THE EFFECTS OF TRIGLYCERIDE ON LYSOSOMAL FUNCTION IN MACROPHAGE FOAM CELLS

By

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To my parents and my husband
for their support, love and encouragement.

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12-O-tetradecanoylphorbol-13-acetate .....TPA

3β-[2-(diethylamino)ethoxy]androst-5-en-17-one.....U1866A

Acetylated LDL .....acLDL

Aceyl-coA:cholesterol aceyltransferase.....ACAT

Aggregated LDL.....aggLDL

Apolipoprotein A.....apoA

Apolipoprotein B.....apoB

Apolipoprotein C.....apoC

Apolipoprotein E .....apoE

ATP binding cassette transporter A1 .....ABCA1

ATP binding cassette transporter G1.....ABCG1

Beta-cyclodextrin .....β-CD

Block lipid transport-1.....BLT-1

Bovine serum albumin .....BSA

CCAAT/-enhancer-binding protein homologous protein .....CHOP

Cholesteryl ester .....CE

Cholesteryl ester-rich lipid dispersion .....CE-DISP

Cholesterol ester transfer protein .....CETP

Cholesterol methyl ether.....CME

Chylomicron .....CM

Chylomicron remnant .....CMR

Cluster of Differentiation 36 .....CD36

Diacylglycerol acyltransferase .....DGAT

Docosahexaenoic acid.....DHA

Early endosomal antigen-1 .....EEA-1

Eicosapentaenoic acid.....EPA

Electron microscopy.....EM

Electron paramagnetic resonance .....EPR

Endoplasmic reticulum .....ER

Ethylenediaminetetraacetic acid .....EDTA

Eukaryotic translation initiation factor 2A.....eIF2α

Fatty acid.....FA

Fetal bovine serum .....FBS

Free cholesterol .....FC

Free fatty acid .....FFA

Gas chromatography.....GC

Glucose regulate protein 78 .....Grp78

Glycerol-3-phosphate .....GP

Heparin sulfate proteoglycan .....HSPG

Hepatic lipase.....HL

Hepatocyte nuclear factor 4 alpha HNF-4α

High density lipoprotein .....HDL

Horseradish peroxidase .....HRP

Hypertriglyceridemeia .....HTG

Interleukin-8.....IL-8

Intermediate density lipoprotein.....IDL

Linoleic acid.....LA

Lipid dispersions.....DISP

Lipoprotein lipase .....LPL

Liver X Receptor .....LXR

Long-chain fatty acid.....LCFA

Low density lipoprotein .....LDL

Low density lipoprotein receptor .....LDL-R

Low density lipoprotein receptor-related protein.....LRP

Lysosomal acid lipase.....LAL

Lysosomal associated membrane protein 1 .....LAMP-1

Lysosomal storage disorders.....LSD

methyl-β-cyclodextrin.....β-CD

Microsomal triglyceride transfer protein .....MTP

Monocyte chemoattractant protein-1.....MCP-1

Neutral cholesteryl ester hydrolase.....NCEH

Niemann Pick type C .....NPC

Oleic acid.....OA

Oxidized LDL.....oxLDL

Palmitic acid .....PA

Peroxisome-Proliferator Activated Receptor .....PPAR

Phosphatidylcholine.....PC

Phosphatidylserine.....PS

Phospholipid .....PL

Phospholipid transfer protein.....PLTP

Plasma membrane .....PM

Post nuclear supernatant.....PNS

Reverse cholesterol transport .....RCT

Sarcoplasmic-endoplasmic reticulum calcium ATPase-2b SERCA2b

Scavenger Receptor type A.....SR-A

Scavenger Receptor type B1 .....SR-B1

Site-1-protease.....S1P

Site-2-protease.....S2P

Stearic acid.....SA

Sterol regulatory element binding proteins .....SREBP

SREBP cleavage-activating protein .....SCAP

Tetrahydrolipstatin .....Orlistat

Thiobarbituric acid-reactive substances.....TBARS

Transmission electron microscopy .....TEM

Trialkylglycerol ether .....TGE

Triglyceride.....TG

Triglyceride-rich lipid dispersions .....TG-DISP

Triglyceride-rich particles .....TRPs

Tris buffered saline .....TBS

Tris buffered saline containing 0.1% TWEEN-20 .....TBST

Vacuolar type H+-ATPase.....v-ATPase

Very long-chain fatty acid .....VLCFA

Very Low Denisity Lipoprotein.....VLDL

CHAPTER I

INTRODUCTION

Objective of Dissertation

As atherosclerotic lesions develop, foam cells in the lesion accumulate significant amounts of cholesterol in large, swollen lysosomes. Previous studies from our laboratory and others using macrophages in culture indicate that cholesterol derived from various cholesteryl ester (CE) sources can mimic this lysosomal accumulation (1-11). Early in atherosclerosis, free cholesterol (FC) is the primary form of cholesterol in lysosomes. However, in later stages, both FC and CE are found trapped in the lysosome (9,12). The late-stage CE accumulation is the result of an inhibition of lysosomal CE hydrolysis (9-11,13). Lysosomal acid lipase (LAL) is the principal enzyme responsible for lysosomal CE hydrolysis. During CE accumulation, there is a concomitant increase in the lysosome pH to a level that is not conducive to LAL activity. This suggests an inverse correlation between lysosomal pH and inhibition of CE hydrolysis.

Cholesterol is trapped within lipid engorged lysosomes. Stimulation of cholesterol efflux removes extralysosomal cholesterol but does not reduce the lysosomal FC and CE stores. However, preliminary studies in our laboratory showed that triglyceride-rich particles (TRPs), similar to those present in the atherosclerotic lesion, have the potential to influence lysosomal and cellular cholesterol metabolism and homeostasis. Based on our preliminary studies and literature suggesting a role of

triglyceride (TG) in macrophage foam cell cholesterol homeostasis, we hypothesized that TG-treatment might enhance CE hydrolysis and FC clearance from lysosomes. One mechanism by which this could occur is by helping lysosomes maintain a low pH and, thus, continue CE hydrolysis. It is also possible that TG treatment might enhance the expression of proteins important in cholesterol metabolism. The objective of this research was to test these hypotheses by investigating the effect of TRP-treatment on lysosomal pH, lysosomal CE hydrolysis, and lysosomal and cellular sterol clearance.

We divided the work into three separate, but related, aims. The first aim was to determine the effect of TG treatment on the lysosomal degradation of CE and mobilization of FC from lysosomes. Preliminary results indicated that cellular cholesterol levels were reduced when treated with TG. Therefore, we sought to investigate the enhancement of lysosomal CE hydrolysis as a potential mechanism for this reduction in cellular cholesterol levels. We employed biochemical and microscopic analyses to examine the cellular and lysosomal hydrolysis of CE and subsequent clearance of FC within the lysosome in cells treated with TG and/or CE-rich lipid particles.

The second aim was to determine the effects of TG treatment on lysosomal acidification. Initial studies suggested that lysosome activity is maintained upon treatment with TRPs, even in the presence of CE-rich vehicles. Given our data indicating a relationship of CE hydrolysis rate and lysosome pH, we sought to determine if TG acted to reestablish normal acidic lysosome pH. Studies were performed to measure lysosomal pH in cells treated with TG and/or CE-rich lipid particles. Additionally, in

order to define the mechanism of the effects of TRPs on lysosomal acidification, biochemical analyses were performed to examine the activity of the lysosomal vacuolar-type H^+-ATPase (v-ATPase), a critical proton pump responsible for maintaining acidic lysosomal pH. We also examined lysosome membrane order in the presence of cholesterol accumulation and after TRP treatment. Membrane order is a known regulator of many membrane proteins (14,15) and, thus, is a possible mediator of the cholesterol and TG effects on lysosomal function.

Our third aim was to determine whether TG hydrolysis was necessary for TRP to affect lysososmal CE metabolism. As described above, preliminary studies showed enhanced cholesterol clearance and improved lysosomal activity within macrophage foam cells treated with TRPs. As part of this goal, it was important to differentiate between lysosomal TG, cytoplasmic TG and/or hydrolytically released fatty acids (FAs) as the mediator of enhanced cholesterol clearance from the lysosome. Biochemical analyses were used to determine if TG and/or hydrolytically released FAs are required for lysosomal and cholesterol homeostatic effects. Additionally, studies examined the potential for TG to mobilize cellular cholesterol by investigating the regulation of cholesterol efflux pathways following TG enrichment.

Cholesterol is an important molecule that influences both structural and functional properties of all membranes and lysosomal cholesterol accumulation is a common attribute of foam cells in atherosclerotic lesions. It is also a major homeostatic signal. The sequestration of cholesterol (both FC and CE) in lysosomes, away from normal cellular sterol metabolic pathways, could have dramatic effects on a number of

cell properties and might lead to cells that are substantially more proatherogenic than foam cells without lysosomal sequestration. Thus, understanding intracellular cholesterol metabolism and the influences of cholesterol sequestration or release on other metabolic pathways are critical to understanding atherosclerosis. To date, most studies of cellular cholesterol accumulation and its effects deal with cells in which cholesterol is the principal lipid being metabolized. However, in atherosclerotic lesions there are a number of TG-rich sources of lipid that can be taken up by foam cells. Our data suggest that TRPs can influence lysosomal cholesterol metabolism and, thus, have consequences for atherogenesis. The studies described in this dissertation have improved our understanding of the nature of the TRP and cholesterol interactions within the lysosomes and the subsequent effects on macrophage foam cells.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Pathogenesis of Atherosclerosis

Atherosclerosis is a complex disease, with multiple factors contributing to its pathogenesis. It is an insidious disease that begins in early childhood and develops over a lifetime, but clinical complications typically become manifest in late adulthood (16,17). Genetic and epidemiologic factors contributing to the development of atherosclerosis are the key to how fast lesions advance to clinical stages. Genetic risk factors, including lipid abnormalities, hypertension, diabetes, and obesity can speed the progression (17-19). Overlying the genetic factors, a number of environmental factors, including smoking, unhealthy diet, and lack of physical activity also contribute to the progression of lesions (17-19). While conventional risk factors determine the time course of lesion development, importantly, the latency of clinical disease onset provides an extended period of time for therapeutic intervention. However, in order to develop effective therapies, it is important to define the key factors involved in atherogenesis.

Early Atherosclerotic Lesions

Early atherosclerotic lesions are characteristically cellular in nature. Although the initiating factors are still debated, it is believed that one of the earliest events in atherogenesis is endothelial cell dysfunction, which initiates the recruitment of

circulating monocytes to the arterial endothelium. Endothelial dysfunction is thought to result from vessel injury, an increase in lipid deposition in the subendothelial space, and/or changes in shear stress within the vasculature (20-22). Monocytes are recruited to the endothelium as part of a normal inflammatory response to endothelial cell damage as well as in response to chemoattractants released from the artery wall, such as monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) (23-28). Recruitment includes upregulation of adhesion molecules on both monocytes and activated arterial endothelial cells (29-32). Monocyte adhesion molecules include β_2-integrin, VLA-4, and PCAM-1 while endothelial cells express the adhesion molecules P-selectin, E-selectin, VCAM-1 and ICAM-1. Following adherence, the monocytes transmigrate into the arterial wall (**figure 1**). Once inside the subendothelial space, monocytes differentiate into macrophages. The infiltration and accumulation of macrophages in the artery wall are the hallmarks of early atherosclerotic lesions (**figures 1 and 2**). This process is known as the "response-to-injury" hypothesis of atherosclerosis (33).

Intermediate Atherosclerotic Lesions

As lesions progress to fatty streaks, lipids play a significant role in lesion advancement. Intermediate atherosclerotic lesion growth occurs primarily by the accumulation of lipid in the subendothelial space. Lipoproteins present in circulation are able to pass into the arterial intima. Once in the artery wall, the cholesterol-rich lipoproteins, primarily low-density lipoproteins (LDLs), are subject to modification, including oxidation and aggregation (**figure 2**)(34,35). While the recruitment of

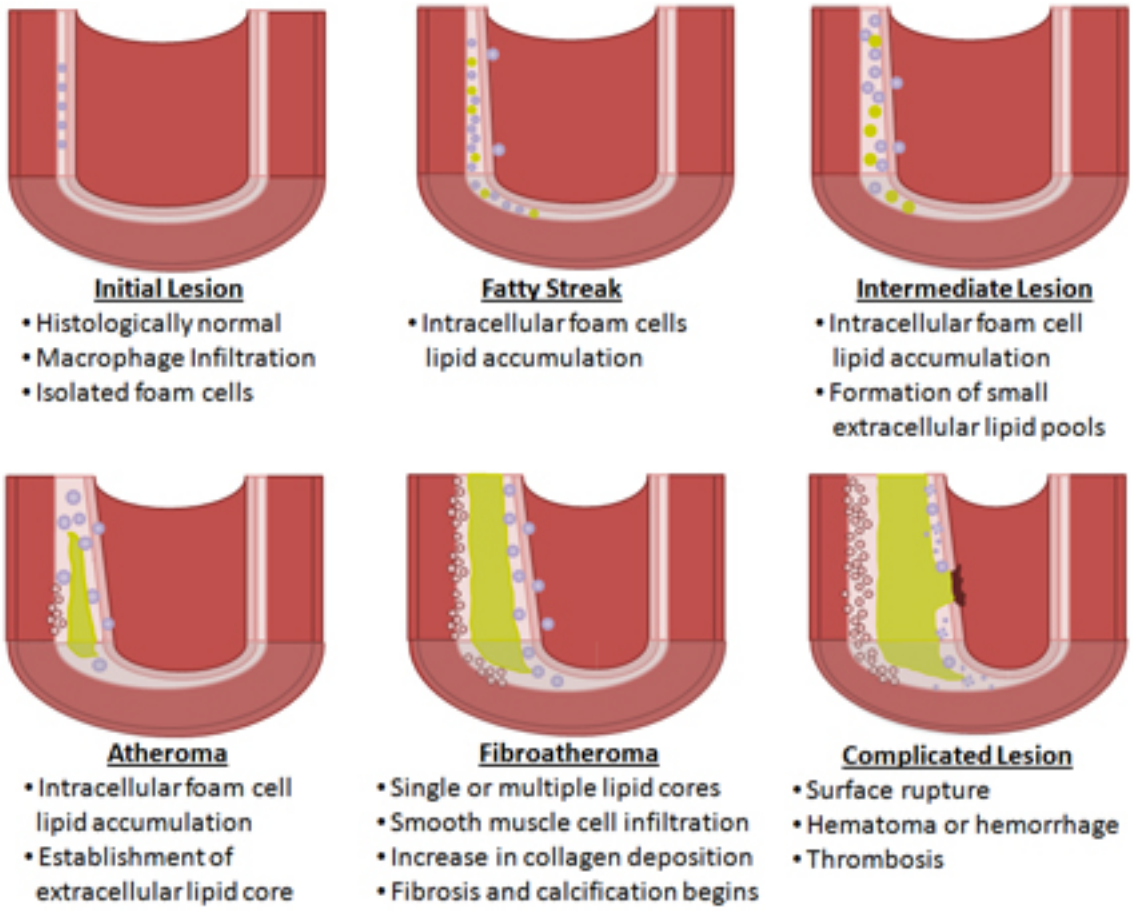


Figure 1: Atherosclerosis is a progressive disease, involving a number of cell types and lipids. Lesions at various stages are classified based on their histology. The progression of early lesion development (initial lesions, fatty streaks, intermediate lesions and atheroma) are predominantly driven by lipid accumulation in the artery wall and in lipid-enriched macrophage foam cells. Clinical manifestations become present at late-stage lesions (fibroatheroma and complicated lesions). Growth of late stage lesions occurs by the infiltration of smooth muscle cells and collagen into the artery wall. Complicated lesions may become unstable, leading to rupture, hemorrhage and thrombosis. Such complications result in clinical ailments, including myocardial infarction and stroke.

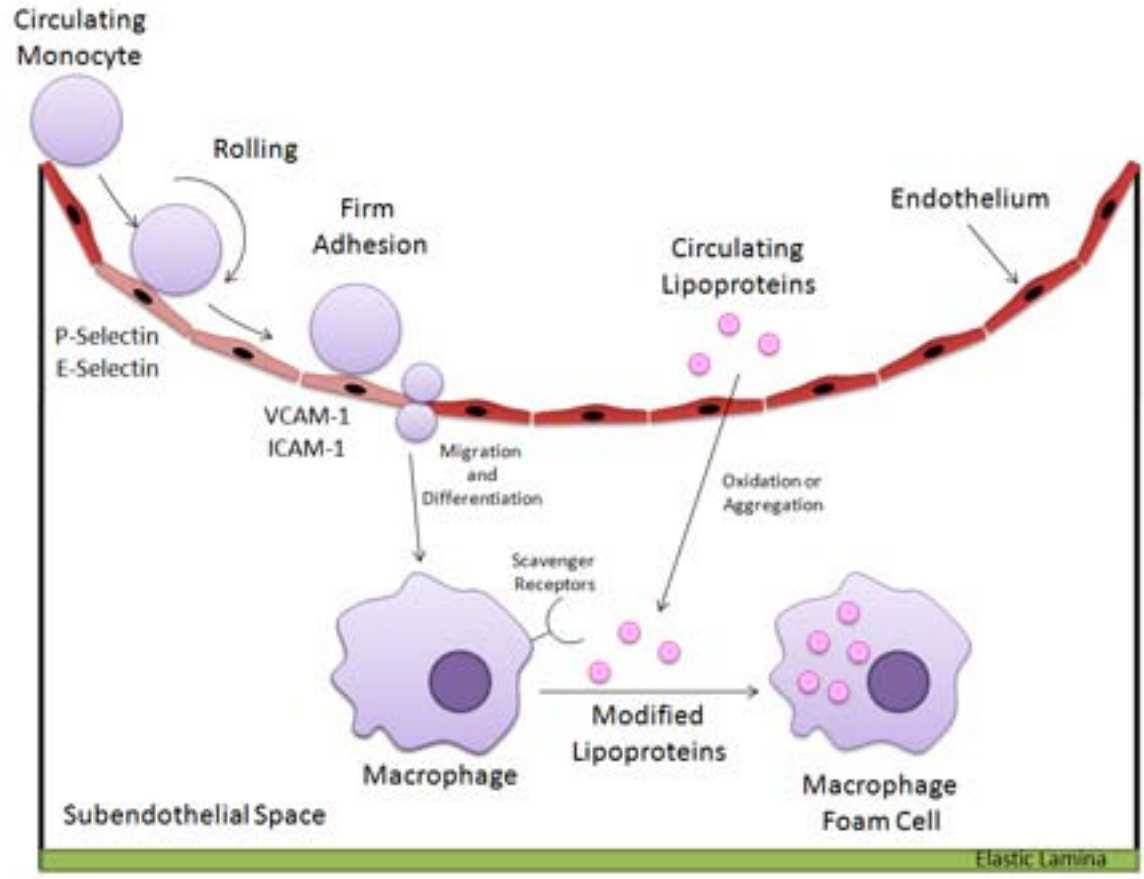


Figure 2: Monocyte-derived macrophages and circulating lipoproteins play predominant roles in atherosclerotic lesion development. The earliest events in atheroma formation include endothelial dysfunction and lipid accumulation in the arterial intima. This results in the recruitment of monocytes to the arterial endothelium. Monocytes migrate into the subendothelial space and differentiate into macrophages. Following differentiation, macrophages ingest large amounts of modified lipoproteins present in the subendothelial space resulting in the formation of macrophage foam cells.

monocytes/macrophages is initially a protective immune response to arterial injury, uptake of modified LDL via macrophage scavenger receptor-mediated endocytosis, and possibly other mechanisms, leads to macrophage foam cell formation. Foam cells, named for the "foamy" appearance created by the severe lipid accumulation, are macrophages that have engulfed large amounts of lipid. Foam cells are a hallmark of the thickened atherosclerotic lesion. Progressive accumulation of modified LDLs in the artery wall and continual formation of foam cells leads to intermediate lesions and atheroma (**figure 1**). During the intermediate stages of atherosclerosis, a core of extracellular lipid develops and grows. The progressive accumulation of lipid and cellular material leads to more advanced and more unstable atherosclerotic lesions.

Late Atherosclerotic Lesions

Late stage lesions are far more complicated than earlier stages of atherosclerosis and, with the increased degree of complexity, clinical complications become manifest. Atherosclerosis remains largely asymptomatic unless the lesion is sufficient to obstruct blood flow in the artery. In these cases, exertion can produce angina due to insufficient blood flow. These larger, late stage lesions include more than just monocyte-derived macrophage foam cells and extracellular lipid. Smooth muscle cells are recruited to the lesion from the underlying medial portion of the artery wall. There is also a concomitant increase in collagen that is secreted from SMCs (36,37). All of these events contribute to arterial wall thickening. At even later stages, the lesion can become fibrotic and/or calcified, which leads to the advancement to complicated, clinically important lesions

(38-40). Complicated lesions often contain a core of necrotic material (17). The necrotic core consists of dead and dying macrophages, macrophage debris and extracellular lipid, including cholesterol crystals (41-46). The size of the necrotic core renders advanced lesions highly unstable and, as a result, they are subject to hemorrhage and thrombosis (45). The rupture of unstable, advanced lesions results in myocardial infarction and stroke, two of the most common clinical manifestations of atherosclerosis and the most common causes of death from atherosclerosis (47-49).

Therapeutic Intervention during the Progression of Atherosclerosis

Atherosclerotic lesions develop over a period of decades. Hence, there is sufficient time for intervention if the lesions are detected. Unfortunately, in the majority of cases, atherosclerosis remains undetected until the presentation of late-stage, clinical lesions. Therefore, the development of therapies to remedy late-stage atherosclerosis is important. Current options for treatment reduce or eliminate exposure to risk factors but do not cure the disease. Therapies include restriction of dietary fat and cholesterol, pharmacologic interventions, aerobic exercise, and, in more severe cases, surgical intervention to place stents in the affected artery or to completely remove portions of occluded arteries. While these therapies will slow lesion progression or protect against rupture, they do not permanently resolve advanced lesions. Therefore, the identification of therapies that will promote lesion regression is most significant in treating clinical atherosclerosis. Recent studies in the literature identify macrophage foam cells as important targets for inducing atherosclerotic lesion regression (50,51).

Specifically, reducing the lipid content of macrophage foam cells *in vivo* can significantly reduce lesion area and, perhaps, help stabilize the plaque. Thus, defining mediators of intracellular cholesterol homeostasis that can be manipulated to treat advanced atherosclerotic lesions is an important therapeutic target.

Dietary Lipids in Physiology

Dietary lipids, including cholesterol and TG, are significant to normal physiology. Cholesterol is an important component of cellular membranes and steroid hormones while TG provides a significant amount of energy required for cellular function. For transportation in the blood and lymph system, cholesterol and TG are packaged into macromolecular complexes of proteins and lipid called lipoproteins. Lipoproteins can be classified based on their hydrated density into five major classes: chylomicrons (CMs), very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Additionally, lipoproteins are defined by their lipid and apoprotein composition (**table 1**). Importantly, apoproteins maintain the solubility of lipid-rich particles in the aqueous environment of the plasma, transport and redistribute lipids among various tissues, function as ligands in the receptor-mediated uptake of lipoproteins, maintain the structure of the lipoproteins, and function as cofactors for enzymes of lipid metabolism (52-55). The production, circulation, and metabolism of circulating lipoproteins are important in normal metabolism and in several pathological conditions, including atherosclerosis.

Table 1: Composition of the major classes of lipoproteins.

Class	Density (g/ml)	Diameter (nm)	Lipid Content			Apoprotein Content	
			% CE	% PL	% TG	% Protein	Apoproteins
CMs	<0.95	100 – 1000	8	7	84	<2	apoAIV, apoB48, apoCI, apoCII, apoCIII
VLDL	0.95 – 1.006	30 – 80	22	18	50	10	apoB100, apoE, apoCI, apoCII, apoCIII
IDL	1.006 – 1.019	25 – 50	29	22	31	18	apoB100, apoE
LDL	1.019 – 1.063	18 – 28	50	21	4	25	apoB100
HDL	>1.063	5 – 15	30	29	8	33	apoAI, apoAII, apoAV, apoD, apoE

Systemic Dietary Lipid Homeostasis

Cholesterol and TG are derived from consumption of animal products and saturated fats and/or synthesized within cells. Following a meal, dietary lipids are absorbed in the intestine, packaged into chylomicrons and secreted into the lymphatic system (**figure 3**). These TRPs deliver TG to peripheral tissues, including adipose and skeletal muscle, for storage or oxidation, respectively. Additionally, whole or remnant CMs deliver lipids to hepatocytes, where dietary and synthesized lipids are assembled into VLDL. After assembly in hepatocytes, VLDL enters the circulation and transfers lipids to extrahepatic tissues, where TG is utilized for cellular energy. However, in the circulation, VLDL undergoes extensive remodeling, which alters the lipid and apoprotein content of the lipoproteins. Specifically, VLDL TGs are degraded by lipases, including lipoprotein lipase (LPL) and hepatic lipase (HL), resulting in the production of IDL, and eventually LDL, the lipoprotein responsible for transferring cholesterol to peripheral tissues (**figure 3**). Furthermore, complex interactions between lipoproteins in circulation can alter their lipid and apoprotein composition. For example, HDL can transfer apoproteins to VLDL through aqueous diffusion and can exchange lipids with VLDL by the activity of phospholipid transfer protein (PLTP) and cholesterol ester transfer protein (CETP) (56,57). Despite the complexity of the interactions between circulating lipoproteins, it is important to note that all of the aforementioned lipoproteins are present in the circulation and the periphery, including within atherosclerotic lesions. The exposure of peripheral cells to circulating lipoproteins is important in normal and pathological conditions, including atherosclerosis.

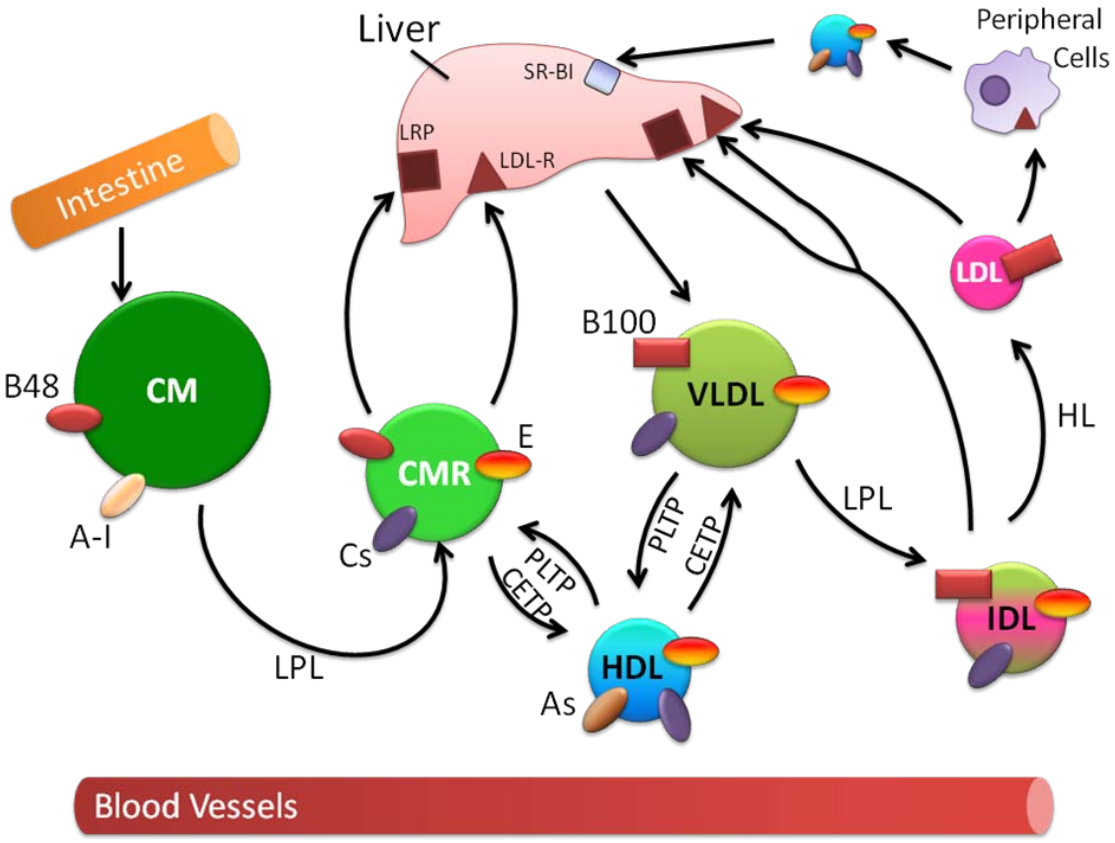


Figure 3: Systemic lipoprotein metabolism. Following a meal, lipids are absorbed in the intestine and packaged into CMs. Hydrolysis of CM-TG in the circulation results in the formation of CM remnants (CMRs). CMRs are delivered to the liver where dietary lipids carried by the CMRs and lipids synthesized by hepatocytes are assembled into VLDL. VLDL enters the circulation and sequential hydrolysis of VLDL-TG results in the formation of IDL and LDL. HDL is also present in the circulation and can interact with other circulating lipoproteins to exchange lipids and apoproteins.

Dietary Lipids and Atherosclerosis

Numerous studies have shown a link between circulating lipoprotein levels and atherosclerosis. Specifically, lipoprotein imbalances are significant risk factors for atherosclerosis. High levels of LDL cholesterol (referred to as "bad cholesterol") and low levels of HDL cholesterol (referred to as "good cholesterol") are clinical hallmarks of the disease as the levels of circulating lipoproteins correlate with the severity of coronary atherosclerosis (58-61). HDL is important in reverse cholesterol transport because of its ability to transport lipids from peripheral cells to the liver for removal. Thus, low circulating levels of HDL-cholesterol are associated with a higher risk for coronary heart disease (62-64). High levels of LDL-cholesterol are proatherogenic, as their presence in the lesion contributes to macrophage foam cell formation. However, the role of circulating TG-rich lipoproteins in atherogenesis is less defined.

Importantly, studies showing a positive correlation between VLDL and atherosclerosis typically examine the effect of β-VLDL on atherogenesis. β-VLDL is an abnormal cholesterol-rich lipoprotein found in hypercholesterolemic animals and is very different from normolipidemic, TG-rich VLDL (65). Some insight into the role of normolipidemic VLDL and CMs in atherogenesis can be gleaned from studies of human patients with LPL deficiency or apolipoprotein C-II (apoC-II) deficiency (66-68). Patients with these conditions have elevated CMs and VLDL, but do not have an increased risk for atherogenesis. In contrast, patients with such deficiencies appear to be protected from atherogenesis. Thus, it is possible that VLDL and CMs can prevent or ameliorate atherosclerotic lesion formation, although the mechanism is unclear. The studies

presented in this dissertation provide critical insight into potential influences of TG-rich lipoproteins on atherogenesis. The current studies focus on the affects of TRPs on macrophage foam cells, one of the critical mediators of atherogenesis.

Atherosclerotic Foam Cell Development

Macrophages are central to the initiation, progression, and development of atherosclerotic plaques. One of the earliest events in atherosclerosis is the development of macrophage foam cells. Most of the sterol in foam cells is derived from the endocytic uptake of modified lipoproteins and extracellular lipid complexes (34,69,70). The critical first step in the cell's attempts to eliminate this sterol is delivery of the particles, via endocytic vesicles, to late endosomes/lysosomes where CEs are hydrolyzed. Under normal conditions, the FC generated from CE hydrolysis, is actively removed from lysosomes and used either for membrane synthesis or stored as cytoplasmic CE (71). Excess FC is toxic and must be cleared from the cell as an intact, hydrophobic molecule. In most cells this clearance is initiated by transport to the plasma membrane (PM) followed by efflux to acceptor particles in the extracellular milieu (72,73). Most of the cholesterol that is not effluxed out of the cell is reesterified to a fatty acid (FA) by acyl-CoA acyltransferase (ACAT) to CE and stored in extralysosomal cytoplasmic inclusions (69,74). In addition to re-esterification, some cholesterol can move into alternative metabolic pools that can act to signal specific changes in the macrophage. Among these are activation of apoptosis pathways or processing to 27-OH cholesterol, a liver X receptor (LXR) agonist (73,75-78). The foam cell is a major regulator of plaque

development (46,79). Thus, the increased cholesterol accumulation observed in foam cells can impact macrophage function in multiple ways that can influence lesion development. For this reason, understanding what mediates foam cell intracellular cholesterol homeostasis is a major goal of atherosclerosis research.

Macrophage Foam Cell Cholesterol Homeostasis

Macrophage cholesterol balance is influenced by cholesterol synthesis, lipoprotein uptake, CE storage, and cholesterol efflux. Several intracellular pathways exist to control each step in cholesterol homeostasis. Importantly, when one homeostatic pathway is disrupted, the other attempt to compensate for the disruption, in order to maintain the balance of intracellular cholesterol levels. Overburdening of the compensatory processes that maintain cellular cholesterol homeostasis results in the massive increases in cholesterol observed in macrophage foam cells.

Cholesterol Synthesis

Macrophages are able to synthesize their own cholesterol in a highly regulated process that recognizes and responds to intracellular cholesterol levels. The endoplasmic reticulum (ER) is the critical site of cholesterol homeostasis, as it is the primary site for cholesterol synthesis and esterification. The rate-limiting enzyme of the pathway is hydroxymethylglutaryl CoA reductase (HMG-CoAR), which generates mevalonate from the reduction of HMG-CoA. Importantly, HMG-CoAR activity is highly regulated through feedback inhibition by cholesterol in a mechanism involving sterol

regulatory element binding proteins (SREBP) and SREBP cleavage activating protein (SCAP). SCAP has a sterol sensing domain that undergoes conformational changes in response to the cholesterol content of the ER membrane. Under conditions of high cholesterol, SCAP confines SREBP to the ER, resulting in the inhibition of cholesterol biosynthesis (80). In contrast, when cholesterol levels are low, SCAP binds to and escorts SREPB to the Golgi where two proteases, site-1-protease (S1P) and site-2-protease (S2P), act sequentially to release the bHLH-Zip domain of the protein. Following its cleavage, the bHLH-Zip domain translocates to the nucleus, where it acts as a transcription factor to activate the genes responsible for cholesterol synthesis (80). Although cholesterol synthesized by the cell can contribute to intracellular lipid homeostasis, it plays a very minor role in the lipid accumulation observed in foam cells because synthesis of cholesterol is tightly regulated and decreases when cholesterol accumulates (81). Thus, internalized cholesterol is the most significant contributor to the lipid burden in macrophage foam cells.

Lipoprotein Cholesterol Uptake

Several mechanisms have been identified for accumulation of exogenous cholesterol by macrophages. LDL receptors (LDL-R) bind LDL particles in clathrin-coated pits for delivery to the endosomal-lysosomal system. LDL is delivered to the lysosome where its lipids are hydrolyzed by acid lipases within the lysosome. Depending on the levels of intracellular cholesterol, LDL-R can escape the lysosome and be recycled to the PM for continual uptake of LDL or, in cases of high cholesterol, the LDL-R can be

delivered to the lysosome for degradation (82). Similarly, LDL-R expression is sensitive to intracellular cholesterol levels, whereby negative feedback mechanisms can downregulate LDL-R expression in conditions of high intracellular cholesterol (83). Macrophages, however, possess alternate receptors for binding and internalizing lipoproteins (84-87). Within the atherosclerotic lesion, macrophages are exposed to a large quantity of modified LDL, including oxidized LDL (oxLDL) and aggregated LDL (aggLDL). The uptake of modified LDL occurs through the activity of macrophage scavenger receptors, including CD36 and scavenger-receptor A (SR-A) (**figure 4**). In contrast to LDL-R, uptake of modified lipoproteins via these alternate receptors is unregulated, resulting in the continual uptake of lipoproteins, even under conditions of high intracellular cholesterol. Accordingly, scavenger receptor-mediated uptake of lipoproteins is the most important lipid uptake pathway contributing to foam cell formation (88).

Intracellular Cholesterol Storage

Excess cellular cholesterol is cytotoxic and, when intracellular cholesterol levels are high, the cell must find a way to safely store or process the excess sterol. Cells do not have the capacity to degrade the complex cholesterol structure. However, the cells have developed a number of mechanisms to store and efflux cholesterol. Cholesterol is an important component of biological membranes and, when intracellular cholesterol levels are high, a significant amount of cholesterol can be incorporated into the plasma and other organelle membranes. However, in foam cells the capacity of membranes to

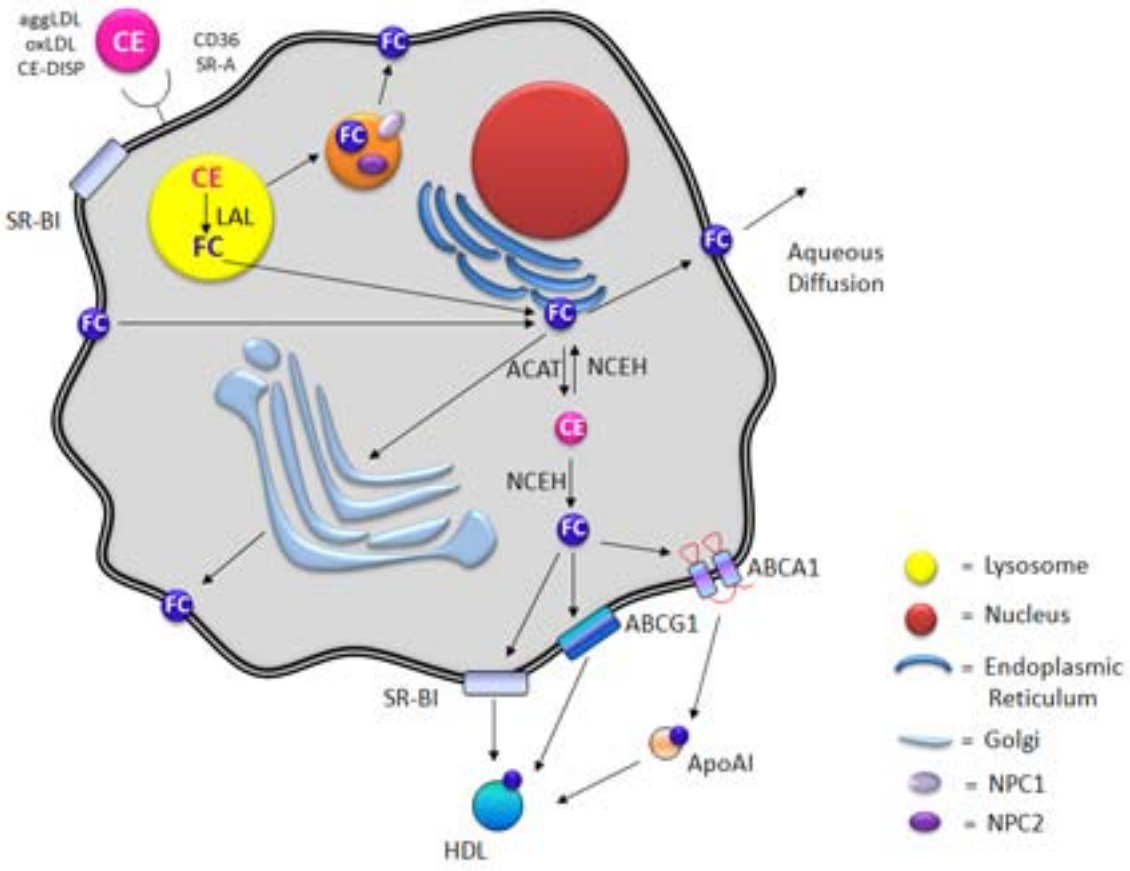

#ggLDL
oxLDL
CE-DISP
CE
CD36
SR-A
FC
CE
LAL
FC
SR-BI
Aqueous
Diffusion
ACAT
NCEH
CE
NCEH
ABCA1
ABCG1
SR-BI
ApoAI
HDL
= Lysosome
= Nucleus
= Endoplasmic Reticulum
= Golgi
= NPC1
= NPC2

Figure 4: Macrophage cholesterol homeostasis is maintained by a number of highly regulated mechanisms. Cholesterol homeostasis involves the balance of cholesterol biosynthesis, cholesterol uptake, intracellular sterol storage, and cholesterol efflux. The key mediators of these pathways are identified in this diagram. Modified cholesterol-rich lipoproteins enter the cell through scavenger receptors such as CD36 and scavenger receptor-A (SR-A). Lipoproteins are delivered to the lysosome where they are degraded by lysosomal proteases and lipases. Importantly, the cholesterol ester (CE) component is hydrolyzed by lysosomal acid lipase (LAL) to generate free cholesterol (FC) and a fatty acid (FA). The FC can be transported to the plasma membrane for cholesterol efflux in a process mediated by the lipid transporters NPC1 and NPC2 and/or non-specific vesicular transport. Alternatively, when cells are exposed to large quantities of cholesterol, excess FC is delievered to the endoplasmic reticulum where it can be esterified to a FA in a process mediated by the enzyme ACAT. Following esterification, the CE can be stored in cytoplasmic droplets. If the cell requires additional cholesterol, or if cholesterol efflux processes are activated, the cytoplasmic CE can be hydrolyzed by neutral CE hydrolase (NCEH). FC released from lysosomes or from cytoplasmic CE hydrolysis can be effluxed from the macrophage to cholesterol acceptors (including HDL and apoAI) through the activity of lipid transporters ABCA1, ABCG1, SR-BI and/or through passive diffusion.

incorporate cholesterol is exceeded and ACAT enzyme activity is activated (89-92). ACAT catalyzes the esterification of FC to FA generating CE, which can be stored safely in the cytoplasm as lipid droplets (69,93). Concomitant with cholesterol esterification and incorporation of CE into cytoplasmic droplets, another enzyme, neutral cholesteryl ester hydrolase (NCEH), can hydrolyze CE back to FC (74,94). This hydrolysis can be stimulated further when FC levels drop, such as when cells are exposed to promoters of cholesterol efflux (**figure 4**)(95). The FC generated by NCEH supplies the cell with a source of cholesterol for use in cellular processes, including membrane synthesis. In the plasma membrane, FC can be effluxed to extracellular acceptors.

Macrophage Cholesterol Efflux

In addition to storing excess cholesterol within the cell, in the presence of high intracellular cholesterol, macrophages are able to efflux excess FC. Macrophage cholesterol efflux can occur through four major pathways, including aqueous diffusion, SR-BI mediated efflux, ABC transporter mediated efflux, and apoE mediated efflux (**figure 4**).

Passive Cholesterol Efflux Mechanisms

Aqueous diffusion is a passive process in which cholesterol spontaneously desorbs from the PM and enters the aqueous phase where the FC can collide with and be absorbed by HDL acceptor particles (72,96,97). Importantly, aqueous diffusion is driven by the cholesterol concentration gradient, meaning that the direction and

transport of cholesterol must occur from areas of high FC concentration to an acceptor with low FC concentration (72,97). Thus, aqueous diffusion is an inefficient, passive cholesterol efflux mechanism, which occurs on the time scale of hours.

Active Cholesterol Efflux Mechanisms

Scavenger-receptor class BI (SR-BI) is responsible for the bidirectional, active transport of cholesterol across the PM. Similar to aqueous diffusion, the net movement of cholesterol via SR-BI is dependent on the cellular cholesterol concentration gradient (98-100). If intracellular levels of cholesterol are low, SR-BI can bind to oxLDL and mediate its influx (72,101-103). In contrast, when intracellular cholesterol levels are high SR-BI can efflux FC to HDL (72,98-100,102,104).

Cholesterol efflux can also occur through the function of ATP-binding cassette transporter AI (ABCA1) or ATP-binding cassette transporter G1 (ABCG1). ABCA1 and ABCG1 are ATPases that hydrolyze ATP to generate energy for the unidirectional transport of lipids to extracellular acceptors. ABCA1 promotes the efflux of FC and phospholipids (PLs) to lipid-poor amphipathic helical proteins such as apolipoprotein AI (apoAI) and apolipoprotein E (apoE). Absorption of PL and FC by apoAI is thought to result in the formation of nascent HDL particles (105,106). ABCG1 promotes the efflux of FC to nascent HDL particles, such as those generated by ABCA1 mediated efflux (107). Thus, it is possible that ABCA1 and ABCG1 can act sequentially, and together they mediate the efflux of 60-70% of FC movement from the cell (108,109).

Apolipoprotein E is a multifunctional protein that is synthesized by many peripheral tissues including macrophages. ApoE can act as a cholesterol acceptor that can mediate efflux even in the absence of additional cholesterol acceptors (110). Addition of exogenous apoE can stimulate cholesterol efflux (111,112). Similarly, endogenous expression and secretion of apoE is known to enhance cholesterol efflux (113,114). Although the exact mechanism by which endogenous apoE enhances efflux is unclear, we know that following secretion from the macrophage, apoE is bound by heparin sulfate proteoglycans (HSPGs) on the cell surface, which tethers the molecule close to the PM. Thus, the retention of apoE in the proximity of the PM can facilitate the diffusion of cholesterol to lipid-poor apoE, which may involve the aforementioned cholesterol transporters ABCA1 and/or SR-BI (115,116).

Cholesterol Efflux from Macrophage Foam Cells is Reduced

The efflux of cholesterol via all known mechanisms is significant in reverse cholesterol transport (RCT). Reduced cholesterol efflux from macrophage foam cells is observed both *in vivo* and *in vitro* (117,118). Mobilization of macrophage cholesterol has previously been shown to reduce lesion area and enhance lesion stability (50). Therefore, defining mechanisms to enhance macrophage cholesterol efflux is critical for lesion regression.

Lysosomal Lipoprotein Processing and Macrophage Foam Cell Cholesterol Homeostasis

A critical first step in the uptake of lipoproteins and their delivery to lysosomes/late endosomes for subsequent processing is receptor-mediated endocytosis. The lysosome is an organelle containing acid hydrolases, important digestive enzymes that degrade a variety of endocytosed material. VLDL, internalized through LRP, and modified LDL, internalized through scavenger receptors, are initially delivered to lysosomes for degradation and processing. Lipoprotein lipids, including both TG and CE, are degraded by LAL in the lysosome. LAL hydrolysis of lipoprotein lipids is significant because the clearance of sterol from the lysosomes requires hydrolysis of CE. FC formed from hydrolysis can be transported out of the lysosome through a number of mechanisms (**figure 4**). While the transport process is ill-defined, it involves both vesicular and non-vesicular pathways and key proteins such as NPC1 and NPC2 (119-122). Importantly, once liberated from the lysosome, sterol is available for utilization as a substrate for cellular processes and signaling pathways, including cholesterol efflux pathways (**figure 4**). Therefore, lysosomal processing of endocytosed lipoproteins is the significant first step for the cell to utilize lipoprotein cholesterol.

Deficiencient Lysosomal Lipoprotein Processing Contributes to Atherosclerosis

During early atherosclerotic lesion development, CE accumulates mainly in cytoplasmic droplets in macrophages (123). Cytoplasmic CE is formed from the ACAT reesterification of FC released from the lysosome following lipoprotein lipid lipolysis. However, as lesions progress to more advanced stages, substantial accumulation of lipid

occurs in foam cell lysosomes (123-125) suggesting an interruption in lysosomal processing and excretion of cholesterol. Studies of foam cells in culture have also demonstrated lysosomal cholesterol accumulation (9-11,126-132). These studies show that the buildup of CE within lysosomes is not the result of defective or insufficient lipase enzyme; however, the actual mechanism remains unclear (126,133). Our work has shown that cholesterol accumulation in lysosomes can inhibit CE hydrolysis (9-11,13). Since CE cannot be cleared, it is trapped in the lysosome.

Multiple studies indicate that lysosomal sequestration of cholesterol can have negative consequences in atherosclerosis (1-8). In addition to a direct effect of accumulating sterol on lysosome function, sterol in lysosomes is trapped and unavailable for participation in other cellular processes (13). Not only can this trapping prevent removal of cholesterol by efflux, but it can also restrict cholesterol movement into alternative metabolic pools that act to signal specific changes in the macrophage. Among these are activation of apoptosis pathways and processing of cholesterol to oxysterols, which are LXR agonists (73,75-78). LXR activation influences a number of lipid homeostatic pathways including cholesterol clearance. Sterol-induced signaling pathways also influence cell viability (73). Thus, the foam cell is a major regulator of plaque development and rupture (46,79). For this reason, understanding the influences on cholesterol metabolism in foam cells is a major goal of atherosclerosis research.

Foam Cell Lysosomes and pH

A number of modified CE-containing particles can induce lysosomal cholesterol accumulation in cultured macrophages (134), including oxLDL, aggLDL, and CE-rich phospholipid dispersions (CE-DISP). Previous studies indicate that, initially, the macrophage can metabolize these particles, but the FC generated from hydrolysis is not efficiently removed from lysosomes. As FC accumulates, CE hydrolysis is inhibited and CE accumulates in lysosomes. Our preliminary evidence indicates that the inhibition of hydrolysis is due, at least in part, to a failure of lysosomes to maintain an acidic pH, perhaps related to the FC accumulation. LAL, the enzyme responsible for lysosomal CE hydrolysis, functions in a very narrow pH range with very little activity above pH 4.5 (135). The optimal pH is established by unique electrogenic pumps called v-ATPases which translocate protons into the lysosome. Lysosomal pH is also determined by the leakiness of the lysosomal membrane (136). A number of factors, including sterols, can affect membrane permeability. FC can stabilize membranes and decrease leakiness, while oxysterols can increase leakiness, thereby affecting pH (71,137). Increased FC content in membranes can also inhibit v-ATPase activity, which potentiates changes in pH (138). Thus, the FC content of lysosomal membranes is a potential mediator of lysosome function.

Importance of Triglycerides in Diseases of Cholesterol Metabolism

Atherosclerosis studies have generally focused on cholesterol, because it is the principal lipid accumulating within the atherosclerotic lesion (139). However, TRPs have

the potential to enter the arterial wall and be incorporated within atherosclerotic plaques (140-145). TG is transported in the bloodstream as a component of TRPs, including CMs and VLDL and their corresponding remnants. TRPs can be internalized by macrophages via interaction of apoE found on their surface with a number of receptors, including LDL-receptor related protein (LRP), the VLDL receptor, and/or HSPGs (146-153). The presence of TRPs in the lesion, as well as the ability of macrophages to internalize and catabolize TRPs, suggests that TGs could influence foam cell lipid metabolism.

The role of TG in atherosclerosis is widely debated and highly controversial. Some studies suggest that elevated circulating TG levels are associated with increased atherosclerotic risk, while others show a benefit of circulating TG levels on cardiovascular disease. Univariate analysis of case-controlled and prospective cohort studies shows a positive correlation between TG levels and coronary artery disease (CAD) risk (154-158). Specifically, high circulating levels of certain TRPs, including small VLDLs and IDL, have been independently linked with the incidence, severity and progression of atherosclerosis (159,160). Small VLDLs and IDL are hypothesized to contribute to lesion progression, plaque rupture, and clinical coronary events through indeterminate mechanisms (158).

One potential mechanism through which elevated circulating levels of VLDL could contribute to atherosclerosis is through their capacity to increase arterial inflammation (161,162). Previous studies show that exposure of vascular endothelial cells to VLDL increases the expression of nuclear factor-kappa B (NF-κB), a critical

transcriptional regulator of inflammation (162,163). This results in the upregulation of proinflammatory proteins such as vascular adhesion molecules and chemokines, which enhance the recruitment of monocytes to the lesion (162,163). Additionally, VLDL can activate proinflammatory gene expression in lesional macrophages, including upregulation of tumor necrosis factor-α (TNF-α), interleukin-1β, ICAM-1, matrix metalloproteinase 3, MCP-1 and macrophage inflammatory protein-1α (MIP-1α) (161,164-166). Thus, previous studies establish a relationship between TRPs and atherogenesis and suggest that increased circulating TRPs could increase CAD risk.

Despite evidence establishing a prospective relationship between elevated circulating TG and increased CAD risk, a number of studies suggest that TG levels do not significantly impact the development of CAD. Specifically, multivariate analyses of epidemiologic data considering other lipid risk factors, such as levels of circulating HDL and small, dense LDLs, do not show a significant relationship between TG levels and risk (154-158,167). The majority of studies that find a correlation between elevated serum TG levels and CAD risk show that the influence of TG is minor compared to the effect of HDL-cholesterol even when serum HDL is included as a covariate (157). Thus, the results of multivariate analyses suggest that TG may not be considered a significant risk factor for CAD (168,169). Additionaly, analysis of the relationship between circulating TG levels and CAD risk is very complex given that TGs are carried in all circulating lipoproteins and present a different risk profile in the postprandial versus fasting state (156,158). Consequently, the relationship between circulating TG levels and atherosclerosis remains ambiguous.

Implications of Triglyceride in Macrophage Cholesterol Metabolism

Primary cultures of human monocyte-derived macrophages, as well as *ex vivo* human foam cell macrophages, contain TGs (144,170-172). Further, TGs are more metabolically active than CE and represent a dynamic lipid pool (118). Thus, the size and metabolic activity of intracellular TG pools may be an important component of macrophage lipid metabolism. Therefore, defining the interaction between TG and intracellular cholesterol pools is critical for understanding the role of TRPs in atherogenesis. Unfortunately, this is an understudied area of atherosclerosis research.

We do know that macrophages metabolize TG from TRP to glycerol and FA through surface hydrolysis and/or internalization and lysosomal hydrolysis (173,174). It is not clear (and somewhat controversial) what the relative importance of each is in determining intracellular TG and FA levels, and the contribution of each may change with TRP composition (173,175-177). However, FAs produced by both pathways are transported to the cytoplasm and can be reformed into cytoplasmic TG and stored within the same cytoplasmic droplets that store CE.

Cellular TG can influence intracellular cholesterol homeostasis. Macrophage lysosomes hydrolyze CE when it is introduced as mixed CE and TG particles compared to CE without TG (128). Studies indicate that this is because TG can alter the physical state of CE keeping it more fluid (128). This physical state effect is not limited to lysosomal hydrolysis. Association of TG with CE in cytoplasmic CE droplets makes the CE more susceptible to hydrolysis by NCEH (178,179). Since hydrolysis of CE is the first step in their mobilization, TG may well enhance cellular cholesterol removal (179).

FAs, hydrolytically released from TG, are also influential in cholesterol homeostasis. FAs are key signaling molecules, which greatly affect the expression of critical genes controlling cellular cholesterol mobilization. FAs can act at the level of nuclear receptors to affect the transcription of a number of genes important in cholesterol homeostasis. For example, the individual or cooperative upregulation of peroxisome-proliferator activated receptor (PPAR) and LXR expression by FA has been shown to regulate expression of a number of cholesterol homeostatic genes including the ATP-binding cassette gene family members A1 and G1 (180), which enhance cholesterol movement and efflux. FA can also influence the synthesis and secretion of apoE from macrophages, providing another mechanism through which FAs influence cholesterol efflux (181). Therefore, TRPs and/or their hydrolytic byproducts have the ability to enhance reverse cholesterol transport from macrophage foam cells, potentially resulting in atherosclerotic lesion regression.

There is an apparent paradox between epidemiologic studies suggesting that hypertriglyceridemia (HTG) may increase atherosclerosis and the cellular studies indicating a role for TG in cholesterol clearance. However, the epidemiologic evidence is controversial and does not define whether HTG has a direct or indirect effect on coronary disease (173). Patients with high TG invariably have other major risk factors for cardiovascular disease, including obesity, diabetes, and/or high blood pressure. Additionally, HTG is associated with low HDL cholesterol. Thus, TG cannot be conclusively identified as an independent risk factor for atherosclerosis. A better understanding of how HTG directly affects cells in the artery wall is required to resolve

this paradox. An important component of this effort is defining the role of TRP as a modulator of cholesterol metabolism and foam cell biology.

Significance

Atherosclerosis is characterized by progressive thickening of the artery wall due, in large part, to the presence of lipid-engorged macrophage foam cells. In late stage disease, foam cell cholesterol is located within large, swollen lysosomes. Importantly, lysosomal cholesterol is trapped and unavailable to pathways of cellular cholesterol efflux. Additionally, the massive cholesterol accumulation within lysosomes renders them inactive by inhibiting the activity of the v-ATPase, which is responsible for maintaining an acidic pH. The majority of studies examining the relationship between lipids and atherosclerotic foam cell formation have focused almost exclusively on the influence of cholesterol-rich lipoproteins. While cholesterol is clearly important in atherogenesis, there are a number of lipids present within the complex atherosclerotic milieu that can influence foam cell lipid homeostasis. Specifically, TRPs are abundant within the artery wall and have the potential to substantially influence atherosclerotic lesion development by modulating foam cell lipid metabolism. A limited number of preliminary studies suggest that TG could potentially influence foam cell cholesterol homeostasis by manipulating lysosomal CE hydrolysis, intracellular trafficking of cholesterol, storage of cholesterol within CE droplets, and cholesterol efflux. The goal of this dissertation research was to define the mechanism(s) by which TG modulates lysosomal and cellular cholesterol clearance from macrophage foam cells.

CHAPTER III

EFFECT OF TRIGLYCERIDE ON LYSOSOMAL CHOLESTEROL METABOLISM IN THE MACROPHAGE FOAM CELL

Introduction

Atherosclerosis is a complex, multi-factorial disease. An early event in atherosclerosis is the development of macrophage foam cells. These are primarily formed through the uptake of modified lipoproteins by macrophages within the artery wall (69,182-184). In late stage atherosclerotic lesions, a large amount of both FC and CE accumulates in foam cell lysosomes. The presence of extensive amounts of CE suggests an interruption in the normal lysosomal hydrolysis of lipid particle-derived CE while the accumulation of FC indicates an interruption in the normal removal of FC from lysosomes to other organelles, primarily the plasma membrane (123-125). Many studies indicate that lysosomal sequestration of cholesterol can have consequences for atherosclerotic lesion development (1-8). In addition to a direct effect of accumulating sterol on lysosome function, trapping of sterol in lysosomes can prevent removal of cholesterol by efflux. In fact, the FC and CE in lysosomes remains trapped in lysosomes, even when further uptake of lipoproteins is halted and acceptor concentrations in the media are increased to levels that remove most of the non-lysosomal CE stores (13). Thus the sequestration of sterol within lysosomes prevents efflux and limits the availability of cholesterol to other intracellular processes (13). With this in mind, factors

that influence the removal of lysosomally sequestered sterol could have profound effects on foam cell biology and atherosclerotic lesion development.

Many studies have examined macrophage foam cell metabolism in the presence of various CE-containing particles (182-184). However, TRPs, including VLDL, are also present within the atherosclerotic lesion and could have an impact on foam cell cholesterol metabolism (145-149). Surprisingly, the influence of TRP on metabolism of CEs has not been extensively studied. Lesion macrophages, primary cultures of human monocyte-derived macrophages, and *ex vivo* human foam cell macrophages, contain TG (144,170-172). Moreover, TGs are rapidly turned over within macrophages and represent a dynamic lipid pool with the potential to influence cellular CE metabolism. Thus, defining the interaction between TG and intracellular cholesterol pools is critical for a full understanding of atherogenesis.

A key observation linking TRP to CE metabolism is that macrophages hydrolyze CE more efficiently when it is introduced into lysosomes as a mixed CE and TG particle, compared to CE-containing particles alone (128). Additionally, it has been shown that TG can alter the physical state of CE by keeping it more fluid and accessible (128). This is consistent with studies of cytoplasmic CE droplet metabolism, which demonstrate increased activity of lipolytic enzymes in the presence of mixed CE and TG droplets compared to CE alone (178,179). Furthermore, FAs generated by lysosomal or extra-lysosomal hydrolysis of TG are known ligands for, and can upregulate, cholesterol homeostatic genes, including LXR and PPAR (180,185-192). Therefore, it is clear that increased levels of TG in cells have the potential to affect macrophage CE metabolism.

In the current study, we investigate the potential for TG to reestablish lysosomal CE hydrolysis and to enhance the mobilization of the resulting FC out of lysosomes. Since CE cannot be cleared from lysosomes, lysosomal CE hydrolysis is the mandatory first step in cellular metabolism of lipoprotein-derived cholesterol and cellular cholesterol utilization or efflux. In this chapter, we show that treatment of macrophages with TRP before, during, or after cholesterol accumulation reduced both lysosomal FC and CE stores and promoted the eventual efflux of sterol from the cells. The alterations in lysosomal CE metabolism occurred, at least in part, through the ability of TGs to maintain normal lysosomal activity. Thus, we conclude that modulation of lysosomal CE metabolism, through alterations of cellular TG levels, has profound influences on the ability of foam cells to clear cholesterol. TRP flux through the atherosclerotic lesion and our studies indicate that uptake of these particles by macrophage foam cells can influence the ability of foam cells to metabolize the extensive lysosomal CE stores found in late stage lesions.

Materials and Methods

Materials

THP-1 human monocytes/macrophages were purchased from ATCC (Manassas, VA). Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), cholesteryl oleate, trioleate, and cholesteryl methyl ether (CME) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). Fetal bovine serum (FBS) was

obtained from Atlanta Biologicals (Norcross, GA), and RPMI, L-glutamine, Eagle's vitamins, streptomycin, and penicillin were purchased from Mediatech (Herndon, VA). All tissue culture plasticware was purchased from Corning (Corning, NY). All other chemical reagents and chemical solvents were obtained from VWR (West Chester, PA).

Lipoprotein isolation and aggregation

Human LDL was isolated from plasma collected from fasted, normocholesterolemic human volunteers who had provided informed consent. Collection of blood followed procedures approved by the Human Subjects Institutional Review Board. LDL (1.006 g/ml < d < 1.063 g/ml) and VLDL (d < 1.006 g/ml) were isolated by sequential ultracentrifugation (13). LDL and VLDL were dialyzed against 0.9% NaCl containing EDTA (0.3 mmol/l) for 72 h, filter-sterilized through a Millipore filter (0.45 µm), and stored under nitrogen at 4°C. Isolated LDL was aggregated by vortex (1 min) followed by sonication with a Branson sonifier (10 min, 50% duty cycle) on ice to break up large aggregates. The resultant aggregates were passed through a 0.45 µm filter, to produce small (~30–75 nm) aggregates that induce maximal uptake and lysosomal delivery. Aggregation of LDL and size were confirmed by negative staining with 2% phosphotungstic acid. Measurement of thiobarbituric acid-reactive substances (TBARS) and conjugated diene levels confirmed the absence of oxidation after the aggregation procedure (193,194).

Preparation of Lipid Dispersions

CE-rich or TG-rich lipid dispersions (DISP) were prepared under sterile conditions, as described by Mahlberg *et al.* (128). Briefly, phosphatidylcholine (1 mg), and phosphatidylserine (0.1 mg) and either cholesteryl oleate (30 mg, anisotropic) or triolein (10.35 mg, isotropic) were combined in a sterile 50 ml Corex glass tube and dried under nitrogen. RPMI medium (17 ml) supplemented with HEPES (12.5 mM) was then added and the suspension was heated in an 80°C water bath for 20 min to melt the dried lipids. The solution was sonicated for 20 min using a Branson sonifier (50% duty cycle). TBARS levels showed no oxidation after sonication of DISP. Dispersion size was confirmed by negative staining electron microscopy (EM) with 2% phosphotungstic acid (**figure 5**).

Cell culture of THP-1 macrophages

THP-1 macrophages were plated onto 35 mm wells or coverslips at a density of 1.5 x 10^6 cells and incubated for 3–4 days at 37°C in RPMI containing 10% FBS and 50 ng/ml phorbol ester (TPA), to allow for differentiation into macrophages. Culture media for all incubations was supplemented with HEPES (20 mmol/l), Eagle's vitamins, L-glutamine (200 mmol/l), streptomycin (100 µg/ml), penicillin (100 IU/ml), and ß-mercaptoethanol (0.008 µl/ml). TPA was included in the incubation medium throughout the duration of the experiments. Macrophages were incubated with medium containing 1% fatty acid-free BSA for 24 h before cholesterol loading to minimize excess TG in cells prior to lipid loading.

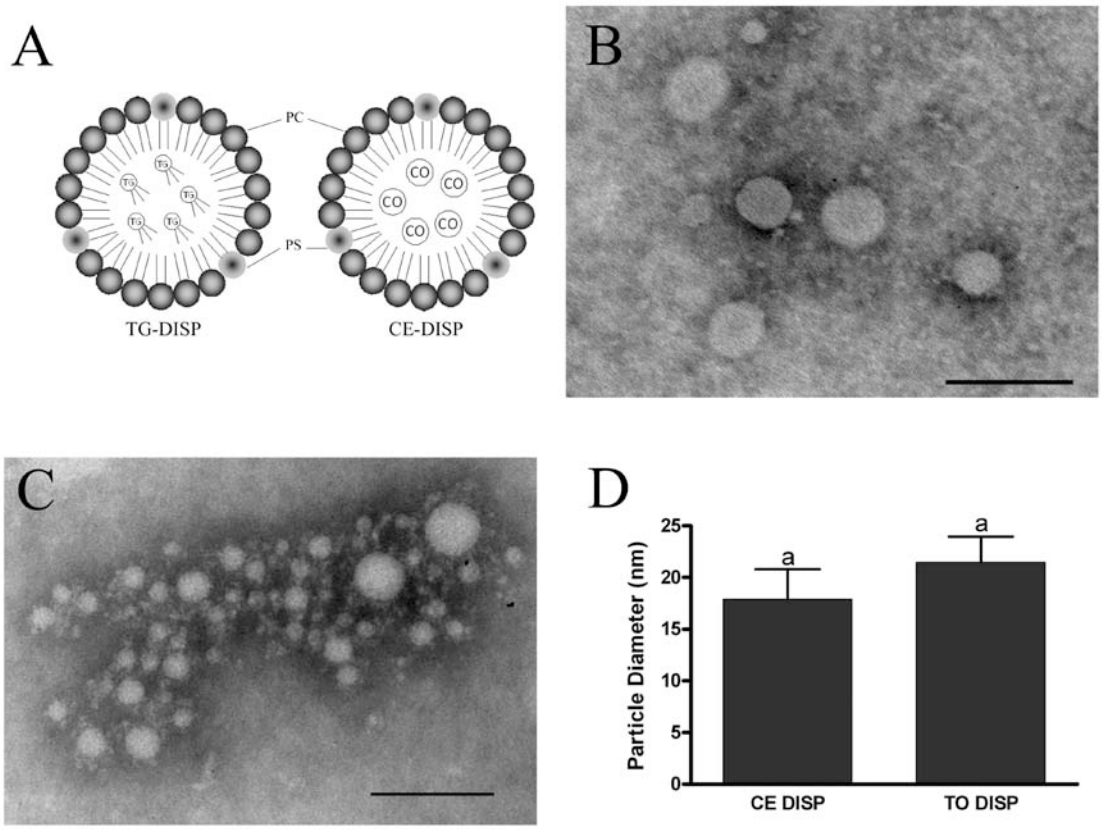


Figure 5: Characterization of lipid dispersions reveals similar sized particles in cholesterol-enriched (CE DISP) or triglyceride-rich (TG DISP) lipid dispersions. A: Diagram of the lipid dispersions. DISPs contain a phospholipid membrane monolayer, that is primarily composed of phosphatidylcholine (PC) and phosphatidylserine (PS) in a 10:1 ratio. TG-DISPs have a TG-enriched neutral lipid core, while CE-DISPs have a CE-enriched neutral lipid core. B-D: Lipid dispersions containing either CE (B) or TG (C) are uniform in size with CE-DISP ranging from 9 – 38 nm (average diameter of 17.8 nm) and TG DISP ranging from 9 – 36 nm (average diameter of 21.5 nm)(panel D). Values are the mean ± SEM for three experiments. Within each panel, bars with the same letter (a) indicate that means were not statistically different.

Lipid loading and analysis

To measure lipid loading, macrophages were incubated for 0–6 days at 37°C in culture medium containing 1% FBS with or without lipid particles, including aggLDL, VLDL, CE-rich DISP, or TG-rich DISP. In some experiments, only one type particle was employed, while in other experiments both CE-rich particles (aggLDL or CE-DISP) and TG-rich particles (VLDL or TG-DISP) were used either simultaneously or sequentially. In sequential treatment during pulse-chase experiments, the pulse media was removed after three days of treatment and cells were washed briefly with 1% FBS prior to addition of the chase media for a further three days. Concentrations and specifics of incubation order are described for each experiment. The lipid loading medium was changed every 3–4 days to fresh medium containing the cholesterol or TG loading vehicle.

Loading with aggLDL or VLDL was done using standard culture techniques. In contrast, experiments using DISP were conducted using an inverted culture technique that has been previously described (128). This method maximizes contact of cells with dispersions and the subsequent internalization of the particles (128). For the inverted technique, cells were first plated on glass coverslips on the bottom of 35 mm dishes. After adherence and differentiation, the coverslips were inverted and placed on sterile rings. Loading medium was then added so that the coverslip was submerged and the floating DISP came into contact with the adherent cells.

For quantification, cellular lipids were extracted from cells with 2 ml isopropanol containing 5–10 µg of CME as an internal standard. Lysosomal lipids were extracted

from isolated lysosomes using the method of Bligh and Dyer with 5 μg of CME as an internal standard (195). The cholesterol content of the lipid extracts were quantified by gas-liquid chromatography according to the procedure of Ishikawa et al. (196) as modified by Klansek *et al.* (197). TG content was quantified using a GPO Trinder assay kit from Raichem, according to the manufacturer's instructions. Cellular proteins and proteins in isolated lysosomes were solublized in 1 N NaOH overnight, and protein content was measured using the method of Lowry *et al*. (198). Cellular lipid values are reported as μg cholesterol or TG normalized to mg cell protein while isolated lysosomal lipid values are reported as μg cholesterol or TG normalized to mg lysosomal protein.

Cell viability during loading was assessed by counts of cell number and by protein levels. Experiments were performed in triplicate to assess experimental variability. The mean value for the three measures was used as the value for that experiment for subsequent statistical analysis of multiple experiments.

Microscopy

After lipid loading, microscopy was used to examine the subcellular localization of the accumulated lipids as well as to analyze changes in lysosomal environment. LysoSensor Yellow/Blue DND-160 staining (Molecular Probes, Eugene, OR) was used to determine changes in lysosomal pH (10,199). This dye fluoresces yellow in an acidic environment but the fluorescence wavelength shifts towards blue as the environment becomes more alkaline. For staining, cells were washed two times in PBS, and the dye was added to cells at a concentration of 5 μM in medium containing 1% FBS. All images

were collected within 10 min after the placement of dye on the cells to avoid artifacts produced by the alkaline properties of the dye. As a positive control, macrophages in which active lysosomes were increased by incubation of macrophage with polystyrene beads rather than lipoproteins were stained with LysoSensor Yellow/Blue DND-160. Images were collected using a Zeiss Axioplan Imaging E fluorescence/brightfield microscope (Zeiss, Germany) equipped with a Photometrics Coolsnap HQ digital camera with a cooled CCD chip (Roper Scientific, Tucson, AZ). Image analysis was conducted using MetaMorph imaging software (Universal Imaging, Downingtown, PA). To quantify changes in the number of active lysosomes, a grid of points was superimposed over each image. This provided an unbiased selection of vesicles to evaluate. As previously published, vesicles with a pH less than 4.8 were classified as active while those with a pH above 4.8 were considered inactive vesicles (10). A pH of 4.8 was chosen because LAL should have no activity above a pH of 4.8 and this value is above the pKa of LysoSensor such that there is a significant blue shift in the fluorescence of the probe. The vesicles in at least 20 cells per condition from three separate experiments were counted.

Negative stain EM was used to determine the ultrastructural characteristics of the isolated lysosomes and to analyze changes in average lysosome diameter with the various treatments. Lysosomal isolates were absorbed to Formvar-caoted grids for 30 seconds and then negatively stained for 20 seconds with 2% phosphotungstic acid, pH 7.0. Digital images were collected using a FEI CM-12 electron microscope operated at 80 keV accelerating voltage and equipped with an AMT cooled CCD camera. To determine the average lysosome diameter, a grid of points was superimposed over the images to

select, in an unbiased fashion, the lysosomes for quantification. The diameter of each selected lysosome was computed as the distance between the two most distant points on the lysosome periphery. At least 100 diameters were computed for each condition.

EM was also used to assess the distribution of lipid in foam cells between lysosomes and cytoplasmic droplets. Lysosomes and related organelles were identified by the presence of acid phosphatase using a modification of the Gomori lead precipitation method (200,201). Beta-glycerol phosphate was used as a substrate, and the reaction control was incubated in identical medium without the enzymatic substrate. After incubation, cells were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Before further staining, the sections were viewed to verify the enzymatic reaction and then counter-stained with uranyl acetate.

Lysosomal isolation and modification

THP-1 macrophages were treated with various lipid particles, as described above, and then isolated as described previously (10). Briefly, the cells were rinsed in cold STE buffer (0.25 M sucrose, 0.01 M Tris-HCl, 1 mM EDTA, and 0.1% ethanol) and scraped into 1 ml/dish of STE buffer containing protease inhibitors (Sigma). The cell suspension was placed in a cell disruption chamber (Kontes) and disrupted using three passes of 20 min each at 150 p.s.i. This method consistently resulted in disruption of > 95% of cells but left lysosomes intact. The suspension was centrifuged at 1500 RPM to separate the post-nuclear supernatant (PNS) from the nuclear pellet. The PNS density was raised to 1.15 g/ml through the addition of sucrose and then applied to a sucrose

density gradient ranging from 1.28 g/ml to 1.00 g/ml. The gradient was centrifuged at 19,400 RPM for 4 hours at 4 °C to separate lysosomal fractions based on their buoyant density. Morphological analysis of the fractions by negative stain electron microscopy (as described above) revealed the presence of a reasonably pure lysosomal population with no apparent structural features of other organelles, including Golgi, rough endoplasmic reticulum, and mitochondria. The purity of the lysosomal populations was assessed further by western blotting for markers of cellular organelles that might share common morphology to lipid-engorged lysosomes, including early endosomes (EEA1) and lipid droplet associated proteins (perilipin A). Fractions were negative for EEA1 and perilipin and were positive for the lysosomal marker (LAMP-1, **figure 6**). Lysosomal recovery was verified using western blotting of both the lysosome isolate and the nuclear pellet for LAMP-1 in comparison with whole cell extracts. LAMP-1 was not detected in the nuclear pellet. The isolated lysosomes were resuspended in buffer containing 150 mM KCl to generate high K^+ levels inside the lysosome, which provided a membrane potential during the stimulation of the v-ATPase with ATP. Aliquots were obtained for cholesterol, TG and protein analyses, as described above.

Vacuolar-type ATPase Activity

Measurement of lysosomal v-ATPase activity was carried out using a modification of a procedure described previously (10,138). Briefly, isolated lysosomes were placed in a cuvette containing activation buffer and 6.7 μM acridine orange. After achieving a steady spectrophotometric baseline, v-ATPases were primed with $MgCl_2$.

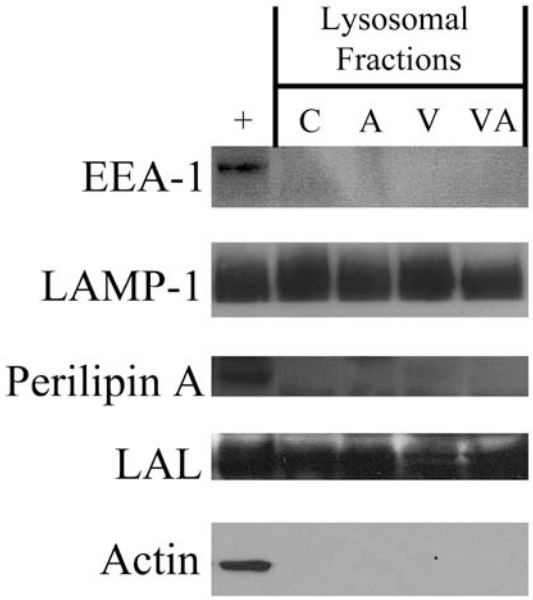


Figure 6: Western blot analysis of lysosomal, endosomal and lipid droplet markers. Analysis of isolated lysosomes showed the absence of markers of early endosomes (EEA-1) and cytoplasmic lipid droplets (perilipin A) and the presence of the lysosomal markers LAMP-1 and LAL. Cells were treated with 100 μg aggLDL protein/ml and/or 100 μg VLDL protein/ml for six days and lysosomes were isolated according to the method described in the Experimental Procedures. Fifteen micrograms of protein was loaded in each lane. Whole control cell fraction was utilized as a positive control (+) for antibody staining. C = Control, V = VLDL-treated, A = aggLDL-treated, VA = VLDL + aggLDL coincubation.

After approximately 60 seconds to allow the baseline to be re-established, v-ATPases were activated by the addition of ATP (1.4 μM final concentration) and valinomycin (to promote the movement of K^+ from inside to out for membrane potential generation). v-ATPase-driven pumping of hydrogen ions into the lysosome lumen, as measured by the quenching of acridine orange fluorescence when excited at 495 nm and recorded at 530 nm, was determined using an SLM Aminco 8100 dual-wavelength spectrophotometer. As a control, lysosomes were activated in the absence of the requisite membrane potential by including valinomycin in the medium.

Statistics

For most analyses, the experiments were repeated at least three separate times. For replicate experiments, a mean, standard deviation, and standard error of the mean were determined. The value used for each separate experiment was the mean value determined from triplicate measures. Two group comparisons used Student's *t* test. For multiple comparisons, following an analysis of variance, group comparisons were performed with the multiple comparison HSD method of Tukey (202). The criterion for significance was set at $P < 0.05$ for a type I error.

Results

TRPs could potentially influence the foam cell metabolism of cholesterol. This is particularly true of the CEs derived from the uptake of CE-rich particles. These CE-containing particles must first be processed within lysosomes. We have previously

shown that that this CE hydrolysis is inhibited in heavily cholesterol-laden foam cells primarily due to the accumulation of FC within the lysosome lumen and lysosome membrane (9,10). The lysosomally sequestered FC and CE are trapped and cannot be removed from the lysosome even under conditions that promote the removal of non-lysosomal cholesterol from membranes and intracellular droplets (13). To determine if TRPs affect the lysosomal metabolism of CE, cellular lipid levels were measured in THP-1 macrophages treated with VLDL and aggLDL at the same time. As controls, macrophages were incubated with aggLDL or VLDL alone. Consistent with what we have shown previously, incubation of THP-1 human macrophages with 100 μg aggLDL protein/ml produced a dramatic accumulation of both FC and CE (**figure 7A**). As in previous studies (9), thin section EM of acid phosphatase stained samples showed that >75% of the lipid volume was within lipid-engorged lysosomes. In contrast, co-incubation of THP-1 with both aggLDL and VLDL resulted in a significant ($p<0.05$) reduction in the accumulation of CE (**figure 7A**). This correlated with a significant increase in cellular TG (**figure 7B**). When cells were incubated with aggLDL alone, significant TG accumulation was not seen. Thus, the presence of TG, delivered to the cell as a component of VLDL, reduced cholesterol accumulation, specifically CE, from aggLDL in THP-1 macrophages.

The simplest explanation for our results would be that TG-rich and CE-rich lipoproteins compete for uptake. In order to determine the extent to which our observations were the result of competition for uptake, THP-1 cells were treated with I^{125} labeled aggLDL (50 μg aggLDL protein/ml) in the presence or absence of increasing

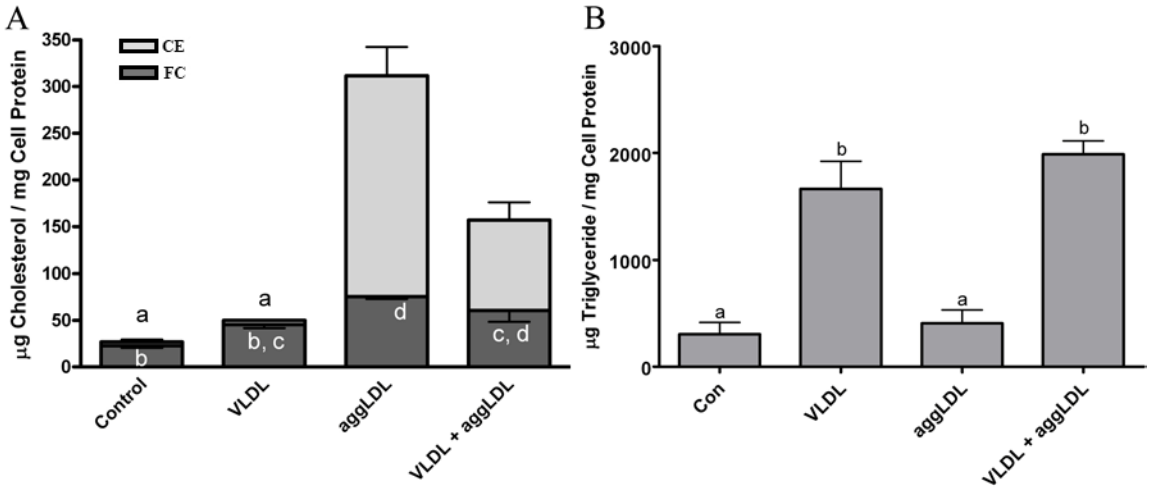


Figure 7: Accumulation of lipids in THP-1 macrophages incubated with aggregated LDL (aggLDL) and/or VLDL. THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or VLDL. The cells were harvested and the cellular lipid levels were determined as described in Materials and Methods. A: Incubation of THP-1 with aggLDL produced a dramatic increase in total cellular cholesterol seen primarily as a significant increase ($p<0.05$) in CE (light gray portion of bar). Although the FC (dark gray portion of bar) increased nearly two-fold compared with control, this difference was not statistically significant. Incubation with both aggLDL and VLDL reduced the cellular CE accumulation compared to that seen with aggLDL alone. B: Incubation of cells with VLDL produced a significant ($p< 0.05$) increase in cellular TG levels compared to control or aggLDL-treated cells both when used alone or in combination with aggLDL. Values are the mean ± SEM for three experiments. Within each panel, bars with the same letter indicate that means were not statistically different. All other comparisons were significantly different ($p<0.05$).

concentrations of VLDL (0, 10 or 50 μg VLDL protein/ml) for 48 hours (**figure 8**). Results show no difference in particle uptake over a range of VLDL concentrations, indicating that VLDL treatment does not reduce uptake of aggLDL. This suggests that the two particles do not compete for uptake and indicates a TRP-specific mechanism by which lysosomal CE metabolism and cellular clearance is enhanced.

Lipoproteins are complex aggregates of lipids and proteins, both of which can influence the uptake and cellular metabolism of internalized material. In order to rule out an effect of lipoprotein-derived protein on the process, protein-free DISP of PL and CE (CE-DISP, 60 μg CE/ml) were substituted for aggLDL and dispersions of PL and TG (TG-DISP, 50 μg TG/ml) replaced VLDL. TG-DISP significantly reduced ($p<0.05$) the CE-DISP-induced accumulation of CE (**figure 9A**). As with lipoproteins, this correlated with a significant increase ($p<0.05$) in cellular TG. There was no significant difference in cellular TG levels between incubation with TG-DISP alone and co-incubation with TG-DISP and CE-DISP, indicating that uptake of CE-DISP and TG-DISP was not significantly affected by co-incubation (**figure 9B**). Thus, it appears that TG is required for the reduction of cellular cholesterol and is the primary mediator of the effects on cellular sterol metabolism. An even more dramatic reduction in cellular cholesterol concentration was accomplished when cells were first loaded with cholesterol from CE-DISP (60 μg CE/ml) and then chased, after removing CE-DISP from the media, with media containing TG-DISP (50 μg CE/ml). The loading with CE-DISP more than doubled the cellular cholesterol

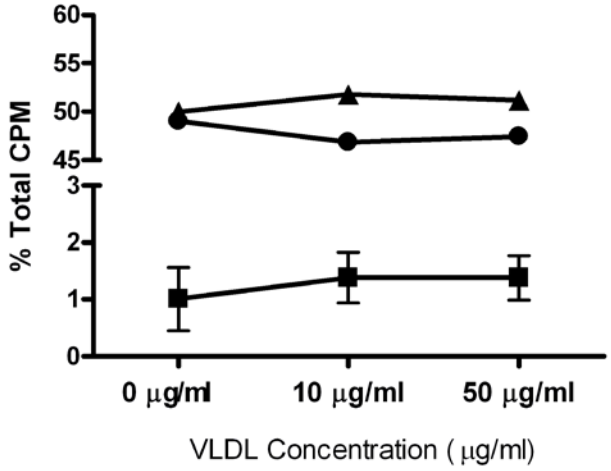


Figure 8: VLDL and aggLDL do not compete for uptake and degradation. THP-1 cells were treated with ^{125}I labeled aggLDL (50 μg aggLDL protein/ml) in the absence or presence of VLDL (0, 10 or 50 μg VLDL protein/ml) for 48 hours. Squares represent internalized (i.e. cell associated) ^{125}I labeled aggLDL, circles represent ^{125}I labeled aggLDL that has been processed by the cells and discarded to the media, and triangles represent non-degraded ^{125}I labeled aggLDL found in the media. Results show no difference in particle uptake along the VLDL concentration gradient, indicating that VLDL does not reduce uptake of aggLDL.

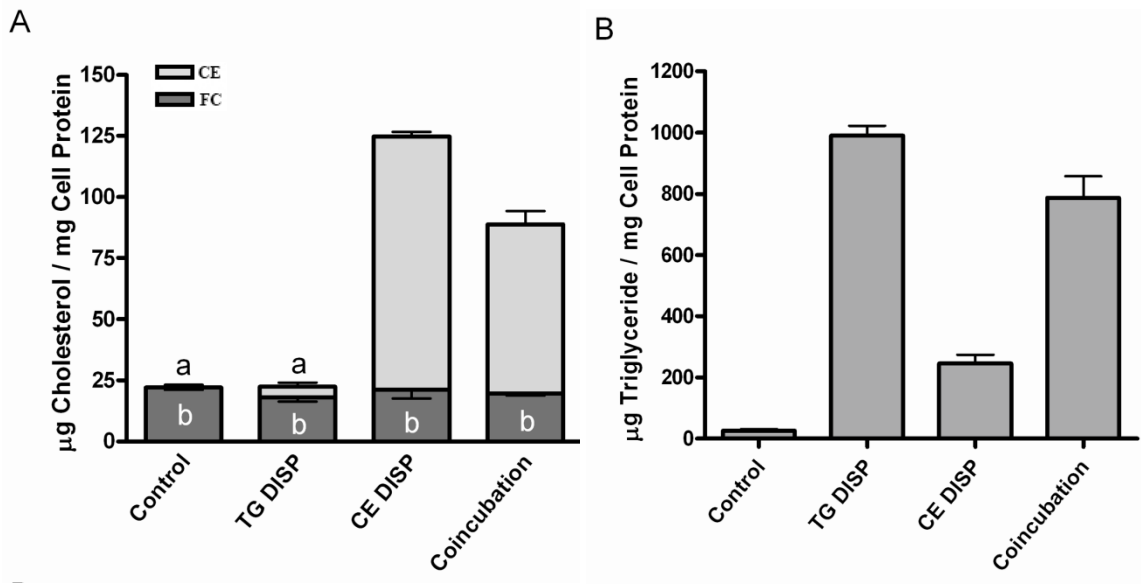


Figure 9: Accumulation of lipids in THP-1 macrophages incubated with TG-DISP and/or CE-DISP. THP-1 macrophages were treated for 48 hours at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with the specified concentrations of lipid dispersions. Cells were cultured utilizing the inverted cell culture technique as described in Materials and Methods. A: Incubation of THP-1 with 50 µg TG/ml TG dispersions (TG-DISP) alone did not significantly ($p<0.05$) alter cellular FC (dark gray portion of bar) or CE (light gray portion of bar). In contrast, incubation of cells with 60 µg CE/ml CE dispersions (CE-DISP) significantly increased the CE stores in cells. Co-incubation of cells with both CE DISP and TG DISP produced a significant ($p<0.05$) decrease in CE accumulation. B: Incubation of THP-1 with 50 µg TG/ml TG DISP either alone or in combination with 60 µg CE/ml CE DISP produced a significant ($p<0.05$) increase in cellular TG compared to control cells or cells treated with CE DISP alone. Values are the mean ± SEM for three experiments. Within each panel, bars with the same letter indicate that means were not statistically different. All other comparisons were significantly different ($p<0.05$).

content and all of the accumulation was as CE. The chase with TG-DISP produced a 60% reduction in cellular total sterol with all of the reduction occurring as loss of CE.

To further confirm that competition for uptake of aggLDL by VLDL was not the explanation for reduced cellular cholesterol and to more completely define the contribution of intracellular TG concentration on cellular lipid levels, we performed pulse-chase experiments with a constant concentration of aggLDL (50 μg aggLDL protein/ml) and varying concentrations of VLDL (5, 20, and 50 μg VLDL protein/ml). TG treatment reduced cholesterol content in aggLDL-treated THP-1 macrophages in a concentration dependent manner (**figures 10A and 10B**). Importantly, the TG-effect was observed when the cells were incubated with VLDL either following (**figure 10A**) or prior to (**figure 10B**) aggLDL incubation. Moreover, the effect on cellular cholesterol was roughly proportional to cellular TG accumulation (**figures 10C and 10D**)**.** Therefore, TG reduces cellular cholesterol levels in a manner that is dependent on the cellular concentration of TG. Furthermore, TG can prevent subsequent cellular cholesterol accumulation and can mobilize preexisting stores of cellular CE, including the large volume of sterol that accumulates in cellular lysosomes (9). This is in stark contrast to direct stimulation of extralysosomal cholesterol mobilization which does not affect the cholesterol trapped within lysosomes (11,13).

We recently demonstrated that lysosomal accumulation of CE occurring in macrophages incubated with CE-rich particles, including oxLDL, aggLDL and CE-DISP, in large part, is the result of inhibition of lysosomal CE hydrolysis in response to an initial accumulation of excess FC within the lysosome (9-11). Lysosomal CE hydrolysis is a key,

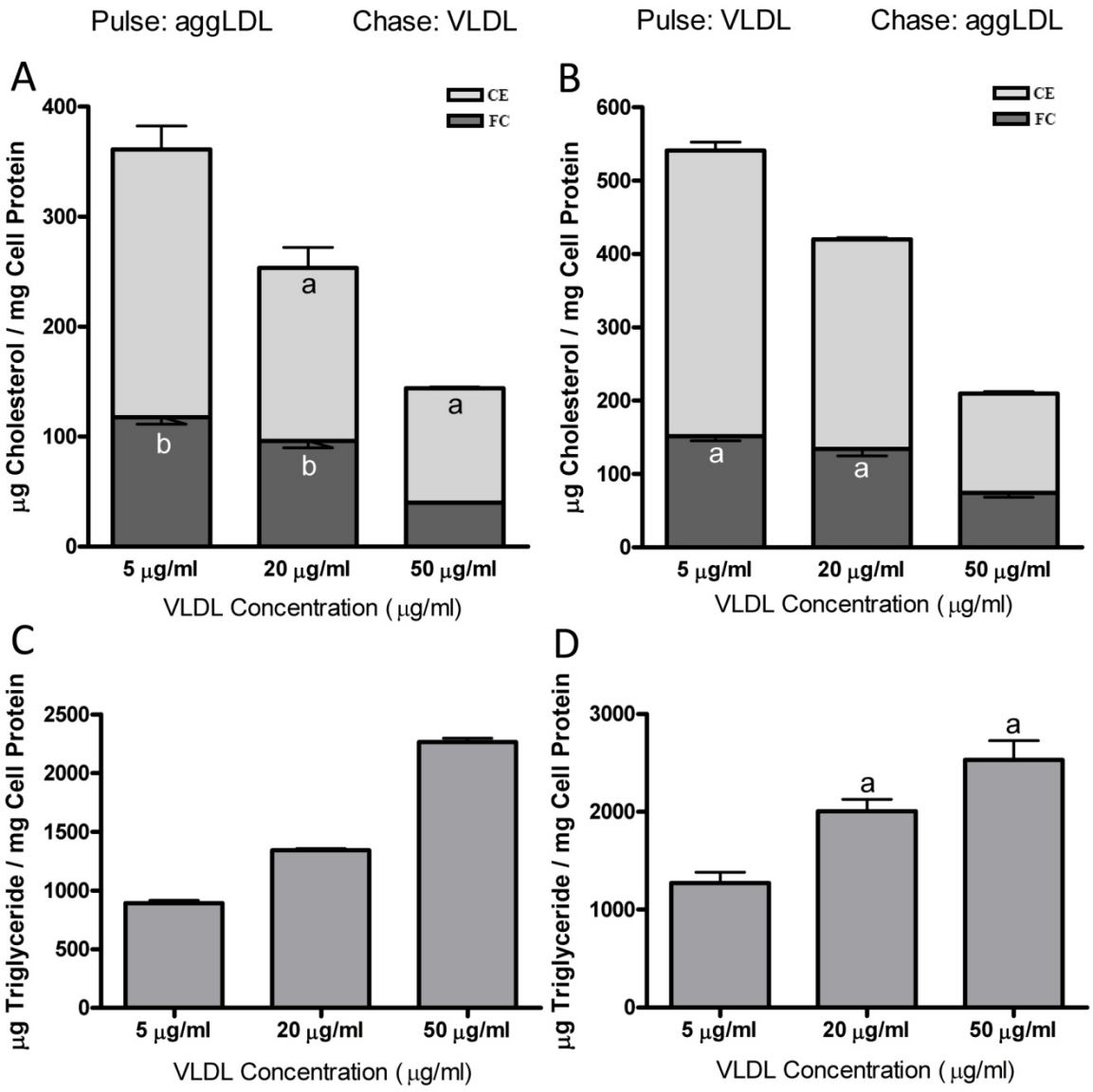

Pulse: aggLDL
Chase: VLDL
Pulse: VLDL
Chase: aggLDL
A
B
C
D
CE
FC
μg Cholesterol / mg Cell Protein
μg Triglyceride / mg Cell Protein
5 μg/ml
20 μg/ml
50 μg/ml
VLDL Concentration (μg/ml)
a
b

Figure 10: Incubation of THP-1 macrophages with VLDL decreases cellular FC and CE in a TG-dose dependent manner. THP-1 macrophages were pulsed for 3 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) with 50 μg protein/ml of aggLDL or the specified concentrations of VLDL (ranging from 5-50 μg protein/ml) followed by a 3 day chase period with either 50 μg protein/ml of aggLDL or the specified concentrations of VLDL. A: In cells preloaded with sterol by incubation with 50 μg aggLDL protein/ml, a chase with TG significantly ($p<0.05$) reduced total cholesterol accumulation over a range of VLDL concentrations (5, 20, and 50 μg protein/ml) in a dose-dependent manner. B: Preincubation of cells with VLDL also produced a significant ($p<0.05$) and dose-dependent reduction in total cellular cholesterol accumulation from subsequent incubation with 50 μg aggLDL protein/ml. C and D: Incubation of THP-1 with increasing concentrations of VLDL produced a stepwise increase in cellular TG levels. Values are the mean ± SEM for three experiments. Within each panel, bars with the same letter indicate that means were not statistically different. All other comparisons were significantly different ($p<0.05$).

rate-limiting step in the cellular clearance of exogenously derived sterol. Thus, the TG-induced enhancement of cellular CE metabolism and loss of sterol from the cell suggests that TG alters lysosomal CE hydrolysis. To study this further, we isolated lysosomes from cholesterol normal cells and cells under our various treatment conditions. EM of negatively stained isolates from various cellular subfractions indicates that the lysosomal fraction contained primarily membrane-limited vesicles having the appearance of lysosomes. Furthermore, these fractions were positive for LAMP-1, a marker for lysosomes/late endosomes (9,10), and LAL, the acid CE hydrolase (**figure 6**). Additionally, these fractions were negative for markers of early endosomes (EEA-1) and cytoplasmic lipid droplets (perilipin A) (**figure 6**). Although the lysosomal fractions from each treatment group contained membrane-limited vesicles, differences in lysosome size and morphology were observed in lysosomes isolated from TG-enriched macrophages compared to those treated with cholesterol-enriched particles. The presence of small LDL-sized particles within the lysosomes isolated from cells incubated with aggLDL (**figure 11**) is consistent with our previous observations that, under conditions of cholesterol enrichment, CE-rich particles continue to be delivered to the lysosomes but digestion of the particles is inhibited (10). In contrast to treatment with cholesterol-rich vehicles (**figure 11B**), cellular TG enrichment results in lysosomes which are much smaller and have relatively homogenous luminal contents, indicative of active lipid particle digestion (**figure 11C**). Additionally, coincubation of aggLDL and VLDL (**figure 11D**) results in lysosomes that are similar in size and morphology to control lysosomes (**figure 11A**). The quantitative differences in lysosomal diameter are shown in

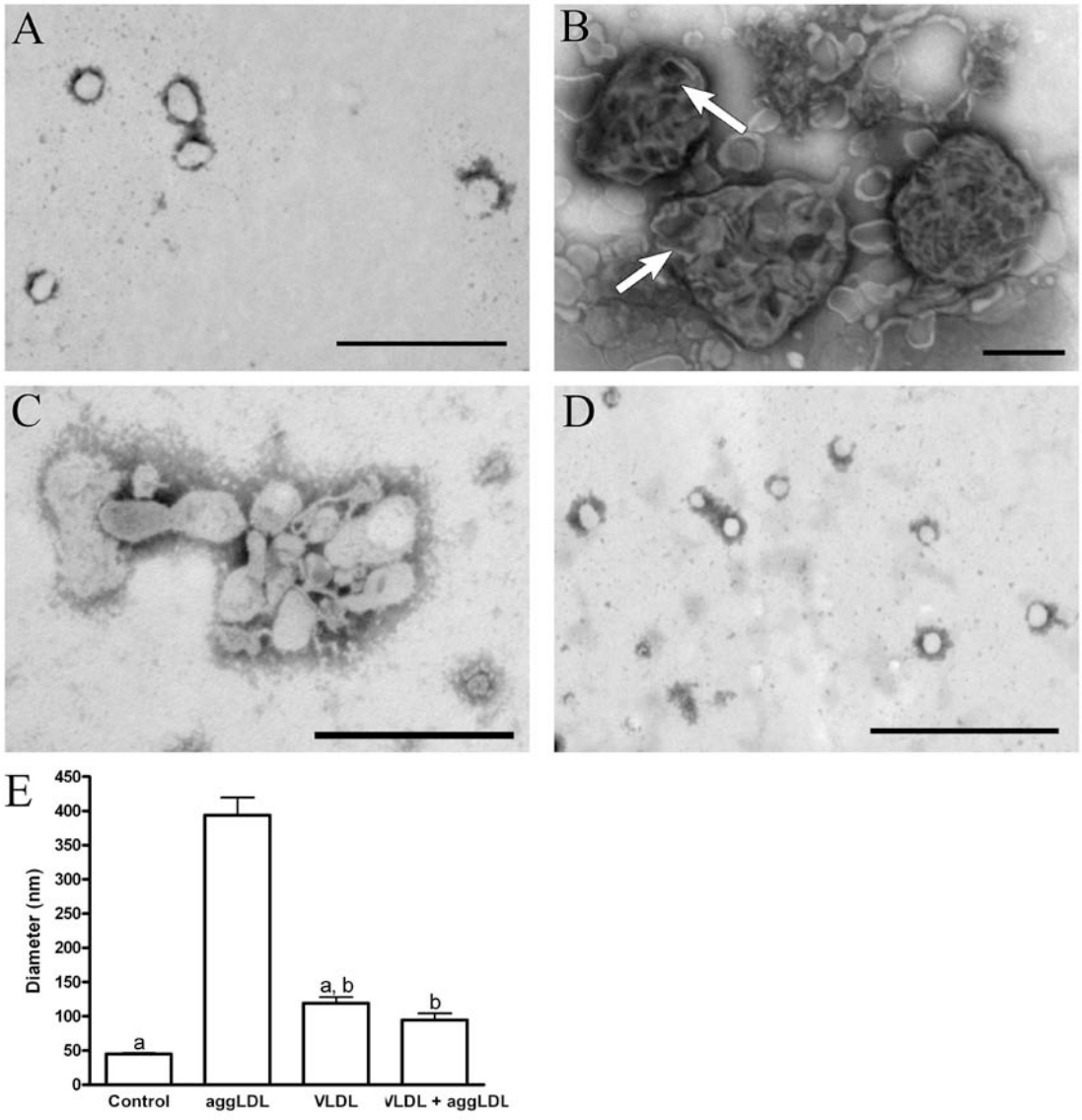

A
B
C
D
E
Diameter (nm)
450
400
350
300
250
200
150
100
50
0
a
a, b
b
Control
aggLDL
VLDL
VLDL + aggLDL

Figure 11: Negative staining electron microscopy of isolated lysosomes. THP-1 macrophages treated with TRPs for 6 days had reduced lysosome diameter compared to macrophages that were only cholesterol enriched. Panels A-D show negative stain EM of lysosomes isolated from control cells (A), aggLDL-treated cells (100 μg aggLDL protein/ml, B), VLDL-treated cells (100 μg VLDL protein/ml, C), or cells co-incubated with aggLDL and VLDL (100 μg aggLDL protein/ml and 100 μg VLDL protein/ml, D). Panel E shows the average lysosomal diameter determined from 3 separate experiments. Lysosomes from control cells were generally small and had a homogenous appearing lumen (A and E). In contrast, lysosomes from aggLDL-treated cells had significantly larger ($p<0.05$) lysosomes which had a variety of appearances but often contained a heterogenous mixture of apparently undigested material (arrows in figure B) within their lumen (B and E). Lysosomes from VLDL-treated cells were small with homogenous lumina similar to those isolated from control cells (C and E). Significantly, when THP-1 were incubated with VLDL in combination with aggLDL, their lysosomes remained small and failed to develop the large, heterogenous appearance of lysosomes isolated from cells incubated with aggLDL alone. Within each panel, bars with the same letter indicate that means were not statistically different. All other comparisons were significantly different ($P < .05$). Magnification for panels A, C and D = 40000X; Magnification for panel B = 25000X; bar = 500 nm.

figure 11E. These results are consistent with our hypothesis that cellular TG enrichment enhances lysosomal activity and clearance of lipid from the lysosome.

Changes in the size and morphology of isolated lysosomes suggest enhanced CE hydrolysis and subsequent clearance of lysosomal sterol upon TG enrichment. To confirm this, we examined the effect of TG specifically on lysosomal sterol content. Treatment of cells with TRPs alone or in combination with cholesterol-rich molecules induced an increase in lysosomal TG levels while treatment with cholesterol-rich molecules alone resulted in increased lysosomal total cholesterol (**figure 12**). However, coincubation of cells with both TRPs and cholesterol-rich molecules resulted in a 33% reduction in lysosomal cholesterol (**figure 12**). As with the analysis on a whole cell basis, the reduction was almost exclusively in CE. This suggests that the reduction in cellular cholesterol begins with TG-induced changes in lysosomal CE hydrolysis.

Conversion of CE to FC is a critical first step in the removal of cholesterol from lysosomes. The hydrolysis of CE is mediated by the enzyme LAL, an acid hydrolase that is only functional at an acidic pH (135). Thus, the observed changes in lysosomal sterol levels are likely the result of effects on LAL. We do not observe changes in the cellular or lysosomal expression of LAL under the various lipid loading conditions (**figure 13**). Therefore, it is likely that the effects of TG involve changes in the environment in which the enzyme must function. Previously, we have shown that accumulation of excess FC in foam cell lysosomes leads to an inhibition of CE hydrolysis as a result of failure of the lysosomes to maintain the correct acid pH. Thus, the most probable candidate for the

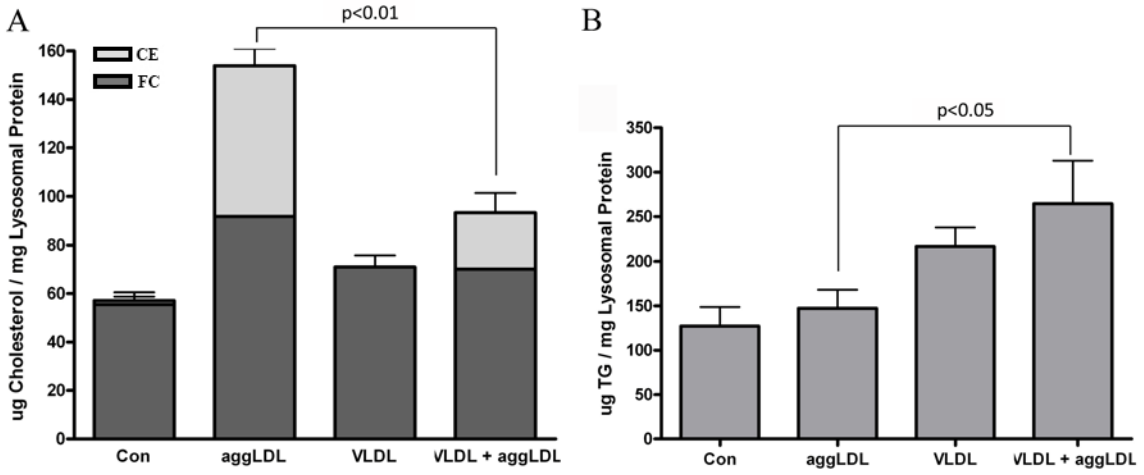


Figure 12: Accumulation of lipids in lysosomes isolated from THP-1 macrophages incubated with aggLDL and/or VLDL. THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or VLDL. The cells were harvested following 6 day lipid accumulation and then lysosomes were isolated. The lysosomal lipid levels were determined as described in Materials and Methods. A: Incubation of cells with aggLDL produced a significant increase ($p<0.05$) in the total cellular cholesterol that was seen primarily as increase in CE. In contrast, incubation of cells with VLDL did not produce an increase in lysosomal sterol. Importantly, co-incubation of THP-1 with both VLDL and aggLDL significantly reduced the lysosomal CE content. B: Incubation of THP-1 with aggLDL and VLDL together significantly increased ($p<0.05$) the amount of TG accumulating within lysosomes. Values are the mean ± SEM for three experiments.

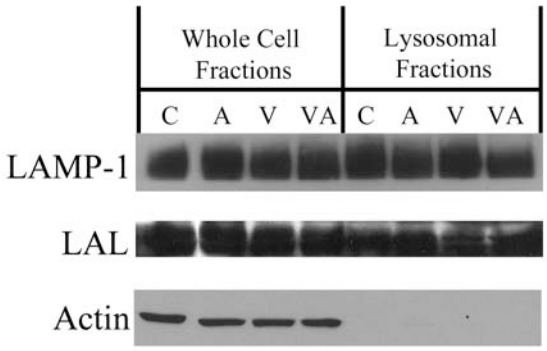


Figure 13: Western blot analysis of LAL and LAMP-1. Analysis of cells treated with aggLDL and VLDL (100 μg lipoprotein/ml each for 6 days) and lysosomes isolated from similarly treated cells showed no difference in the expression of LAL between TG and/or CE loaded cells. Fifteen micrograms of protein was loaded in each lane and incubation conditions were as described in Experimental Procedures. Cells loaded with inert beads acted as a control for lysosome stimulation. ß-Actin was used as a loading control for whole cell fractions while LAMP-1 was used as a control for lysosomal fractions. C = Control, V = VLDL-treated, A = aggLDL-treated, VA = VLDL + aggLDL coincubation.

TG reversal of the inhibition of CE hydrolysis would be the reestablishment of the acid pH to the lysosome.

We measured the pH of vesicles within cells under our various treatments using Lysosensor Yellow/Blue DND-160, a pH sensitive dye (10). LysoSensor Yellow/Blue DND-160 fluoresces yellow in an active lysosomal environment and has a significant blue shift as pH approaches neutrality (199,203). We classified intracellular vesicles as active if the pH was < 4.8 and inactive if the pH was > 4.8. This pH is at the upper limit of the accepted normal lysosomal pH and is well above the narrow active pH range of human LAL, which displays peak activity at pH 3.8–4.0 and possesses little activity above pH 4.5 (135). Consistent with our previous studies, the majority of vesicles in untreated macrophages exhibited an active pH (**figures 14A and 14F**). Additionally, the majority of vesicles in cholesterol-enriched macrophages were inactive (**figures 14B and 14F**). However, examination of the vesicle pH in TG-enriched macrophage foam cells revealed differences in pH upon treatment with CE-rich compared to TG-rich lipoproteins. In contrast to cholesterol-rich foam cells (**figure 14B**), macrophages enriched with TG via treatment with VLDL did not display an alteration in the number of active vesicles (**figures 14C and 14F**). In fact, the presence of TG prevented cholesterol-induced lysosome neutralization when TG enrichment occurred concurrent with aggLDL accumulation (**figures 14D and 14F**). Moreover, when cells were loaded with sterol via aggLDL, subsequent treatment with VLDL reestablished an active pH to the previously inhibited vesicles (f**igures 14E and 14F**). To confirm that most of the vesicles we analyzed were lysosomes, in separate experiments we used LAMP-1 staining to

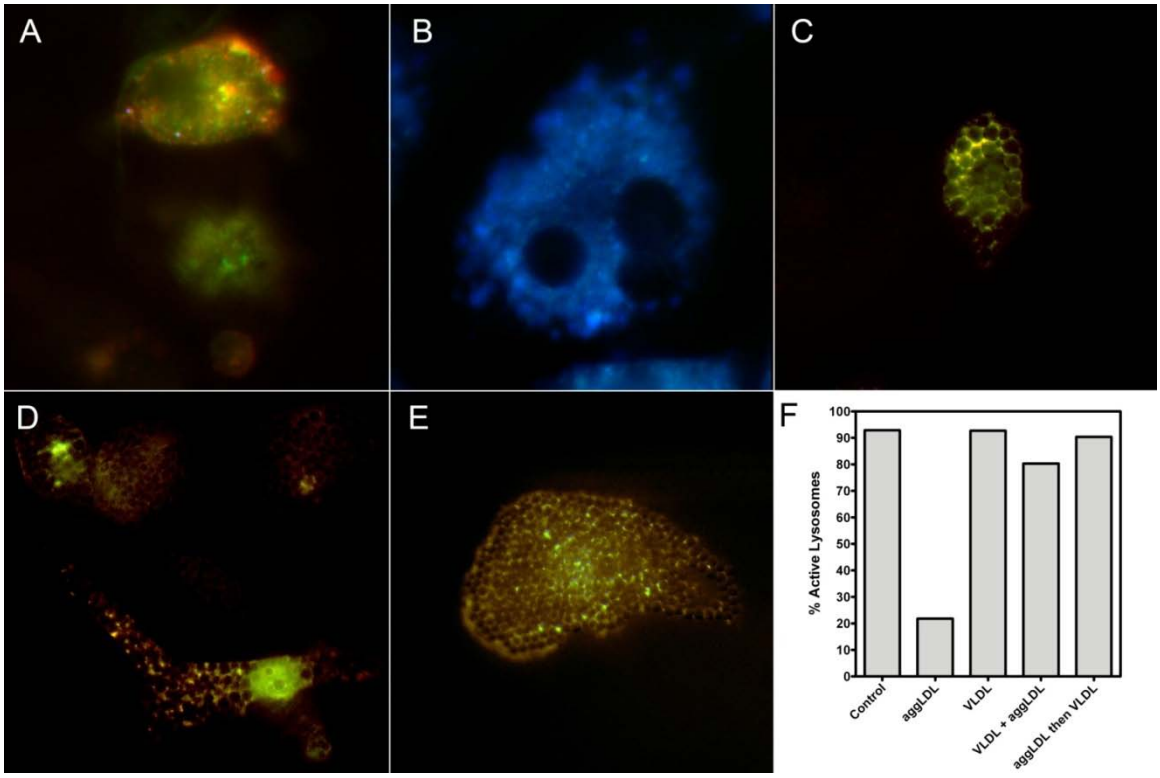


Figure 14: LysoSensor Yellow/Blue DND-160 staining of lipid loaded macrophages. Enrichment of THP-1 macrophages with TG maintains and/or restores active lysosome pH. A: After 6 days in culture, untreated lysosomes maintained an active (pH < 4.8), as indicated by a yellow fluorescence pattern. B: After 6 days of incubation with 100 μg aggLDL protein/ml, most lysosomes displayed a predominantly blue fluorescence, indicating an increase in pH to levels above 4.8. At this pH CE hydrolysis is inhibited. C: Macrophages treated with 100 μg VLDL protein/ml maintained a large population of active lysosomes (pH < 4.8). D: Similarly, when cells were simultaneously incubated with 100 μg aggLDL protein/ml and 100 μg VLDL protein/ml, the lysosomes maintained activity, as evidenced by predominantly yellow fluorescence. E: When macrophages were incubated with 100 μg VLDL protein/ml after the cells were first incubated with 100 μg aggLDL protein/ml, the VLDL-treatment was able to restore the pH of the lysosomes back to normal levels F: Quantification of the percentage of active (pH < 4.8) vesicles following the various treatment conditions Magnification of panels A-E = 500X.

investigate changes in the number of LAMP-1 positive vesicles under our various treatment conditions. Greater than 75% of vesicles in THP-1 foam cells were LAMP-1 positive and the number of LAMP-1 positive vesicles did not significantly change ($p<0.05$) with treatment. Thus, the shift in percentage of active lysosomes was not the result of changes in lysosome number but rather represents the ability of lysosomes to maintain an active pH. Control cells incubated with polystyrene beads did not show a change in pH indicating that time duration was not the cause of changes in pH measurement; this was consistent with previous observations (10). EM analysis of acid-phosphatase stained cells also indicates that most of the lipid accumulation was in lysosomes and the number of lysosomes was not appreciably altered with our various treatments.

We have previously shown that cholesterol-induced inhibition of lysosomal acidification occurs primarily through the inhibition of the vacuolar ATPase, an integral membrane protein responsible for pumping H^+ ions into the lysosomal lumen (10). Loss of v-ATPase function occurred concurrently with the accumulation of FC in the lysosome and, specifically, within the lysosomal membrane. Our LysoSensor yellow/blue data suggest that TRPs accentuate CE hydrolysis by restoring lysosome pH. We hypothesized that the reduction in pH was due to TG-induced restoration of lysosomal v-ATPase activity. To confirm this, we examined the activity of the lysosomal v-ATPase in isolated lysosomes from cells treated with TG, cholesterol, or both. Quenching of acridine orange fluorescence was our measure of the v-ATPase proton pump activity (10,138). Consistent with our published results (10), treatment with aggLDL alone (50 μg

aggLDL/ml, 6 days) resulted in deficient v-ATPase activity (**figure 15**). However, lysosomes isolated from cells treated with both aggLDL and VLDL (50 μg lipoprotein/ml each, 6 days) exhibited a rapid quenching of the acridine orange, comparable to control lysosomes (f**igure 15**), indicating that the v-ATPases remained active. This suggests that TRP can help lysosomes to maintain an active pH by overcoming cholesterol's normal ability to suppress the v-ATPases proton pumping.

Discussion

Much of our understanding of atherosclerotic foam cells focuses on the study of CE metabolism and accumulation, and most tissue culture models limit themselves to the study of the metabolism of cholesterol-rich particles. However, extracellular areas of atherosclerotic lesions contain a complex milieu with multiple lipid species, each of which could have a distinct influence on foam cell biology. In the present study, the influence of TRPs on macrophage CE metabolism was examined. TG was introduced through incubation of cells with VLDL or artificial TG-rich dispersions. Treatment with TRPs led to a reduction in total cellular cholesterol but more importantly, in lysosomal CE, in macrophage foam cells. This suggests that TG (or a metabolite of TG) is the mediating agent inducing the removal of sequestered sterol from foam cell lysosomes.

Our data and that from previous studies suggest that the size and metabolic activity of intracellular TG pools can be an important component of macrophage lipid metabolism. Thus, it is important to define the interaction between TG and intracellular cholesterol pools in order to fully understand the flux of lipids within foam cells present

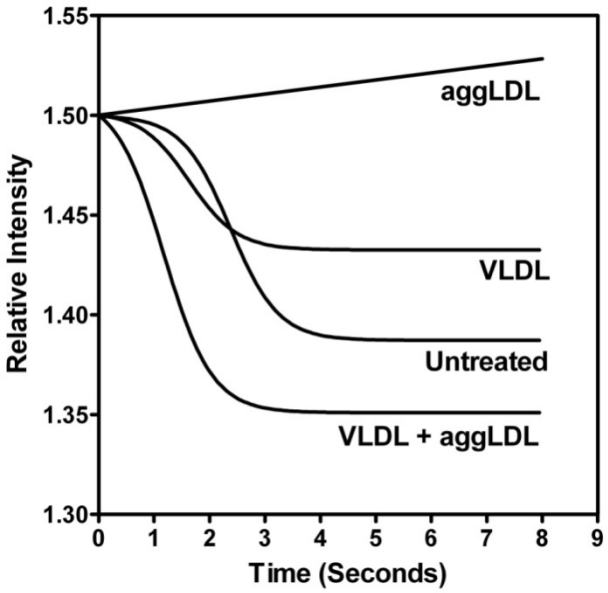


Figure 15: Activation of v-ATPases in isolated lysosomes following TG and/or cholesterol enrichment. Untreated lysosomes exhibited activation of v-ATPase and the pumping of hydrogen ions into the lysosomal lumen when stimulated by the addition of ATP and valinomycin (time 0), as indicated by the decrease in the relative fluorescence intensity of acridine orange. Lysosomes from macrophages that had been treated with 100 μg aggLDL protein/ml exhibited a lack of v-ATPase activity as evidenced by no reduction in the relative fluorescence intensity. In contrast, lysosomes from cells treated with 100 μg VLDL protein/ml, either alone or in combination with 100 μg aggLDL protein/ml, exhibited rapid quenching of acridine orange fluorescence, indicating active v-ATPases. Data is a representative of example chosen from multiple separate experiments.

in atherosclerotic lesions. However, despite some provocative reports (179,204), the role of TRP in foam cell cholesterol metabolism has not been studied extensively. In this chapter, we have demonstrated that cellular TG greatly influences lysosomal cholesterol homeostasis and significantly impacts atherosclerotic macrophage foam cell CE accumulation. Previous research on the interaction of TG-rich and cholesterol-rich lipoproteins has indicated a complex interaction between lipids which can have multiple effects within the macrophage. These potential interactions include the possibility that TG or its metabolites can alter the physical properties of the mixed lipid particles to enhance metabolism, can affect the affinity and activity of the enzymes responsible for lipolysis, and can regulate cholesterol metabolic metabolism and trafficking (128,178,179). For instance, cellular TG can increase the rate and efficiency of CE hydrolysis in macrophage lysosomes when the CE resides in a mixed lipid pool containing TG with lysosomes (128). This results, at least in part, from the ability of TG to alter the physical state of CE, keeping it more fluid (128). This physical state effect is not limited to lysosomal hydrolysis, as association of TG with cytoplasmic CE droplets has previously been shown to make CE more susceptible to hydrolysis by NCEH in the cytoplasm (178,179). Since the mobilization of cytoplasmic and lysosomal CE stores is a key mechanism for cellular cholesterol clearance (179), the ability of TG to mobilize intracellular cholesterol is highly significant.

In this chapter, we have shown that increases in cellular TG drastically reduce cellular and, specifically, lysosomal cholesterol levels by maintaining the activity of foam cell lysosomes. Thus, our results are in line with previous studies demonstrating that TG

plays a significant role in altering the metabolism of cholesterol in macrophage foam cells. However, our studies are the first to reveal a reduction in lysosomal cholesterol levels in response to increased cellular and lysosomal TG content. This is not an unimportant finding. In advanced atherosclerotic lesions, it is estimated that greater than 70% of the lipid in a macrophage foam cell is sequestered in lipid enriched lysosomes (125,205). Moreover, our previous studies indicate that the cholesterol within lysosomes is trapped and is extremely resistant to treatments that mobilize and efflux other intracellular cholesterol stores (5,13). We and others have shown that enhancement of efflux promoters, including increases in ABCA1 or the extracellular concentration of free apoproteins or HDL, does not reduce lysosomal cholesterol stores (13,206). Therefore, TG-containing particles appear to be unique in their ability to promote lysosomal cholesterol clearance.

Our studies also suggest that the effect of TG on lysosomal CE metabolism and cholesterol clearance from the lysosome is more complex than just alterations in enzymatic hydrolysis as evidenced by the influence of TG on lysosomal v-ATPase activity. The lysosomal v-ATPases are critical lysosome integral membrane proteins responsible for pumping H^+ into the lysosomal lumen in order to maintain the acidic pH necessary for the function of lysosomal lipolytic enzymes. It is not clear whether TG directly or indirectly affects the pumps. We have previously shown that the activity of v-ATPases is sensitive to the FC content of the lysosome membrane (10). This is consistent with similar studies which have shown that the macrophage ER calcium pump, sarcoplasmic-endoplasmic reticulum calcium ATPase-2b, is also sensitive to changes in membrane

fluidity induced by cholesterol content (207). Therefore, it is possible that the TG-effect, at least in part, is mediated through lysosome membrane changes, which enhance proton pumping, lysosomal acidification, and lipolytic enzyme activity. This possibility is addressed in chapter IV.

It is also possible that TG, either directly or through its metabolites, influences the removal of cholesterol from lysosomes and into cholesterol efflux pathways. In this regard, while the egression of cholesterol out of lysosomes is not a well defined process, it is known that it primarily involves the intercalation of FC into lysosomal membrane followed by its subsequent removal via formation of FC-enriched vesicles that traffic to and fuse with other cellular membranes (208). Thus, TG-mediated effects on lysosomal membrane properties have the potential to influence this aspect of lysosomal cholesterol clearance. It remains to be determined how lysosomal TG influences lysosomal cholesterol clearance and to what extent membrane alterations are required. Insights into this question are addressed in subsequent chapters. However, the current study does indicate that the presence of lysosomal TG enhances the metabolism and removal of cholesterol both from the lysosome and, ultimately, from the cell. Further study is required to define the precise mechanism or mechanisms which drive the enhanced clearance. Our studies to date have shown that TG can restore lysosome function in foam cells by reestablishing the ability of lysosomes to maintain an acid pH. This leads to increased CE hydrolysis, as the initial step, in enhanced sterol clearance both from lysosomes and from the cell.

In addition to the potential modulation of the activity of lipolytic enzymes, the metabolic byproducts of TG could affect lysosomal and cellular lipid metabolism. Macrophages have previously been shown to metabolize TG from TRPs to glycerol and FA through surface hydrolysis and by internalization of TRPs and lysosomal hydrolysis (173). Thus, TG and its metabolic products would be found in lysosomes. The TG pool is metabolized more efficiently than CE (209). This suggests other ways that TG metabolites might impact lysosomal function. For instance, FAs generated from hydrolysis of TG are known to be influential in cholesterol homeostasis. FAs are key signaling molecules that greatly affect the expression of critical genes controlling cellular cholesterol mobilization (180,185-192). FAs can act at the level of nuclear receptors to affect the transcription of a number of genes important in cholesterol homeostasis (180,185-192). In particular, the individual or cooperative upregulation of PPAR and LXR expression by FA has been shown to regulate the expression of a number of cholesterol homeostatic genes, including the ATP-binding cassette gene family members A1 and G1, which enhance cholesterol movement and efflux (180). Free FAs might also elicit an effect within the lysosome. Additionally, it is possible that the FAs generated from the lipolysis of TG within the lysosome may influence lysosomal membrane properties either by direct intercalation into the membrane or by influencing the acyl chains, which are present on the lysosomal membrane phospholipids. This modification in the FA composition of the lysosomal membrane might improve membrane fluidity and enhance the activity of lysosomal integral membrane proteins, including the lysosomal v-ATPase (210-213). Previously, we have shown that increased membrane cholesterol,

which decreases membrane fluidity, can inhibit lysosomal proton pumping (10). These data taken together suggest several explanations for how TG uptake into lysosomes might influence macrophage foam cell lysosomal CE metabolism. Additional experimentation will be required to define the mechanisms underlying TG-induced enhanced lysosomal CE hydrolysis and clearance. Initial studies examining these possibilities are presented in the following chapters.

There is an apparent contradiction between published epidemiologic studies suggesting that HTG may increase atherosclerosis, and our current cellular studies, which indicate a role for TG in cholesterol clearance. A number of points are worth noting in this regard. First, the epidemiologic evidence is controversial and does not determine whether HTG has a direct or indirect effect on coronary disease (173). Specifically, HTG has not been established an independent risk factor for atherosclerosis. High TG levels are typically associated with low HDL-cholesterol (169,214) and, when HDL-cholesterol levels are considered, the significance of HTG in atherogenesis is reduced or nullified (215). Therefore, it is unclear if HTG contributes to lesion formation separate from its effects on other atherosclerotic risk factors. A better understanding of how HTG affects cells in the artery wall is required to resolve this aspect of the paradox. An important component of that effort is defining the role of TRP as a modulator of CE metabolism and foam cell biology. Secondly, it remains to be determined if the increased lysosomal cholesterol clearance induced by TG has a positive or negative impact on macrophage foam cell biology and, ultimately, lesion development. Although in most settings removal of foam cell cholesterol is thought to

have a positive impact, the massive removal of cholesterol from previously engorged lysosomes may generate high levels of cellular FC that overwhelm the normal homeostatic mechanisms. Furthermore, it is known that high FC levels within certain cellular pools are harmful to macrophages (46). Thus, cellular health is regulated not only by the levels, but also the cellular location, of cholesterol. As such, FC is essential for proper cellular growth and membrane stability, but excess cellular free cholesterol in the wrong location is cytotoxic (73,216). The sequestration of cholesterol within the lysosomal compartment may be a protective measure to save the cell from the toxic effects of accumulated FC. Further studies are required to sort out the relative risks and benefits of unleashing lysosomally-trapped cholesterol.

Lysosomal cholesterol is a major constituent of clinically important atherosclerotic macrophage foam cells. Most importantly, the lysosomally sequestered FC and CE has been shown to be highly resistant to removal, even under conditions that promote extralysosomal cholesterol efflux (13,134). In the current study we have shown for the first time that in the presence of TG, cholesterol that had previously been sequestered in foam cell lysosomes, is removed from both the lysosome and the cell. Thus, the TG-induced removal of sequestered lysosomal cholesterol allows this sterol pool to be available for cholesterol efflux. Therefore, our data suggest that TG-induced removal of cholesterol from foam cell lysosomes, if properly managed, may prove to have a positive benefit. This may be important in developing therapies for atherosclerotic lesion regression, as the removal of cholesterol from macrophage foam cells has previously been shown to reduce lesion area and enhance lesion stability (50).

CHAPTER IV

MECHANISM OF THE EFFECTS OF TRIGLYCERIDE ON LYSOSOMAL METABOLISM: ANALYSIS OF LYSOSOMAL MEMBRANE FLUIDITY AND LYSOSOME MEMBRANE PHOSPHOLIPID FATTY ACID COMPOSITION

Our studies have shown that TG enrichment mobilizes lysosomal cholesterol in foam cells and restores lysosome function (chapter III); however, the mechanism by which these effects occur remains unclear. The studies described in this chapter show that TG can reverse the cholesterol-induced changes in lysosome membrane stiffness and restore the normal fluidity of the lysosomal membrane, shedding some light on how TG might operate in the lysosome.

Introduction

V-ATPase activity is the driving force behind lysosome acidification. ATPases are a class of enzymes that catalyze the hydrolysis of ATP to produce energy for subsequent chemical reactions. The majority of ATPases are transmembrane proteins that use the energy generated from ATP hydrolysis to move solutes across biological membranes against a concentration gradient (217). The complex structure of ATPases consists of cytoplasmic and transmembrane domains, each of which play distinct, yet important roles in protein function. The cytoplasmic domain is the site of ATP hydrolysis, while the transmembrane domain consists of a channel that transports solutes, ions and other materials using the energy from ATP hydrolysis (217). The transmembrane domains of

the three major classes of ATPases, (v-, F- and A-ATPases) contain “rotary engines” that convert the energy generated from ATP hydrolysis into mechanical energy. This energy is required for the movement of protons into the luminal space (217,218). Importantly, the activity of integral membrane proteins, including ATPases, is sensitive to the composition of their membrane environment (207,219,220). Increasing membrane cholesterol content abolishes the activity of many ATPase isoforms, and the mechanism is likely due to the ability of cholesterol to change the properties of biological membranes (207,221-224).

Previous studies from our laboratory show that when cholesterol accumulates in the lysosome, the ability of the lysosome to decrease its pH is impaired. This occurs primarily because cholesterol inhibits the function of v-ATPase, the integral membrane protein responsible for pumping H^+ ions into the lysosomal lumen. Furthermore, this loss of v-ATPase function allows FC to accumulate within the lysosomal membrane (10). Excess membrane cholesterol likely inhibits v-ATPase function by exerting physical restrictions on the rotation of the transmembrane motors (207,221,222) by increasing the order and rigidity of the membrane. This assessment, along with the TRP-induced changes in the lipid content of the lysosomal membrane we reported in chapter III (**figures 12 and 15**), forms the foundation of our hypothesis: TG enrichment restores the activity of the v-ATPase by increasing the fluidity of the lysosomal membrane via TRP.

A number of factors can influence the fluidity of cellular membranes, including membrane cholesterol levels and the FA composition of membrane PLs (219,225). Thus, in addition to analyzing foam cell lysosomes for their fluidic properties, we also

investigated the effects of TG on the lysosomal membrane. TRPs deliver TG to the cell where lipases on either the cell surface or in lysosomes can metabolize the TG from TRPs (177,226). Cellular lipids rapidly reincorporate the FAs generated by either of these groups of lipases into cellular lipids, including CE, TG and PLs. Thus, treatment with TRPs could alter the FA composition of membrane PLs by introducing this alternative pool of FAs (227).

During membrane PL synthesis, cellular FAs function as substrates. The acyl chains of cellular PL reflect the available pool of FAs. In turn, the acyl chains of PL can influence membrane properties. For instance, PLs containing shorter acyl side chains and/or acyl side chains with a higher degree of saturation reduce the melting point of cellular membranes and, as a result, reduce membrane fluidity (228,229). We hypothesize that treating macrophages with TRPs alters the PL and FA composition of lysosomal membranes, influencing the fluidity of the lysosomal membrane. These FA-induced changes could explain the alterations in lysosomal v-ATPase activity that occur following treatment with lipoproteins (chapter III, **figure 15**).

Materials and Methods

Membrane Order

We analyzed membrane order by electron paramagnetic resonance (EPR) spectroscopy, as described by Li *et al.* (207). Specifically, we used 5 μl of a 0.28 mM 16-doxyl-phosphatidylcholine (16-doxyl PC) spin label probe (Avanti Polar Lipids, Alabaster, AL) in methanol, adding it to a 250 μl, 1mg/ml solution of isolated lysosomal membrane

fractions in sucrose buffer. In order to allow spin label incorporation into lysosomal membranes, we rotated the solution end-over-end at room temperature for 30 min. We then isolated the lysosomes by ultracentrifugation at 14,000 x *g* for 10 min at 4^{o} C, and transferred the pelleted material to a quartz capillary tube for EPR measurements at 37^{o} C. Using a Bruker Variable-Temperature Unit, (Billerica, MA), we obtained EPR spectra and performed calculations according to published methods (207). As described in the previous chapter, we correlated ESR measurements to measurements of lipid mass, including TG and cholesterol. We carried out the analyses in collaboration with Eric Hustedt and the Vanderbilt University Center for Structural Biology.

FA composition of isolated lysosomes

We extracted lipids using the method of Bligh and Dyer (195), separating individual lipid classes by thin layer chromatography using Silica Gel 60 A plates developed in petroleum ether, ethyl ether, acetic acid (80:20:1) and visualized by rhodamine 6G. We scraped PLs, diglycerides, TGs and CEs from the plates and methylated them using BF_3 /methanol as described by Morrison and Smith (230) and analyzed the methylated FAs were extracted and analyzed by gas chromatography using an HP 5890 gas chromatograph equipped with flame ionization detectors, an HP 3365 Chemstation, and a capillary column (SP2380, 0.25 mm x 30 m, 0.25 µm film, Supelco, Bellefonte, PA). Helium was the carrier gas we used, and we programmed the oven temperature to rise from 160 °C to 230 °C at 4 °C/min. By comparing the retention times to those of known standards we were able to identify FA methyl esters, and we

could quantify the amount of lipid in the sample by including lipid standards with odd chain FAs. We used dipentadecanoyl phosphatidylcholine (C15:0), diheptadecanoin (C17:0), trieicosenoin (C20:1), and cholesteryl eicosenoate (C20:1) as standards.

Results

Although previous studies have proven EPR spectroscopy useful for the measurement of the order of membrane lipids in the endoplasmic reticulum (207), it was necessary to determine whether the technique could be effective for measuring lysosomal membranes. We treated THP-1 macrophages with varying combinations of CE or TG-rich vehicles (aggLDL and VLDL, respectively) to induce varying degrees of cellular and lysosomal cholesterol and TG accumulation. EPR analysis of isolated lysosomal membranes revealed characteristic two component spectra (**figure 16**), with "hyperfine" and "broad" components. The broad spectral component appears in the analysis due to the tendency of the 16-doxyl PC molecules to cluster together, causing strong interactions between spin-labels. The broad component remains constant across all treatments, as sample membrane composition does not influence the interaction between spin labels. However, the hyperfine component of the spectra specifically represents the fluidity of sample membranes. Thus, we focused our analysis on the hyperfine spectral component to determine if there are specific changes in lysosomal membrane fluidity following lipid enrichment.

The EPR curves of lysosomes treated with the various lipids are noticeably different from one another (**figure 16**). Treatment with TRPs (50 μg VLDL protein / ml) in

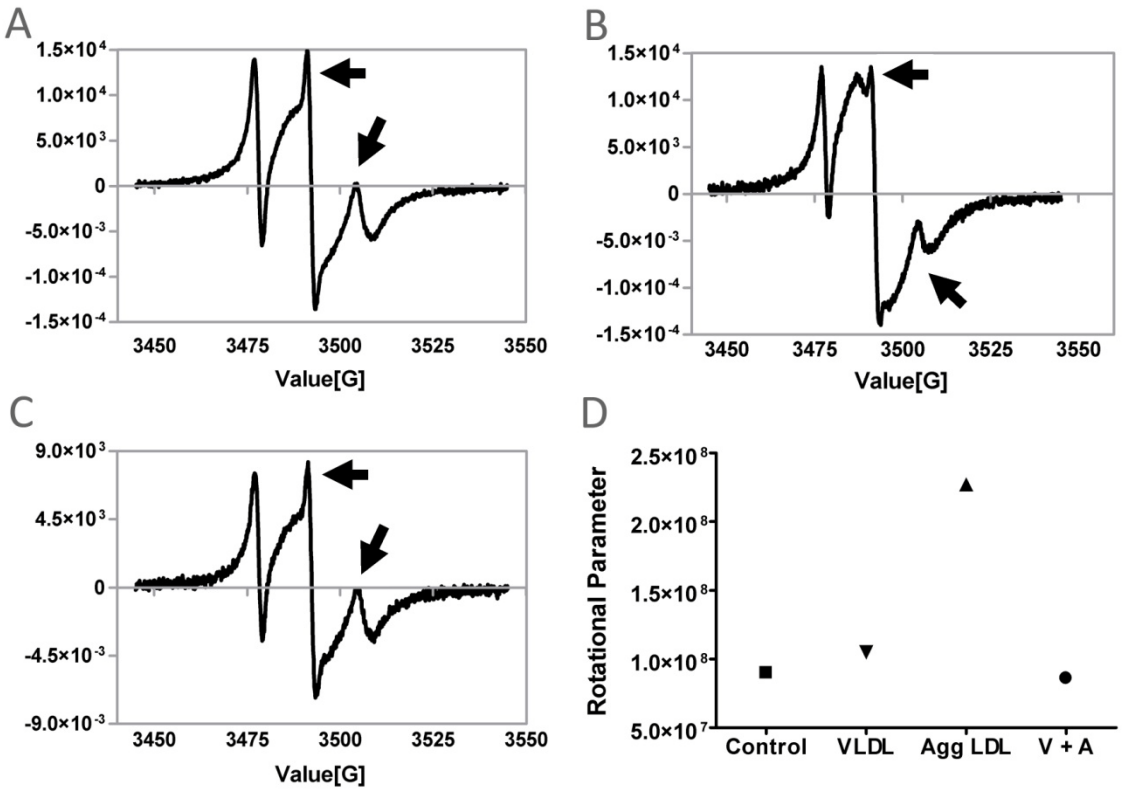


Figure 16: EPR spectra of isolated lysosomal membranes. We treated THP-1 macrophages with media alone (A), aggLDL (50 μg aggLDL protein /ml, B), or aggLDL and VLDL (50 μg lipoprotein / ml) for 6 days (C). Arrowheads indicate the portions of the spectra that are representative of membrane fluidity. Compared to a normal membrane fluidity profile (A), enrichment of lysosomes with cholesterol produced a spectra characteristic of a more rigid membrane, with the loss of integrity of spectral peaks. Coincubation of cholesterol and TG produces lysosomes with normal membrane fluidity. Quantification of EPR rotational parameter following EPR analysis of lysosomes treated with TG, cholesterol or both lipids shows similar membrane fluidity in TG-enriched versus normal lysosomes (D). However, enrichment with cholesterol increases the rotational parameter, indicating a more rigid lysosomal membrane.

combination with cholesterol-enrichment (50 μg aggLDL protein / ml) produced spectral curves indicating normal lysosome fluidity, similar to lysosomes isolated from non-lipid-loaded control cells (**figures 16A and 16C**). This is in sharp contrast to treatment with aggLDL alone (50 μg aggLDL protein / ml), which results in a less defined curve, indicative of a rigid membrane structure (**figure 16B**). Therefore, qualitative analysis of EPR spectra suggests a more rigid lysosomal membrane composition following enrichment with cholesterol, but the presence of TG can maintain normal membrane fluidity during cholesterol loading.

In order to assess quantitative changes in membrane fluidity, we analyzed our EPR spectra to determine the rotational parameter, which is a measurement of the rotational diffusion of the spin label electrons within the lysosomal membrane bilayer. Essentially, the more restricted the spin label is within the membrane, the higher the rotational parameter, indicating reduced movement of the spin label. Analysis of the EPR spectra revealed a 2.5-fold increase in the rotational parameter in cholesterol-enriched cells, indicating a more rigid lysosomal membrane following sterol-enrichment (**figure 16D**). In contrast, when we treated the lysosomes with cholesterol-rich and TG-rich particles simultaneously, the rotational parameter was similar to non-lipid treated cells, indicating no difference in the membrane order (**figure 16D**). This supports our hypothesis that, while membrane fluidity decreases with sterol accumulation, TRP-treatment protects the lysosomal membrane from the cholesterol-induced alterations.

A number of factors can influence membrane fluidity, including membrane cholesterol levels and the fatty acid composition of membrane PL side chains. We have

previously shown a change in lysosomal cholesterol levels upon exposure to TG (shown in chapter III, **figure 12**). However, it is possible that more than one mechanism could contribute to our observed changes in membrane fluidity. For instance, exposure to TRPs may alter the composition of cellular PLs. Thus, we measured the FA composition of PL acyl side chains from isolated lysosomes to determine if there are changes following exposure to lipoproteins. We treated cells for six days with aggLDL and/or VLDL (100 μg lipoprotein / ml) and isolated lysosomes as described previously (chapter III). Results in **table 2** show that each lipoprotein treatment altered the lysosomal FA profile. Both aggLDL and VLDL either alone or together induced a reduction in the percentage of PLs containing oleic acid (18:01) and docosahexaenoic acid (22:06). Similarly, lipid enrichment increased the concentration of linoleic acid (18:02) acyl side chains (**table 2**). In contrast, control and aggLDL treated lysosomes contained PLs with palmitoleic acid (16:01), eicosatrienoic acid (20:3w6) and docosapentaenoic acid (22:05) while those enriched in TG did not (**table 2**).

The results in **table 2** confirm that the various lipid treatments alter the acyl chain composition of membrane phospholipids. Importantly, the composition of membrane PL acyl side chains can greatly influence membrane fluidity. Membranes containing PLs with shorter-chain FAs or a lower degree of saturation are typically more fluid, due to a reduction in the interactions between side chains. However, our analysis showed that lysosomes treated with aggLDL had the highest composition of polyunsaturated FAs, while lysosomes treated with VLDL had the highest composition of saturated FAs (**table 3**). Additionally, we did not observe significant changes in side

Table 2: Analysis of PL acyl side chains from isolated lysosomes. THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or VLDL. The cells were harvested following the 6 day lipid incubation and then lysosomes were isolated. The PL acyl side chain composition was determined as described in Materials and Methods. Values are expressed as percentage of total FA found within the PL fraction. ND = not detected.

	Con	aggLDL	VLDL	V + A
14:00	ND	0.59	ND	ND
16:00	20.68	15.98	21.77	21.61
16:01	2.83	0.86	ND	ND
18:00	16.03	16.90	20.77	18.11
18:01	25.06	14.56	18.71	18.34
18:02	6.07	30.47	24.58	27.09
20:3w6	3.63	2.23	ND	ND
20:04	10.25	11.95	8.84	10.03
22:05	4.10	1.59	ND	ND
22:06	11.37	4.87	5.33	4.82

Table 3: Analysis of PL acyl side chains from isolated lysosomes, characterized by degree of saturation. THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or VLDL. The cells were harvested following the 6 day lipid incubation and then lysosomes were isolated. The PL acyl side chain composition was determined as described in Materials and Methods. Values are expressed as percentage of total FA found within the PL fraction in each sample based on degree of side chain saturation.

	Con	aggLDL	VLDL	V + A
Saturated	36.71	33.47	42.54	39.72
Monounsaturated	27.89	15.42	18.71	18.34
Polyunsaturated	35.42	51.11	38.75	41.94

chain length among the various lipid treatments (**table 4**). This is in sharp contrast to our membrane fluidity data that show a clear increase in membrane fluidity when TG is present and a clear decrease in membrane fluidity following treatment with cholesterol (**figure 16**). Thus, it appears that while the PL-FA composition may exhibit an effect on foam cell membrane properties, the membrane fluidity is highly dependent on the FC content of the lysosomal membrane.

Discussion

Lipids are the major constituent of all biological membranes, and they undergo considerable metabolic turnover, as they are constantly degraded and resynthesized within the cell. When macrophages encounter excessive amounts of dietary lipids, such as those found within the atherosclerotic lesion, the surplus lipids are catabolized completely and are readily incorporated into cellular lipids, including PLs. In foam cells, the substrates for lipid synthesis include TG, FAs and FC released from the hydrolysis of lipoprotein lipids. Thus, exposure of cells to large amounts of lipoprotein-derived lipid can influence the composition of cellular lipids, including the PLs found within cellular membranes. Our results show that under our treatment conditions, FA substrates dissociate and rapidly reincorporate into other cellular lipids in ways that could influence cholesterol metabolism.

Table 4: Analysis of PL acyl side chains from isolated lysosomes, characterized by chain length. THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or VLDL. The cells were harvested following the 6 day lipid incubaton and then lysosomes were isolated. The PL acyl side chain composition was determined as described in Materials and Methods. Values are expressed as percentage of total FA found within the PL fraction in each sample based on the length of the PL side chain. ND = not detected.

		Con	aggLDL	VLDL	V+A
Long Chain Fatty Acids	14 C	ND	0.59	ND	ND
	16 C	23.51	16.84	21.77	21.61
	18 C	47.16	61.93	64.06	63.54
	20 C	13.88	14.18	8.84	10.03
	22 C	15.47	6.46	5.33	4.82

Membrane Fluidity

One way cholesterol metabolism can be affected by the fluctuations in the composition of FAs throughout the cell is through changes in membrane fluidity. Four major factors affect membrane fluidity (**figure 17)**:

- temperature
- membrane PL acyl side chain length
- degree of saturation of the membrane PL acyl side chain tails

  and
- membrane cholesterol content

Since atherosclerotic lesions exist at physiological temperatures, fluctuations in temperature do not play a role in our observed changes in membrane fluidity. However, in foam cells, the macrophages constantly interact with a variety of lipids including TG, cholesterol, CE, PL and, notably, FAs of varying degrees of saturation and side chain length, all of which can alter the other three key factors.

Alterations in membrane PL and/or cholesterol content increase or decrease membrane fluidity by changing the complex interactions between lipids within the bilayer. To understand how these alterations occur, it is helpful to review how lipids interact in a membrane. Electrostatic and hydrogen bonds between head groups and van der Waals forces between the hydrocarbon tails hold together lipids in a membrane (231). Van der Waals attractive forces stabilize the interaction between PL side chains, and the flexibility of the membrane decreases as the number of van der Waals interactions increases (231). For example, lipids with longer tails possess more surface

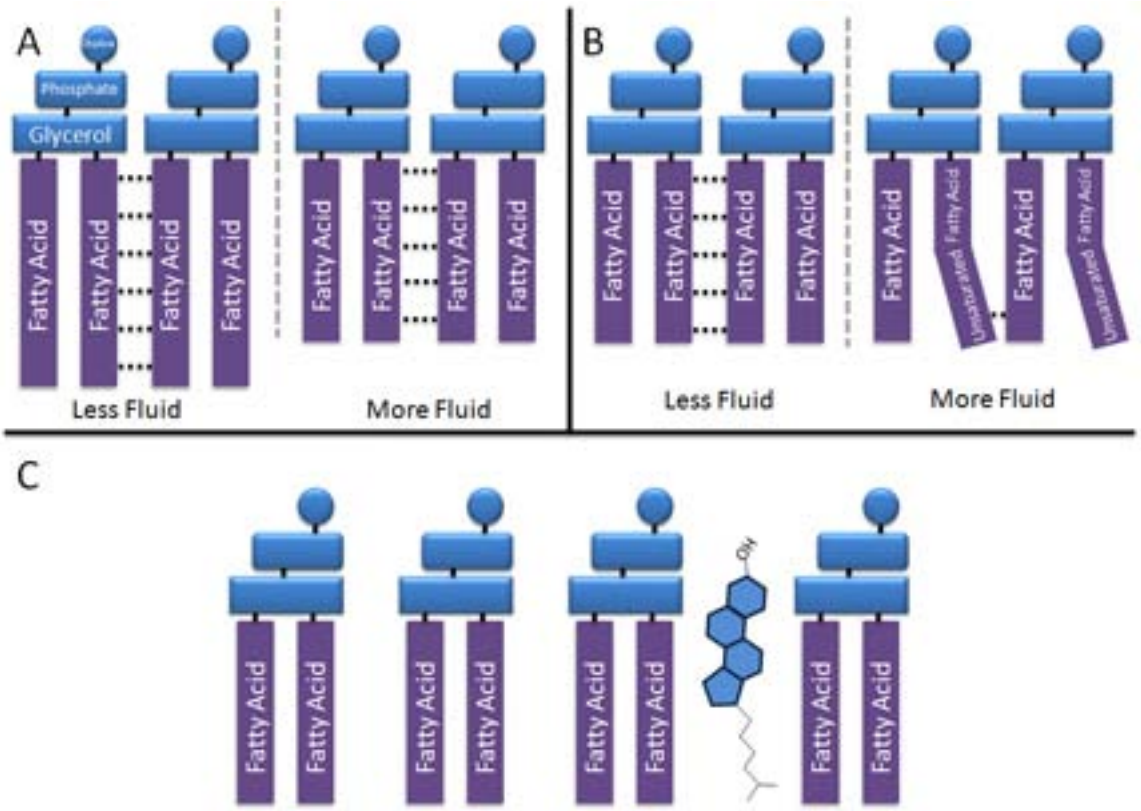


Figure 17: Diagram of PL and/or cholesterol interactions in cellular membranes that contribute to membrane fluidity. A: Chain length is an important determinant of membrane fluidity. Longer-chain FAs have stronger van der Waals forces (represented by dashed lines) than shorter-chain FAs due to increased surface area for interactions. B: Degree of FA side chain saturation influences membrane fluidity. The presence of double bonds in unsaturated FAs introduces kinks in the FA side chains, reducing the area for van der Waals interactions with neighboring PL side chains. C: Increased cholesterol content of the membrane decreases membrane fluidity by strengthening interactions between neighboring PLs.

area to interact with surrounding PLs. Thus, the PL side chains have stronger van der Waals interactions with surrounding lipids and exhibit more drag as they move through the membrane (**figure 17**)(231). However, the studies presented here show that the lysosomal membrane is composed of predominantly long-chain FAs (LCFAs)(**table 4**). Therefore, it does not appear that changes in PL side chain length explain the alterations in membrane fluidity we observe with lipid enrichment of lysosomes.

The degree of saturation of the PL acyl side chains is another important contributor to membrane fluidity. The extent of PL saturation affects membrane fluidity because unsaturated lipid tails, which contain one or more double bonds, are not able to pack as tightly as saturated lipid tails, which do not have double bonds (**figure 17**). The "kinks" present in unsaturated FA side chains reduce the attractive van der Waals forces between lipids in the membrane and, thus, increase membrane fluidity. We analyzed changes in saturation by comparing control lysosomes to cholesterol enriched lysosomes. We found more PUFAs in cholesterol enriched lysosomes and more saturated FAs in TG enriched lysosomes (**table 3**). Thus, if the degree of FA saturation was the key determinant of membrane fluidity, we would expect that TG-enriched lysosomes would be stiffer. This is the opposite of what we observe with EPR analysis. Thus, the changes we observe in PL acyl side chain composition are inconsistent with the hypothesis that degree of PL acyl chain saturation is a major determinant of lysosomal membrane fluidity in foam cells, at least in hypercholesterolemic cells.

Altogether, the results of lysosomal PL side chain analysis and EPR analysis suggest that, in hypercholesterolemic cells, cholesterol may have a stronger influence

on lysosomal membrane fluidity than any effect of PL acyl chain saturation. Cholesterol inserts itself into biological membranes and fits into gaps between membrane PL side chains, where the steroid ring interacts with and immobilizes PL fatty acids (231). Thus, at physiological temperatures, the strong, additional van der Waals forces between cholesterol and membrane PLs would decrease membrane fluidity by stabilizing the interactions between membrane PLs. In chapter III, we showed significant changes in lysosomal cholesterol following lipid enrichment. In particular, treatment with cholesterol-rich lipoproteins doubles the lysosomal cholesterol content, which correlates to the decrease in lysosome membrane fluidity observed in our EPR analyses (chapter III, **figure 12 and figure 16**). Importantly, we showed a 33% reduction in lysosomal cholesterol upon enrichment of foam cells with TG (chapter III, **figure 12**). This reduction in the cholesterol content of the lysosome and, specifically in the lysosomal membrane, would dramatically increase the fluidity of the lysosomal membrane.

v-ATPase Activity

In order to deal with stresses such as increases in membrane cholesterol, cells have developed a number of mechanisms to achieve constant membrane fluidity. Such mechanisms consist of the regulation of membrane lipid composition. For example, increased PL synthesis can counteract an increase in membrane cholesterol. The changes in membrane PL content following lipoprotein treatment suggest that PL synthesis remains active following lipid enrichment, and could potentially serve as a

compensatory mechanism in response to the substantial influx of lipids. However, given the abnormally high lipid levels present in a macrophage foam cell, it appears that it is difficult for the cell to effectively compensate for the substantial increases in membrane cholesterol. As a result, the membrane lipids become restricted resulting in the inhibition of integral membrane protein function. We hypothesized that in the macrophage foam cell, the inhibition of v-ATPase rendered the lysosome incapable of degrading endocytosed-lipoproteins, resulting in a large accumulation of lipids. Therefore, the ability of TG to restore lysosomal membrane fluidity and function could significantly improve the ability of foam cells to process and mobilize lipoproteins. This would enhance the ability of atherosclerotic foam cells to efflux cholesterol, resulting in increased reverse cholesterol transport and potential lesion regression. Our data support this assessment, as treatment of cholesterol-enriched macrophage foam cells with TG restores lysosomal v-ATPase activity (chapter III, **figure 15**).

A number of potential mechanisms could contribute to the increase in proton pumping including:

- Increased v-ATPase protein expression
- increased v-ATPase assembly at the lysosomal membrane
- increased recruitment of v-ATPases to lysosomes

  and/or

- changes in the v-ATPase membrane environment

We have previously shown similar v-ATPase expression in cholesterol-enriched foam cells versus normal macrophages. This suggests that expression of v-ATPases in

macrophages remains unchanged regardless of lipid loading (10), and indicates that the first mechanism is not relevant to our study. Additionally, the cellular localization of v-ATPases and the recruitment of subunits to lysosomes were unchanged in foam cells compared to normal lysosomes, suggesting that the v-ATPases are present and appropriately assembled in lipid engorged lysosomes. This seems to rule out the second and third mechanisms. Finally, v-ATPase activity is significantly impaired in foam cells (10), which points to changes in the v-ATPase membrane environment as the dominant mechanism in the regulation of lysosomal function.

The activity of integral membrane proteins, including v-ATPase, is very sensitive to membrane fluidity. Previous studies have shown that a number of ATPases with similarities to the lysosomal v-ATPase are greatly affected by the FC content of their membrane environment (220). For example, FC enrichment of ER membranes inhibits the activity of the macrophage ER calcium pump, sarcoplasmic-endoplasmic reticulum calcium ATPase-2b (SERCA2b) (207). Importantly, the inhibition of SERCA2b activity occurred in macrophage foam cells with similar lipid levels to the foam cells examined in the current study (207). Thus, our results are consistent with previous studies suggesting that changes in organelle membrane fluidity in macrophage foam cells greatly influence the activity of integral membrane proteins.

Further Studies

The quantitative measurements presented in this chapter involved analysis of the rotational parameter, which is one of many quantitative measurements of EPR spectra that we would require to reach a conclusion about membrane fluidity. Two

other very important values are the order parameters (S_0 and S_2). Order parameters are indicators of the degree rotation and amount of "wobble" of the spin label within the lipid bilayer. Measurement of order parameter in our lipid enriched lysosomes tended to be variable with no clear definition between various lipid treatments. S_0, which should be a positive value, is negative in many of our analyses, indicating random motion of the unpaired electron moiety within the bilayer. Because of this random motion, we believe the difficulties obtaining reliable order parameter measurements are likely due to the location of the doxyl group on the spin label PL. In the case of 16-doxyl PC, the moiety with the unpaired electron sits deep within the membrane bilayer and its motion is less restricted. Thus, its position in the bilayer results in less sensitivity to changes in membrane fluidity upon cholesterol and TG enrichment. Future studies will be performed using alternative spin labels to probe different regions of the bilayer (**figure 18**). However, the preliminary studies presented in this chapter show promising evidence suggesting that enrichment with cholesterol-rich lipoproteins increases lysosomal membrane stiffness while enrichment with TRPs reverses such changes, resulting in normal membrane fluidity.

Conclusion

Although there are some reservations, the results presented in this chapter appear to strongly suggest a relationship between lysosomal v-ATPase activity and lysosomal membrane fluidity. Increased membrane cholesterol leads to protein aggregation, increased lipid bilayer thickness, separation of lipid domains within the

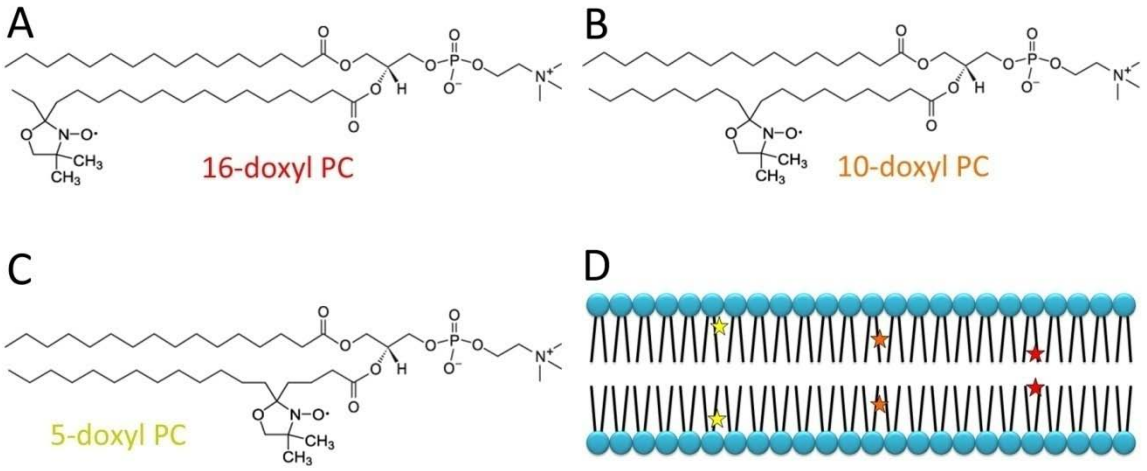


Figure 18: Structure of the doxyl-PC spin label probes used for analyzing membrane fluidity. Panel A depicts the structure of 16-doxyl-PC that was used in the ESR experiments described in this chapter. Panels B and C indicate the structure of alternative PC-derived spin labels with different electron positioning on the PL fatty acids. Panel D depicts the location of the doxyl moiety within the lipid bilayer with red representing 16-doxyl-PC, orange representing 10-doxyl-PC and yellow representing 5-doxyl-PC.

bilayer as well as restriction of the conformational freedom of proteins (220,232-234), and the FC-induced inhibition in SERCA2b activity in the ER of foam cells is likely due to the physical restriction of conformational changes required for functional ATPase activity (207). Given the similarities in lipid content of the ER from the Li *et al*. study and the lipid content of the lysosomes shown here, it is likely that the activity of both ATPases are regulated through similar mechanisms. Thus, increases in lysosomal cholesterol content introduce physical limitations on the v-ATPases, preventing them from functioning (**figure 19**). The transmembrane domain of both SERCA2b and the lysosomal v-ATPase consists of a "rotary motor" which requires rotation of the transmembrane domain for the translocation of protons into the luminal space (218). In the case of v-ATPase, the rotation of the transmembrane domain generates a proton motive force leading to the pumping of two hydrogen ions into the lysosomal lumen (217). Reduced membrane fluidity can restrict the rotational movement of the transmembrane domain (207,221,222). Accordingly, the current studies show an apparent correlation between the fluidity of the lysosomal membrane, lysosomal cholesterol content and v-ATPase activity.

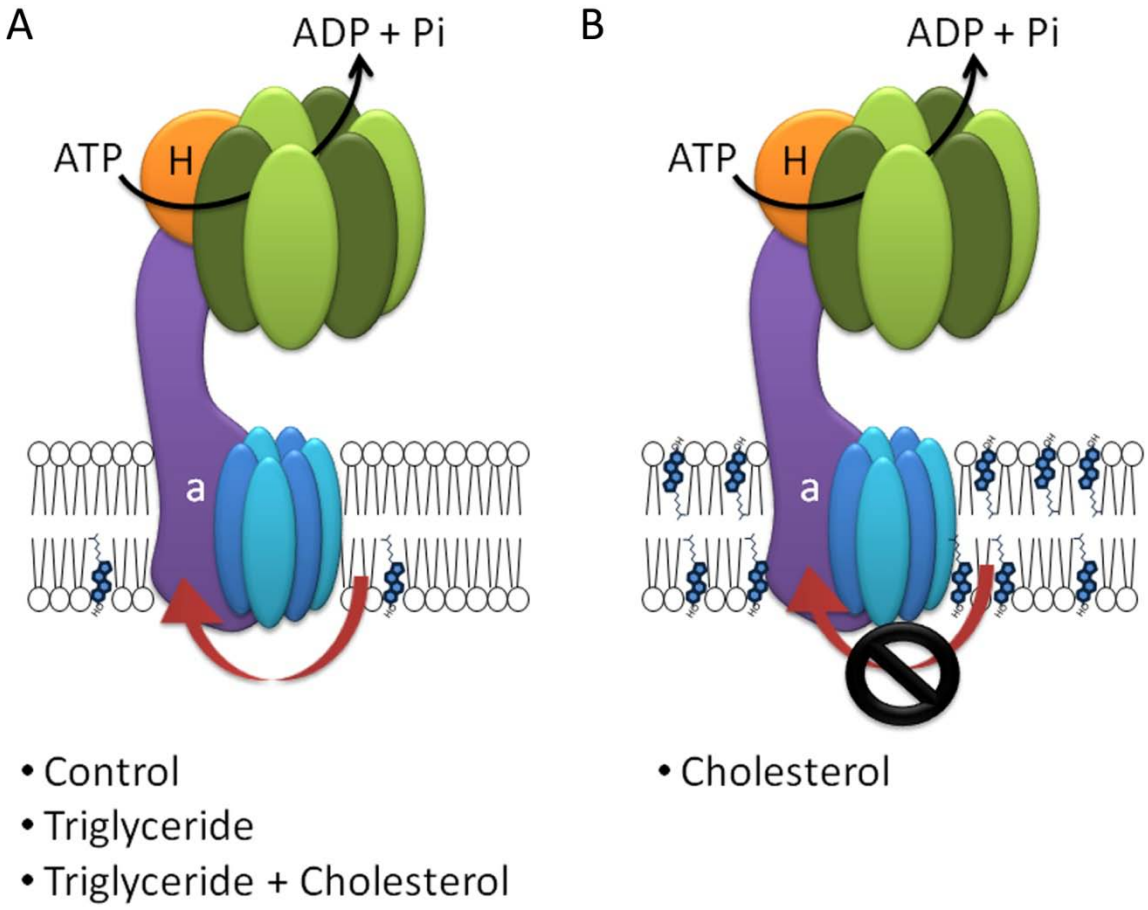


Figure 19: Summary of the effects of lipid enrichment on membrane fluidity and v-ATPase activity. Compared to lysosomes from control cells (A), enrichment of the lysosomal membrane with cholesterol results in a decrease in membrane fluidity that restricts the rotation of the transmembrane domain of the lysosomal v-ATPase (B), rendering the v-ATPases inactive. Treatment of foam cells with TG restores lysosomal membrane fluidity and v-ATPase activity.

CHAPTER V

EFFECTS OF FATTY ACIDS ON MACROPHAGE CHOLESTEROL METABOLISM

Enrichment of macrophage foam cells with TG mobilizes lysosomal and cellular cholesterol. Moreover, studies in chapter IV show significant changes in the FA composition of cellular lipids. Although we were unable to show a correlation between PL acyl chain composition and our observed alterations in lysosome function, it remains plausible that FAs generated from lysosomal and/or surface hydrolysis of TRPs could influence other aspects of foam cell cholesterol metabolism. Thus, this chapter examines the effects of FAs on overall cellular cholesterol content.

Introduction

Because of their high TG content, VLDL and CMs are the primary transporters of dietary fat and are highly concentrated stores of metabolic energy. The lipolysis of TG within a cell releases glycerol and FAs. The released FAs are degraded in a stepwise oxidative process (β-oxidation) that generates metabolic energy, the products of which are either used as substrates for cellular lipid synthesis or act as important activators of many cellular signaling pathways. Thus, TGs and hydrolytically released FAs represent a dynamic lipid pool that can greatly affect both the composition and turnover of cellular lipids. Given the rapid utilization of TG by peripheral cells, the flux of TG through macrophages may serve an important role in the mobilization of lysosomal and cellular

cholesterol rather than simply leading to the accumulation of intracellular TG. Therefore, it is important to consider the role of FAs generated from lipolysis of TRPs in the mobilization of intracellular cholesterol.

FAs released following TG hydrolysis elicit important metabolic effects with the potential to influence atherogenesis, including activation of cholesterol and FA homeostatic signaling pathways. FAs modulate LDL receptor activity, ACAT activity, and the distribution of intracellular cholesterol (235-241). Additionally, FAs regulate the expression of genes involved in lipid and energy metabolism (**table 5**). FAs generated by lysosomal or extralysosomal hydrolysis of TG regulate the activity of several transcription factors important in cellular lipid homeostasis, including the nuclear receptors PPAR, LXR, and hepatocyte nuclear factor 4 alpha (HNF-4α) (180,185-192). By regulating the activity of these nuclear receptors, FAs control the expression of downstream cholesterol homeostatic genes, including cholesterol efflux transporters (ABCA1, ABCG1, SR-BI) and apoproteins (apoA1, apoE, apoCII, apoCIII) (**table 5**).

FAs acquired from dietary sources also serve as important substrates for intracellular lipid synthesis. One of the most significant roles of FAs within cells is the synthesis of biological membranes. FAs are incorporated into membrane PLs by transfer to a glycerol backbone in a process mediated by the enzymes acyl-CoA:glycerol-3-phosphate (GP) acyltransferase and acyl-CoA:l-acyl-GP acyltransferase (242,243). Similarly, FAs can be utilized for TG synthesis by diacylglycerol acyltransferase (DGAT) and CE synthesis via the activity of ACAT (244,245). Both TG and CE are present in cytoplasmic lipid droplets from advanced macrophage foam cells.

Table 5: Cholesterol homeostatic genes regulated by FAs.

Nuclear Receptor	Ligand(s)	Target Gene(s)
PPARs	Saturated FAs	LPL
	Unsaturated FAs	CD36
	Omega-3 FAs	Cyp4a, 4b
	Conjugated Linoleic Acids	ABCA1
	LPL-treated VLDL	ABCB4, ABCD2, ABCD3
	VLDL	LXR (α + β)
LXRα, β	Unsaturated FAs (antagonizes)	ABCA1
	PUFAs (minimally)	ABCG1
	Oxysterols	apoE
		ABCG5
		ABCG6
		SREBP-1c
		Cyp27A1
		SR-B1
HNF-4α, γ	Saturated FAs (agonize)	apo CII
	Monounsaturated FAs (antagonize)	apo CIII
	PUFAs (antagonize)	apo AI
		apo AII
		apoAiV
		Cyp7a1

In the previous chapters, we showed an effect of TG on cellular cholesterol levels. However, it is unclear if TRPs reduce cellular cholesterol via direct effects of TG on cholesterol metabolism or through metabolic byproducts of TG. Therefore, the studies presented in this chapter sought to determine if treatment with FA could induce lysosomal and cellular cholesterol mobilization similar to TG.

Materials and Methods

Materials

Tetrahydrolipstatin (orlistat), oleic acid (OA), linoleic acid (LA), and palmitic acid (PA) were purchased from Sigma-Aldrich (St. Louis, MO).

Orlistat Treatment

To inhibit the surface hydrolysis of VLDL TG, cells were treated with the lipoprotein lipase (LPL) inhibitor Orlistat. Three hours prior to lipid loading, cells were treated with 10 μM Orlistat in ethanol or vehicle control (100% ethanol). Following the three-hour preincubation, cells were treated with lipoproteins (VLDL and/or aggLDL, 100 μg lipoprotein / ml media) for 24 hours. The concentration of Orlistat was maintained throughout lipid loading.

Preparation of FA-BSA Conjugates

FA-albumin conjugates were created using a modification of a procedure described previously (246). Briefly, OA, LA, and/or PA (12.0 mg each) were dissolved in 5

ml of 95% ethanol and were titrated with 1 N NaOH to a phenolphthalein end point (pH > 10). The ethanol solution was evaporated to dryness under nitrogen with warming at 37 °C. Bovine serum albumin (FA poor, Sigma, 4.25 g) was dissolved in 25 ml of 0.9% NaCl for a final solution of 17% albumin. The pH was adjusted to 7.4 with a few drops of 1 N NaOH. A volume of 17% albumin solution was added to the dry, warm FAs for a final molar ratio of 6:1 FA:albumin. OA and LA solutions were incubated at 40 °C for 2 hours in a water bath with gentle shaking. PA required stirring during the 40 °C incubation to prevent precipitation.

Cell Culture and Lipid Loading of THP-1 Macrophages

THP-1 macrophages were maintained as described in chapter II. To measure lipid loading, macrophages were incubated for 0 - 6 days at 37 °C in culture medium containing 1% FBS with or without lipid particles, including aggLDL as a cholesterol source and OA-, LA-, and/or PA-BSA conjugates as a source of FA. In some experiments, only one type of particle was employed, while in other experiments both CE-rich particles (aggLDL) and FAs (OA, LA, and/or PA) were used either simultaneously or sequentially. In sequential treatments for pulse-chase experiments, the pulse media was removed after three days of treatment and cells were washed briefly with 1% FBS prior to addition of the chase media for an additional three days. Concentrations and specifics of incubation order are described for each experiment. The lipid-loading medium was changed every 3 - 4 days with fresh medium containing the cholesterol or FA loading vehicle. Lipids and proteins were quantified as described in chapter III.

Results

VLDL TG can be hydrolyzed at the cell surface by lipases and the FAs released by this hydrolysis are transported into the cell where they are resynthesized into cellular lipids, primarily TGs (174,226,247), resulting in intracellular TG accumulation. To determine if surface hydrolysis of TG influences cellular cholesterol levels, we used tetrahydrolipstatin (Orlistat, 10 μM) to inhibit LPL activity. In cells receiving Orlistat treatment, any cellular TG accumulation must occur through whole-particle uptake via the endosomal-lysosomal pathway. Treatment with TG-DISP in the presence of Orlistat had no effect on the accumulation of TG in the cell (**figure 20B**). Thus, TG-DISP enter the cell as whole particles via the endosomal-lysosomal system. Accordingly, Orlistat treatment did not inhibit the ability of TG-DISP to reduce cellular cholesterol accumulation in experiments where both TG-DISP and CE-DISP were included (**figure 20A**). In contrast, when TG was delivered as VLDL, Orlistat did exhibit an effect on cholesterol accumulation from aggLDL. Consistent with previous studies (174,226), treatment of macrophages with VLDL in the presence of Orlistat reduced cellular TG accumulation by 60% (**figure 20D**). In cells coincubated with VLDL and aggLDL, the Orlistat-mediated reduction in cellular TG resulted in an increase in cellular cholesterol levels, similar to that observed with aggLDL treatment alone (**figure 20A**). Thus, the uptake of FAs, such as those generated by surface hydrolysis of VLDL TGs, could play a role in the TRP-mediated clearance of cholesterol. However, Orlistat produced a significant reduction in cellular TG. Thus, it is not clear whether the effect of FAs on

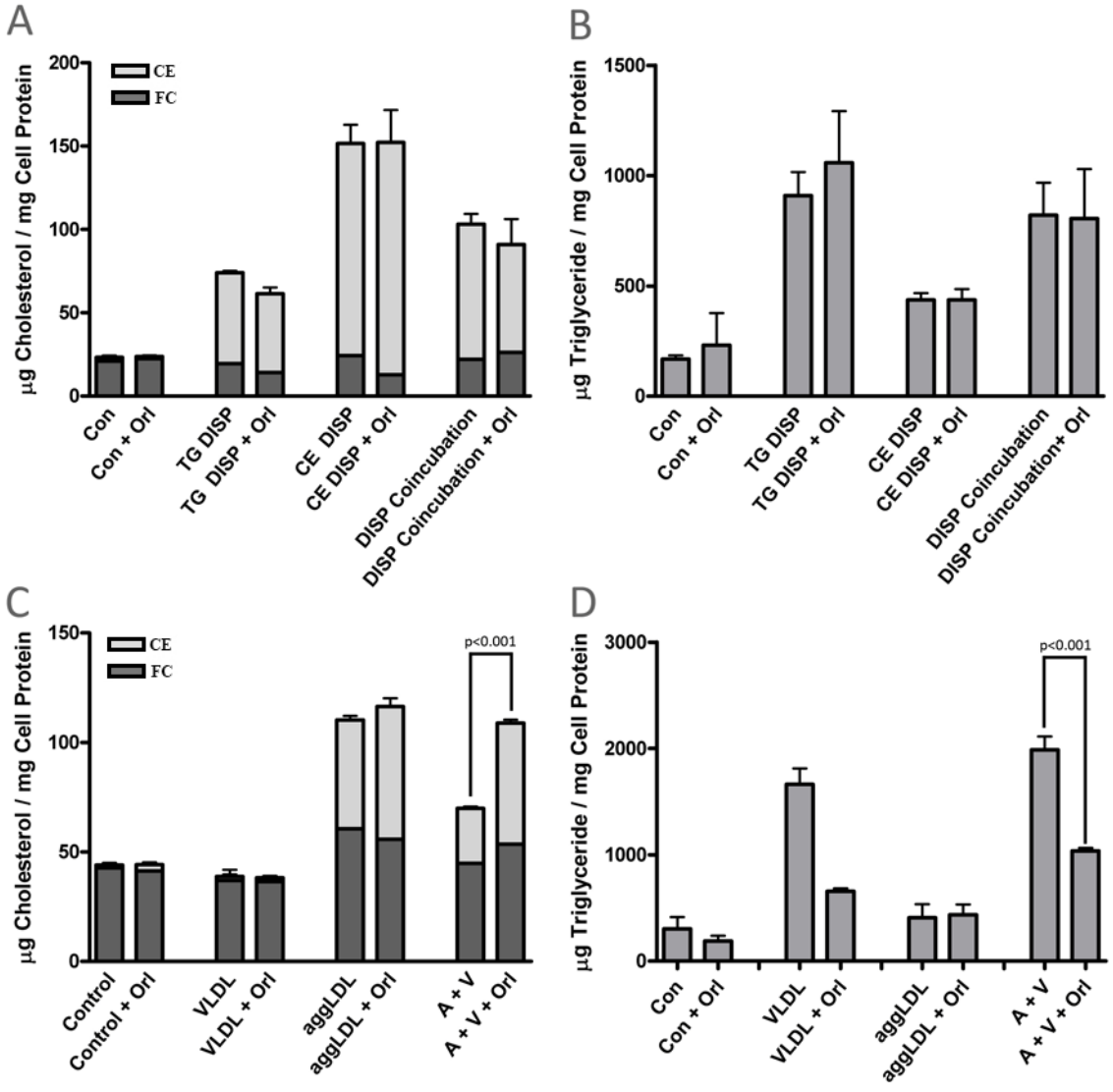


Figure 20: Accumulation of lipids in THP-1 macrophages incubated with TRPs and cholesterol-rich lipoproteins with and without LPL activity. Cells were either treated with TG-rich and/or CE-rich dispersions (A and B) or VLDL and/or aggLDL (C and D) and the cellular total cholesterol (A and C) or total TG (B and D) determined. Incubations were done in the absence or presence (Orl) of 10 μM tetrahydrolipstatin (Orlistat). When cells were lipid loaded using dispersions (DISP), inhibition of surface TG hydrolysis by LPL had no effect on either cellular cholesterol or TG accumulation during any of the treatment regimens (A and B). In contrast, Orlistat treatment reduced the ability of VLDL to promote clearance of cellular cholesterol from aggLDL, potentially by significantly reducing cellular TG levels (C and D). Values are the mean ± SEM for three experiments.

cellular cholesterol metabolism is direct or mediated through their reincorporation into cellular TG.

To further define the effects of FAs on cholesterol metabolism, we treated macrophages with FA-albumin complexes in the presence or absence of cholesterol-enrichment to determine if treatment with FAs could induce the clearance of cholesterol. Treatment of THP-1 macrophages for three days with OA-BSA complexes (250 μM OA +/- 50 μg/ml aggLDL) enhanced cholesterol clearance, as evidenced by a reduction in cellular cholesterol levels (**figure 21A**). OA was rapidly incorporated into TG within the cell and the cholesterol-reducing effect correlated with cellular TG levels (**figure 21B**). Importantly, OA is a monounsaturated FA that comprises approximately 45% of our dietary fat (248). However, macrophages in a lesion are exposed to a range of FAs, including saturated, monounsaturated, and polyunsaturated species. Therefore, our initial analysis was limited in that it only examined the effects of one FA species on cellular cholesterol levels. To determine if the effects on cellular cholesterol were related to the degree of saturation of the FA, we performed studies using two additional FAs that are prevalent in circulating human plasma: LA, a polyunsaturated FA, and PA, a saturated FA. LA treatment (250 μM) produced similar cholesterol-lowering effects as OA (250 μM, **figure 21B**), and both induced similar increases in cellular TG (**figure 21B**). However, PA did not reduce the cholesterol content of the cells (**figure 21A**). Thus, unsaturated FAs may favorably influence cholesterol metabolism and efflux while saturated FAs do not.

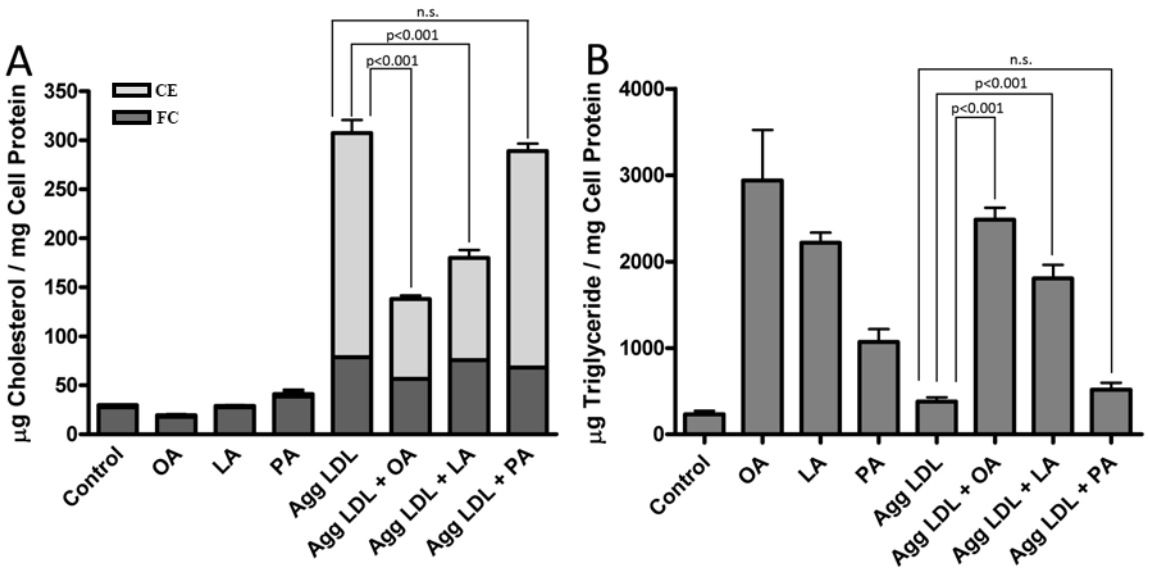


Figure 21: Accumulation of lipids in THP-1 macrophages incubated with aggregated LDL (aggLDL) and/or oleic acid (OA), linoleic acid (LA) or palmitic acid (PA). THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 μg protein/ml of aggLDL and/or 250 μM FA. The cells were harvested and the cellular lipid levels were determined as described in Materials and Methods. A: Incubation of THP-1 with aggLDL produced a dramatic increase in total cellular cholesterol observed primarily as a significant increase ($p<0.05$) in CE (light gray portion of bar). Incubation with both aggLDL and OA or LA reduced the cellular CE accumulation compared to that observed with aggLDL treatment alone. In contrast, coincubation with aggLDL and PA did not affect cellular cholesterol levels ($p>0.05$). Dark gray bars indicate FC levels, which were only minorly affected by FA enrichment. B: Concubation of cells with OA or LA with aggLDL produced a significant increase ($p<0.001$) in cellular TG levels compared to aggLDL-treated cells. However, coinubation of PA and aggLDL did not significantly increase cellular TG levels compared to aggLDL-treated cells ($p>0.05$). Values are the mean ± SEM for three experiments (n.s. = not significant).

The variance in the ability of FAs to mobilize cellular cholesterol appears to depend on the ability of each FA to be incorporated into cellular TG. PA is a poor substrate for TG synthesis while OA and LA are preferentially incorporated into cellular TG (249-255)(**figure 21B**). We hypothesized that the effects of FA on cellular cholesterol levels are dependent on their ability to be synthesized into cellular TG. To address this hypothesis, we treated cells with a mixture of three FAs (PA, OA, and LA). Coincubation of PA with unsaturated FAs results in the incorporation of PA into cellular TGs (250). Therefore, we anticipated that treatment with the mixture of three FAs would increase cellular TG and induce the mobilization of intracellular cholesterol. Consistent with our hypothesis, treatment with the mixture of FAs reduced cellular cholesterol to a similar extent as treatment with OA and LA individually (**figure 22A**). This correlated with an increase in cellular TG to similar levels observed with unsaturated FA treatment (**figure 22B**). The FA-mediated reduction in cellular cholesterol levels appears to occur in correlation with cellular TG levels, indicating that *de novo* synthesis of TG is important for eliciting the cholesterol-reducing effects of FAs.

Although PA did not have an effect on cellular cholesterol levels, it could still elicit an effect on intracellular cholesterol trafficking. We hypothesized that PA could mobilize lysosomal cholesterol, but following its release, the lysosomal FC liberated by PA would be subject to ACAT reesterifcation to PA in the cytoplasm. Thus, the liberated lysosomal FC would not leave the cell but would be stored as cytoplasmic CE. To determine if PA mobilizes lysosomal cholesterol, we treated cells with PA for six days and then chased with the cholesterol acceptor β-cyclodextrin (β-CD) for 24 hours.

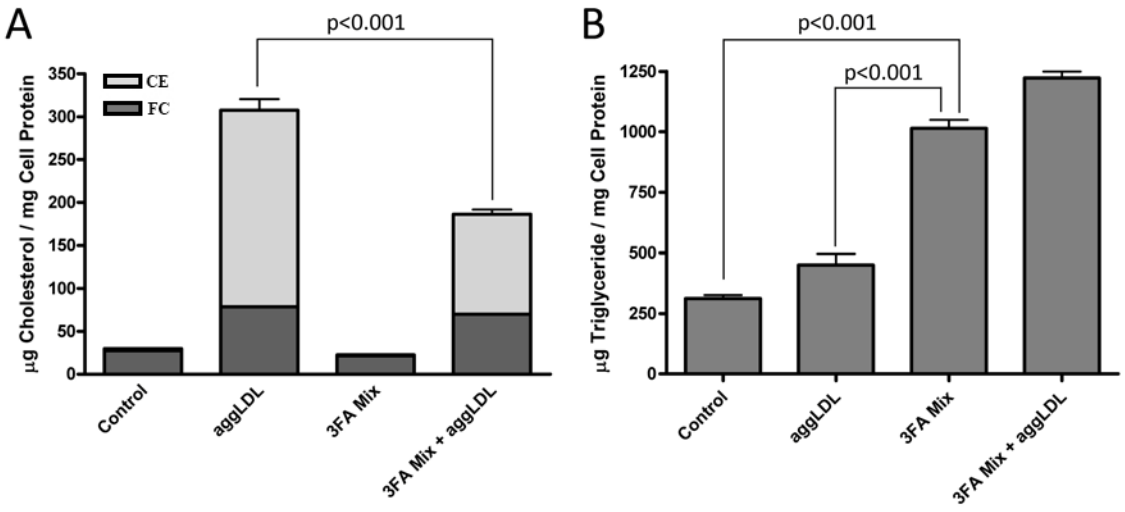


Figure 22: Accumulation of lipids in THP-1 macrophages incubated with aggLDL and/or a mixture of OA, LA, and PA (3FA Mix). THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or a mixture of 250 µM of each FA (750 µM total concentration). The cells were harvested and the cellular lipid levels were determined as described in Materials and Methods. A: Incubation of THP-1 macrophages with the 3FA Mix significantly decreased total cholesterol and CE (light gray portion of bar) compared to treatment with aggLDL alone ($p<0.001$). B: Treatment with the 3FA Mix increased cellular TG levels compared to non-lipid loaded control cells and treatment with aggLDL alone. The results of the 3FA mixture treatment are similar to the results achieved with individual treatment of the unsaturated FAs (OA and LA, **figure 21**). Values are the mean ± SEM for three experiments.

Treatment with β-CD removes all cellular cholesterol with the exception of the sterol sequestered in lysosomes (13). Thus, if PA mobilizes cholesterol from the lysosome to the cytoplasmic lipid droplets, it will be in a pool accessible for removal from the cell via cholesterol efflux to β-CD. In contrast, if PA does not mobilize lysosomal cholesterol, it will not be available for efflux to β-CD and we would not observe an effect on cellular cholesterol levels following treatment with β-CD. Treatment of cells for six hours with aggLDL and PA, followed by a 24-hour β-CD chase, resulted in the clearance of a significant amount of cellular cholesterol (**figure 23A**). Thus, treatment with PA mobilizes lysosomal cholesterol, similar to unsaturated FAs. However, following its release, lysosomal sterol is reesterified to PA and stored as cytoplasmic CE droplets. The effect of FAs on lysosomal and cellular cholesterol metabolism appears to be complex and involves multiple steps, including generation of FAs and *de novo* TG synthesis.

Discussion

TRP-derived TG can enter macrophages through a number of pathways, including surface hydrolysis (LPL), receptor-mediated endocytosis (VLDL receptor and LRP), and/or whole particle phagocytosis (174,226,247)(**figure 24**). Since the mechanism of particle uptake could influence the ability of TG to alter macrophage cholesterol metabolism, we sought to define how the TRPs used in our studies were internalized by THP-1 macrophages. Consistent with previous studies, approximately 60% of intracellular TG in the macrophage was derived from surface hydrolysis of the VLDL TGs, as evidenced by a reduction in cellular TG upon treatment with the LPL-inhibitor,

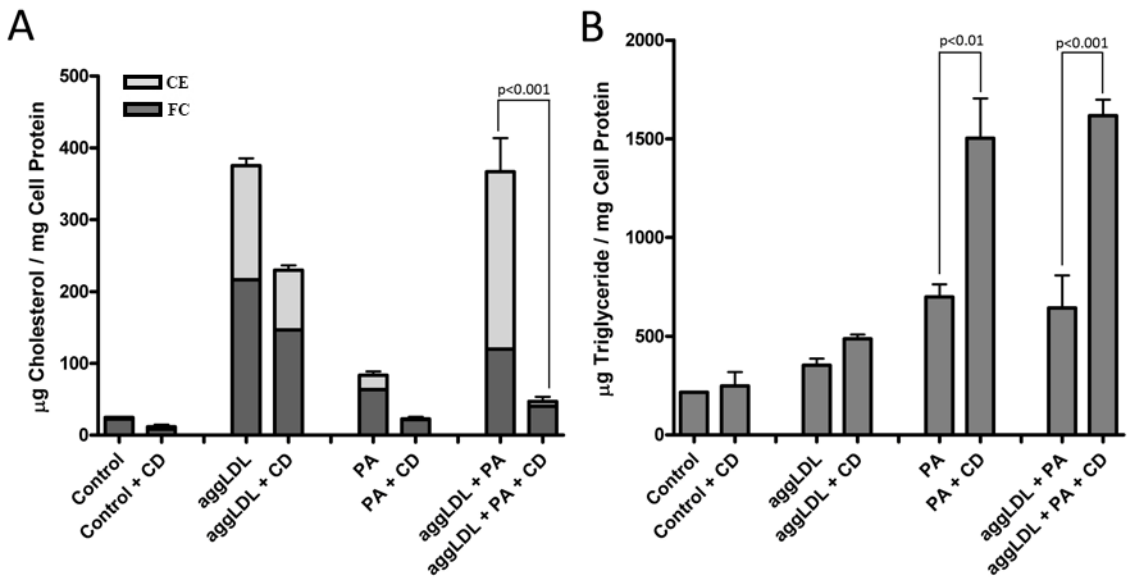


Figure 23: Accumulation of lipids in THP-1 macrophages incubated with aggLDL and/or PA with (+CD) or without a 24 hour chase with β-CD. THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or 250 µM PA. Following the 6 day incubation, cells were chased for 24 hours with 1% media alone or containing β-CD. The cells were harvested and the cellular lipid levels were determined as described in Materials and Methods. A: Incubation of THP-1 macrophages with PA (250 µM each, 6 days) followed by a β-CD chase decreased free cholesterol (dark gray portion of bar) and cholesterol ester (light gray portion of bar) compared to treatment with aggLDL ($p<0.001$). This suggests that the cholesterol in PA treated cells is accessible to cholesterol efflux mechanisms. B: Cells incubated with PA alone or in combination with aggLDL followed by β-CD treatment exhibited higher TG levels compared to similar treatment groups that did not receive a β-CD chase. Values are the mean ± SEM for three experiments.

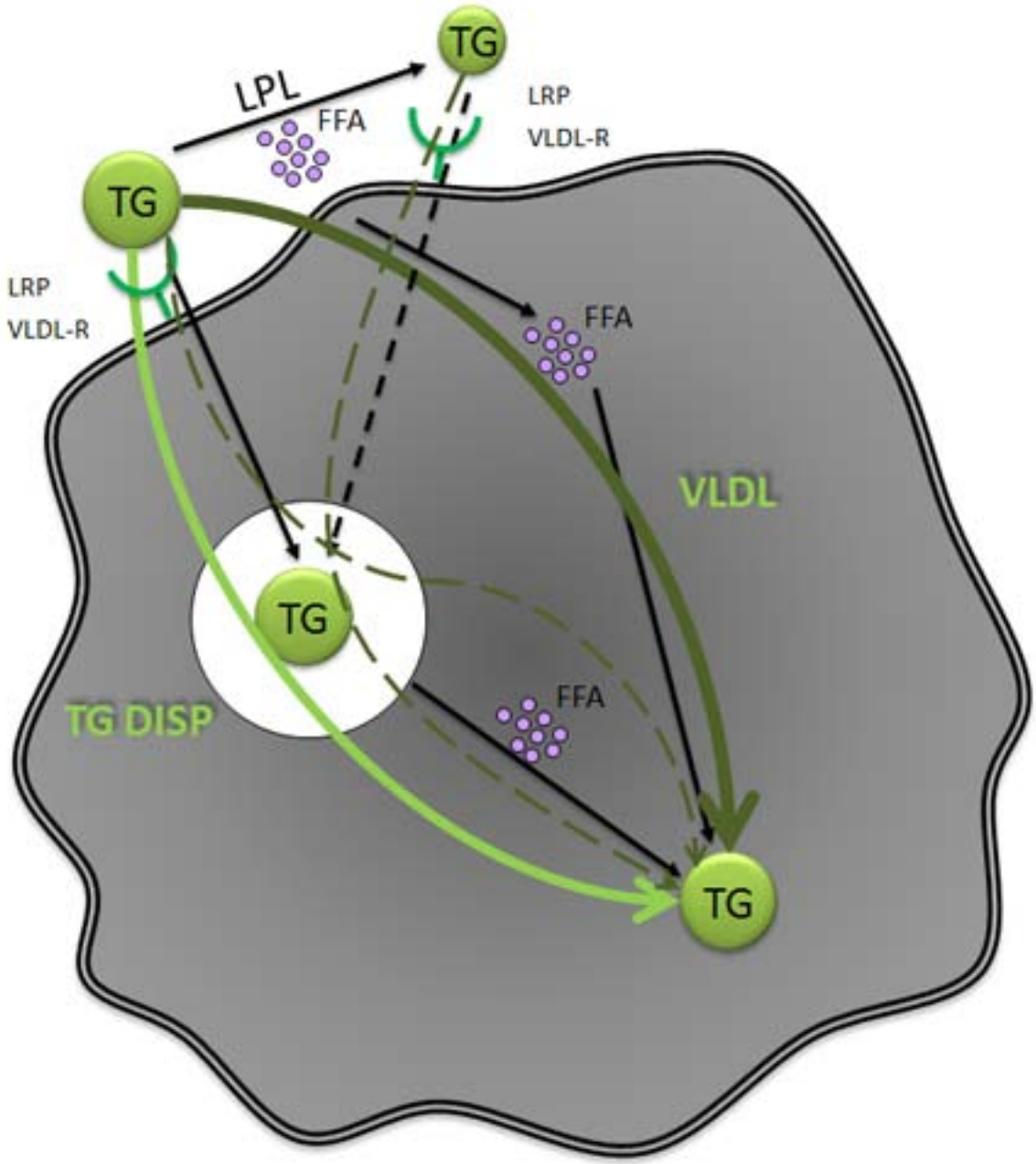

TG
LPL
FFA
LRP
VLDL-R
TG
LRP
VLDL-R
FFA
VLDL
TG
TG DISP
FFA
TG

Figure 24: TRPs enter macrophages via a number of pathways. TG-DISPs (light green) are internalized predominantly as whole particles (without prior hydrolysis of their TG) and are delivered to the lysosome for hydrolysis (light green arrows). The uptake of TG DISP occurs primarily through whole particle phagocytosis. In comparison, TG from VLDL (dark green arrows) enters the cell via three distinct pathways: whole-particle receptor-mediated endocytosis (LRP, VLDL-R), hydrolysis of TG via LPL and internalization of FA, and receptor-mediated endocytosis (LPL, VLDL-R) of remnant VLDL after initial processing via LPL. Endocytosed particles are delivered to the lysosome where their TGs are degraded by lipolyic enzymes, including LAL. The FAs generated from this lipolysis are transported to the cytoplasm. Additionally, FAs generated from surface hydrolysis of VLDL are transported into the cell. FAs generated by lysosomal and/or surface TG hydrolysis are utilized by the cell as substrates for lipid synthesis, including the TG. Each of these intracellular TG and FA lipid pools could influence cellular cholesterol metabolism.

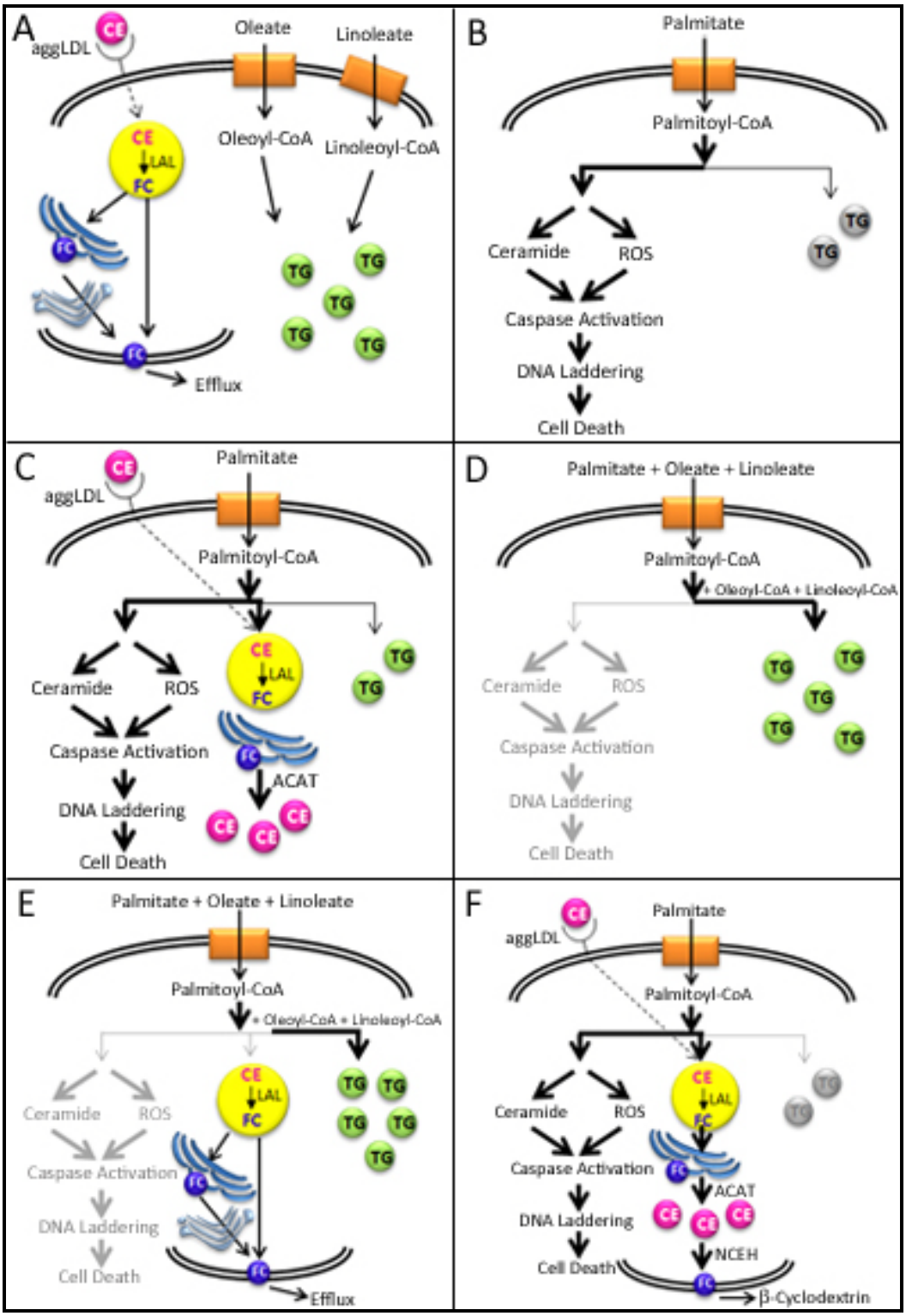
Orlistat (174,226). In this case, FAs generated by the hydrolytic activity of TG are internalized and used for intracellular TG synthesis (**figure 24**). Comparing the effects of DISP delivery of CE and TG (which appears to work exclusively through whole particle uptake) compared to that from native lipoproteins (where surface TG hydrolysis can occur) indicates that surface hydrolysis of TG is not absolutely required for TG to exhibit an effect on foam cell cholesterol levels but TG degraded via surface hydrolysis may contribute to the overall lysosomal hydrolysis of CE and clearance of sterol from cells. How these two pathways may interact remains to be elucidated.

FAs generated from the hydrolysis of TG and/or circulating in the plasma influence macrophage foam cell biology. FAs are quickly utilized for cellular lipid synthesis, including TG, PL, and/or CE. Additionally, FAs are found in circulation and are present in the artery wall in atherosclerotic lesions (256,257). FA could elicit similar effects on cellular cholesterol metabolism compared to treatment with TRPs, either associated with or distinct from the observed effects of TG. The studies presented in this chapter show that treatment with unsaturated FAs reduces cellular cholesterol, similar to the effect observed upon treatment with TRPs. The reduction in cholesterol occurs in tandem with an increase in cellular TG, suggesting that *de novo* TG synthesis is important for mobilizing cellular cholesterol. This is consistent with the TRP experiments presented in chapter III.

The studies described in this chapter provide preliminary evidence that FAs induce a similar reduction in cellular cholesterol levels compared to TRPs. Future studies are required to expand on these initial observations and to confirm the differential

effects of saturated versus unsaturated FAs on cholesterol metabolism. For our initial studies, we selected FAs based on their importance in human nutrition, since they are abundant in milk products (PA), meat (PA), and oils (OA and LA)(258). PA, OA, and LA are the most abundant saturated, monounsaturated, and polyunsaturated FAs (respectively) in our diet (258,259). Furthermore, we treated cells with concentrations consistent with circulating plasma free FA levels in the postprandial state (260). Therefore, we believe our results are physiologically relevant and may represent interactions occurring *in vivo.*

The differences between PA, OA, and LA in the mobilization of lysosomal and cellular cholesterol suggest the possibility of a multistep process for the removal of trapped cholesterol from the lysosome and the cell. Importantly, treatment with PA, a saturated FA that is a poor substrate for TG synthesis, did not reduce cellular cholesterol (**figure 25C**). However, treatment with β-CD was able to remove cholesterol from cells co-incubated with PA and aggLDL. Lysosomal cholesterol is not accessible for cholesterol efflux, even in the presence of efflux promoters, including β-CD (13). Specifically, entrapment of FC and CE in lipid-engorged lysosomes prevents sterol from being mobilized to the plasma membrane for efflux. Thus, PA treatment mobilizes lysosomal cholesterol to a cellular pool that is accessible to cholesterol efflux to β-CD (**figure 25F**). Therefore, our working hypothesis is that, in contrast to treatment with aggLDL alone, coincubation with PA mobilizes lysosomal cholesterol to the ER where it is reesterified to palmityl-CoA by ACAT. PA is able to mobilize cholesterol from the lysosome but liberated sterol is stored as cytoplasmic CE rather than being mobilized to the PM for


A
aggLDL
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Palmitate
Palmitoyl-CoA
Ceramide
ROS
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Caspase Activation
DNA Laddering
Cell Death
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ACAT
DNA Laddering
CE
CE
CE
Cell Death
NCEH
FC
β-Cyclodextrin

Figure 25: Summary of FA experiments. A: OA and LA are rapidly incorporated into cellular TG. The increase in cellular TG corresponds to a decrease in cellular cholesterol in cells coincubated with aggLDL and OA and/or LA-BSA conjugates. B: PA is poorly incorporated into cellular TG and as a result leads to cell death. Our studies support these previous studies and show poor incorporation of PA into cellular TG. C: Cells coincubated with PA and aggLDL do not exhibit the clearance of cellular cholesterol that occurs in cells treated with unsaturated FAs. Accordingly, coincubated cells have reduced cellular TG and increased cellular cholesterol, suggesting that in cholesterol enriched cells, PA is incorporated into cellular cholesteryl ester. D: Consistent with previous studies, coincubation of cells with OA and LA appears to shuttle more PA into TG synthetic pathways. E: When cells are coincubated with a mixture of PA, OA, and LA in combination with aggLDL, we observe an increase in cellular TG that results from the ability of unsaturated FAs to shuttle PA into TG synthetic pathways. Importantly, the increase in cellular TG correlates with a decrease in cellular cholesterol. This emphasizes the importance of intracellular TG synthesis in our effects on cellular cholesterol clearance. F: Treatment of cells coincubated with PA and aggLDL for six days with β-cyclodextrin for 24 hours mobilizes intracellular cholesterol. Lysosomal cholesterol is not accessible to cholesterol efflux, even in the presence of efflux promoters, including β-cyclodextrin. Thus, PA mobilizes lysosomal cholesterol to an intracellular pool that is accessible to cholesterol efflux (i.e. cytoplasmic CE).

efflux. Lysosomal cholesterol is effluxed in two steps: the initial mobilization of cholesterol from the lysosome and the efflux of liberated cholesterol from the cell. A potential model for multistep cholesterol mobilization is described in **figure 26**. FAs generated by surface and/or lysosomal hydrolysis have the potential to mobilize lysosomal and cellular cholesterol. Importantly, the generation of FAs, including both saturated and unsaturated species, mobilizes lysosomal cholesterol through a mechanism that remains to be determined (**figures 25A, 25D, 25E**). However, the mobilization of liberated cholesterol from the cell is dependent upon the ability of FAs to be incorporated into cellular TG. Since unsaturated FAs are rapidly incorporated into cellular TG they are unavailable for reesterification to liberated cholesterol (**figure 26A**). Saturated FAs, which are poor substrates for TG synthesis, are preferentially reesterified to the liberated cholesterol and thus sequester the sterol in cytoplasmic CE droplets (**figure 26B**).

The studies presented in this chapter emphasize that intracellular TG may provide positive and in some cases protective benefits in many cell types, including macrophage foam cells. It is unclear why certain saturated FAs, including PA, are poor substrates for TG synthesis. The differential partitioning of FAs into cellular lipids occurs as the result of the preference of some enzymes in the lipid synthetic pathways for unsaturated versus saturated FA substrates (261,262). Importantly, the inability of cell to incorporate PA into cellular TG pools is cytotoxic (249-255). PA induces apoptosis through a number of mechanisms, including ceramide synthesis, direct effects on mitochondrial permeabilization, ROS generation, and activation of ER stress

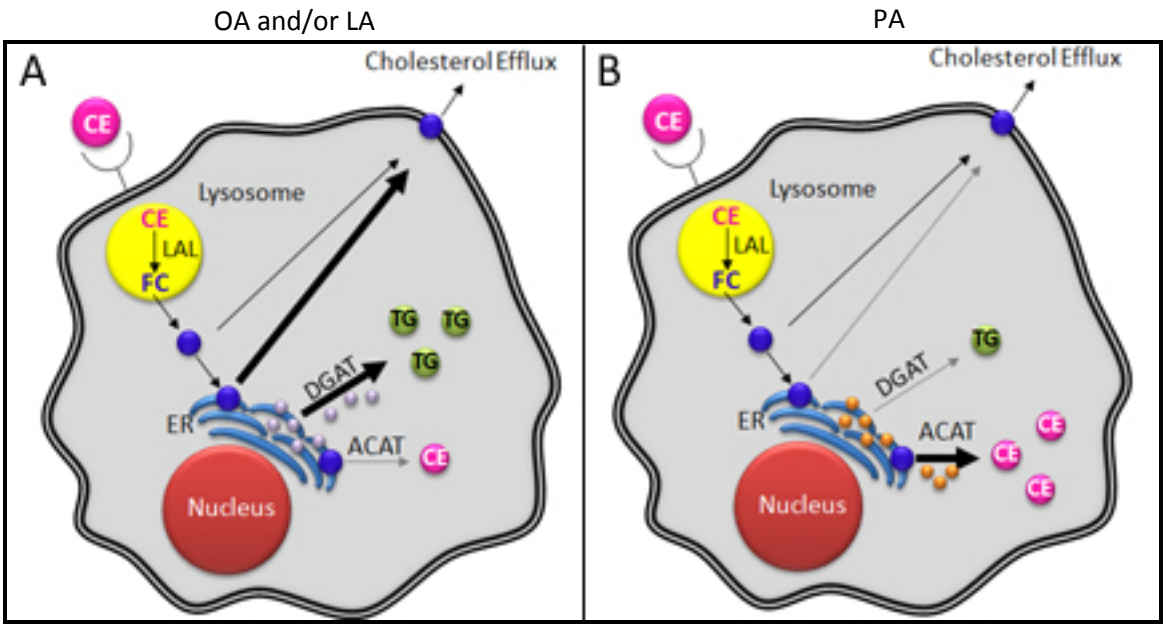

Figure 26: Model for the multistep removal of cholesterol induced by FA incubation. OA and LA (purple), which are rapidly incorporated into cellular TG, induce the removal of cholesterol from the lysosome and shuttle mobilized sterol to the plasma membrane for cholesterol efflux. Similarly, PA (orange), which is a poor substrate for TG synthesis, mobilizes lysosomal cholesterol. However, in the presence of increased FC in the ER, PA is reesterified to FC by ACAT, preventing its removal from the cell.

(252,253,263,264). However, intracellular TG accumulation is protective against FA-induced lipotoxicity. Specifically, cosupplementation of unsaturated FAs rescues the lipotoxic effects of PA by shuttling PA into TG synthetic pathways (249,250,255,263,265,266). Our data combined with the results of these previous studies suggest that intracellular TG synthesis can have a number of positive effects on macrophage foam cells, including reducing the cytotoxic effects of saturated FAs and mobilizing intracellular cholesterol into reverse cholesterol transport pathways.

CHAPTER VI

PILOT STUDIES EXAMINING THE EXTRALYSOSOMAL EFFECTS OF TRIGLYCERIDE IN MACROPHAGE FOAM CELLS

Our studies have shown that enrichment of macrophage foam cells with TG mobilizes lysosomal cholesterol and reduces the cellular cholesterol content. This indicates that, in addition to lysosomal CE hydrolysis and free sterol clearance, the extralysosomal trafficking and efflux of sterol out of the cell may also be enhanced. However, the pathways by which FC is released from the lysosome and ultimately removed from the cell are unknown. We have examined the extralysosomal effects of released sterol, and the mechanism by which liberated lysosomal sterol exits the cell.

Introduction

We have previously shown that cholesterol is sequestered in the lysosome upon treatment with cholesterol-rich lipoproteins (9-13,267). The current studies have shown that TG has the ability to mobilize significant quantities of sequestered cholesterol from the lysosome and, ultimately, from the cell. The mobilization of such large amounts of FC could have drastic effects on cellular homeostasis, some of which are diagrammed in **figure 27**. For example, FC is known to be cytotoxic and a potent activator of apoptotic pathways (44,46,73,268), and FC released from the lysosome must be processed into a non-toxic metabolite in order to prevent cytotoxic effects. Thus, it is possible that once

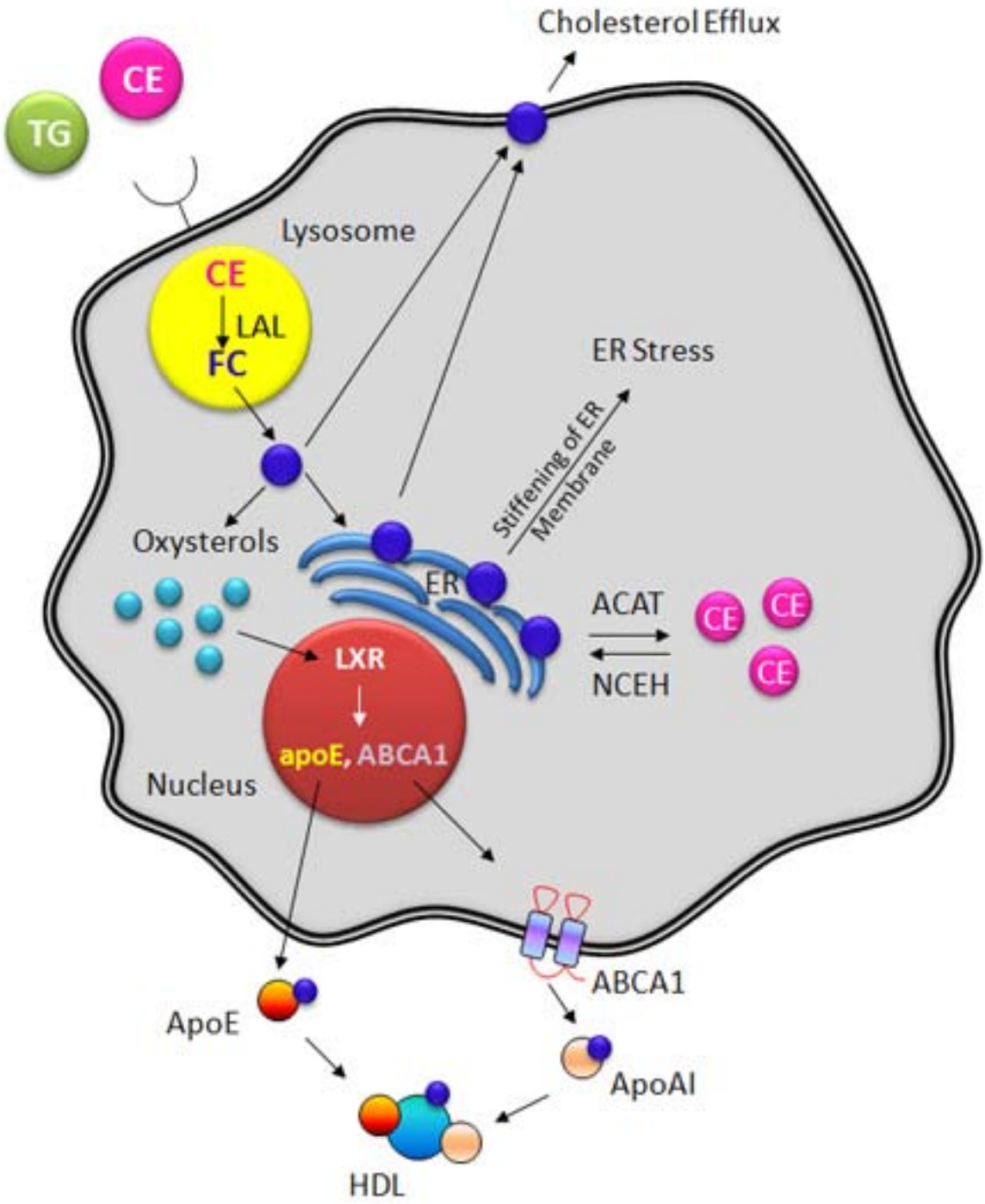

Figure 27: Diagram of the extralysosomal effects of FC. Following release from the lysosome, FC can be trafficked to the ER and/or the plasma membrane. Mobilization of FC to the ER increases the ER cholesterol content and initiates mechanisms of FC processing and/or mobilization. FC can be processed by ACAT to form cytoplasmic CE droplets. If not properly managed, increase in ER cholesterol results in the activation of ER stress and, ultimately, cell death via apoptosis.

released from the lysosome, FC is trafficked to the ER, where it is reesterified by ACAT, moved to mitochondria where it can be converted to oxysterol, or transported to the PM for efflux. Additionally, if the flux of FC from the lysosome to the ER is too great, and if the FC is not mobilized or metabolized, the resulting increase in ER cholesterol could activate ER stress and, consequently, cell death via apoptosis.

The studies presented in chapter III show a clear reduction in cellular cholesterol levels following treatment with TRPs (chapter III, **figure 7 and figure 9**) indicating enhanced efflux of sterol. While the mobilization of lysosomal cholesterol is an important first step, it is not sufficient to induce cellular cholesterol efflux. Importantly, the TG-induced mobilization of foam cell cholesterol occurs in the absence of extracellular cholesterol acceptors, which would enhance the movement of cholesterol from the cell. This suggests that TG enrichment could activate and/or upregulate promoters of cellular cholesterol efflux including SR-BI, ABCA1, ABCG1, and/or apoE.

In sum, the studies presented in this chapter have sought to define the extralysosomal effects of lysosomal sterol mobilized following TG enrichment. We systematically examined the potential downstream effects of liberated lysosomal FC including alterations in cholesterol homeostatsis (ACAT, ABCA1, ABCG1, SR-BI, apoE) and/or activation of ER stress (CHOP, phospho-eIF2a).

Materials and Methods

Cell Culture

Cells were cultured as described in chapter III. In some incubations, the ACAT inhibitor compound CP113,818 (1.5 μg/ml medium) was included in the culture medium to prevent reesterification of cholesterol in the cytoplasm. Comparison of FC and CE in cells with and without ACAT inhibition provided a means of determining whether CE in uninhibited cells was derived from ACAT or, rather, was unhydrolyzed lysosomal CE. Additionally, use of ACAT inhibitors allowed us to determine if lysosomally liberated cholesterol was trafficked to the ER for reesterification. Treatment with acetylated LDL (acLDL) with or without ACAT inhibitors was used as a positive control, as cholesterol derived from acLDL is known to be rapidly transported into cytoplasmic lipid pools that are accessible to ACAT activity.

Western Blotting

Following incubation with aggLDL and/or VLDL (100 μg lipoprotein/ml), cells were washed three times in ice-cold PBS and solubilized in ice-cold buffer containing 150 mM NaCl, 100 mM NaF, 50 mM tris-HCl (pH 8.0), 35 mM sodium deoxycholate, 5 mM EDTA, 1% triton X-100, 0.1% SDS and 1X Protease Inhibitor Cocktail. Cell lysates were scraped into Epppendorf tubes, sonicated, rotated end-over-end for 30 min at 4 ^{o}C and then centrifuged for 10 min to pellet insoluble material. The protein concentration was determined by the Lowry method, as described in chapter III. Lysates were either used immediately or frozen as single-use aliquots at -80 ^{o}C for subsequent immunoblot

analysis. Lysates were separated by electrophoresis on a 4-12% gradient Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA) for 15 min at 100 V and 90 min at 120 V. Proteins were transferred to 0.45 μM pore nitrocellulose for 90 min at 25 V. Membranes were blocked for 1 hour at 4 ^{o}C in Tris-buffered saline (25 mM Tris, 1.3 mM KCl and 137 mM NaCl) containing 0.1% TWEEN-20 (TBST) and 5% non-fat milk. Blots were probed with primary antibody to ABCA1 (dilution 1:750)(Novus Biologicals, Littleton, CO), ABCG1 (dilution 1:750)(GeneTex Inc, Irvine, CA), SR-BI (dilution 1:1000)(Novus Biologicals, Littleton, CO), apoE (dilution 1:50000)(a gift from Dr. Larry Swift, Vanderbilt University), or β-actin (1:5000) (Santa Cruz, Santa Cruz, CA) overnight at 4 ^{o}C and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Promega, Madison, WI) for 1 hour at room temperature. Protein bands were visualized on Kodak Biomax film using enhanced chemiluminescence detection methods.

Results

There are a number of potential pathways liberated FC could enter following release from the lysosome (**figure 27**). Importantly, mobilized lysosomal FC can be trafficked to the ER for sorting and processing, including reesterification by ACAT resulting in the generation of cytoplasmic CE (269,270). In order to determine if FC is transported to ACAT for reesterification to form cytoplasmic CE droplets, we repeated our lipid loading experiments with the inclusion of the ACAT inhibitor CP113,818. The lysosomal sequestration that occurs following treatment with cholesterol-rich particles renders this sterol unavailable as a substrate for ACAT. Thus, the majority of the CE that

accumulates following treatment with cholesterol-rich particles is localized specifically to the lysosome. Consistent with our previous results, treatment with cholesterol-rich particles (aggLDL or CE-DISP) induced similar levels of cholesterol loading that did not change upon inhibition of ACAT (**figures 28A and 28C**). Inhibition of ACAT had minimal effects on TG levels in cells treated with TG-rich lipid dispersions and/or VLDL (**figures 28B and 28D**). Additionally, inhibition of ACAT did not influence cholesterol levels in TG-enriched cells (**figures 28A and 28C**). This indicates that the cholesterol mobilized from lysosomes following TG-enrichment is not reesterfied by ACAT. Thus, liberated lysosomal cholesterol is not stored intracellularly but, rather, is mobilized from the cell.

FC released from lysosomal processing of endocytosed lipoproteins has been shown to activate ER stress (46,73,207,271). Therefore, if the massive amounts of FC released from lysosomes following TG enrichment is trafficked to the ER and not properly managed, ER stress could be activated. Activation of ER stress can lead to the activation of apoptosis. In order to determine the fate of liberated FC with respect to apoptotic signaling, we measured the expression of ER stress mediators following loading with TRPs and/or cholesterol-rich lipoproteins. Importantly, we examined markers of early (phospho-eIF2α) and late (CHOP) portions of the ER stress signaling cascade to determine if the pathway is both initiated and advanced by liberated lysosomal FC. Treatment with CE-DISP for six days (60 μg CE/ml) induced the expression of CHOP as well as the phosporylation of eIF2α (**figure 29**). Interestingly, inclusion of TG-DISP in combination with CE-DISP reduces the expression of CHOP and phospho-

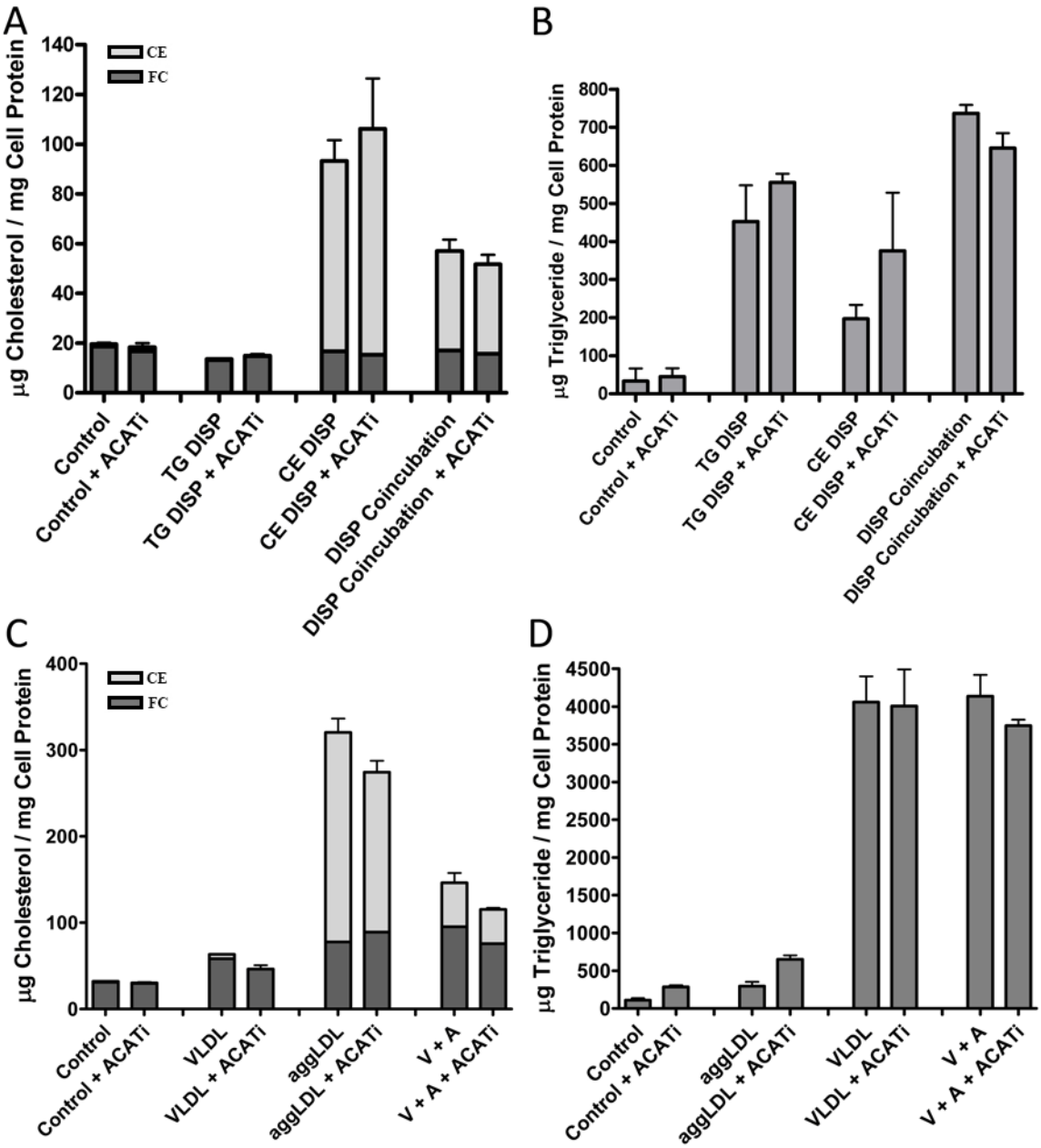
A
μg Cholesterol / mg Cell Protein
CE
FC
Control
Control + ACATi
TG DISP
TG DISP + ACATi
CE DISP
CE DISP + ACATi
DISP Coincubation
DISP Coincubation + ACATi
B
μg Triglyceride / mg Cell Protein
C
μg Cholesterol / mg Cell Protein
VLDL
VLDL + ACATi
aggLDL
aggLDL + ACATi
V + A
V + A + ACATi
D
μg Triglyceride / mg Cell Protein

Figure 28: Accumulation of lipids in THP-1 macrophages incubated with TRPs and/or cholesterol-rich lipoproteins with and without the ACAT inhibitor CP113,818 (1.5 μg/ml medium). THP-1 macrophages were treated for 6 days at 37°C in RPMI containing 1% FBS and TPA (50 ng/ml) alone or containing CE DISP (60 μg CE/ml) and/or TG DISP (50 μg TG/ml) or 100 μg protein/ml aggLDL and/or VLDL. The cells were harvested and the cellular lipid levels were determined as described in Materials and Methods. Inhibition of ACAT has no effect on TG levels in cells treated with TG-rich lipid dispersions (B) or VLDL (D) ($p>0.05$). Additionally, inhibition of ACAT does not affect cholesterol loading in all conditions ($p>0.05$). Importantly, inhibiting ACAT does not change the reduction in cellular cholesterol observed in TG-enriched cells (A, C) ($p>0.05$). This indicates that neither the cholesterol accumulation observed with CE loading nor the mobilization by TG-enrichment involves increased cholesterol reesterification via ACAT. Values are the mean ± SEM for three experiments. Comparisons between matched conditions (similar lipid loading with and without ACAT inhibitor) were not statistically significant ($p>0.05$)

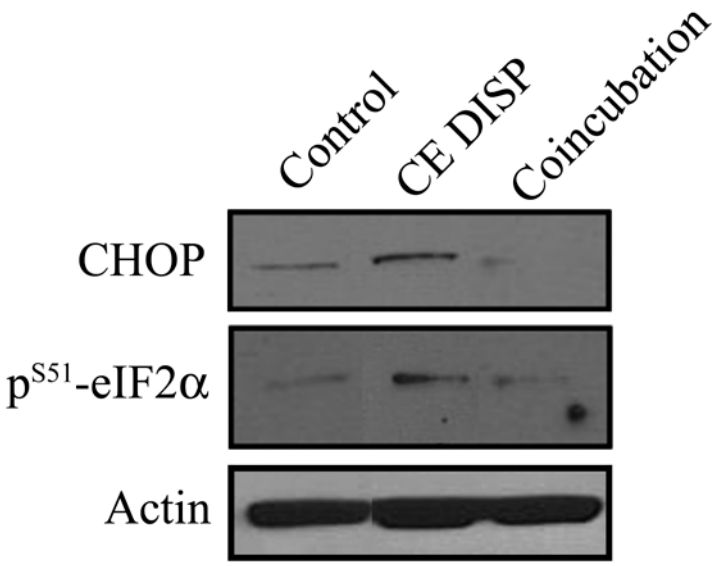

Figure 29: Western blot analysis of mediators of ER stress. Analysis of cells treated with CE DISP (60 μg TG/ml) with and without TG DISP (50 μg TG/ml) for 6 days shows differential expression of ER stress proteins CHOP and phospho-eIF2α. Treatment with cholesterol-rich particles alone induced the expression of ER stress proteins including those expressed at late (CHOP) and early (phospho-eIF2α) stages of the ER stress response. Similar to previous studies, this indicates that ER stress is both initiated and maintained in cholesterol enriched macrophage foam cells. However, coincubation with TRPs reduced the expression of ER stress proteins. Fifteen micrograms of protein was loaded in each lane and incubation conditions were as described in Experimental Procedures.

eIF2α to control levels (**figure 29**), indicating the absence of ER stress in coincubated cells.

The absence of ACAT reesterification and lack of ER stress following TG enrichment suggests that FC liberated from lysosomes could be rapidly transported to the PM for cholesterol efflux. Moreover, our results not only show that lysosomal clearance of sterol (FC and CE) is accentuated with TRP treatment but also indicate a decrease in total cellular cholesterol levels. Surprisingly, this occurs even in the absence of exogenously added extracellular cholesterol acceptors. Thus, cellular cholesterol mobilization occurred in media containing only 1% serum (chapter III, **figure 7 and figure 9**) and also in serum free media (**figure 30**). The efflux of cholesterol from the cell could occur through a number of processes including both passive cholesterol diffusion from the plasma membrane to the surrounding media or through active processes, including those mediated by ABCA1, ABCG1, SR-BI and/or apoE. Many of these, however, require extracellular acceptors for maximal efflux. In order to define the mechanism of cholesterol efflux in TG-enriched macrophages, we examined cellular protein levels of ABCA1, ABCG1, SR-B1 and ApoE by western blot. Compared to non-loaded control cells, no differences were observed in the protein levels of ABCA1, ABCG1 and SR-B1 in TG-enriched cells (**figure 31**). However, coincubation of cells with VLDL and aggLDL resulted in a drastic reduction in cell-associated apoE protein levels (**figure 31**). We reason that this is likely due to secretion of apoE from the cell. This is a potentially important finding because, following its release from the cell, secreted apoE can mediate the efflux of cholesterol. Thus, it is plausible that apoE released from

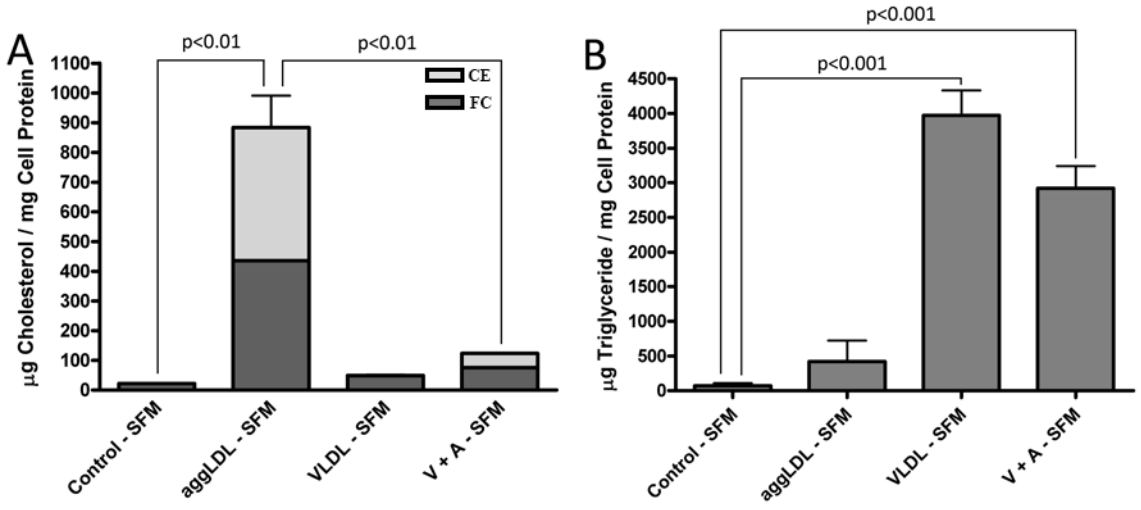

Figure 30: Accumulation of lipids in THP-1 macrophages incubated with aggLDL and/or VLDL in serum free media. THP-1 macrophages were treated for 6 days at 37°C in macrophage serum free media and TPA (50 ng/ml) alone or with 100 µg protein/ml of aggLDL and/or VLDL. The cells were harvested and the cellular lipid levels were determined as described in Materials and Methods. A: Incubation of THP-1 macrophages with aggLDL produced a significant increase ($p<0.01$) in total cholesterol, free cholesterol (dark gray portion of the bar) and esterified cholesterol (light gray portion of the bar). Incubation with both aggLDL and VLDL significantly reduced the cellular CE accumulation compared to that seen with aggLDL alone ($p<0.01$). B: Incubation of cells with VLDL produced a significant increase in cellular TG levels compared to control or aggLDL-treated cells both when used alone or in combination with aggLDL ($p<0.001$). Treatment of macrophages in SFM are similar to the results achieved in cells treated in 1% FBS (**figure 7**), indicating that the serum concentration of the media does not affect lipid loading. Values are the mean ± SEM for three independent experiments.

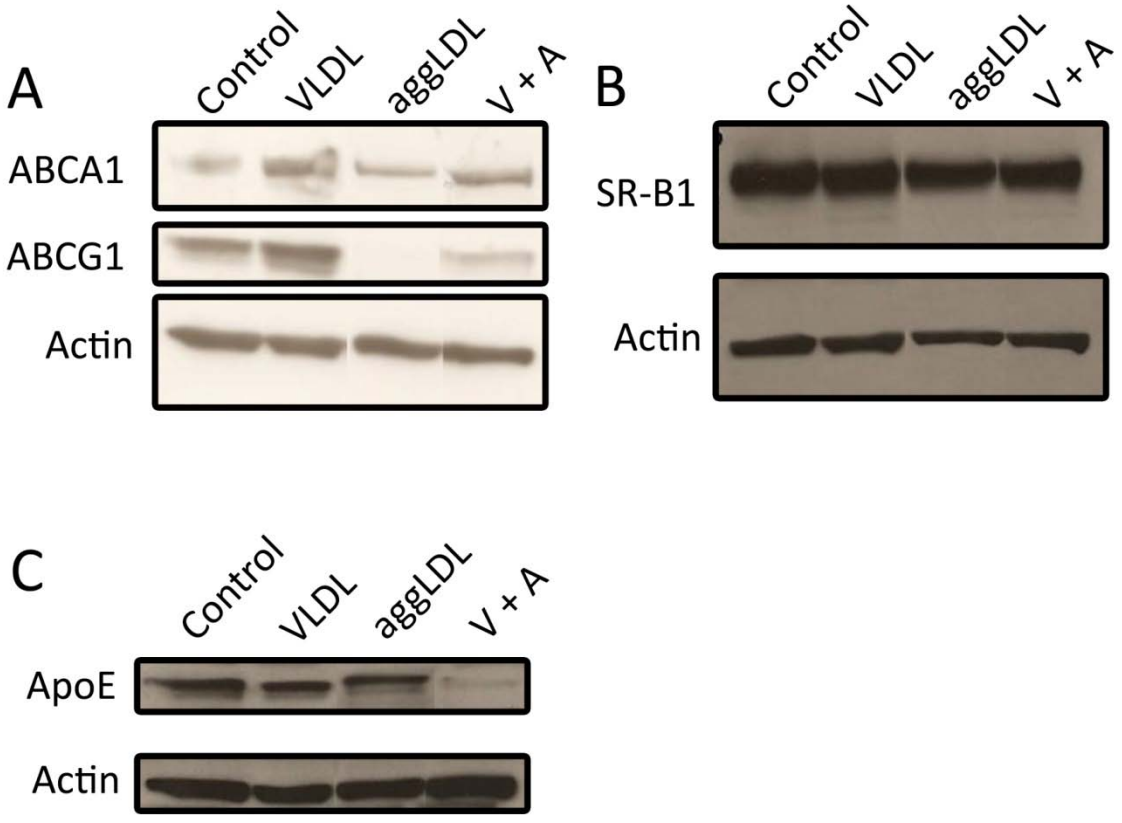

Figure 31: Western blot analysis of key mediators of cholesterol efflux. THP-1 macrophages were treated for 6 days with VLDL and/or aggLDL (50 μg lipoprotein/ml). Compared to non-loaded control cells, no differences were observed in the protein levels of ABCA1 (**A**), ABCG1 (**A**) and SR-B1 (**B**) in TG-enriched cells. Coincubation of cells with VLDL and aggLDL resulted in a drastic reduction in cell-associated apoE protein levels (**C**), indicating that apoE could be secreted from the cell where it could serve as an extracellular cholesterol acceptor.

lysosomes following TG enrichment could act as an extracellular cholesterol acceptor (as diagrammed in **figure 32**).

Discussion:

The current studies suggest that FC liberated from lysosomes following TG treatment of foam cells is trafficked to the PM for efflux and does not contribute to intracellular cholesterol signaling. Previous studies have shown that FC liberated from lysosomes can be trafficked to the ER for processing (207) (**figure 27**). In foam cells, this massive movement of cholesterol into the ER, the site of cholesterol homeostatic enzymes, could initiate signaling pathways to process cytotoxic FC into an inert form for storage in a process catalyzed by the enzyme ACAT. The absence of ACAT-derived CE in our TRP treated cells suggests that following its release, lysosomally sequestered cholesterol is not trafficked to the ER. This is supported by the absence of ER stress signaling that occurs following FC enrichment of ER membranes. Recent studies suggest that trafficking of lysosomally processed FC to the ER contributes to the activation of ER stress (46,73,207,271). However, we do not observe activation of ER stress following TG-induced liberation of lysosomal cholesterol. The absence of ACAT reesterification and activation of ER stress in combination with the massive reduction in cellular cholesterol upon TG-enrichment suggests that sterol mobilized from lysosomes following treatment with TRPs is rapidly transported to the PM for efflux.

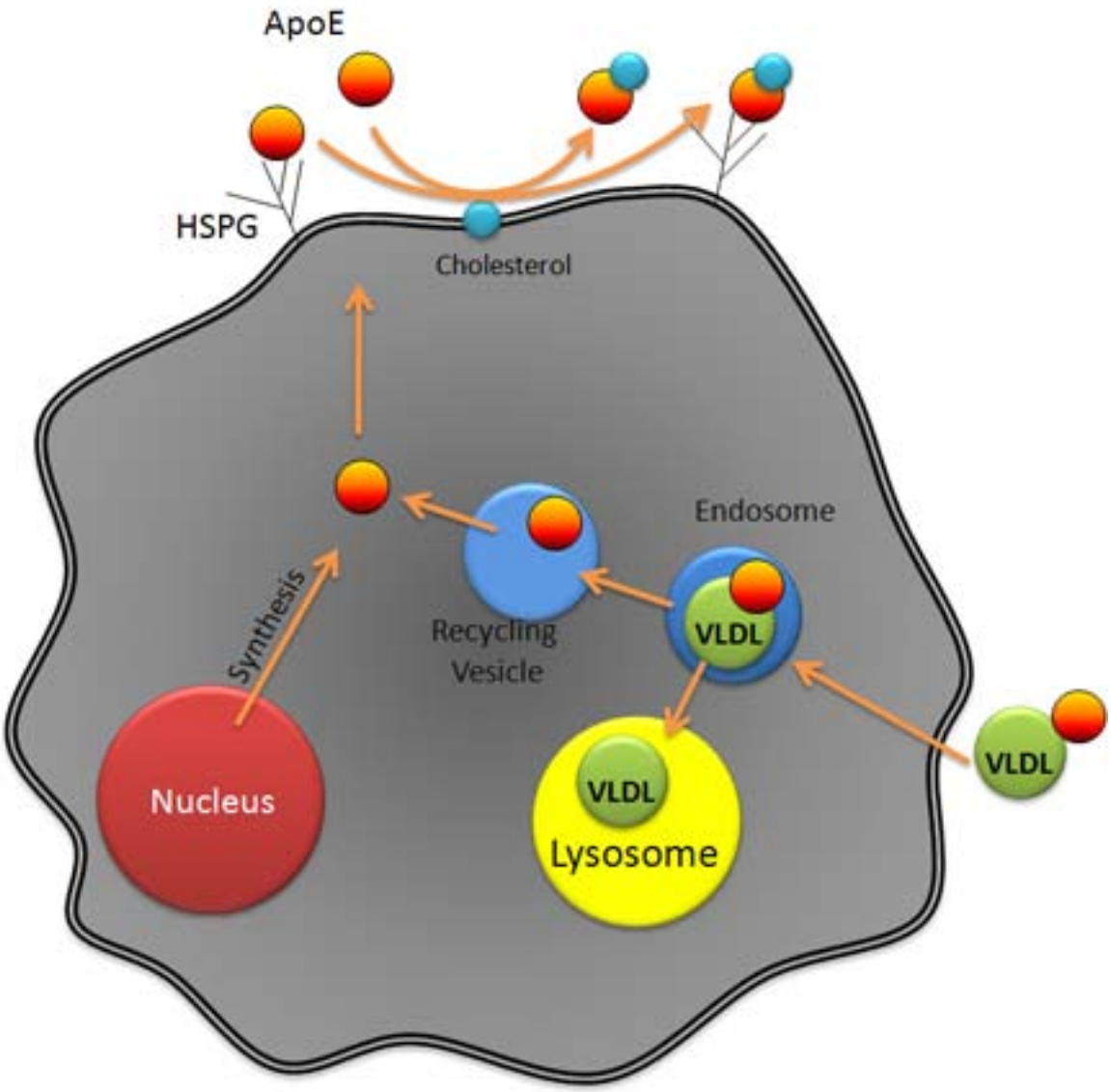

Figure 32: Diagram of the metabolism of apolipoprotein E (apoE). ApoE is an important mediator of cholesterol homeostasis. Macrophages are able to synthesize apoE. Additionally, macrophages can acquire apoE from exogenous sources, including from endocytosed apoE-containing lipoproteins, such as VLDL. ApoE derived from lipoproteins can escape degradation in the lysosome and be recycled to the cell surface where it can act as an acceptor of effluxed cholesterol.

As mentioned previously, exposure of foam cells to promoters of cholesterol efflux is not sufficient to mobilize lysosomal cholesterol (13). Therefore, TRPs must exhibit multiple effects on the mobilization of macrophage cholesterol including the restoration of lysosomal function, enhanced lysosomal sterol hydrolysis, mobilization of lysosomal sterol to the PM and promotion of efflux from the cell. The analysis of the major pathways of cholesterol efflux presented in this chapter suggests an important role for apoE in the removal of cholesterol from TG-enriched foam cells. Cell-associated apoE is reduced in cellular fractions of macrophages coincubated with aggLDL and VLDL, suggesting that apoE is secreted and, as a result, is able to mediate the removal of cholesterol from the TG-enriched macrophages. This is consistent with previous studies showing that endogenous apoE plays a critical role in cholesterol efflux from macrophages (110,112,272-276). Specifically, *in vitro* studies show that induction of apoE expression in cultured macrophages enhances cholesterol efflux, even in the absence of other extracellular cholesterol acceptors (110). Furthermore, exogenous apoE could contribute to the cholesterol mobilization observed in TRP-treated macrophages. ApoE is an important apoprotein component of TRPs, including VLDL (277,278). Previous studies show that apoE acquired from exogenous sources, such as internalization as a component of VLDL, is spared degradation in the lysosome and is recycled to the to the extracellular space, where it can act as a cholesterol acceptor (177,279-289). Thus, apoE recycled from endocytosed VLDL may be important in inducing cholesterol removal in TG-enriched foam cells. Although further studies are required in order to distinguish the contribution(s) of exogenous versus endogenous

apoE in the TG-induced cholesterol mobilization, the studies shown here suggest a significant role of apoE in cholesterol efflux of mobilized lysosomal sterol.

Surprisingly, we did not see an upregulation of protein expression for other efflux promoters (including ABCA1, ABCG1, and SR-BI) following TG enrichment. The expression of these promoters is regulated by LXR gene expression, which can be activated by FA, such as those generated by TRP hydrolysis and FFA found in the circulation (290-296). ABCA1 and ABCG1 are transmembrane proteins that utilize the energy released from ATP-hydrolysis to transport PLs and cholesterol across the plasma membrane to their acceptors, lipid-poor apoA1 and nascent HDL, respectively (72,107,109,297). However, there is crossreactivity between cholesterol efflux pathways which could significantly impact cholesterol efflux to apoE. For example, ABCA1 can transfer lipids to apoE, and SR-BI can mediate cellular levels and secretion of apoE (274,298,299). Therefore, it is possible that TG-enrichment enhances the activity of efflux promoters without changing their protein expression.

Future studies should specifically measure the activity of ABCA1, ABCG1 and SR-BI in TG-enriched and/or cholesterol-enriched macrophages. Specifically, studies should be performed in the presence of cholesterol acceptors, including apoA1 and nascent HDL to examine the transfer of cholesterol through ABCA1, ABCG1 and SR-BI mediated pathways. ABCA1 effluxes cholesterol to lipid-poor apoproteins including apoA1 (300). Therefore, if activity of ABCA1 is increased in the presence of TG, there should be measureable efflux to apoA1.

Measurement of the activity of ABCG1 and SR-BI is more complex because both transporters efflux cholesterol to the same acceptor: nascent HDL. Therefore, to analyze the activity of each efflux promoter individually, studies must be performed using specific inhibitors of each pathway in the presence of nascent HDL. The activity of SR-BI can be inhibited by treatment with the inhibitor block lipid transport-1 (BLT-1) while the activity of ABCA1 can be inhibited by treatment with cyclosporin A (301,302). Since the studies shown in this chapter suggest that apoE is the mediator of cholesterol efflux, studies examining ABCA1, ABCG1 and SR-BI activity should be perfomed with THP-1 macrophages that have been treated with apoE siRNA constructs. Comparison of this cell line to normal THP-1 macrophages will allow us to determine the role of apoE in the efflux of cholesterol through each lipid transporter.

Our current data strongly suggests that, in the presence of intracellular TG, cholesterol mobilized from the lysosome is removed from the cell through active cholesterol efflux mechanisms involving apoE. The results indicate that in addition to improving the ability of the foam cell to process endocytosed cholesterol, TG can induce efflux of liberated cholesterol. Therefore, TG or its metabolites significantly improve the ability of macrophage foam cells to mobilize intracellular cholesterol to efflux pathways. Accordingly, TG has the potential to greatly improve atherosclerotic lesion regression.

CHAPTER VII.

SUMMARY, DISCUSSION AND FUTURE DIRECTIONS: TRIGLYCERIDE-RICH PARTICLES MAINTAIN LYSOSOME ACTIVITY, MOBILIZE LYSOSOMAL CHOLESTEROL AND ENHANCE THE EFFLUX OF CHOLESTEROL FROM MACROPHAGE FOAM CELLS IN A TRIGLYCERIDE DEPENDENT MANNER

The studies presented in this dissertation probe the complex interactions between lipids and macrophage foam cells. A majority of previous studies focused on the effects of a single lipid species (i.e. cholesterol) in macrophage foam cell formation and do not consider the intricate relations between the variety of lipoproteins and extracellular lipids found within the lesion. The presence of other lipids can clearly influence macrophage cholesterol metabolism and as shown by our studies can induce reverse cholesterol transport from foam cells. Previous studies using genetic manipulation of intracellular cholesterol metabolism show that enhanced clearance of cholesterol from foam cells initiates lesion regression (50). However, in clinically important lesions, greater than 70% of the sterol within a foam cell is trapped within lysosomes and unavailable to reverse cholesterol transport mechanisms (5,13,125,205). To date very little progress has been made in the discovery of treatments that would mobilize cholesterol from the lysosome. Our finding that significant mobilization of lysosomal cholesterol occurs following TG treatment is a groundbreaking discovery that could prove to be significant in lesions as a means to increase reverse cholesterol transport as well as influence lesion remodeling and regression.

Intracellular TG Mobilizes Lysosomal and Cellular Cholesterol

The data presented in this dissertation suggest that intracellular TG elicits a principal influence on lysosomal and cellular cholesterol homeostasis. While the magnitude of the effect varies based on cell passage number and the source of lipoproteins we consistently see an increase in cellular TG following treatment with TRPs that results in a decrease in cellular and lysosomal cholesterol levels. This effect is not due to competition between cholesterol-rich and TG-rich particles for uptake; however, it appears to be directly related to the concentration of TG within the cell. Furthermore, the studies in chapter III show that by altering lysosomal cholesterol content TG restores and/or prevents the loss of lysosomal v-ATPase activity, which is a novel finding.

The effect of TG on the metabolism of lysosomal and cellular cholesterol is clearly more complex than we initially anticipated. Our studies establish a novel relationship between intracellular TG levels and cholesterol mobilization. However, additional studies are required to ascertain the specific details of the intracellular interactions of the two lipids. For example, the relative contributions of lysosomal versus cytoplasmic TG to the effects on cellular cholesterol mobilization remain unclear. TG can accumulate in cells through two distinct pathways: surface hydrolysis of TRPs followed by uptake of FAs and reesterification back to TG within the cell cytoplasm and/or receptor-mediated endocytosis of particles (chapter V, **figure 26**). Consistent with previous reports, FAs generated by LPL-mediated surface hydrolysis of VLDL are rapidly resynthesized into cellular TG in our THP-1 macrophage foam cells. In contrast, TG-DISPs, which are representative of the extracellular lipid pools found within an

atherosclerotic lesion, are not affected by surface LPL hydrolysis. Rather, these particles are engulfed through the endosomal-lysosomal system and the TG accumulation from this mechanism is solely dependent on lysosomal processing of the particles. The disparate pathways through which various TRPs enter the cell suggest that within the TG-enriched foam cell there are distinct intracellular pools of TG and FAs that can affect lysosomal and cellular cholesterol metabolism. An important concern to be addressed in the future is to ascertain the differences and similarities in the ability of these intracellular pools to modulate intracellular cholesterol metabolism.

While the studies presented in this dissertation make significant progress in defining a mechanism for the influence of TG on macrophage cholesterol homeostasis a specific mechanism has not been conclusively identified. At least four distinct pools of TG and/or the proteolytic byproducts of TG could be involved in the mobilization of lysosomal and cellular cholesterol, including lysosomal TG, cytoplasmic TG, lysosomally generated FAs, and/or surface-generated FAs. For example, accumulation of lysosomal TG could change the physical properties of the lipids within the lysosome, rendering them more amenable to hydrolysis by lysosomal lipases. Similarly, generation of FA by lysosomal and/or surface hydrolysis of TG would introduce potent activators of lipid homeostatic genes and could enhance the mobilization of lysosomal and cellular cholesterol. Future studies should explore the contribution of lysosomal versus cytoplasmic TG in the mobilization of lysosomal cholesterol. One potential experimental strategy would be to treat cells with a nondegradable triether analogue of TG, trialkylglycerol ether (TGE), to determine the significance of TG hydrolysis and

resynthesis on sterol metabolism (303,304). TGE could be delivered to the cell as a component of TG-DISPs and, since it is not hydrolytically cleaved by LPL or LAL, it would be delivered to the lysosome where it would accumulate due to the lack of hydrolysis. Additionally, substrates for intracellular TG synthesis (i.e. glycerol and FAs) would not be generated because TGE is not hydrolyzed and, as a result, treatment with TGE would serve as a negative control for TG resynthesis. Therefore, by employing TGE we would be able to determine the influence of lysosomal TG versus cytoplasmic TG pools on cholesterol clearance. We anticipate that multiple intracellular pools of lipid contribute to the mobilization of lysosomal and cellular cholesterol since treatment with TG-DISP, VLDL and FAs exhibit similar effects on cholesterol homeostasis in spite of distinct uptake and processing mechanisms for each molecule.

The ability of TG to alter lysosomal function is significant. Recent studies suggest lysosomes are critical mediators of intracellular lipid metabolism extending far beyond their role in lipoprotein endocytosis (305-307). A newly defined autophagic process, known as macrolipophagy is responsible for the degradation of intracellular lipid deposits. Macroautophagy is a conserved process that allows the cell to repair itself by degrading cytoplasmic components, including long-lived proteins and excess or dysfunctional organelles (308). The process involves the sequestration of cytoplasmic material within a double-membrane vesicle and fusion of the vesicle with lysosomes to degrade the faulty material (308). However, recent studies by Singh *et al*. show the recruitment of LC3 and other autophagy related proteins to lipid droplets, where they form a double membrane autophagosome structure that encloses portions of

intracellular lipid droplets (**figure 33**)(305). Similar to macroautophagy, the newly formed autophagosome delivers these sequestered portions of the cytoplasmic lipid droplets to the lysosome for degradation (305). While macrolipophagy has only been examined in hepatocytes it could prove to be a very important process in macrophage lipid homeostasis (**figure 33**). The inability of macrophages to mobilize intracellular lipids results in the formation of foam cells. If macrolipophagy is active in macrophage foam cells inert cytoplasmic lipid droplets could be redistributed to the lysosome for processing and clearance. Importantly, macrolipophagy is activated by cellular TG enrichment. Thus, the ability of TRPs to mobilize lysosomal cholesterol in a TG-dependent manner could be partially related to the activation of macrolipophagy in foam cells. Future studies examining the expression of markers of macrolipophagy in relationship to intracellular lipid deposits in TG-enriched foam cells will be highly relevant.

Triglyceride Increases Lysosomal Membrane Fluidity

We previously showed that increased cholesterol in lysosome membranes can disrupt lysosomal function by inhibiting lysosomal v-ATPases (10). In this dissertation, we have extended these studies to show that increasing intracellular TG restores lysosomal v-ATPase activity, and this occurs in relation to an increase in the fluidity of lysosomal membranes (chapters III and IV). While the concept of lipoprotein-derived lipids altering membrane fluidity is not novel the studies presented in this dissertation are the first to show changes in lysosomal membrane fluidity following TG and/or

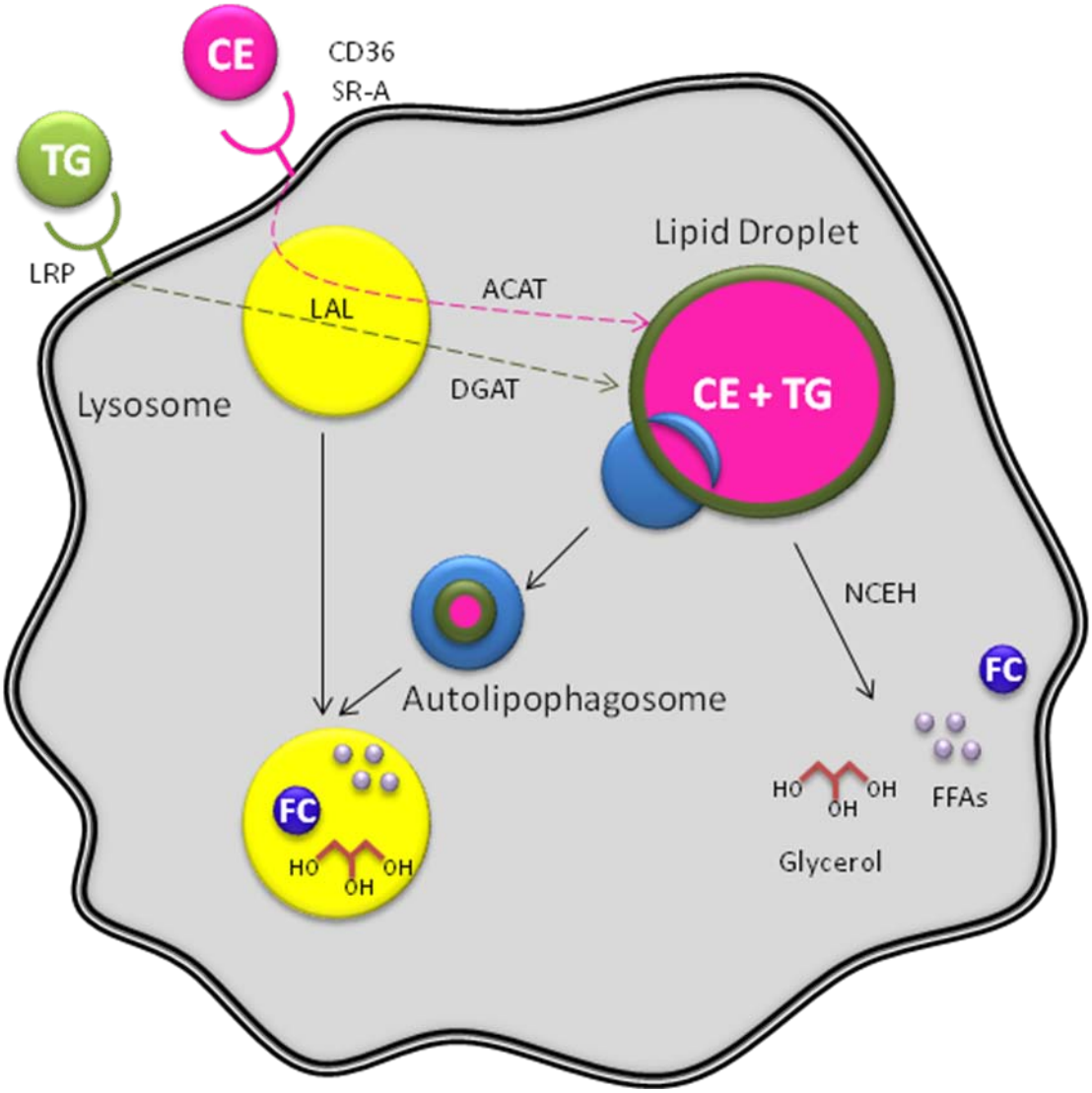

Figure 33: The potential role of macrolipophagy in macrophage foam cells. Macrolipophagy was recently identified to play a critical role in intracellular lipid metabolism in hepatocytes. In this process, portions of intracellular lipid droplets are sequestered in autophagosomes and delivered to the lysosome for processing. While this process is newly discovered to contribute to a significant portion of the lipid turnover in hepatocytes it could play an even more significant role in foam cells, where the lipid burden is often too great for hydrolytic enzymes, including NCEH, to compensate.

cholesterol enrichment in macrophage foam cells. Importantly, the fluidity of the lysosomal membrane is closely related to lysosomal function. Thus, alterations in lysosomal lipid content can significantly impact the capacity of the lysosome to degrade endocytosed material.

Our studies suggest that, at least in foam cell lysosomes, cholesterol plays a more prominent role in determining membrane fluidity than the PL acyl side-chain composition. The increase in lysosomal membrane cholesterol content is likely too great for the cholesterol-enriched foam cell to completely compensate for through normal processes. However, our data suggests that manipulation of the lysosomal membrane fluidity could significantly improve the ability of foam cells to mobilize intracellular cholesterol. The ability of TG to influence membrane fluidity could have important implications in other cellular organelles, including the ER. In addition to reducing the sterol content of foam cell lysosomes, TG mobilizes total cellular cholesterol resulting in increased efflux. Presumably this reduction in cellular cholesterol content could impact the cholesterol content of all organelle membranes, including the ER. Previous studies show that the inhibition of SERCA2b, the ER calcium pump responsible for maintaining ER calcium stores, occurs in relation to an increase in ER cholesterol (207). The loss of SERCA2b activity following FC enrichment leads to the depletion of ER calcium stores and results in the induction of ER stress and, ultimately, foam cell apoptosis. Therefore, reducing the FC content of the ER membrane in foam cells would restore SERCA2b activity, prevent the activation of ER stress and reduce FC-mediated foam cell death. Accordingly, we show a reduction in the expression of

mediators of ER stress in response to TG enrichment (Chapter VI, **Figure 29**). One interpretation of these results shown here is that TG is able to reduce the cholesterol content of all organelle membranes by promoting the mobilization of cholesterol from foam cells. In this regard, TG enrichment might reverse or prevent FC accumulation in the ER and, as a result, eliminate macrophage apoptosis via ER stress mechanisms. The potential for TG enrichment to induce cell wide changes in membrane fluidity suggests that the effect of TG on foam cells extends far beyond alterations in lysosomal lipid processing. Future studies are required to explore this hypothesis.

Cholesterol Efflux Following Macrophage TRP Exposure Occurs via Apolipoprotein E

The essential first step in the mobilization of cholesterol from macrophage foam cells in late stage lesions is the movement of cholesterol from the lysosome (5,13,125,205). Accordingly, the studies in this dissertation show that TRPs can mobilize lysosomal sterol. Our studies also demonstrate the massive mobilization of cholesterol from the macrophage following enrichment with TRPs. While release of cholesterol from the lysosome will make sterol available to a number of cellular pathways, the mobilized cholesterol must be transported to and across the PM to an acceptor in the surrounding media in order to be effluxed from the foam cell. The most efficient cholesterol efflux mechanisms involve active transport of cholesterol out of the cell. In the presence of TRPs, cholesterol efflux promoters are likely activated in order to initiate reverse cholesterol transport. Preliminary results indicate that most known sterol trafficking and efflux pathways are unaffected by TG enrichment. However, levels

of cell-associated apoE are decreased following TG-enrichment, leading us to propose that apoE-dependent pathways may be the major mechanism in the TG-induced clearance of cholesterol following release from the lysosome. Future studies will focus on defining the relationship between intracellular TG, apoE uptake, synthesis and/or secretion and the ability of TRPs to induce cholesterol mobilization. Importantly, measurement of apoE protein levels in the media should be performed following treatment with TRPs to verify increased apoE secretion following TG enrichment. Additionally, further analysis is required to define the contribution of endogenous (i.e. synthesized) apoE and exogenous (i.e. VLDL-derived) apoE in the removal of cholesterol from TG-enriched macrophages. An experimental strategy to address the role of exogenous apoE in the effects of TG on foam cell cholesterol mobilization would be to silence apoE expression in THP-1 macrophages using siRNA technology. This would eliminate the contribution of endogenously synthesized apoE. Exogenous apoE could be delivered to the cells as a component of VLDL. Since treatment with protein-free TG-DISPs is able to induce cholesterol efflux to a similar extent as VLDL, exogenous apoE is not likely to contribute significantly to the mobilization of cellular cholesterol. Thus, we hypothesize that increased endogenous apoE synthesis and/or secretion plays the predominant role in the mobilization of cellular cholesterol following TG enrichment.

The specific relationship between increased foam cell TG and increased synthesis and/or secretion of apoE is not clear. Similar to ABCA1 and ABCG1, the expression of apoE is controlled by the nuclear receptors LXRα and LXRβ (309). Since the transcription of ABCA1, ABCG1 and apoE are controlled by the same regulatory pathways involving

LXR, the absence of changes in ABCA1 and ABCG1 expression following TG enrichment suggests that apoE expression is not modified. Therefore, it is possible that TG may enhance uptake/and or secretion of apoE rather than increasing its synthesis. Interestingly, previous studies show that OA treatment can enhance the secretion of apoE from macrophages (181,310). OA specifically modulates the post-translational glycosylation of apoE in the Golgi, which significantly increases apoE stability and secretion (181,311). This suggests that the FA generated by TRP hydrolysis could promote the secretion of endogenously synthesized apoE and as a result it enhances the mobilization of cellular cholesterol from TG-enriched foam cells.

Alternatively, the decrease in cell-associated apoE following TG enrichment could indicate that macrophages synthesize and secrete apoE-containing lipoprotein particles in order to facilitate the transport of lipids to the liver for excretion. The assembly of apoE containing particles by macrophages could occur through a mechanism similar to the assembly and secretion of VLDL by hepatocytes. Briefly, in hepatocytes, microsomal triglyceride transfer protein (MTP) transfers neutral lipids to nascent apoB (312,313) (**figure 34A**). MTP continues to add TG to the growing lipoprotein particle, resulting in the assembly of mature VLDL, which is secreted into the circulation. Importantly, macrophages express the enzymes required for lipoprotein assembly, including MTP (Dr. Larry Swift, personal communication). Accordingly, previous studies have shown that macrophages are able to secrete apoE-lipid complexes in response to increased intracellular lipid concentration (110,314). It is possible that the increase in cellular TG observed in our TRP-treated macrophages stimulates MTP to

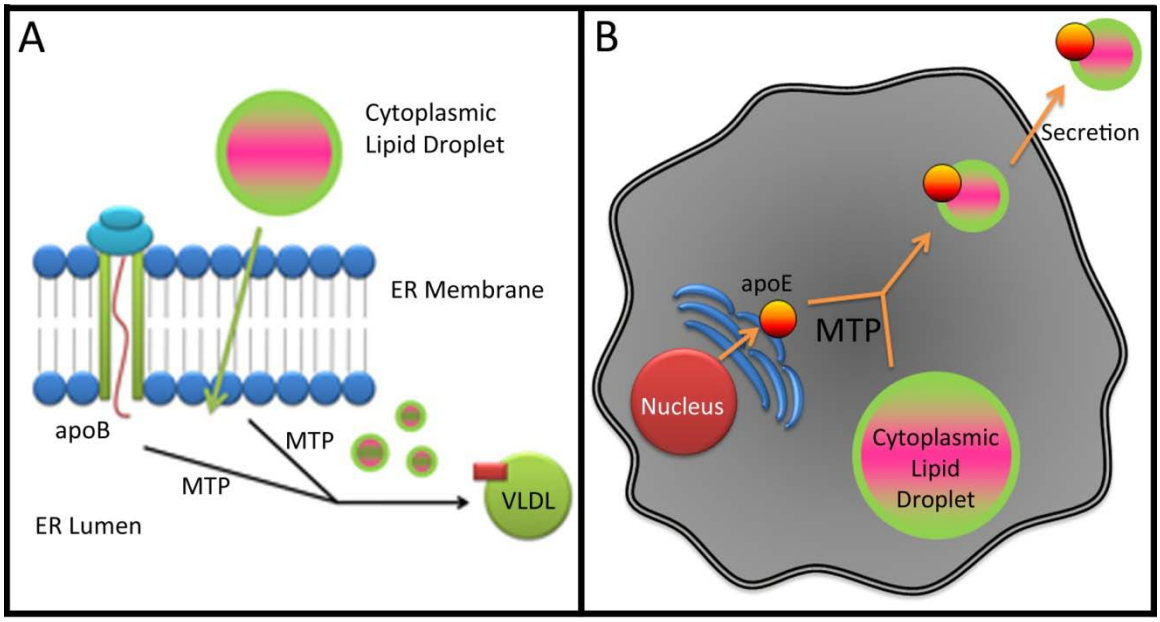

Figure 34: Lipoprotein assembly in hepatocytes and macrophages. A: VLDL is assembled in hepatocytes through the activity of MTP, which sequentially transfers neutral lipid first to nascent apoB and then to the precursor lipoprotein. B: Macrophages could potentially assemble and secrete apoE-containing lipoproteins in response to increased cellular TG following TRP treatment. The process could proceed similar to hepatocytes, with MTP transferring neutral lipid to apoE synthesized by the macrophage.

transfer cellular lipids to newly synthesized apoE (**figure 34B**). Thus, the assembly and secretion of apoE-containing lipoproteins from macrophages could be an additional mechanism through which TG reduces foam cell cholesterol and contributes to reverse cholesterol transport.

FA Induce Cholesterol Mobilization in a TG-dependent Manner

The studies shown in chapter V suggest that monounsaturated and polyunsaturated FAs are able to replicate the effects of TRPs on cellular cholesterol mobilization. However, the reduction in cellular cholesterol occurs concurrently with an increase in cellular TG. Accordingly, treatment with PA, a FA that is not readily incorporated into cellular TG, mobilizes lysosomal cholesterol but does not induce cellular cholesterol efflux. This emphasizes that intracellular TG synthesis is important in the ability of TRPs and FAs to mobilize cellular cholesterol. Additionally, while the studies presented here show that FA treatment is able to reduce cellular cholesterol levels, it will be important to examine the effects of particular FAs on the lysosome in future studies. Specifically, it will be important to verify that FA supplementation restores lysosome function and enhances lysosomal cholesterol clearance. The results of these future studies have significance in specifically identifying the role of FAs and/or intracellular TG levels on cholesterol homeostasis. Understanding the complex interactions between lipids within the macrophage is crucial in developing therapeutic strategies to induce reverse cholesterol transport from atherosclerotic foam cells.

The preliminary studies presented here establish the ability of specific FAs to mobilize cellular cholesterol from macrophage foam cells. In order to define more clearly the contribution of FAs to the effects on cholesterol mobilization it will be necessary to examine the effects of other common dietary FAs on foam cell cholesterol homeostasis including saturated, monounsaturated and polyunsaturated FAs of various chain lengths (**table 6**). Stearic acid (SA) would be the most logical saturated FA to analyze since it is poorly incorporated into TG and does not induce apoptosis as dramatically as PA (315-317). SA is a reasonable choice since it would present a saturated FA similar to PA and is less cytotoxic than PA. However, one limitation to the use of SA as a representative saturated FA is that SA is rapidly converted to OA by stearoyl-CoA desaturase (SCD) following internalization (318,319). Therefore, experiments with SA would need to be performed in the presence of SCD inhibitors, including sterculate (320).

An important class of FAs are those derived from fish oils. It would be informative to determine if the PUFAs found in fish oils, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are able to reproduce the effects of OA and LA on cellular cholesterol homeostasis. Current dietary recommendations of the American Heart Association suggest that dietary supplementation of EPA and DHA, either through consumption of fish two times a week or through fish oil capsules, leads to lower cardiovascular risk (321). Thus, part of the ability of EPA and DHA to reduce cardiovascular risk may be due to the cholesterol mobilization effects presented in this dissertation. Examination of the effects of additional dietary fatty acids on the

Table 6: Most common dietary fatty acids. Future studies are required to define the role of dietary FAs in foam cell cholesterol mobilization. The FAs listed here are the most common FAs found in the average American diet. Information compiled from the International Food Information Council at http://www.ific.org/.

Common Name	# of Carbon Atoms	# of Double Bonds	Structure	Dietary Source
Common Saturated FAs				
Caproic Acid	6	0	$CH_3(CH_2)_4COOH$	Butterfat, Coconut Oils
Caprylic Acid	8	0	$CH_3(CH_2)_6COOH$	
Capric Acid	10	0	$CH_3(CH_2)_8COOH$	
Lauric Acid	12	0	$CH_3(CH_2)_{10}COOH$	
Myristic Acid	14	0	$CH_3(CH_2)_{12}COOH$	
Palmitic Acid	16	0	$CH_3(CH_2)_{14}COOH$	Cottonseed, Palm Oils
Stearic Acid	18	0	$CH_3(CH_2)_{16}COOH$	Cocoa Butter, Animal Fat
Arachidic Acid	20	0	$CH_3(CH_2)_{18}COOH$	Peanut Oil
Common Unsaturated FAs				
Palmitoleic Acid	16	1	$CH_3(CH_2)_5CH\text{-}CH(CH_2)_7COOH$	Fish Oils, Beef Fat
Oleic Acid	18	1	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	Olive Oil, Canola Oil
Linoleic Acid	18	2	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COOH$	Soybean Oil, Corn Oil
α-Linoleic Acid	18	3	$CH_3(CH_2)_4(CH{=}CHCH_2)_3(CH_2)_6COOH$	Soybean Oil, Canola Oil
Arachidonic Acid	20	4	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COOH$	Lard
EPA	20	5	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_2COOH$	Fish Oils
DHA	22	6	$CH_3CH_2(CH{=}CHCH_2)_6(CH_2)COOH$	Fish Oils

mobilization of intracellular foam cell cholesterol would broaden our knowledge of the relationship between circulating dietary lipids and atherogenesis.

Despite initial evidence suggesting that TG plays a more significant role than FA in influencing the reduction of cellular cholesterol, the data presented here do not conclusively rule out the potential for transient effects of FA on cholesterol metabolism. Given the rapid turnover of TG, the important effects of TG on the foam cell may be the result of cholesterol flux through the cell rather than cellular TG accumulation. Thus, it is possible that short term exposure to FAs generated by hydrolysis of TRPs could activate cell signaling pathways that alter cellular sterol metabolism prior to their incorporation into cellular TGs. Studies using inhibitors of cellular TG synthesis could address this possibility. Triacsin C, an inhibitor of long-chain fatty acyl CoA synthetase, blocks the de novo synthesis of TG, CE and diglycerides. Our preliminary studies show that triacsin C inhibits the incorporation of FAs into all cellular lipids and, as a result, is not useful in distinguishing FA versus TG effects on cholesterol metabolism. However, use of specific inhibitors of TG synthesis, including inhibition of DGAT, the key enzyme catalyzing the final step in known pathways of TG synthesis, could specifically examine the role of TG synthesis in effects on cellular cholesterol homeostasis. This could prove to be difficult due to the lack of synthetic inhibitors of DGAT. *In vitro* studies have identified several naturally occurring compounds that have potent DGAT inhibitory properties including xanthohumols, roselipins, prenylflavonoids, polyacetylenes and tanshinones (322-326). Additionally, siRNA technology could be used to knock down the expression of DGAT in the THP-1 macrophage cell line. In DGAT silenced cells, FAs would not be utilized for TG

synthesis and, as a result, we would be able to determine the significance of TG synthesis in the mobilization of lysosomal and cellular cholesterol from foam cells. Defining the contribution of TG and FAs in cholesterol homeostasis is important to design effective therapies to modulate reverse cholesterol transport in macrophage foam cells.

Potential Therapies Involving Alterations in Lesion TRPs and FAs

The studies presented in this dissertation suggest TG has the potential to induce reverse cholesterol transport and, as a result, can promote lesion regression. Importantly, high TG levels are correlated to some diseases including obesity and diabetes. Therefore, systemic increases in TG could be detrimental. However, specifically increasing TG levels within the atherosclerotic lesion may prove to have a therapeutic benefit. Cancer biologists have utilized homing mechanisms to tumors in order to specifically target the tumor for drug delivery, therapeutic moieties, and imaging agents (327-329). Such homing strategies are based on disease-specific molecular tags expressed in the vasculature, which is referred to as a vascular 'zip code' system (327-329). Pathological hallmarks of atherosclerotic lesion vasculature, mainly the dysfunction of endothelial cells, are currently being studied for their vascular homing properties (330-332). Recent studies have identified targeting moieties that home specifically to the developing atherosclerotic plaque. Using phage display *in vivo* biopanning, Thapa *et al*. have discovered a novel peptide (CLWTVGGGC) that is specifically targeted to the atherosclerotic endothelium (332). Once delivered to the

lesion the peptide is able to deliver attached cargo, including fluorescent tracers, into the subendothelial space (332). Thus, conjugation of an artificial TG particle, similar to a TG-DISP, to the targeting peptide would allow the specific delivery of TG to the interior of the atherosclerotic lesion (**figure 35**). Although utilizing such mechanisms for atherosclerotic lesion regression could take years to develop, the delivery of TG to the lesion using such mechanisms could clearly have an important therapeutic benefit. In the short term, utilization of homing peptides to deliver TRPs to the artery wall could be a tool to investigate the effects of TG on lesion regression *in vivo* using mouse models of atherosclerosis. Such *in vivo* studies defining the role of TG in foam cell cholesterol homeostasis within the atherosclerotic lesion would be highly relevant.

Importantly, the studies shown here reveal that unsaturated FAs are also able to induce reverse cholesterol transport. Therefore, FA supplementation could be a more promising alternative for therapy, given their ability to reduce a number of atherosclerotic risk factors. Current dietary recommendations of the World Health Organization and American Heart Association suggest substitution of PUFAs for saturated FAs as part of a healthy diet that reduces the risk of developing coronary heart disease (321,333,334). Dietary supplementation with fish oil, which contains the omega-3 PUFAs EPA and DHA, reduces the incidence of cardiac death by 30 – 45% (321,335,336). Furthermore, mono- and polyunsaturated FAs have the ability to lower serum total and LDL-cholesterol levels (337-339). An increase in dietary n-3 PUFAs is known to increase macrophage cholesterol efflux by altering the physical state of cholesterol within the cell and increasing CE hydrolysis, as well as increasing the fluidity

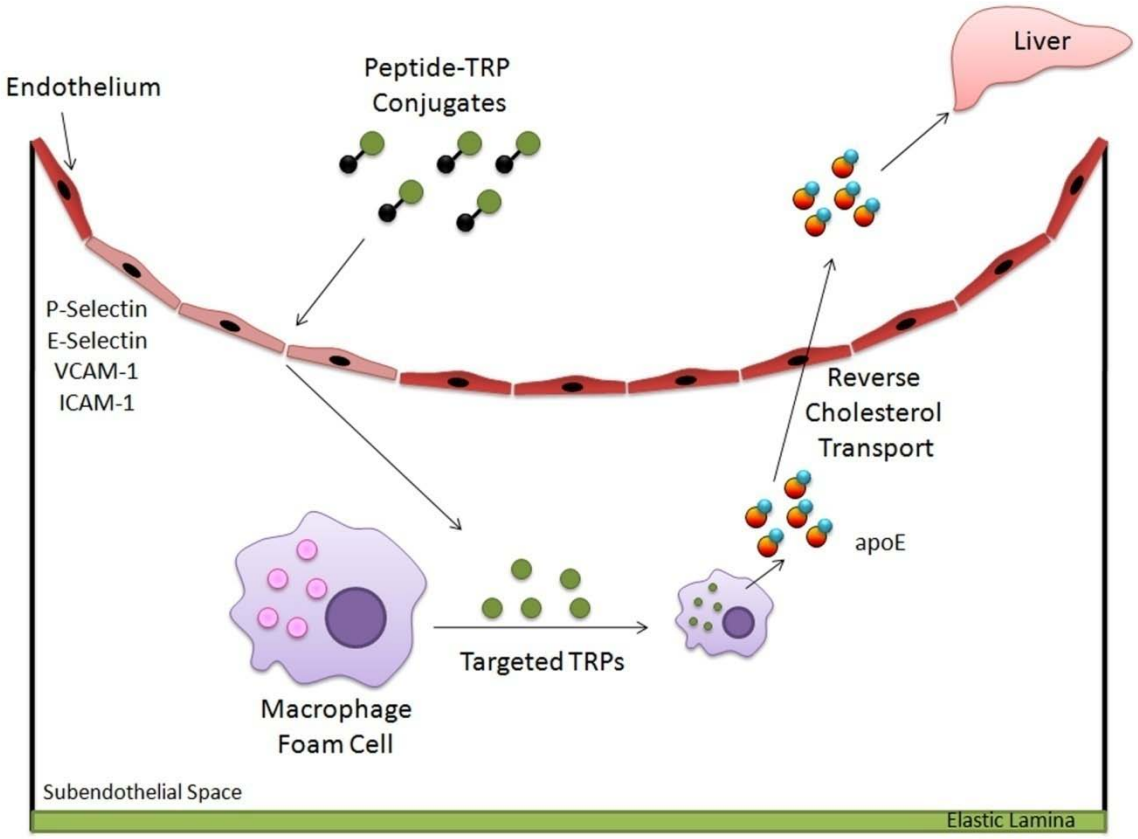

Figure 35: A potential therapeutic strategy involving directed TG delivery to the atherosclerotic lesion. While systemic increases in TG could have potentially harmful side effects the targeted delivery of TG to the atherosclerotic lesion could result in macrophage foam cell cholesterol efflux and, as a result, lesion regression. Peptides that specifically deliver molecules, including drugs and fluorescent imaging probes, are currently in development. Initial studies suggest that peptides designed to recognize the dysfunctional endothelial cells that overlay an atherosclerotic lesion deliver cargo specifically to the subendothelial space. Utilization of this technology could prove useful in delivering TG to the lesion, which could initiate reverse cholesterol transport from atherosclerotic foam cells by similar mechanisms to those defined in this dissertation.

of cellular membranes (227,340,341). Additionally, FA supplementation has been shown to produce anti-inflammatory effects (342). Thus, the potential of FAs to enhance lysosomal sterol movement to pathways responsible for reverse cholesterol transport, as suggested by the studies in this dissertation, could prove to be another therapeutic benefit of FA supplementation.

Consistent with our studies dietary FAs, including OA, have been shown to contribute to lesion regression (343). Previous studies performed in Golden Syrian Hamsters exhibiting advanced atherosclerotic lesions showed that switching dietary lipids from a saturated (e.g. coconut oil) to monounsaturated (e.g. olive oil) composition results in dramatic reduction in aortic arch lesion area which is consistent with enhanced lesion regression (343). The effects of altered circulating FA on lesion regression appear to be partially mediated by the ability of MUFAs and PUFAs to reduce circulating LDL levels. However, clinical studies examining the effects of FA supplementation and/or dietary changes on lesion regression have not examined the potential for FA to influence the individual components of the lesion, including the potential for FA supplementation to mobilize foam cell sterol. Consequently, studies in primates show reduction of coronary artery atherosclerosis following dietary supplementation with polyunsaturated FA that occurs independently from affects on circulating lipoproteins (344). PUFA supplementation has also been shown to reduce cardiovascular disease independently of effects on serum cholesterol in human populations (333,345). Dietary consumption of PUFAs is associated with a reduction in arterial stiffness, which is thought to occur due to the ability of PUFAs to alter the

structural properties of the atherosclerotic lesion (346). The PUFA-induced reduction in arterial stiffness suggests that a diet high in PUFAs could prevent the calcification and loss of elasticity present in advanced atherosclerotic lesions. Although the effectiveness of DHA and EPA in mobilizing foam cell lysosomal and cellular cholesterol must be examined the promising preliminary results achieved following treatment with OA and LA suggest that a portion of the lesion regression and remodeling observed following PUFA treatment *in vivo* is due to the mobilization of foam cell cholesterol (**figure 36**). Thus, the studies presented here establish a potential mechanism for the observed benefit of FA supplementation on atherosclerotic lesion regression.

Closing

Clinical studies examining the influence of circulating lipid profiles and risk of cardiovascular disease suggest high plasma TG levels are a risk factor for atherosclerosis. However, the relationship between hypertriglyceridemeia and atherogenesis remains undefined and highly controversial. The work presented here argues in favor of TG having positive effects on atherosclerosis. TG significantly mobilizes lysosomal cholesterol and is the only molecule shown to do so to date. Considering that over 70% of the cholesterol in late-stage atherosclerotic macrophage foam cells can be trapped in lipid engorged lysosomes, the influence of TG on intracellular cholesterol metabolism is extremely important. Not only does TG increase the clearance of lysosomal cholesterol but enrichment of macrophages with TG enhances mobilization of extralysosomal cholesterol as well. Therefore, the results of our studies provide strong evidence that

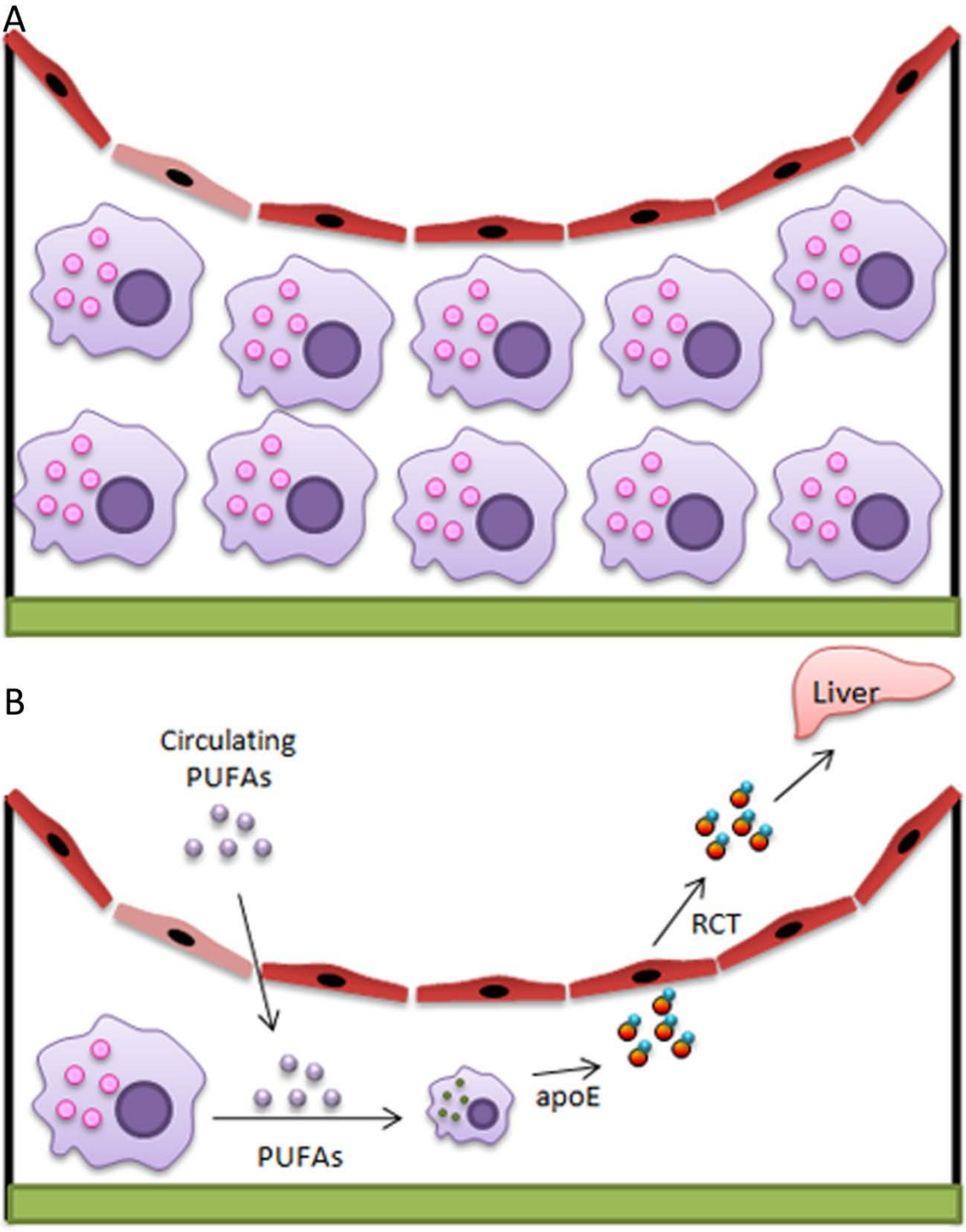
A
B
Liver
Circulating
PUFAs
RCT
apoE
PUFAs

Figure 36: The potential therapeutic value of PUFA supplementation. We have established a role for PUFAs in mobilizing foam cell cholesterol *in vitro.* Given the ability of PUFAs to induce the release of sequestered lysosomal sterol and efflux of cholesterol from the macrophage PUFA supplementation has the potential to induce reverse holesterol transport from the atherosclerotic lesion. Thus, in comparison to the foam cell enriched atherosclerotic lesion (A), treatment with PUFAs could reduce lesion area by inducing lysosomal and cellular cholesterol mobilization (B).

TG can mobilize cholesterol from macrophage foam cells which could ultimately lead to atherosclerotic lesion regression and remodeling.

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