mM MOPS/1 mM EDTA) and 200 μL of internal standard (2.0 mg/mL eicosanol in 2:1 $MeCl_2$: MeOH). Samples were extracted with 2.0 mL of (2:1 $MeCl_2$: MeOH) with vortex and subsequent centrifugation at 1500 rpm to separate and remove the lower organic layer. The extraction procedure was repeated with 2.0 mL of (2:1 $MeCl_2$: MeOH). Organic extracts were combined, washed with 1.0 mL 0.9% NaCl and the solution was dried under N_2. The final dried sample was reconstituted with 1.0 mL TDM (95:5:5 trimethylpentane:$MeCl_2$: MeOH). Standard curves were based on solutions of eicosanol (internal standard), cholesterol ester, cholesterol and triglyceride – with initial stock solutions prepared at 1.0 mg/mL in TDM. Standard curves were generated by serial dilution of 1mg/mL stock solutions to working concentrations ranging from 0.030 – 0.600 mg/mL.

Ten microliters of liver extract, internal standard, or standard curve samples were injected onto an Agilent Series 1100 HPLC system and separated over a 5μ, 100 x 4.6 mm ID Spherisorb S5W Silica gel column (Waters). The following gradient mobile phase was employed to separate lipid species: Solvent A contained 99:1 Isooctane: THF. Solvent B contained 2:1 Acetone: $MeCl_2$. Solvent C contained 85:15 Isopropanol: 7.5 mM GlHOAc/7.5 mM ethanolamine in water. The flow rate was a 1.6 mL/min with the gradient: 100% solvent A initially, progressing to a mixture of 40:42:18 (solvent A: solvent B: solvent C; respectively) over 9.0 minutes. Lipids were detected using a SEDEX evaporative light scattering detector (ELSD) with settings of P=1.5 Bar; T=32°C; Gain=4. Each sample was analyzed in duplicate.

7.2.7. In vivo studies

In vivo studies were carried out using both the non-radioisotopic as well as radioisotopic methods. For nonradioactive fecal cholesterol transport assay, male C57Bl mice Mice (n=8-10 in each group) were used. Typically, mice were teated with the test agent for one week followed by collection of feces over 24 h period of time. The feces were extracted and cholesterol measured as dscribed in the materials and methods section. LXR agonists are frequently used as a tool compound to study reverse cholesterol transport in animal models (117,118). Here also a pan LXR agonist, T0901317, was used. Groups of male C57Bl mice were treated with T0901317 for 7 days. At the final day of dosing, plasma, liver, and feces were isolated 2 h post-dose and analyzed. RCT studies in apoA-I transgenic, ob/ob, and $LDLr^{-/-}$ were done just like C57Bl mice with some modifications as described in the results section.

Radioisotopic study was done as described (117,196) and shown in figure 7. For this assay, J774 cells were grown, treated with acetylated LDL and then ^{3}[H]-cholesterol was loaded as drscribed (117,196). First, mice were treated with the test agent for indicated time period followed by injection of radioisotopic cholesterol loaded J774 cells intraperitonially. After 24 and 48 hours, feces, plasma, and liver, and in some studies bile, were isolated. Radioisotoe was counted in all the fractions as drscribed (196).

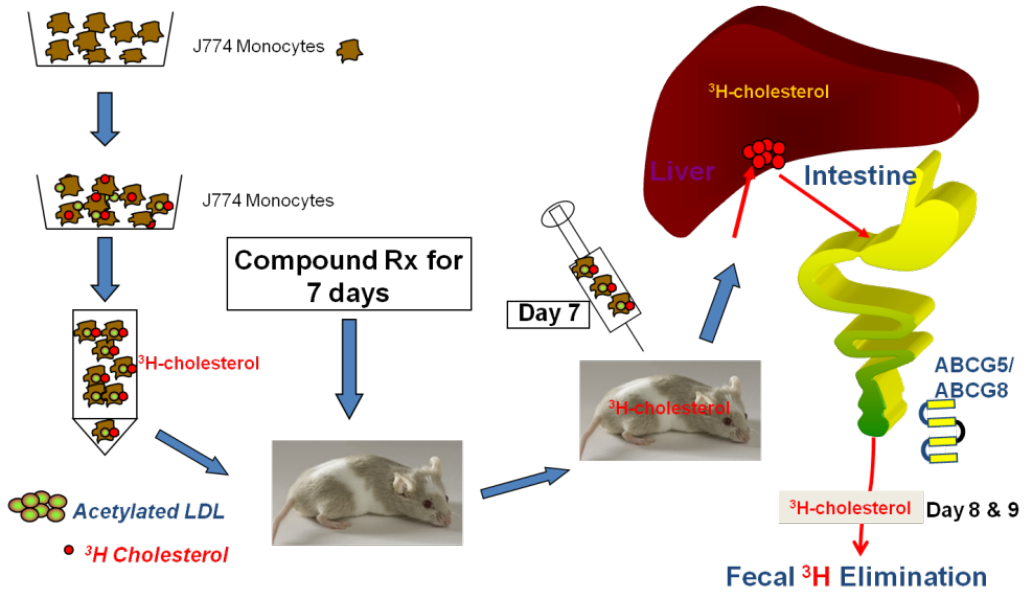


Fig 7. In vivo macrophage-specific reverse cholesterol transport. In this assay first J774 cells are grown in complete medium and then differentiated into macrophages using acetylated LDL followed by loading with radioisopic cholesterol (^{3}H-cholesterol). An aliquot of this preparation is counted on liquid scintillation counter to determine the volum of macrophage to be administered in each mouse. Groups of mice are separately treated with the desired therapeutic agent for 7 days followed by intrperitoneal administration of differentiated and ^{3}H-cholesterol loaded macrophages. After 24 h of treatment, liver, blood and feces are collected for radioactive counting.

In the Leptin$^{-/-}$ /LDLr$^{-/-}$ double knockout mice, glucose, insulin and atherosclerosis were measured as described (132). Cholesterol efflux was measured by the method of Smith et al (56).

8. RESULTS

8.1 Regulation of ATP-binding Cassette Transporter A1 (ABCA1) by cyclic-AMP in Stably Transfected 293 Cells

Eukaryotic cells maintain cholesterol homeostasis by regulated cholesterol synthesis, uptake, efflux, transport, and removal. Imbalance of any of these regulated pathways leads to cholesterol abnormalities and susceptibility to diseases. ATP-binding cassette transporter A1 (ABCA1) was identified as the mutant molecule causing a severe HDL deficiency syndrome known as Tangier disease (TD) (62-64). These TD patients have cholesteryl esters accumulation in tissues, and fibroblasts of these patients are unable to facilitate cholesterol and phospholipids efflux, leading to increased cardiovascular disease risk. Mice lacking ABCA1 showed similar phenotypes observed in TD patients with low levels of HDL (23). Further understanding of the roles of ABCA1 in lipid transport and atherosclerosis became clear from studies with ABCA1 transgenic mice (23). These mice showed enhanced cholesterol efflux from macrophages and reduced atherosclerotic lesion formation.

Regulation of ABCA1 occurs by the transcriptional (111,114) as well post-transcriptional mechanism (154). cAMP inducible cholesterol efflux to apoA-I in macrophages was attributed to the transcriptional up-regulation of the ABCA1. Studies in mice confirmed that LXR is an important activator of ABCA1 (23). To understand the regulation of ABCA1 protein and mRNA to maintain cellular cholesterol homeostasis and requirement of cAMP response element for the cAMP-induced upregulation of the ABCA1, a transfected cell line expressing ABCA1 under the control of CMV promoter that harbors cAMP response element was used. In this cell line, the cAMP-mediated regulation of the ABCA1 gene was studied.

cAMP Induces ABCA1 Expression in Transfected 293 Cells

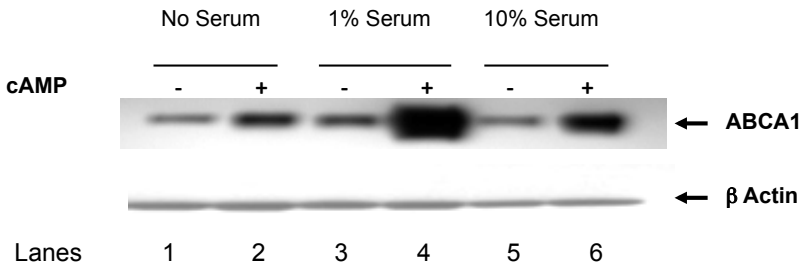


Fig 8. Effect of serum on 8-Br-cAMP-induced regulation of ABCA1. The tranasfected cell line was incubated in he absence or presence of serum with or without 8-Br-cAMP. 8-Br-cAMP treatment was done for 24 h. Cell lysate (25 μg protein) was separated on 8-16% Tris- Glycine gel and transferred to nitrocellulose membrane followed by detection with either ABCA1 antibody or a polyclonal anti-beta actin antibody. ABCA1 and β-actin proteins bands are shown by arrows.

ApoA-I-mediated Increased Cholesterol Efflux in Transfected 293 Cells

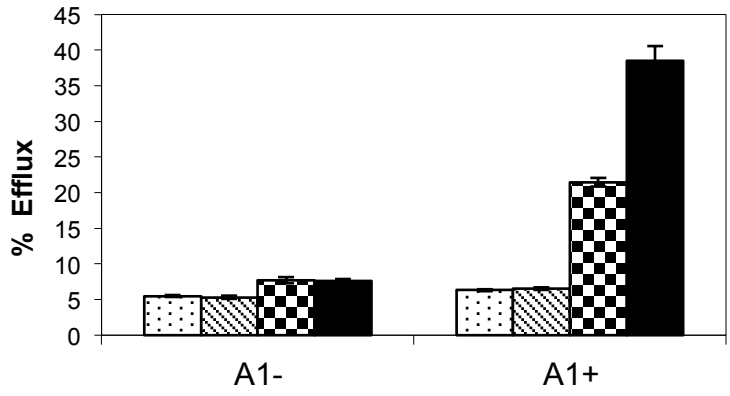


Fig 9. Effect of cAMP on cholesterol efflux in WT 293 cells, and in transfected 293 cells in the presence and absence of apoAI and 8-Br-cAMP. The details of the efflux assay are provided in the methods section. Dotted bars, 293 cells, shaded bars, 293 cells with 8-Br-cAMP, big dotted bars, transfected 293 cells, filled bars, transfected 293 cells with 8-Br-cAMP.

Since cAMP induces ABCA1 and cholesterol efflux (56,66), the stably transfected 293 cells were first examined for their ability to respond to cAMP in inducing ABCA1 in culture media containing 1% or 10% serum. As seen in Fig 8, treatment with 8-Br-cAMP for 24 h showed marked induction of ABCA1 protein in 1% serum containing media. However, increasing serum concentration to 10% caused blunted induction, showing <50% ABCA1 protein as compared to induction in the presence of

1% serum. The stably transfected cells were also examined for requirement of apoA-I as cellular cholesterol acceptor. As shown in Fig 9, apoA-I protein is needed as an acceptor in the process of ABCA1-mediated cellular cholesterol efflux.

cAMP Withdrawal Rapidly Diminishes ABCA1 Protein

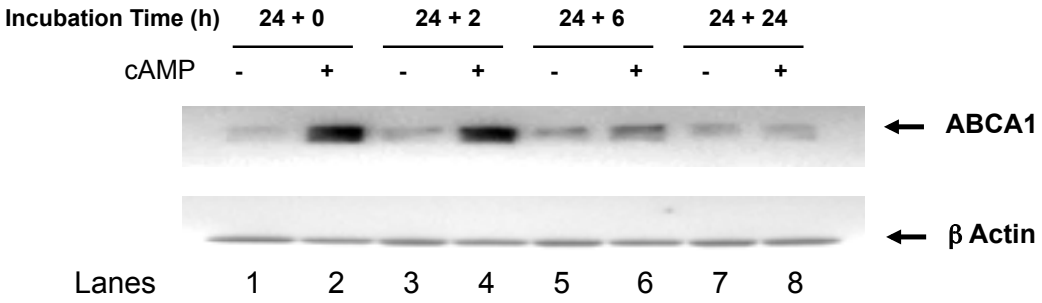


Fig 10. Effect of 8-Br-cAMP withdrawal on ABCA1 protein levels in the 293 cells stably transfected with hABCA1. The transfected cells were treated with or without cAMP for 24 hours, the media was removed and cells were further incubated in the same media but without cAMP for 0 h, 2 h, 6h, and 24 h as indicated after the + sign. The cell lysates were prepared and ABCA1 and β-actin proteins were measured by Western blotting. The respective protein bands of ABCA1 and β-actin are indicated by arrows.

In a separate study, effect of cAMP withdrawal on ABCA1 induction was studied. As shown in Fig 10, cells were treated for 24 h with 8-Br-cAMP and then washed with media and incubated for various time without 8-Br-cAMP. As shown, ABCA1 protein drops to basal level within 6 h of 8-Br-cAMP withdrawal.

To investigate the effect of 8-Br-cAMP occurs at transcriptional level or posttranslational level, cells were harvested at different time points and total mRNA was prepared. The concentration of ABCA1 mRNA was quantitated using Q-PCR. The results in Fig 11, suggests that 8-Br-cAMP withdrawal reduces ABCA1 mRNA to basal level within 6 h, similar to ABCA1 protein. Similar phenomenon was noticed in the nontransfected 293 cells (Fig 12), suggesting that transfected cells are behaving just like untransfected cells in regulating 8-Br-cAMP-mediated ABCA1 regulation. Measurements of cholesterol efflux also showed that within 6 h of 8-Br-cAMP withdrawal, the ABCA1 function in mediating cholesterol efflux reduces to basal level in stably transfected 293

cells (Fig 13). These results clearly show that withdrawal of 8-Br-cAMP shuts down ABCA1 transcription followed by rapid diminution of ABCA1 protein and cholesterol efflux. Thus, 8-Br-cAMP appears to upregulate ABCA1 transcription and does not influence ABCA1 protein turn over.

cAMP Withdrawal Rapidly Diminishes ABCA1 mRNA

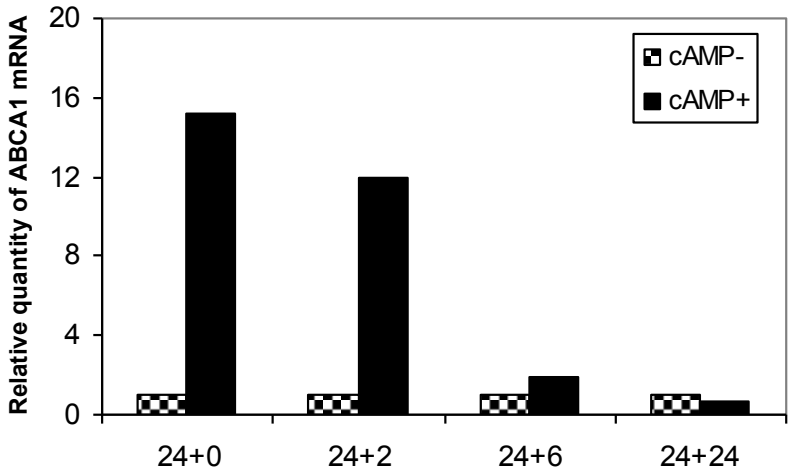


Fig 11. Effect of 8-Br-cAMP withdrawal on ABCA1 mRNA levels in the 293 cells stably transfected with hABCA1. Cells were treated with or without 8-Br-cAMP as described in the legends to figure 3, and RNA was prepared from the cells. Sixty nanogram of the total RNA were taken for ABCA1 mRNA quantitation by real-time RT-PCR using Taqman 7900HT. As a control 18S RNA was also quantitated. The numbers after the + sign indicates time in h after cAMP treatment. cAMP- indicates without cAMP, and cAMP + indicates with cAMP.

Diminished Levels of ABCA1 as a Result of cAMP Withdrawal Reduces Chol Efflux

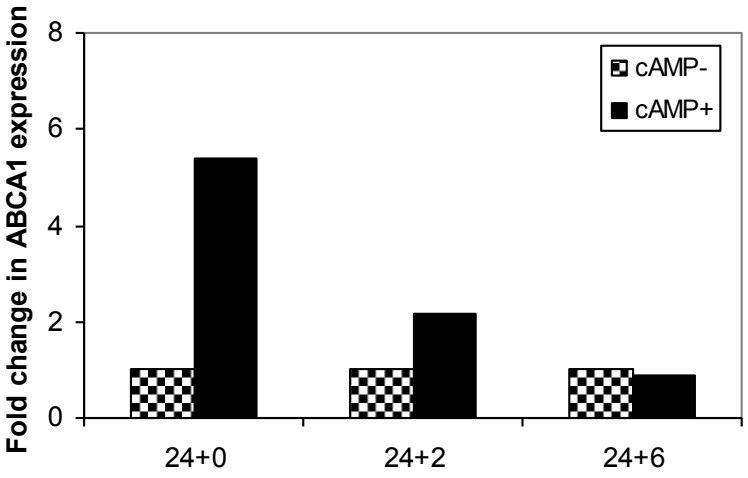


Fig 12. Effect of 8-Br-cAMP withdrawal on ABCA1 mRNA in the wild type 293 cells. Cells were treated with or without 8-Br-cAMP for 24 h, the media was removed and incubated in the media without 8-Br-cAMP for 2 and 6 h, respectively, as indicated. The numbers after the + sign indicates time in h after cAMP treatment. cAMP- indicates without cAMP, and cAMP + indicates with cAMP.

Diminished Levels of ABCA1 Caused by cAMP Withdrawal Reduces Chol Efflux in Transfected Cells

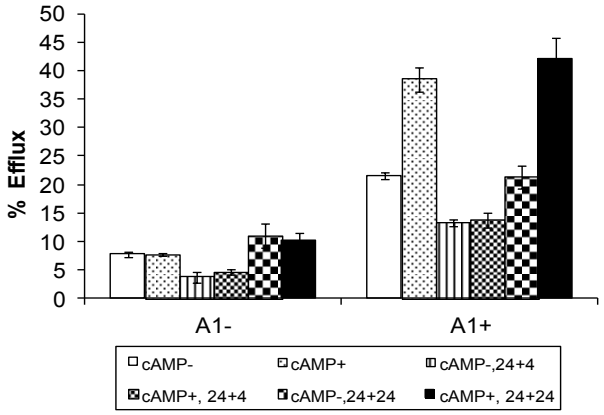


Fig 13. Effect of cAMP withdrawal on the cholesterol efflux. Stably transfected 293 cells were seeded in triplicate in the 24-well plates and cholesterol assay done in the absence and presence of 8-Br-cAMP as described in the methods section. – indicates without cAMP and + indicates with cyclic AMP. The indicated numbers after the + sign in the caption box indicates the time of incubation after cAMP treatments.

The mechanism of cAMP-induced upregulation of ABCA1 transcription was investigated. First the role of PKA in the transcriptional regulation of ABCA1 was

examined in the presence of PKA inhibitor. PKA inhibitor blocked the 8-Br-cAMP – mediated induction of ABCA1 protein (Fig 14). Three PKA inhibitors, as shown in Fig 15, completely blocked the cAMP-mediated ABCA1 mRNA (Fig 15), suggesting the requirement of PKA for the cAMP-mediated transcriptional regulation of ABCA1 gene.

PKA Inhibitor Blocks cAMP-mediated Induction of ABCA1

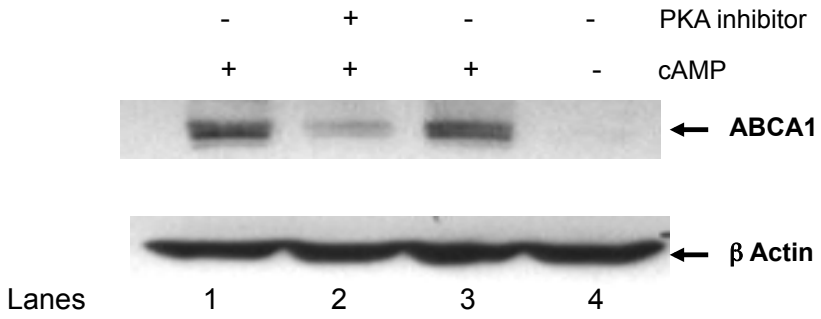


Fig 14. Effect of PKA inhibitor on cAMP-induced regulation of ABCA1 in stably transfected 293 cells. Stably transfected cells were treated with PKA inhibitor as described in the methods. Cells were washed after the treatments and cell lysates were prepared. An aliquot of the cell lysates were subjected to SDS-PAGE to detect ABCA1 protein and β-actin protein as an internal standard.

PKA Inhibitor Blocks cAMP-mediated Induction of ABCA1 mRNA

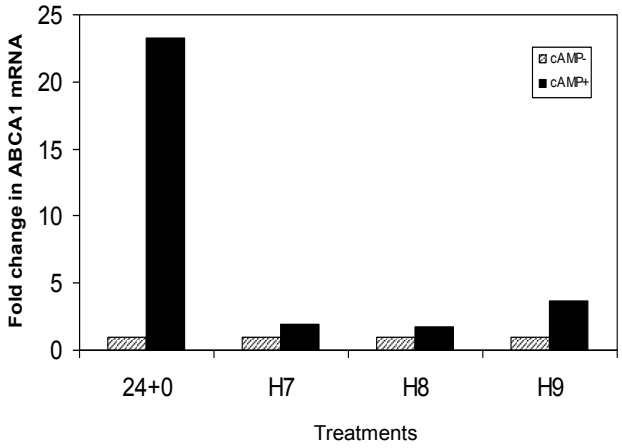


Fig 15. Effect of protein kinase inhibitors on the cAMP-induced ABCA1 mRNA in the stably transfected cells. The stably transfected cells were treated with three different protein kinase inhibitors, followed by washing and RNA preparation. In each RNA samples, ABCA1 mRNA were quantitated. Values shown in the figure are relative to 18S RNA.

Cholesterol efflux was measured to examine if PKA inhibitor blocks cellular cholesterol efflux to acceptor apoA-I. As shown in Fig 16, cholesterol efflux also showed reduction in the presence of PKA inhibitor, further demonstrating that PKA is required for cAMP-mediated induction of ABCA1 and cholesterol efflux.

PKA Inhibitor Blocks cAMP-mediated Induction of Chol Efflux

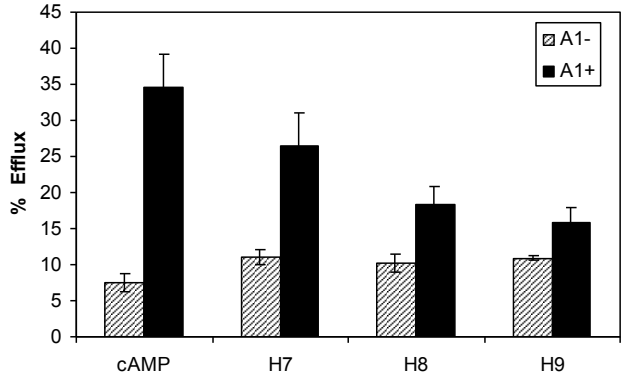


Fig 16. Effect of protein kinase inhibitors on the cholesterol efflux in the 293 cells stably transfected with hABCA1. Stably transfected cells were treated with protein kinase inhibitors as indicated and cholesterol efflux measured following the method described in the methods section.

In summary, the in vitro study with stably transfected 293 cells with ABCA1 construct showed high levels of ABCA1 expression, and treatments of ABCA1 overexpressing cells with cAMP induced expression of ABCA1 mRNA and protein. As expected, cAMP-mediated enhanced expression of ABCA1 resulted in increased cholesterol efflux. Withdrawal of cAMP caused time-dependent rapid comcomitant diminution of ABCA1 protein and mRNA, suggesting transcriptional regulation of ABCA1. To gain insights into the cAMP-mediated induction of cholesterol efflux, ABCA1 expressing 293 cells were treated with protein kinase inhibitors that abolished cAMP-mediated induction of ABCA1 and cholesterol efflux. These results demonstrate that cAMP regulates ABCA1 gene expression by transcriptional mechanism and continued transcription of ABCA1 is required to maintain cellular level of ABCA1. Furhermore, cAMP-mediated upregulation of ABCA1 requires protein kinase-dependent mechanism. And finally, overexpressing ABCA1 cells mimics similar cAMP response as

with cells having natural ABCA1 promoter. Thus, the present studies demonstrate that the transfected cell line shows similar cAMP-induced regulation of ABCA1 as in the WT macrophages, and this effect occurs via PK-mediated pathway. It is also shown that the continued transcription of the ABCA1 is required in order to maintain the physiological level of intracellular ABCA1, which in turn leads to maintain the intracellular cholesterol balance.

8.2 Optimization of In Vivo Reverse Cholesterol Transport Assay

8.2.1 Non-radioisotopic Assay

As shown in figure 17, treatment with LXR agonist, an ABCA1 inducer, increases both LDL and HDL as well as prebeta particles. Quntitation of various liporptoein fractions (figure 17 & 18) validated the LXR agonists effect in mice, that is increased level of both apoA-I and apoB-containing lipoproteins. LXR agonists are known to have lipogenic effects leading to increases in the plasma levels of triglycerides. Indeed, triglyceride levels were found to increase in the LXR agonist-treated mice (Figure 18). Fecal cholesterol showed increases following T0901317 treatment. A 20 mg/kg/d dose of T0901317 showed a robust effect on excretion of cholesterol appearing in the feces (Figure 19).

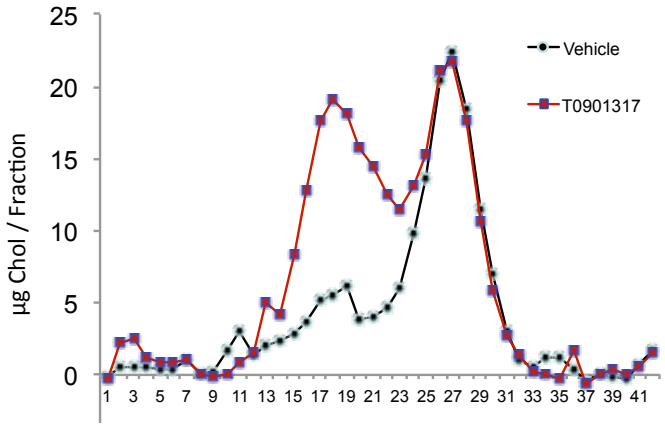


Fig 17. Treatment of C57Bl mice with 20 mg/kg/d LXR Agonist, T0901317, increases LDL-C and raises HDL-C as measured by FPLC Profile of Plasma from Vehicle- and LXR Agonist Treated Mice. Repeat dosing was performed by oral gavage once daily in the morning between 8-9 AM for 7 days. On the eighth day mice were fasted for 6 h and blood was withdrawn to prepare plasma. Results show plasma lipoptoein profile in the vehicle-treated and LXR pan agonist treated mice.

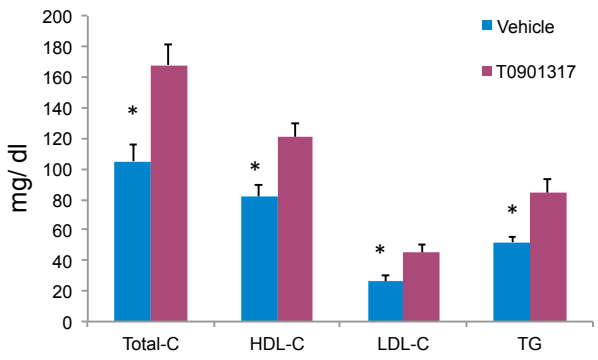


Fig 18. Treatment of C57Bl mice with 20 mpk LXR Agonist, T0901317, for 7 days increases LDL-C and raises HDL-C and TG, consistent with the effect of LXR agonists on lipoproteins.

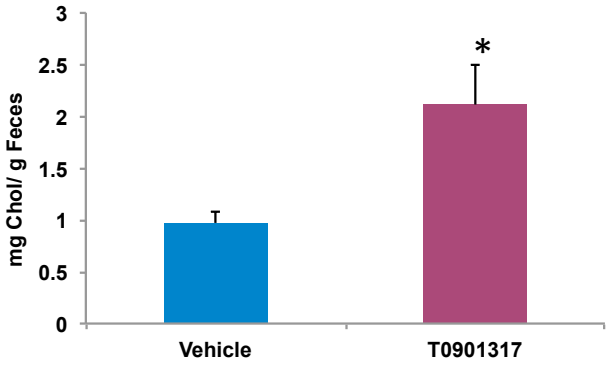


Fig 19. Fecal Cholesterol in C57Bl Mice Treated with 20 mg/kg/d T0901317. This was a non-radioisotopic study where radioisotope was not used. Feces were collected 24 h post 7-day treatment and subjected to quantitation of cholesterol as described in the methods section.

To further gain confidence in the measurements of fecal cholesterol following treatment with LXR pan agonist, a dose response study was performed. In this study, groups of mice were treated with incrasing dose of LXR agonist (3, 10, and 30 mg/kg/day), and fecal cholesterol quantitated. As shown in Figure 20, there was a nice dose-response seen from 3mg/kg/d to 30mg/kg/d dose range.

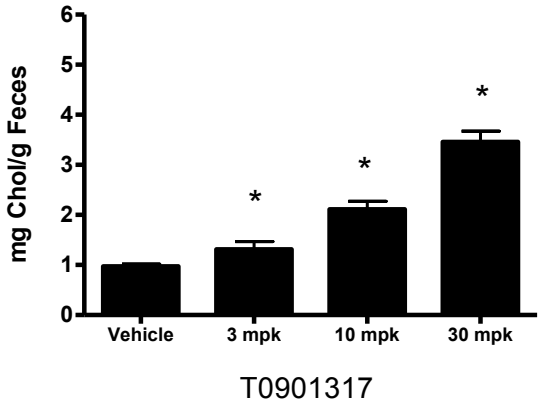


Fig 20. LXR Agonist T0901317 Increases Fecal Cholesterol in a Dose-dependent Manner. Male C57Bl mice were treatd with either vehicle or various concentrations of LXR agonist, T0901317, (3 mpk to 30mpk) for 7 days. Feces collected over 24 h were used for cholesterol quantitation.

Enhancement in the reverse cholesterol transport by LXR agonist occurs via ABCA1 and ABCG1 gene induction (197). Therefore, to validate LXR agonist effect, ABCA1 and G1 mRNA quantitations were done in the peripheral blood monocytes. As shown in Figure 21, a 4-6-fold induction of ABCA1 and G1 was noticed, suggesting that LXR agonist induced increases in the fecal cholesterol.

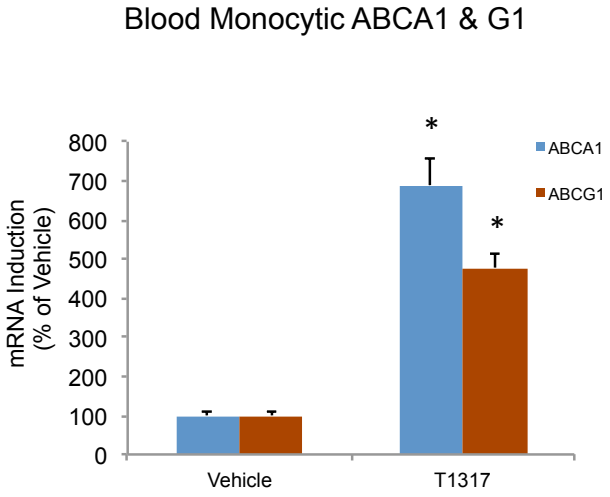


Fig 21. LXR Agonist, T0901317 Shows Induction of Target Genes, ABCA1 and G1. Male C57Bl mice were treatd with either vehicle or various concentrations of LXR agonist, T0901317, ranging from 3 mpk to 30mpk for 7 days followed by the collection of blood and preparation of total RNA to quantitate ABCA1 and G1 mRNA

8.2.2 Radio-isotopic Assay

Along with non-radioisotopic method of measuring RCT, a radiosiotopic method was also carried out. This was done to compare both methods and determine if both methods could be used interchangeably depending upon the purpose of a given study. For istance, if mRNA profiling is planned in a study, it is better to use non-radioisotope method to evaluate RCT and measure gene expression in various tissues. This avoids contamination of labwares. A schematic presentation of the macrophage-specific RCT is depicted in Figure 7, which is basically a method developed by Dan Rader's group (196,198).

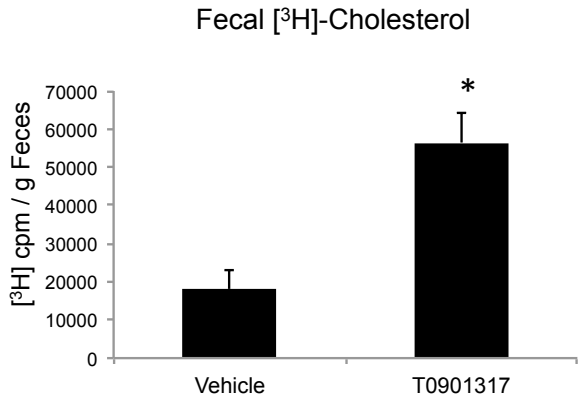


Fig 22. LXR pan agonist, T0901317, shows increases in fecal [^{3}H]-cholesterol counts in macrophage-specific Reverse Cholesterol Transport in a Radioisotopic Study.

Thus, both the non-radioisotopic and radioisotopic methods showed increases in the fecal cholesterol following reatment with an ABCA1 inducer, LXR agonist.

8.3 Effect of ABCA1 Inducers, PPAR-α and LXR Agonists, on RCT in C57Bl and ApoA-I Transgenic Mice: Non-radioisotopic Study

Since both PPAR-α and LXR Agonists have been shown to induce macrophage ABCA1 and reverse cholesterol transport (120,199) and action of PPAR-α occurs via LXR (200), it was hypothesized that both PPAR-α and LXR agonists should have additive/synergistic effect in inducing macrophage specific cholesterol transport. To test this hypothesis, C57Bl/6 mice were treated with PPAR-α and LXR agonists separately, and also in combination. The results of such study are shown in Figures 23 & 24. As

expected, LXR agonist increased total-, HDL-, and LDL-C as well as triglycerides. PPAR-α agonist, fenofibrate, on the other hand, showed reductions in HDL-C, LDL-C, and triglycerides. The reductions in HDL-C by fenofibrate was a result of rodent-specific effect of PPAR-α agonist as described before (133).

Measurement of fecal cholesterol clarly showed induction of RCT by both PPAR-α and LXR agonists (Fig 24), both of which have been shown to induce ABCA1 (120,201). A combination of PPAR-α and LXR agonists showed massive increase in the fecal cholesterol excretion (Fig 24), suggesting a synergistic effect and confirming the results of Nakaya et al (200) that PPAR-α requires LXR to nduce macrophage reverse cholesterol transport.

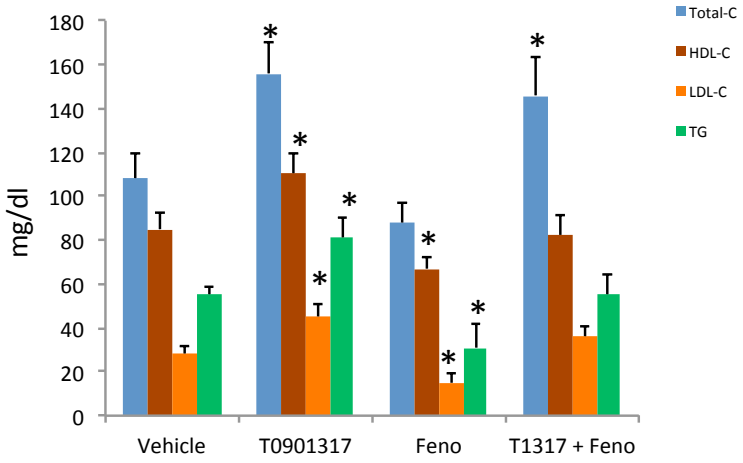


Fig 23. Plasma Lipids in LXR, PPAR-α, and Combination of LXR and PPAR-α Treated C57Bl Mice.

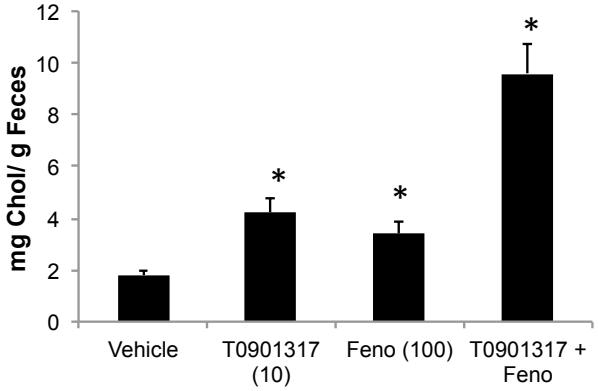


Fig 24. PPAR-α and LXR Agonist Enhance RCT in C57Bl Mice as Measured by Nonradioisotopic Assay.

ApoA-I transgenic mice show increased levels of HDL-C (202) and expression of apoA-I in apoE-deficient mice attenuates atherosclerotic lesion formation (43). Since overexpression of apoA-I promotes reverse cholesterol transport from macrophage to feces (196), it is possible that the antiatherosclerotic efficacy of PPAR-α and LXR agonists (131) occur via enhanced reverse cholesterol transport.

The two ABCA1 inducers, PPAR-α and LXR agonists, were also tested in apoA-I transgenic mice for their efficacy in promoting reverse cholesterol transport. As shown in figures 25 and 26, treatment of apoA-I transgenic mice with ABCA1 inducers, fenofibrte and T0901317, further increased plasma levels of HDL. A combination of PPAR-α and LXR agonist (Feno + T1317) showed improved lipid profile when compared to vehicle treated group. Because mouse is an HDL animal transporting majority of their cholesterol as HDL partcles and, unlike humans, non-HDL cholesterol is only a fraction of the total cholesterol, expressing apoA-I in HDL increases HDL-C further, but not in the proportion of plasma levels of apoA-I. HDL increases only to certain level as evidenced by combined treatment of apoA-I transgenic mice with two HDL elevators, PPAR-α and LXR agonists (Fig. 25 & 26).

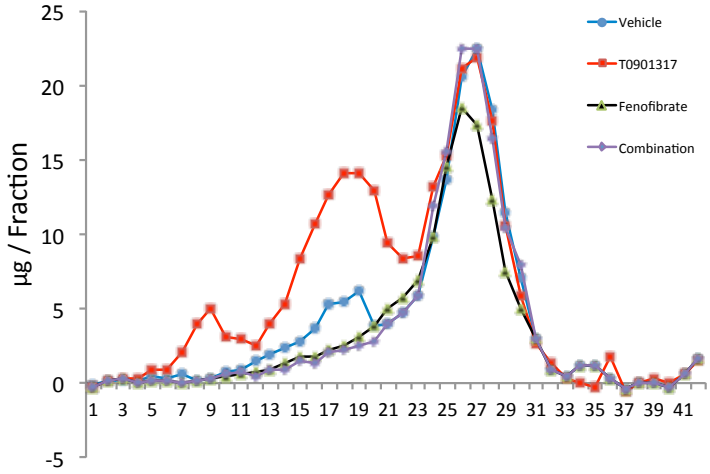


Fig 25. Plasma Lipoprotein Profile in LXR, PPAR-α, and Combination of LXR and PPAR-α Treated ApoAI-Tg Mice

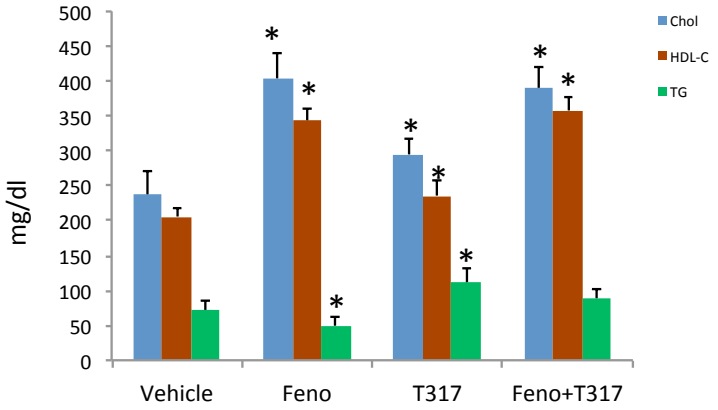


Fig 26. Plasma Lipids in LXR, PPAR-α, and Combination of LXR and PPAR-α Treated ApoAI-Tg Mice. The apoA-I transgenic mouse used in the present study has been described elsewhere (202).

Measurements of fecal cholesterol further demonstrated that expression of apoA-I in apoA-I transgenic mice increase reverse cholesterol transport as seen with increases in fecal cholesterol (Fig 27). Both PPAR-α and LXR agonists showed similar efficacy in terms of fecal cholesterol excretion. Again, the combinationof PPAR-α and LXR agonists show synergistic effects on fecal cholesterol excretion

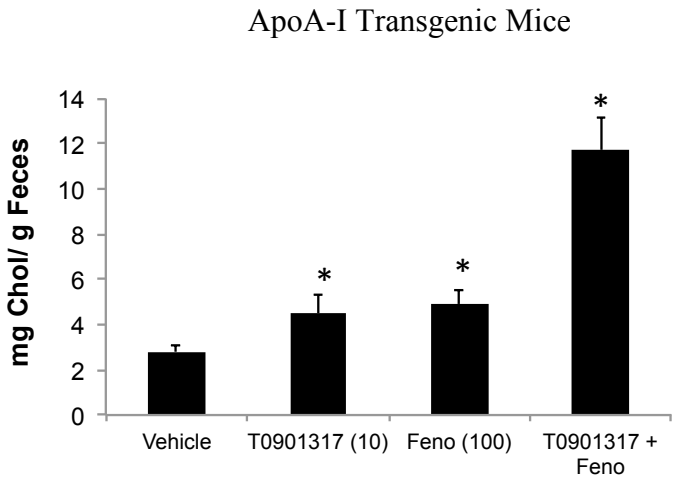


Fig 27. PPAR-α and LXR Agonists Enhance RCT in ApoA-I-tg Mice in Nonradioisotopic Study. Fecal cholesterol was collected and cholesterol measured as described in the materials and methods section.

8.4 Effect of PPAR-α and LXR Agonists on RCT in C57Bl Mice: Radioisotopic Study

Fecal cholesterol excretion was further verified by doing a radioisotopic assay in which ^{3}[H]-cholesterol was loaded to J774 cells, injected intraperitoneally and radioisotope followed in the feces over 24 h time. As shown in Fig 28, the radioisotope assay corroborated non-radioisotope assay (Fig 27) in terms of synergistic effects of PPAR-α and LXR agonists in promoting macrophage cholesterol excretion through the feces.

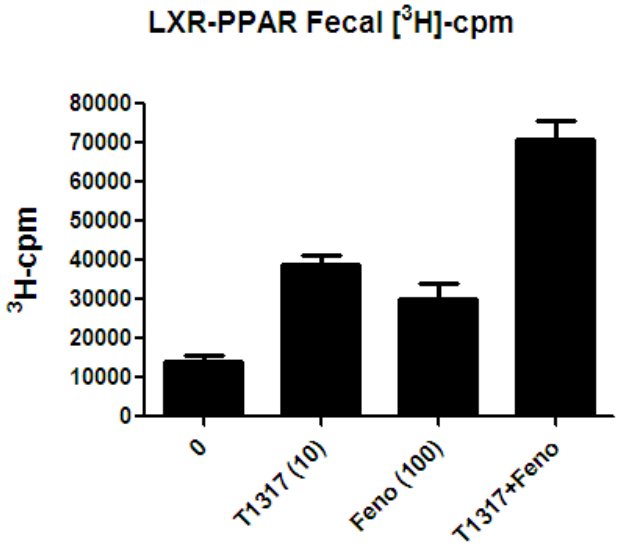


Fig 28. PPAR-α and LXR Agonists Enhance RCT in C57Bl Mice as Measured by Radioisotopic Assay. The details of experiment and fecal cholesterol isolation and determination of fecal radioisotope counts are described in the materials and methods section.

8.5 Effect of Antiatherosclerotic Agents on Reverse Cholesterol Transport in Hyperlipidemic Animal Models

Hyperlipidemic mouse models like ob/ob$^{lep/lep}$ (leptin-deficient) and LDLr$^{-/-}$ (LDLr-deficient) mice are used for studying pathophysiology of diabetes and atherosclerosis, respectively (132). These mouse models have also been used to evaluate efficacy of hypolipidemic agents (203). Although several agents show attenuation of atherosclerotic lesion in LDLr $^{-/-}$ mice, it is not known if this ccurs exclusively in an LDL-driven mechanism or RCT also plays a role. As shown in Figure 29, both PPAR-α

(GW9578) and LXR (T0901317) agonists show cholesterol mobilization in LDLr $^{-/-}$ mice as shown by plasma ^{3}H counts. Fecal counts also showed increases; LXR agonist, T0901317, showing greater enhancement in RCT compared to PPAR-α agonist, GW9578.

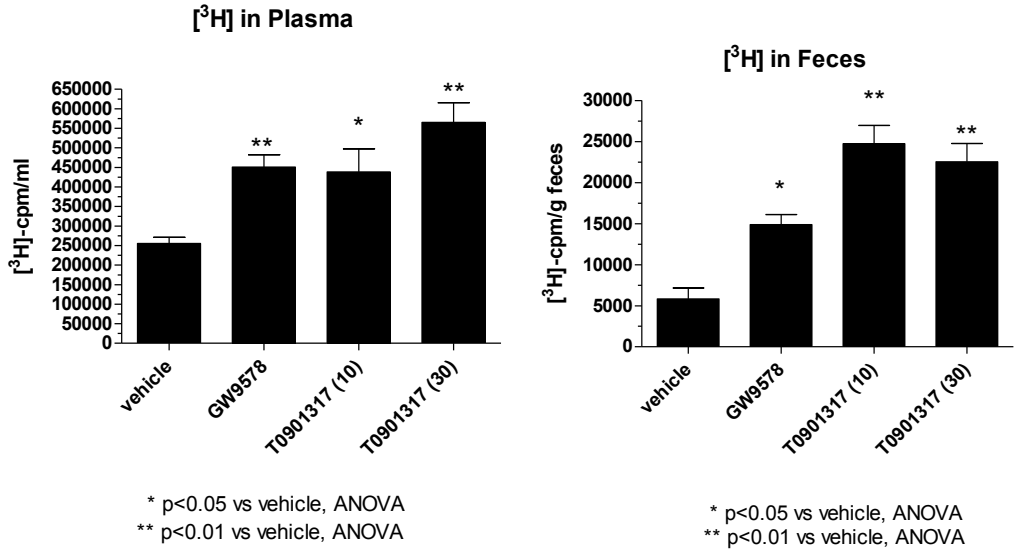


Fig. 29. Mobilization of administered radioisotopic cholesterol as macrophages in LDLr $^{-/-}$ mice by LXR and PPAR-α agonists, T0901317 and GW9578, respectively. Mice were treated with these agonists as indicated for 7 days followed by administration of radioisotopic cholesterol loaded macrophages via intraperotoneal injection. Mice were sacrificed 24 h post macrophage administration and blood, liver and feces were collected for radioactive counting.

A non-isotopic RCT was perfromd in ob/ob mice. These mice are hyperglycemic and hyperinsulinemic. Treatements with PPAR-α agonist (GW9578), PPAR-γ agonist (rosiglitazone) LXR agonists (GW3965, T01317) or a combination of both PPAR-α and LXR agonists (GW3965 + T01317) showed induction of RCT pathway leading to excretion of cholesterol in the feces. As shown in figure 30, rosiglitazone did not enhance RCT in this model, whereas, both PPAR-α and LXR agonists showed marked increased in RCT, measured as fecal cholesterol. Two combinations of PPAR-α and LXR agonists, one less potent (GW3965) and one more potent (T01317) were evaluated in this model. While PPAR-α and LXR agonists show with less potent as well as with more potent LXR agonists.

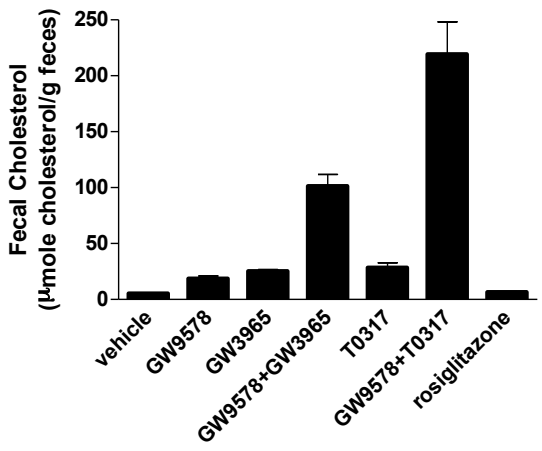
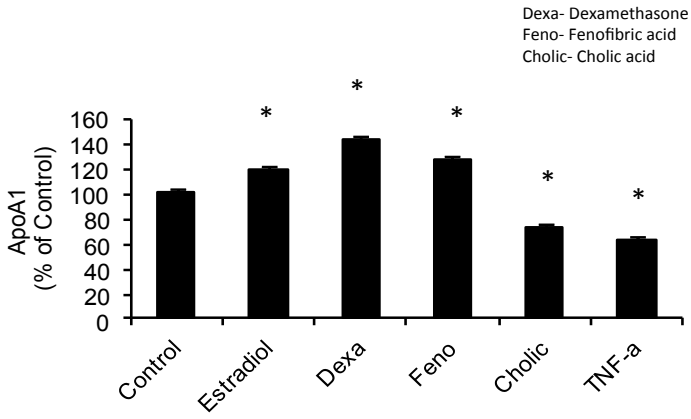


Fig 30. Fecal cholesterol excretion in ob/ob mice treated with PPAR-α agonist (GW3965), PPAR-γ agonist (rosiglitazone), LXR agonists (GW9578 & T01317), and a combination of PPAR- and LXR agonists (GW9578 + T01317). All procedures were done as described in Figure 29 except the administration of radioisotpic cholesterol loaded macrophages. In this assay only the feces were collected for cholesterol quantitation using LC-MS.

8.6 Inflammation and Reverse Cholesterol Transport

8.6.1 In vitro Study: TNF- α-mediated apoA-I Regulation

An inverse correlation has been noticed between proinflammatory cytokines, and reverse cholesterol transport (204) and inflammation impairs reverse cholesterol transport in animal models (185) and in humans (205). Several studies have reported inflammation leading to low levels of apoA-I and HDL-C. Indeed, administration of apoA-I attenuates LPS-induced endotoxemia (206). A direct correlation of TNF-α-induced lowering of apoA-I was demonstrated (207-209). We looked into the molecular mechanism of apoA-I down-regulation by proinflammatory cytokine, TNF-α. First, we examined if TNF-α lowers apoA-I mRNA in cell model of hepatocytes, HepG2 cell. As seen in Figures 31 and 32, TNF-α dowregulates apoA-I protein and mRNA, respectively. TNF-α was found to decrease apoA-I in the spent media by about 50% (Fig 31). ApoA-I mRNA also showed a reduction of 50% following treatment with TNF-α.



HepG2 cells were grown to subconfluence, and plated in 100 mm petridish. Cells were allowed to recover overnight and grown for 2 days. At the end of two days media were removed and concentrated using centricon filter, and used for apoA1 protein measurement by Western blotting. Cells were scrapped off from the plate, washed with cold PBS, and total RNA prepared. One hundred nanogram total RNA was taken for the measurements of apoA1 mRNA by real time PCR.

Fig 31. HepG2 cells, a model of human hepatocytes, were treated with agents as described in the figure. As controls, both inducers and repressors of apoA-I were used as tool agents. As shown, TNF-α reduced apoA-I protein in the media.

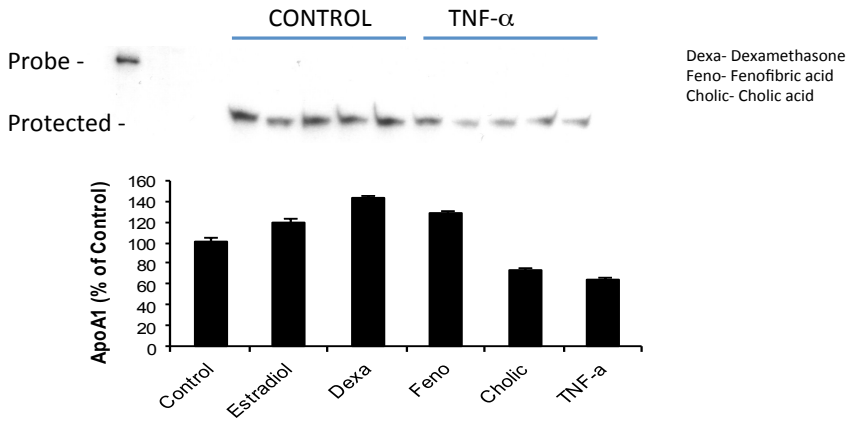


Fig 32. HepG2 cells were grown in culture as described in the legends to Fig 31. Cells were harvested to prepare total RNA and apoA-I mRNA quantitated by RNAse protection assay.

8.6.2 In vivo Study: LPS-induced Inflammation and Cholesterol Efflux

To corroborate cell-based assay, in vivo studies were carried out to examine levels of apoA-I and HDL-C in mice previously treated with LPS and compared with sham-treated group of mice. As shown in Fig 33, LPS treatmentshowed marked increases in the VLDL peak and significant reductions in HDL peak, suggesting that LPS-induced

increases in the proinflammatory cytokines resulted in a proatherogenic lipid profile with 2-fold increases in apoB-lipoproteins and significant reductions in apoA-I particles (Fig 34).

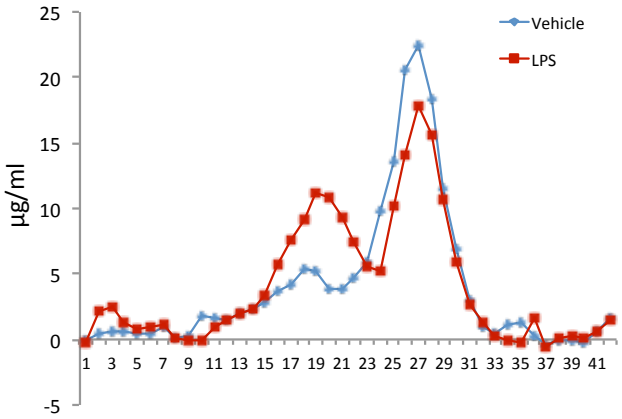


Fig 33. FPLC profile of plasma isolated from control and LPS-treated mice. Blue trinagles represent control and red circles indicate FPLC-treated group. Mice were treated with LPS (5 µg/kg/day) followed by blood withdrawal and lipoprotein profile analysis. As shown, LPS treatment markedly increased triglycerides-rich lipoprotein particles and lowered apoA-I containing lipoproteins.

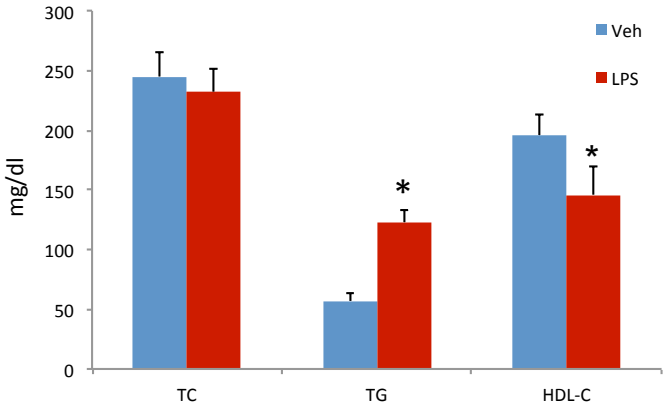


Fig 34. Fractions obtained in the FPLC profile (shown in Fig 33) were collected and peaks corresponding to non-HDL and HDL fractions were pooled and cholesterol concentration quantitated. Blue bars represent control and red bars represent LPS-treated mice.

To examine if increased levels of proinflammatory cytokines as a result of LPS treatment leads to impaired reverse cholesterol transport, plasma obtained from control

and LPS-treated mice were apoB-depleted and cholesterol efflux measured as described in Section 7.2.2. As seen in figure 35, cholesterol efflux capability was found to be compromised in the LPS-treated mice when compared to the control mice, suggesting that cholesterol efflux capability of plasma is impaired in proinflammatory state. Further evaluation of impaired cholesterol efflux in LPS-treated mice was done by measuring fecal cholesterol counts in macrophage-reverse choletserol transport in radioisotopic assay. As seen in figure 36, both plasma and fecal counts decreased, suggesting impaired reverse cholesterol transport.

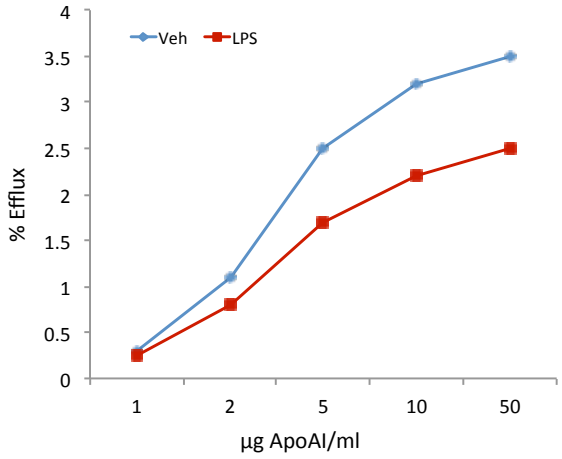


Fig 35 Cholesterol efflux in apoB-depleted plasma. As shown increasing concentration of acceptor apoA-I was used in the media. Blue line represents control and red line represents LPS-treated group.

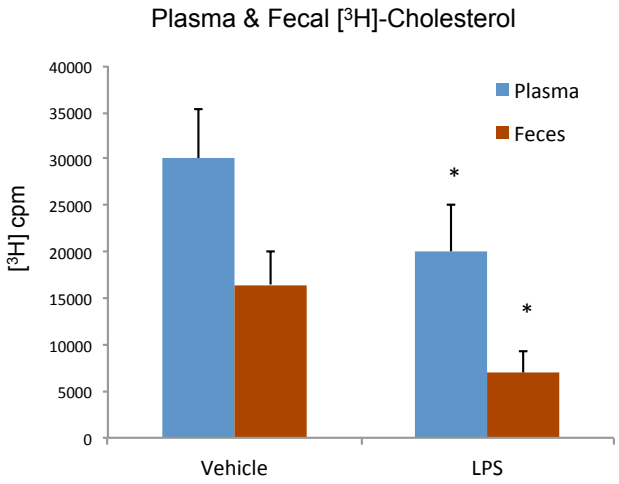


Fig 36. Macrophage-specific reverse cholesterol transport in mice treated with LPS. Groups of mice were either sham-treated or LPS treated and injected IP with activated macrophages induced by ac-LDL and loaded with radioisotopic cholesterol. After 48 h, plasma and feces were isolated and radioactive counts were measured. Blue bars represent control and red bars represent LPS-treated.

8.7 HDL Functionality in Animal Model of Hyperlipidemia, Diabetes, Low-grade Inflammation and Atherosclerosis

Since inflammation impaires cholesterol efflux and reverse cholesterol transport, a process needed to remove atherogenic cholesterol accumulation in the peripheral tissues, including macrophages entrapped in the subendothelial space, it was hypothesized that disease conditions that leads to oxidative stress and inflammation may increase arterial atherosclerosis burden. Hyperlipidemia and diabetes mellitus are such disease conditions in which increased cholesterol, free fatty acids, glycated end products, and MPO, lead to inflammation and oxidative stress. To test this hypothesis, a double knockout mouse was generated by crossing hyperlipidemic LDLr-deficient mouse with leptin-deficient (ob/ob) hyperglycemic diabetic mouse. These mice were screened for insulin, hyperglycemia and hyperlipidemia. Feeding hyperlipidemic diet for 10 weeks makes them severely hyperlipidemic, hyperglycemic, and hyperinsulinemic. These profiles are highly atherogenic and lead to aortic lipid deposition and lesion formation. After feeding hyperlipidemic Western Diet for 10 weeks, mice were treated for 6 weeks with LXR agonist, T0901317, a reference agent known to show anti-inflammatory and antiatherosclerotic activities. Another reference agent, metformin, was used as an

antidiabetic agent (210,211). As shown, these mice show high TG and cholesterol levels (Fig 37, upper 2 panels) and also increased levels of glucose and insulin (Fig 37, lower two panels) as compared to LDLr-deficient or ob/ob mouse (compare blue and orange bars with green bar in the vehicle-tretaed group only).

Treatment of the double knockout mice (DKO) with anti-inflammatory agent LXR agonist, T0901317, known to inhibit progression of atherosclerosis (131), with AMPK activator, metformin, known to have antidiabetic effect (203), and a combination (COMB) of metformin, quercetin (212), and curcumin (213), which are anti-inflammatory agents. Both quercetin and curcumin show antiatherosclerotic activity as well (214-216) showed improvements in lipid and glycemic profile. As seen in Figure 37, treatment with COMB reduced total cholesterol, triglycerides, glucose and insulin more than T1317 (used as a reference) and metformin alone.

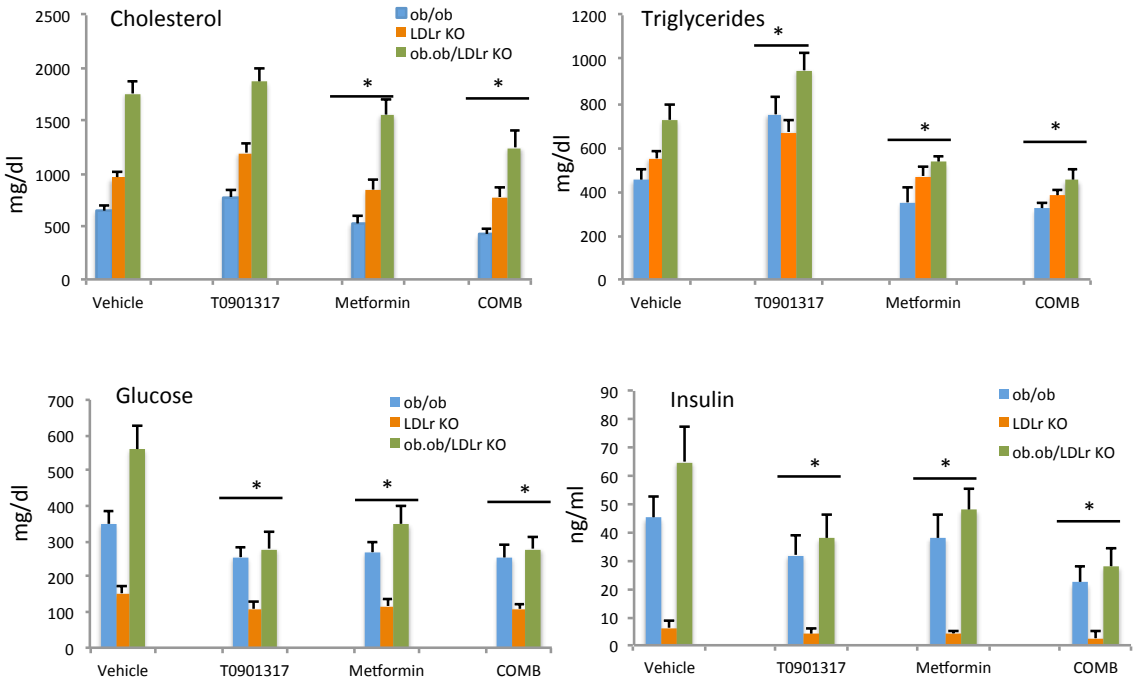


Fig 37. Lipid, glucose and insulin levels in ob/ob (blue), LDLr KO (orange) and ob.ob/LDLr DKO (green) mice. Animals were fed WD for 10 weeks followed by 6 weeks treatment with LXR agonist, T0901317 (20 mg/kg/day, qd) Metformin (300 mg/kg/day, bid), and COMB (Met, 300 mg/d/kg; quercetin 200 mg/d/kg.Curcumin 400 mg/d/kg, bid). * $p<0.025$ compared to Vehicle control.

To examine effect of COMB on attenuation of inflammation, proinflammatory cytokines and haptoglobin were quantitated. Haptogobin is an acute phase protein (217), synthesized primarily in the liver (218), and associates with HDL (219). Humans with cardiovascular disease have increased levels of haptoglobin (220-222), and anti-inflammatory activity of HDL isolated from mice lacking haptoglobin is compromised (219). COMB reduced proinflammatory cytokines IL-6 and TNF-α, adhesion molecule VCAM1, and haptoglobin (Fig 38).

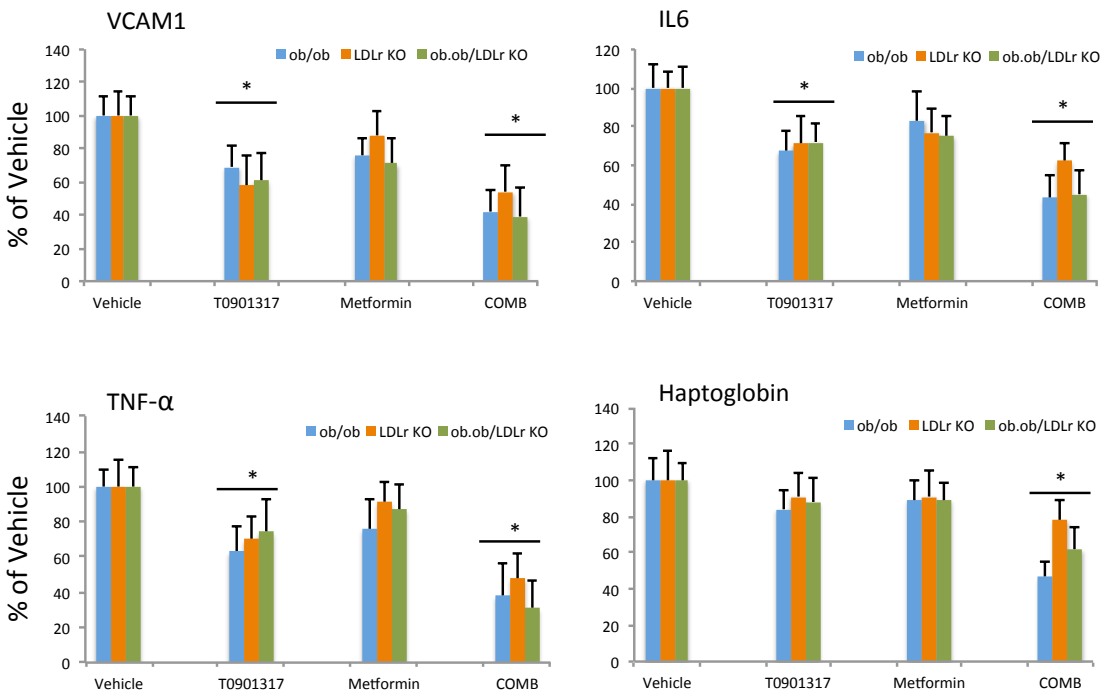


Fig 38. Animals were fed WD for 10 weeks followed by 6 weeks treatment with LXR agonist, T0901317 (20 mg/kg/day, qd) Metformin (300 mg/kg/day, bid), and COMB (Met, 300 mg/d/kg; quercetin 200 mg/d/kg; Curcumin 400 mg/d/kg, bid). * $p<0.025$ compared to Vehicle control. Proinflammatory cytokines and haptoglobin were measured in the plasma

We hypothesized that attenuation of hyperlipidemia and hyperglycemia-induced proinflammatory cytokines, and hyperglycemia in DKO would result into dampening of HDL function through impairement of cholesterol efflux. We therefore, measured both cholesterol efflux in the apoB-depleted plasma and in a separate experiment carried out macrophage-specific reverse cholesterol transport using radioisotopic assay. The results of these studies are shown in Figure 39. Attenuation of cholesterol efflux (Fig 39, Left Panel) as well as increases in fecal cholesterol as a result of enhanced RCT following treatments with COMB, were observed (Fig 39, Right Panel).

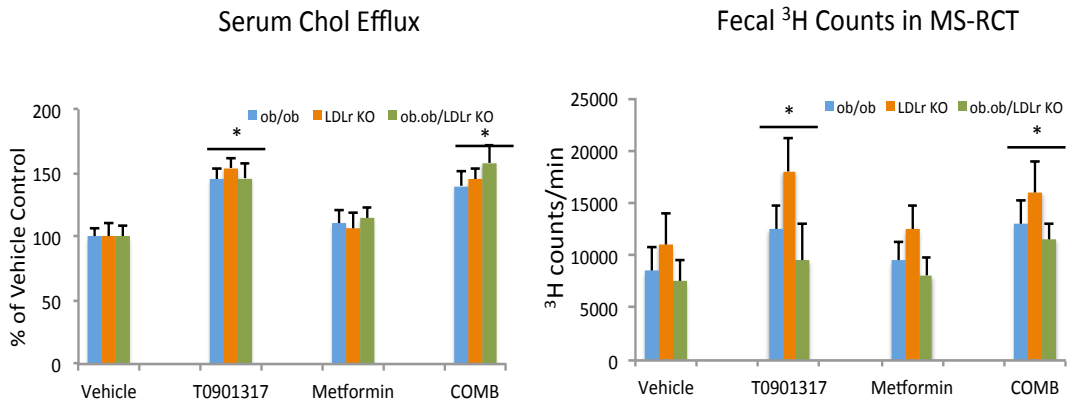


Fig 39. Animals were fed WD for 10 weeks followed by 6 weeks treatment as shown in the legend to Fig 38. * p<0.025 compared to Vehicle control. For serum chol efflux, serum wasapoB-depleted and cholesterol efflux measured. For MS-RCT, radioisotopic assay was performed.

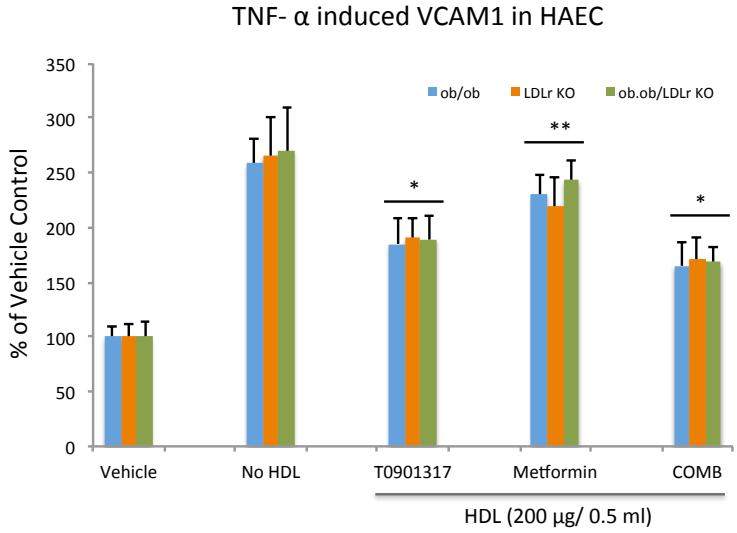


Fig 40. Animals were fed WD for 10 weeks followed by 6 weeks treatment with LXR agonist, T0901317 (20 mg/kg/day, qd) Metformin (300 mg/kg/day, bid), and COMB (Met, 300 mg/d/kg; quercetin 200 mg/d/kg; Curcumin 400 mg/d/kg, bid). * p<0.025 compared to Vehicle control. At the time of necropsy, aliquot of plasma from each muse from each group was pooled and HDL isolated, protein content determined, and used for assay.

A portion of the plasma was used to isolate HDL fraction to evaluate anti-inflammatory activity by measuring VCAM1 in TNF-α-induced human aortic endothelial cells (HAEC). As shown in Figure 40, TNF-α showed induction of VCAM1 as compared to cells with no treatment with TNF-α. In the presence of HDL isolated from treated

groups, a clear anti-inflammatory activity was noticed in all groups, but attenuation of TNF-α-induced inflammation was greater in the COMB group as compared to other two treatment groups.

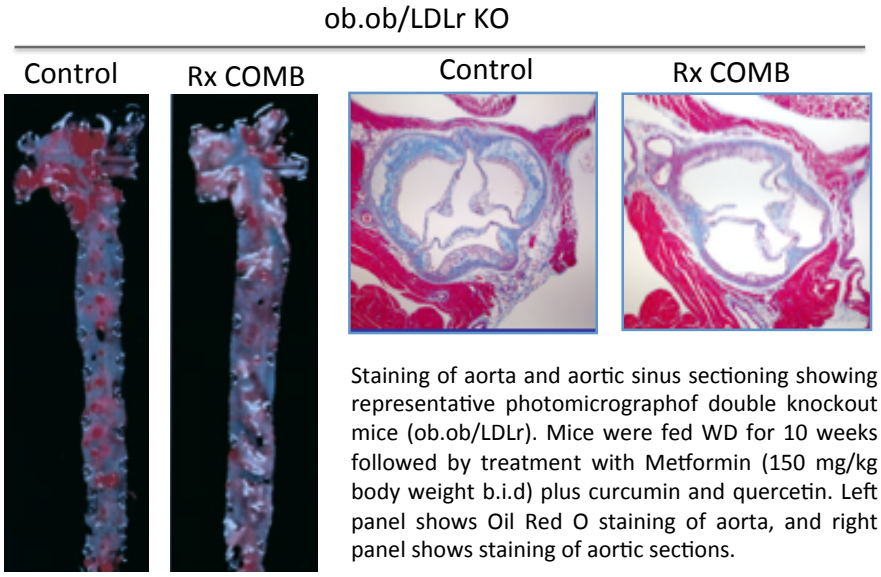


Fig 41. Oil-Red O stained aorta (Left) and sections of aortic sinus (right) of COMB and control group.

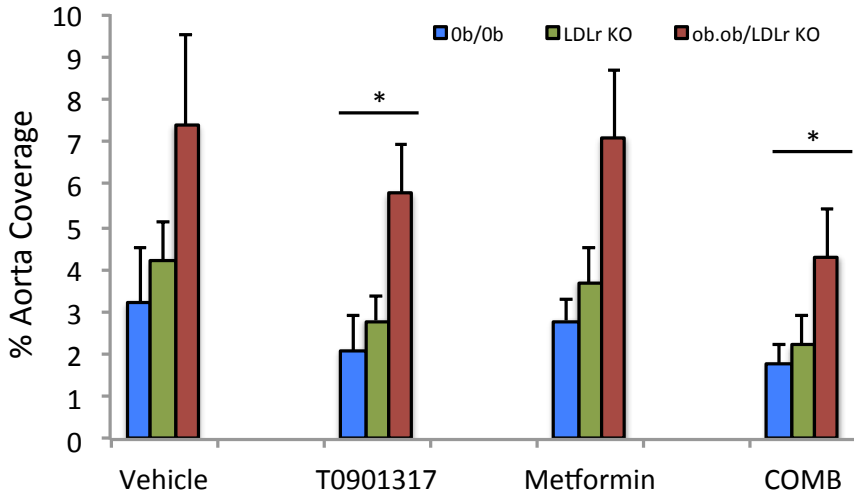


Fig 42. Oil-Red O stained area shown in Figure 41 was measured and plotted.

To examine the effect of HDL function attenuation on atherosclerotic lesion area, aorta was isolated from each mouse from all groups and lesion area quantitated by Oil-Red O staining. Additionally, aortic sinus sectioning was done and stained with Oil-Red O. Greater effects on reducing lesion area was seen in COMB as compared to other two groups (Figs. 41, 42). The anti-inflammatory activity of HDL, cholesterol efflux capability, and lipid profile, all correlated with the extent of lesion formation. COMB showed greater reductions in lipids, glucose and proinflammatory cytokines and at the same time showed increased cholesterol efflux together with enhanced macrophage-specific reverse cholesterol transport.

9. DISCUSSION

The primary objective of this dissertation was to have an in-depth understanding of HDL functionality and how it leads to modulation of reverse cholesterol transport and eventually aortic lipid deposition in pathophysiological conditions of hyperlipidemia, diabetes, and inflammation. Given the higher prevalence of cardiovascular morbidity and mortality in diabetics, it is important to understand if HDL dysfunction is one of the factors causing arterial lipid deposition in diabetics. Recent cell-based studies do suggest a link between HDL and glucose metabolism through both insulin-dependent (137,138) and –independent mechanisms (139). Indeed, HDL has been shown to modulate glucose metabolism in type 2 diabetic patients (161), and infusion studies with recombinant and reconstituted HDL (rHDL) demonstrated attenuation of coronary plaque morphology and volume (47,141,223) through enhanced RCT and improved endothelial function in type 2 diabetes mellitus (142).

The two major components of the HDL-mediated RCT are apoA-I, the main protein constituent of HDL particles, and a membrane protein, ABCA1, that facilitates the cellular cholesterol efflux across cell membrane to the acceptor nascent lipid-poor apoA-I, a preferred acceptor of ABCA1-mediated lipid efflux. Since apoA-I has been shown to specifically bind to ABCA1 (66), the lipid-poor apoA1 (preβ-HDL) functions as an acceptor of cholesterol and phospholipid in an ABCA1-dependent manner resulting the formation of mature cholesterol ester rich spherical α-HDL particles. Thus, a functional apoA-I is important in the process of RCT. A modified apoA-I as a result of oxidative damage may lead to dysfunctional HDL (189,191). Indeed, oxidatively modified dysfunctional apoA-I has been identified in human atherosclerotic plaques (152). The importance of apoA-I and ABCA1 in reverse cholesterol transport has been thoroughly studied and demonstrated in knockout and transgenic mouse models (42,224). ApoA-I KO mice have very low levels of HDL and are susceptible to develop atherosclerosis (42) while apoA-I transgenic mice show resistant to atherosclerosis (224). Overexpression of ABCA1 increased plasma levels of HDL and increased apoA-I-mediated cholesterol efflux from macrophages (78). The data from two different groups

(77,78) are in agreement with regard to the elevated levels of HDL, cholesterol efflux and inhibition of atherogenesis in ABCA1 transgenic mice (22), and in A1-CIII-AIV transgenic mice (83). Similarly, *Abca1*-deficient mice were severely hypolipidemic with almost complete absence of HDL, a condition observed in TD (62). Thus, modulation of ABCA1 is an important factor in the overall reverse cholesterol transport in the process of cholesterol removal from the peripheral tissues and arterial wall. ABCA1 regulation is of utmost importance in driving the RCT and sparing cells from toxicity arising from intracellular cholesterol accumulation.

In a series of studies carried out in ABCA1 overexpressing cell line, it is demonstrated that ABCA1 regulation is highly controlled at the transcriptional level, since upregulation of ABCA1 increased ABCA1 protein in the same proportion as the mRNA, and withdrawal of ABCA1 transcription inducer resulted in proportional reductions in both the mRNA and protein. Further studies showed that transcriptional regulation of ABCA1 was dependent on a phosphorylation step. Thus, ABCA1 regulation is tightly regulated at the transcriptional level where intracellular cholesterol serves as a sensor to turn cholesterol efflux on and off in order to maintain adequate intracellular cholesterol for cellular needs. Studies in RAW264.7 macrophages identified CRE-responsive element, and detected a phosphorylated CRE (110). They also identified in RAW264.7 cells a STAT3/4 site near the b'CRE, which appeared to be required for cAMP-mediated activation of ABCA1 transcription. The present study further extends the understanding of cellular ABCA1 regulation and identifies PKA as a player in the phosphorylation of CRE. Present study also demonstrates, in a non-macrophage cell line, that CMV promoter-mediated transcription of ABCA1 possibly does not require a STAT3/4 site, and that transcription and translation are tightly regulated and linked to the level of cellular ABCA1 protein in a non-macrophage cell line. While ABCA1 phosphorylation was noted in the cAMP-induced cholesterol efflux (108), a direct correlation between inhibition of phosphorylation and ABCA1 transcription is demonstrated in the present study.

In optimized non-radioisotopic and radioisotopic assays, a number of agents

known to modulate lipid metabolism and reverse cholesterol transport were evaluated. ApoA-I functions as an acceptor of cellular cholesterol in ABCA1-mediated pathways and plays an important role in RCT. ApoA-I transgenic mice show increased levels of HDL-C (202) and expression of apoA-I in apoE-deficient mice attenuates atherosclerotic lesion formation (43). Results presented demonstrate that overexpression of apoA-I enhances reverse cholesterol transport, consistent with the role of apoA-I in promoting reverse cholesterol transport from macrophage to feces (196). By the same mechanism, it is possible that the antiatherosclerotic efficacy of PPAR-α and LXR agonists (131) occur via enhanced reverse cholesterol transport, since both of these agents increase apoA-I and HDL. LXR pan agonist, T0901317, known to up-regulate ABCA1 & G1 gene expression showed a nice dose-dependent increase in the fecal cholesterol which was associated with increases in HDL and monocytic ABCA1 mRNA, consistent with the notion that ABCA1 facilitates excretion of cholesterol through RCT. An interesting observation made in the present study was the synergistic effect of LXR and PPAR-α in inducing fecal cholesterol excretion. Given the requirement of LXR for the PPAR-α -mediated enhancement in RCT (200), it is understandable why a synergistic effect was noticed in mice treated with PPAR-α and LXR agonists simultaneously.

The ATP-binding cassette transporter A1 (ABCA1), an important player in the cholesterol efflux that mediates transfer of intracellular cholesterol to lipid-poor HDL as an acceptor, has been shown to modulate insulin secretion (138). HDL reverses the deleterious effects of oxidized low-density lipoprotein (LDL) on insulin secretion by pancreatic beta cells (137). Additionally, it is suggested that HDL and its major apolipoprotein, apoA-I, activate the key metabolic regulatory enzyme, AMP-activated protein kinase (AMPK), in endothelial cells and are critical for the nitric oxide–mediated vasodilatory effects of HDL (140). Indeed, activation of AMPK induces cholesterol efflux from macrophage-derived foam cells leading to reductions in lesion burden in apoE-deficient mice (225). Taken together, it appears that AMPK activators may not only have benefits from its antidiabetic effect, but also may impart additional beneficial effects through influencing RCT. Therefore, a double knock mouse was produced that exhibits

characteristics of hyperlipidemia, hyperglycemia, low-grade inflammation, and develop atherosclerotic lesion on hyperlipidemic diet. Studies performed in this mouse model with an anti-inflammatory agent, T0901317, an AMPK activator, metformin, and a combination of anti-inflammatory agent and AMPK activator clearly suggest that attenuation of hyperglycemia, insulin resistance, hyperlipidemia and low-grade inflammation improves HDL functionality as measured by cholesterol efflux and macrophage-specific reverse cholesterol transport. This improvement in HDL functionality translated into attenuation of aortic lipid deposition as measured by lipid staining. These data clearly demonstrate that improvement in HDL functionality may benefit diabetic patients who have elevated oxidative stress and inflammation with compromised HDL function (182).

Poor glycemic control in type-1 diabetes is associated with accelerated oxidative damage to apolipoprotein (apo) A-I, the main protein component of HDL (150) and advanced glycated albumin diminishes anti-inflammatory properties of HDL (179,180). Reconstituted HDL shows anti-inflammatory activity in humans (164,168,181). ABCA1-mediated cholesterol efflux capability of HDL is compromised in type 2 diabetes patients (182), possibly as a result of oxidative damage to apoA-I (183). Since antioxidative and antiinflammatory properties of HDL are impaired in diabetics (184), oxidative stress and inflammation may contribute to HDL dysfunction (185). HDL undergoes modification and multiple structural changes in an inflammatory condition and transforms normal functional HDL into “acute phase HDL”, enriched in free fatty acids, triglycerides, serum amyloid A (SAA), and decreased anti-inflammatory enzymes, including paraoxanase 1 (186,187). One such acute phase protein is haptoglobin (217). This protein is synthesized primarily in the liver (218) and secreted in the circulation where it associates with HDL (219). In humans, increased levels of haptoglobin are associated with cardiovascular disease (220-222). HDL isolated from mice lacking haptoglobin show anti-inflammatory activity compared to WT mice (219), suggesting a proinflammatory role of haptoglobin. In the present study, treatment of DKO mice with COMB lowered haptoglobin concentration in the blood, and possibly contributed to attenuation of HDL functionality

as measured by cholesterol efflux. In addition, inflammation induces secretion of myeloperoxidase (MPO), which has been shown to modify apolipoprotein A-I (apoAI) and impair its ability to accept cholesterol (151,188-191). MPO-mediated oxidation of apoA-I makes it proinflammatory (192). Tryptophan substitution with phenylalanine in apoA-I renders it resistance to MPO oxidation (193). All these studies suggest that the fundamental changes due to lipid and carbohydrate dysregulation together with increase in inflammation in diabetes mellitus leads to HDL dysfunction.

The present study demonstrates that apoA-I and ABCA-I are important players in the RCT, and attenuation of oxidative stress, inflammation, and lowering of liplid and glucose, restores HDL function, and exert beneficial effect in the process of lipid removal from lipid-laden macrophage in the arterial wall.

PROPOSED WORKING HYPOTHESIS

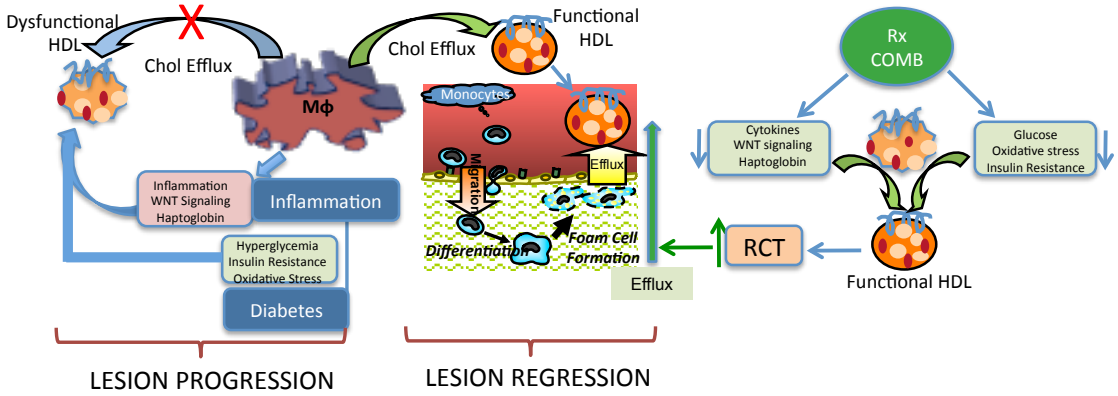


Fig 43. Working hypothesis showing factors causing HDL dysfunction leading to arterial lipid deposition and restoration of HDL dysfunction following treatment with hypolipidemic, anti-inflammatory, and antidiabetic agents, termed as COMB. As shown, in normal physiological conditions, HDL carried out its function of cholesterol efflux in the arterial wall and inhibits accumulation of lipids. In pathophysiological conditions where oxidative stress and inflammation are elevated, HDL undergoes modifications. The functions of modified HDL are compromised leading to dampened cholesterol efflux capability. Agents that attenuate inflammation, hyperglycemia, and oxidative stress improve HDL functionality and restores cholesterol effluc capability leading to reductions in arterial lipids.

As a working hypothesis, the biologic sequence of events that leads to HDL dysfunction is shown in figure 43. HDL performs its normal function by removing cholesterol from lipid-laden macrophages in the arterial wall, thus causing lesion regression. The inflamed macrophages entrapped in the subendothelial space secrete proinflammatory cytokines and haptoglobin, and activates wnt signaling through LRP5/6 as a result of uptake of aggregated LDL (226) in hyperlipidemic conditions. Wnt/β-catanin signaling has been shown to induce proliferation of vascular smooth muscle cells (227), which may lead to narrowing of artery lumen and eventually causing occlusion. High oxidative stress, diabetes, and inflammation cause dysfunctional HDL, leading to dampening of cholesterol efflux capability and impaired arterial cholesterol removal. Treatment with COMB attenuates hyperlipidemia, oxidative stress and inflammation, which lead to improvement of HDL function and removal of cholesterol from lipid-ladden macrophages entrapped in the sub-endothelial space.

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